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HLA-G MOLECULES IN INFECTION AND AUTOIMMUNE DISEASES

Settore Scientifico Disciplinare MED/07

Dottorando
Dott. Bortolotti Daria

Tutore
Dott. Rizzo Roberta

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"It is good to have an end to journey toward;

but it is the journey that matters, in the end."

— Ernest Hemingway

Preface:

The work reported in this thesis was performed during my Ph.D. studies at the Department of Medical Sciences – Section of Microbiology.
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I also like to say thanks to Prof. Carosella, that gave me the opportunity to experience research in his laboratory and all the collaborators that supported all my work.

Last, but not least, thanks with all my heart to my Mum, hoping that she will be proud of me and to Alessandro, always sustain me in my side during this experience.

Thank you all.

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The present thesis is based on the following publications:

I. **Bortolotti D**, Gentili V, Rotola A, Di Luca D, Rizzo R. (2014). Implication of HLA-G 3' untranslated region polymorphisms in human papillomavirus infection. Tissue Antigens. 83(2):113-8.

II. Laaribi AB, Zidi I, Hannachi N, Ben Yahia H, Chaouch H, **Bortolotti D**, Zidi N, Letaief A, Yacoub S, Boudabous A, Rizzo R, Boukadida J.Association HLA-G 14-bp Insertion/Deletion polymorphism with high HBV replication in chronic hepatitis. (2014) Journal of Viral Hepatitis (in press)

III. Rizzo R, Gabrielli L, **Bortolotti D**, et al., "Soluble HLA-G1 as a novel prognostic biomarker of symptomatic congenital cytomegalovirus infection". (Paper in preparation)

IV. R. Rizzo, Malagutti N, **Bortolotti D**, Gentili V, Rotola A, Fainardi E, Pezzolo T, Aimoni C, Pelucchi S, Di Luca D, and Pastore A. (2014). Infection and HLA-G Molecules in Nasal Polyposis. Journal of Immunology Research. 2014:40743

V. Rizzo R, Bergamini G, **Bortolotti D**, Leal T, D'Orazio C, Pintani E, Melchiorri L, Zavatti E, Assael BM, Sorio C, Melotti P. (2015) HLA-G expression and regulation in cystic fibrosis during Pseudomonas aeruginosa infection. Submitted to Journal of Cystic Fibrosis.

VI. Pseudomonas aeruginosa quorum sensing molecule N-(3-oxododecanoyl)-homoserine-L-lactone (3-o-C12-HSL) induces HLA-G expression in human immune cells. (Paper in preparation)

VII. Rizzo R., Farina I., **Bortolotti D**., Galuppi E., Rotola A., Melchiorri L., Ciancio G., Di Luca D., Govoni M. (2013). HLA-G may predict the disease course in patients with early rheumatoid arthritis. Human Immunology, 74:425-432.

VIII. Rizzo R., **Bortolotti D**., Baricordi O., Farina I., Padovan M., Govoni M. (2013). Can HLA-G predict disease course in RA patients?. Int J Clin Rheumatol, 8(6): 627-38.

IX. Rizzo R., **Bortolotti D**., Fredj NB., Rotola A., Cura F., Castellazzi M., Tamborino C., Seraceni S., Baldi E., Melchiorri L., Tola M., Granieri E., Baricordi O., Fainardi E. (2012). Role of HLA-G 14bp deletion/insertion and +3142C>G polymorphisms in the production of sHLA-G molecules in relapsing-remitting multiple sclerosis. Human Immunology, 73:1140-1146.

X. Fainardi E, **Bortolotti D**, Bolzani S, Castellazzi M, Tamborino C, Roversi G, Baldi E, Caniatti ML, Casetta I, Gentili V, Granieri E, Rizzo R, and the ERMES study group .Cerebrospinal fluid amounts of HLA-G in dimeric form are strongly associated to patients with MRI inactive multiple sclerosis (2015) submitted to Multiple Sclerosi Journal.

XI. Zidi I, Ben Yahia H, **Bortolotti D**, Mouelhi L, Laaribi AB, Ayadi S, Zidi N, Houissa F, Debbech R, Boudabous A, Najjar T, Di Luca Dand Rizzo R. Association between sHLA-G and HLA-G 14-bp deletion/insertion polymorphism in Crohn's disease. Int. Immunol (in press)

XII. Borghi A, Rizzo R, Corazza M, Bertoldi AM, **Bortolotti D**, Sturabotti G, Virgili A, Di Luca D. (2014) HLA-G 14-bp polymorphism: a possible marker of systemic treatment response in psoriasis vulgaris? Preliminary results of a retrospective study. Dermatol Ther 27(5):284-9

Contents

HLA-G molecules in infections and autoimmune diseases

Abstract

Human leukocyte antigen-G (HLA-G) is a non-classical HLA class I molecule that differs from classical HLA class I molecules for low allelic polymorphism and restricted tissue distribution. HLA-G is a tolerogenic molecule with an immune-modulatory and anti-inflammatory function on both innate and adaptative immunity. This peculiar characteristic of HLA-G molecule has led to evaluate its role in pathological conditions in order to define a possible use in diagnosis, prevention and treatment of diseases. Recently, HLA-G molecule has been shown to have an important implication in different inflammatory and autoimmune diseases, pregnancy complications, tumor development and severity, and susceptibility to viral infections. HLA-G molecules have been reported to be present differences at both genetic and protein levels in different disease situations, supporting its crucial role as a suitable key actor in pathological conditions. In fact, specific diseases show altered levels of soluble (s)HLA-G and different HLA-G gene polymorphisms correlated with disease outcome. Thus, HLA-G may exhibit two distinct effects in pathological conditions: it could be protective in inflammatory and autoimmune diseases or it could be dangerous, for example, in tumors or infectious diseases.

In this thesis, I report my results on HLA-G molecule analysis in different pathological conditions: microbiological infections and autoimmune diseases.

Introduction

1. Human Major Histocompatibility Complex

The human Major Histocompatibility Complex (MHC) is composed by a series of molecules encoded by a set of genes (~130) located on the short arm of chromosome 6 (6p) that are responsible for lymphocyte recognition, "antigen presentation" and immune response regulation. This gene complex comprises several distinct loci that in humans are called HLA, which stands for Human Leucocyte Antigens. MHC antigens can be subdivided into three major classes: class I, class II and class III (**Figure 1**). The class I and class II antigens are expressed on cells and tissues while class III antigens are present mainly as serum and body fluid proteins (*e.g.*C4, C2, factor B, TNF, complement components). In the class I gene complex are present three major loci: A, B and C. Each of these loci encodes for an alpha-chain polypeptide that associates to ß2-microglobulin, encoded by a gene on chromosome 15. The class II gene complex contains at least three loci, DP, DQ and DR, which encode for one alpha- and one beta-chain polypeptide associated together to form the class II antigens. All together, MHC class I A, B and C are considered as “classical” MHC class I antigens (MHC Ia). In fact, in addiction to those molecules, another group of MHC molecules, called non classical MHC class I molecules or MHC Ib (HLA Ib in humans) started to be studied in the end of the 80’s [1,2].

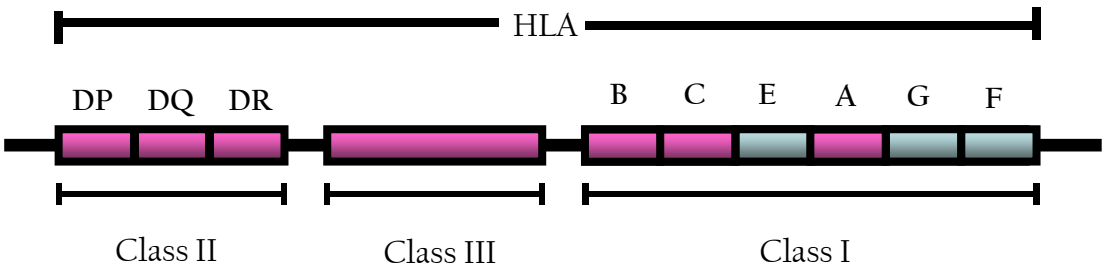


Figure 1. Schematic representation of the HLA region on chromosome 6. The non classical HLA class I gene are represented in grey.

2. The non classical HLA class Ib genes

Non-classical MHC class Ib molecules are closely homologous to classical class Ia molecules but are distinguished by their limited polymorphism and low cell surface expression. The group class Ib is composed by three molecules: HLA-E, HLA-F and HLA-G. The class Ib molecules do not represent just vestigial evolutionary remnants of classical class Ia molecules; rather some exhert highly specialized functions, as testified by their conservation between different species. In particular, human HLA-E (Qa-1 in mouse) and HLA-G (Qa-2 in mouse) constitutes a clear homology between species. In 1993, Warner et al. [3] demonstrated a reproductive advantage in mice encoding Qa-2 molecules, evidencing in this way the importance of the molecule in pregnancy outcome [4]. On the basis of these characteristics, Qa-2 and HLA-G antigens seem to share not only structural but also functional similarities in the regulation of immune response, through interaction with both inhibitory and activatory receptors [5,6].

3.The HLA-G antigen

HLA-G antigen is a non-classical HLA class I molecule characterized by (i) a low allelic polymorphism, (ii) a restricted tissue distribution, (iii) mRNA alternative splicing that generates seven proteic isoforms and (iv) a tolerogenic and anti-inflammatory biological function [7].
The basic characteristics of HLA-G biology have been further described at different levels [8 paper attached]

3.1. The *HLA-G* gene

The *HLA-G* gene shows low allelic polymorphisms with 50 HLA-G alleles acknowledged in the coding region (IMGT HLA database, October 2014). *HLA-G* gene has also polymorphic sites at the 5'-upstream regulatory region (5' URR) and at the 3'-untranslated region (3' UTR) which may contribute to the regulation of *HLA-G* expression [9]. For example, one of the most studied HLA-G polymorphism is a 14 bp insertion/deletion polymorphism (rs16375) [10] in exon 8 in the 3' UTR which is associated with mRNA stability and HLA-G protein expression [11, 12] (**Figure 2**). The presence of an insertion of 14 bp in the gene identify the

allele *ins* that has been associated with lower levels of HLA-G expression than the allele with the 14 bp deleted (*del*) [12-14]. An additional alternatively spliced *HLA-G* transcripts lacking 92 bp of the first part of exon 8 is observed within the insertion of 14 bp allele and is characterized by a more stable transcript [15]. Another polymorphism widely studied regards one single nucleotide polymorphism (SNP) C>G at the +3142bp position (rs1063320) that has been explored by Tan and coauthors [16]. The presence of a guanine at the +3142 position may influence the expression of the HLA-G locus by increasing the affinity of this region for the microRNAs miR-148a, miR-148b and miR-152, therefore decreasing the mRNA availability for translation by mRNA degradation and translation suppression. The influence of the +3142G allele has been demonstrated by a functional study in which HLA-G high-expressing JEG-3 choriocarcinoma-derived cells have been transfected with miR-148a, decreasing soluble HLA-G levels. The contrasting results obtained by Manaster and coauthors [17], who have reported the absence of +3142C>G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression.

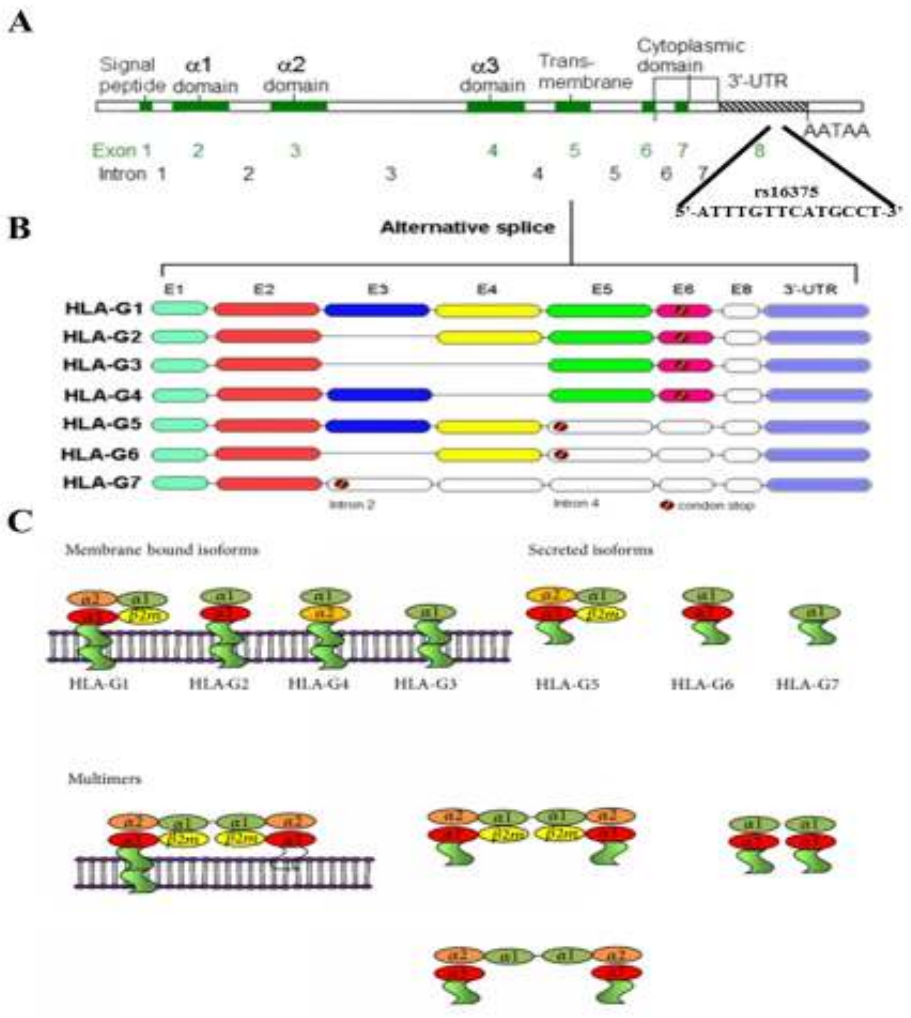


Figure 2. **Multiple HLA-G proteins derived from alternative splicing of HLA-G mRNA.**
A: The HLA-G gene is composed of 8 exons. The α, intracellular and transmembrane domains and the 14 bp insertion/deletion polymorphism (rs16375) in exon 8 in the 3' untranslated region (UTR) are represented.
B: The gene is alternatively spliced to yield 7 transcripts. In two of these, a stop sequence in intron 4 results in soluble isoforms.
C: The 7 HLA-G proteic isoforms: four membrane-bound (HLA-G1, -G2, -G3, -G4) and three soluble (HLA-G5, -G6, -G7) molecules; multimeric conformations of HLA-G molecules

Classical MHC class I genes are transactivated by two groups of juxtaposed cis -acting regulatory modules: (i) the up-stream enhancer A and ISRE (interferon-sensitive response

element) which mediate the constitutive and cytokine-induced expression; (ii) the S-X-Y module which controls the constitutive and CIITA (class II transactivator) mediated transactivation. These modules are divergent in *HLA-G* gene that is unresponsive to NF-kappaB (nuclear factor-kappaB), IRF-1 (interferon regulatory factor 1), and CIITA mediated induction pathways [18, 19]. The *HLA-G* gene promoter contains a putative interferon-regulatory factor (IRF)-1 binding site 746 base pairs upstream from ATG, which is distinct from the interferon-responsive element within proximal class Ia gene promoters. This control region is the probable element, which mediates interferon beta-induced expression of the *HLA-G* gene [20].

It was also reported that IFN-β enhances HLA-G expression throught the interaction with a ISRE present next to the nonfunctional interferon-gamma activated site (GAS) element at positions −754 to −743 [21]. HLA-G enhancer A and ISRE seem to bind only the constitutively expressed factor Sp1 (also known as Specificity Protein 1) [22, 23] but this binding of Sp1 does not modulate the constitutive or IFN-induced transactivation of HLA-G [24]. Furthermore, in the *HLA-G* promoter are present three cAMP/PMA response elements (CRE/TRE) with binding affinity for REB (rice endosperm bZIP)/ATF (activating transcription factor-2) and Fos/Jun proteins. It has been reported that *HLA-G* transactivation is regulated by CREB (cAMP-response element-binding protein), CREB-binding protein (CBP), and p300. These features represent the unique regulation of *HLA-G* transcription among the MHC class I genes [25] (**Figure 3**)

HLA-G expression is also regulated by epigenetic mechanisms [26, 27], such as DNA methylation. In fact, the role of methylation on *HLA-G* expression has been tested in human tumours considering the effect of the methylation inhibitor 5-aza-deoxycytidine on the CpG-enriched regulatory region of the *HLA-G* gene. The 5-aza-dC treatment results in hypomethylation of putative control sequences within the 5' regulatory region of *HLA-G* and these changes in methylation correlate with a significant increase in expression [28, 29].

The *HLA-G* gene seems not to undergo genomic imprinting, in fact it is co-dominantly expressed on trophoblast cells [30].

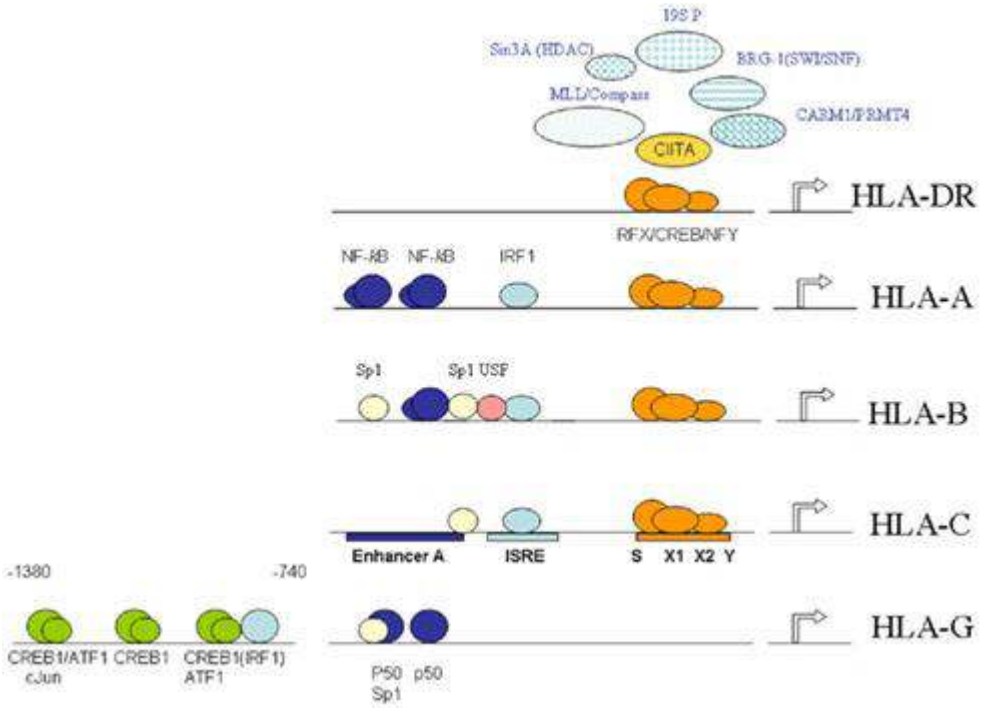


Figure 3. Regulation of HLA-I promoters

HLA-G molecules are also regulated by post-transcriptional mechanisms [31], including alternative splicing, mRNA stability, translation and protein transport to the cell surface [30-32]. This is possible due to the expression in advance of the molecules that are essential for cell surface expression of class I molecules, ß$_2$-microglobulin (ß$_2$m) and the transporter for antigen processing proteins (TAP1 and TAP2).

3.2. HLA-G expression and function

In physiological conditions HLA-G protein presence is restricted to certain tissues such as trophoblast, thymus, cornea, pancreas, proximal nail matrix, erythroblast, endothelial precursors and mesenchymal stem cells [33-35].

Unlike HLA class Ia antigens, seven HLA-G isoforms are generated by alternative splicing of its primary transcript and differential association with β2-microglobulin (β2-m) [36]: four, HLAG1,-G2, -G3 and -G4, are membrane-bound, while three, HLA-G5, -G6 and -G7, are soluble molecules (**Figure 2**). The soluble isoforms retain the intron 4, which includes a stop codon and leads to the termination of the mRNA translation before the transmembrane domain. The HLA-G1 and HLA-G5, the most analysed isoforms, are the only structures that

contain three alpha domains, while the other isoforms lack one or more globular domain. Furthermore, the proteolytical cleavage of surface isoform HLA-G1 generates the soluble HLA-G1 form (sHLAG1) [37]. Several evidences indicate that the soluble HLA-G1 (sHLA-G1) form is generated through the shedding of the membrane bound HLA-G1 by metalloproteinase (MMP) [38, 39]. In particular, our group identify that Matrix Metalloproteinase-2 (MMP-2), a zinc-containing and calcium-requiring endopeptidase known for the ability to cleave several extracellular matrix constituents, as well as non-matrix proteins, is responsible for HLA-G1 membrane-shedding via three possible highly specific cleavage sites [40 paper attached].

An in frame termination codon in *HLA-G* exon 6 leads to a truncated cytoplasmic tail which is 19 amino acids shorter than the corresponding tails of HLA-A, -B and –C proteins. This feature prevents the signal transduction from the cell surface to the nucleus. However, the membrane-bound HLA-G can localize in lipid rafts and can act as a signaling molecule, via modification of the phosphorylation state of raft-localized proteins [41]. This differs from classical HLA-I, which are recruited in lipid rafts upon receptor engagement [42].

The HLA-G production could be enhanced by different stimuli, such as interleukin (interleukin-10), interferon gamma and hormone molecules [43].

The anti-inflammatory and immunosuppressive Interleukin (IL)-10 has been correlated with concomitant HLA-G expression [13]. Transactivation of HLA-G transcription has also been demonstrated by leukemia inhibitory factor (LIF) [44] and methotrexate cell exposure [45]. Furthermore, interferon (IFN)-α, -ß and –γ enhance HLA-G cell-surface expression by tumors or monocytes [46, 47]. HLA-G expression could be acquired by trogocytosis [48], where a “donor” cell that expresses membrane HLA-G exchanges membrane parts containing HLA-G with a “recipient” cell that is not expressing HLA-G molecules. In this particular situation, “recipient” cells will acquire and make use of membrane HLA-G molecules from a “donor” HLA-G positive cell without the activation of HLA-G gene transduction into protein. HLA-G molecules undergo the trogocytosis mechanism: some effectors CD4 and CD8 T lymphocytes acquire immunosuppressive HLA-G1 molecules from antigen presenting cell membranes and reverse their function from effectors to regulatory cells [49].

Trogocytosis of HLA-G from antigen presenting cell (APC) by T cells in humans makes these T cells unresponsive [48]. It has been shown that NK cells can acquire HLA-G1 from tumor cells, which provokes an arrest of NK cells proliferation and cytotoxic activity, behaving like suppressor cells capable of inhibiting other NK-cell functions [50].

Membrane-bound HLA-G1 and soluble HLA-G (HLA-G5 and sHLA-G1) molecules exert immunosuppressive effects: (i) inhibit the cytotoxic activity of CD8 positive T lymphocytes (CTL) and Natural Killer (NK) cells [51], (ii) induce the apoptosis of NK and activated cytotoxic T cells [52], (iii) inhibit the allogeneic CD4 positive T-cell proliferation and interfere with naïve CD4 T-cell priming [53], (iv) inhibit antigen presenting cell and B lymphocyte differentiation [54], (v) induce regulatory T cells [55] (**Figure 4**). Furthermore, sHLA-G affects angiogenesis interacting with endothelial cells [56] and induces resting NK cells to produce chemokines and cytokines [12].

HLA-G exerts its immunomodulatory functions through the interaction with multiple receptors expressed by immune cells (**Figure 4**). HLA-G is known to interact with NK receptor [57, 58], as KIR2DL4 [5, 12] and leukocyte inhibitory receptors (LILRs) / immunoglobulin-like transcripts (ILT) [6, 13] as LILRB1 (LIR-1/ILT2/CD85j), which is highly expressed on T and B-lymphocytes and with LILRB2 (LIR-2/ILT4/CD85d), present mainly in monocytes/macrophages. The alpha3 domain of HLA-G is the putative binding site for ILT receptors [59] while the residues Met76 and Gln79 in the alpha1 domain play a critical role in the recognition of KIR2DL4 receptor [60]. The KIR2DL4, receptor of the killer cell immunoglobulin-like receptors (KIR) family, is expressed in all NK cell types and has unique structural properties among the rest of KIR receptors: it possesses a long cytoplasmic tail characteristic of inhibitory receptors, a charged amino acid in the transmembrane domain similarly to activating KIR receptors [61], and a mix structure in the extracellular part with D0 and D2 domains. Contrary to other KIR receptors, KIR2DL4 expression is transitory on NK cell surface, with a main expression in endosomes, reached by an endocytic process. KIR2DL4 seems to participate to HLA-G endocytosis when it is transitory expressed on NK cell surface, as both HLA-G and KIR2DL4 can be simultaneously co-localized in endosomes [5]. KIR2DL4 expression can be induced by IL-2 and its activation upon antibodies engagement provokes a weak cytotoxic activity with a strong IFN-γ production [62]. In-vitro studies have shown that KIR2DL4 is able to interact with β2-m free HLA-G molecules, inducing IFN-γ production [63] and increasing NK cell cytotoxicity [64]. Contrary to LILR receptors, KIR does not bind HLA-I molecules through its α3 domain, but through α1 and α2 domains which are much more polymorphic than α3 domain [65, 66]. This could account for the broader specificity of LILR receptors in comparison with KIR2DL4, that binds specifically HLA-G and not other HLA-I molecules.

The expression of LILRB1, LILRB2 and KIR2DL4 can be induced by HLA-G without any co-stimulatory requirement, which indicates that it can occur independently from any immune response [67].

Soluble HLA-G has potentially a higher range of activity than membrane-bound HLA-G. The circulating isoforms could bind to the same sets of leukocytes and perform exactly the same functions also systemically.

The membrane-bound and soluble HLA-G proteins have monomer, dimer, and oligomer forms (**Figure 2**); the dimer seems to have a dominant effect on the LILRB signaling.

In fact, in vitro studies have reported that HLA-G1 is able to form disulphide-linked homodimers on cell surface [68-70] that bind ILT2 receptors with more avidity compared to monomers [71]. On the contrary, structural studies suggest that KIR2DL4 cannot bind HLA-G dimers due to steric reasons [72].

A disulfide-bonded dimer conformation is possible for the presence of a cysteine 42 residue that is present only in the heavy chain α1 domain of HLA-G [59, 68]. Besides Cys residues in α 2 and α 3 domains that allow intramolecular disulphide bonds, HLA-G molecule presents other important Cys residues. Cys42 in α1 domain and Cys147 in α 2 domain can form intermolecular disulphide bonds giving rise to HLA-G dimers that can be observed by SDS/PAGE under non-reducing conditions [69]. These structures have been observed for all HLA-G isoforms except HLA-G3 [73].

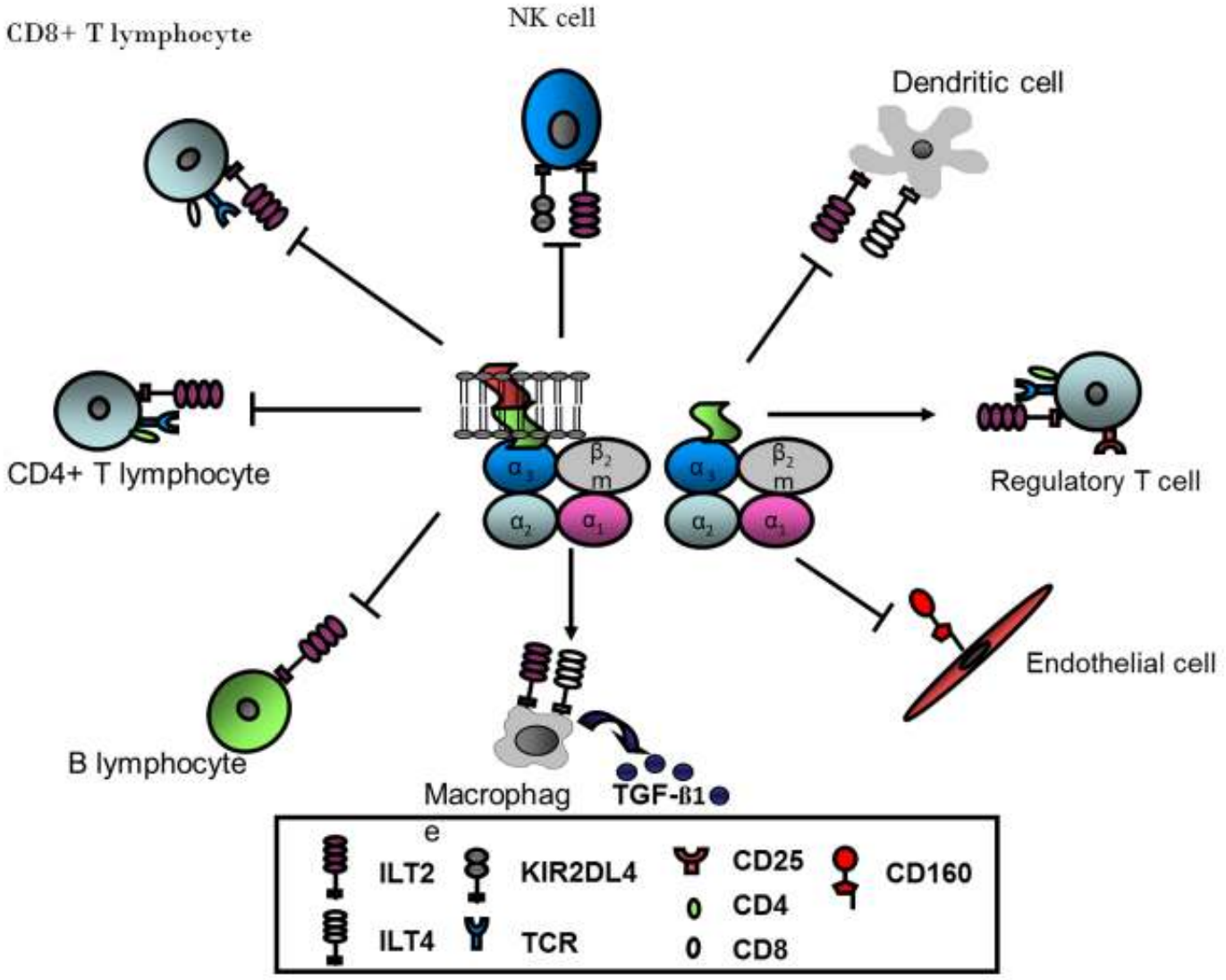
Dimers of HLA-G have been observed on the surface of transfected cells [68], on choriocarcinoma cell line JEG-3 [74], on first trimester trophoblast cells [75] and on malignant cells [76]. Furthermore, a recent work of Ezeakile M et al. reported the importance of HLA-G dimers in kidney allograft outcome [77]. In fact, the authors decribed the presence of HLA-G dimers in plasma of kindney transplant patients together with an increased expression of membrane-bound HLA-G in association with prolongation of allograft survive.

Alegre E. et al also reported that other HLA-G complexes exist in vivo as ubiquinated protein in exosomes [78]

Soluble HLA-G1 is able to inhibit endothelial cells through specific interaction with the CD160 molecule, a glycosylphosphatidylinositol-anchored, major histocompatibility complex (MHC) Class I-dependent, immunoglobulin-like receptor, that is expressed by activated endothelial cells [56] (**Figure 4**). This interaction seems to lead to apoptosis of endothelial cells required for normal placental development.

Figure 4. HLA-G receptors. HLA-G receptors expressed on immune (CD8 T and CD4 T cells, B cells, natural killer (NK) cell, macropages, dendritic cells) and endothelial cells. ILT: immunoglobulin-like transcript; KIR: killer inhibitory receptor; TCR: T cell receptor.

Figure 4.


CD8+ T lymphocyte
NK cell
Dendritic cell
CD4+ T lymphocyte
Regulatory T cell
Endothelial cell
B lymphocyte
Macrophage
TGF-ß1
ILT2
KIR2DL4
CD25
CD160
CD4
ILT4
TCR
CD8

Results

1. Role of HLA-G molecules in pathological conditions

The HLA-G expression has been analyzed in different pathological conditions, such as transplantation, oncology, viral infections, inflammatory and autoimmune diseases indicating that HLA-G can favour graft tolerance, tumor cell and virally infected immune escape and control the inflammatory conditions [79-82 paper attached, 83].
I will report my results, obtained during my PhD course, on HLA-G analysis in different pathological conditions: microbiological infections and autoimmune diseases.

1.1 HLA-G and infections

Host immune defence is normally able to eliminate the majority of viral and bacterial infections. However, it is known that some pathogens, in particular viruses, have developed different mechanisms for subverting host immune defences, thus facilitating their spread in the host [84]. For instance, viruses can protected themselves against attack by NK cells by expressing HLA-G on the infected cell surface providing in this way an immunosuppressive effect. Anyway, the diminished immune function induced by HLA-G in the host could represent an advantage for virus progression by helping viruses subvert the host's antiviral defences [85]. HLA-G can affect all the phases in the immune response: differentiation, proliferation, cytolysis, cytokine secretion, and immunoglobulin production. In fact, during infections, HLA-G modulates adaptive and innate immunity by interacting with T or B-lymphocytes and NK cells or polymorphonuclear cells (**Figure 1**).

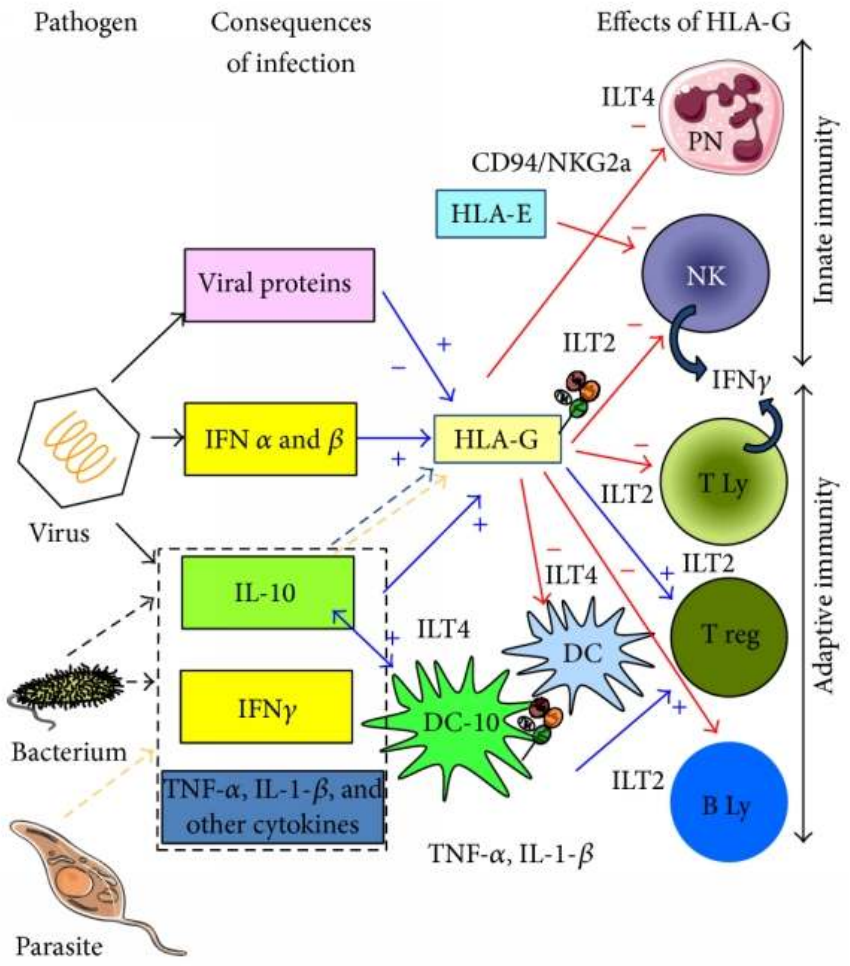


Figure 1: **Causes and consequences of HLA-G modulation in infectious diseases**. Positive and negative effects of HLA-G are shown in blue and red, respectively. Parasites, bacteria, or viruses induce the secretion of various cytokines, including IL-10 and interferon (-γ for bacterium and IFN-α and -β for virus). These cytokines upregulate the expression or secretion of HLA-G. In addition, IL-10 induces IL-10-producing human dendritic cells (DCs), termed DC-10, expressing HLA-G and ILT4. HLA-G induces tolerogenic DC in addition to DC-10 and regulatory cells via direct interaction with ILT2 and/or ILT4. HLA-G, through direct interaction with ILT2, inhibits the function of T and NK cells and B cells, whereas it inhibits the function of granulocytes and myeloid DC via direct interaction with ILT4. Indirect effects of HLA-G are mediated by the induction of HLA-E cell surface expression, which inhibits CD94/NKG2a on NK and T cells.The consequence of HLA-G action is a downregulation of innate and adaptive immunity. *(Immunomodulatory Properties of HLA-G in Infectious Diseases, Amiot L et al. J. Immunol Res 2014 [86]).*

Even if host immune system presents several mechanisms to control infections, the viruses, in particular, have developed several strategies to counteract host immune defenses [84], for example by using HLA-G functions in viral immune-escape from immune cells [85]. HLA-G modulation exherted by pathogens has the aim to affect its expression in different ways, for example exploiting gene polymorphysms and alleles, modifying the exposition or the secrection of the protein or producing homologue proteins to human cytokines [86].

1.1.1 HLA-G and viral infections

During viral immune-escape, HLA-G could be modulated at different levels affecting the expression of HLA-G on the infected cells [86]. Firstly, HLA-G polymorphism is involved in susceptibility to viral infections. In fact, its implication in viral immune-escape is deeply described for example during HIV 1 infection [87-92]. These studies reported that some HLA-G alleles (in particular the G*010108 allele) and polymorphisms (i.e. 14bp ins/del and +3142 C>G) that can influence HLA-G expression have been associated with a greater risk of HIV infection and also of horizontal transmission. Concerning the role of HLA-G, and in particular its polymorphisms during viral infections, we identify two polymorphisms in the 3' untranslated region of the HLA-G gene (3' UTR) (14 bp ins/del, +3142C>G) that are involved in susceptibility to Human papillomavirus (HPV) infection [93 paper attached] reporting characteristic patterns among the different kind of lesion developed. It is well known that HPV is necessary but not sufficient to induce cancer development [94] and that it interferes with host immune response by modifying the expression of human leukocyte antigen-G (HLA-G) [95]. Moreover, previous studies already confirmed that HLA-G polymorphisms could influence HPV infections susceptibility and lesion development [96-99]. Indeed, we reported that the 14 bp del allele is associated with a high risk of HPV infection, and the del/C haplotype facilitates the development of invasive cervical cancer [93 paper attached], suggesting in this way that HLA-G polymorphisms could represent a risk factor for neoplastic transformation expecially in high-risk HPV positive subjects, that are known to be more likely to cancer progression [100]. It is known by literature that HLA-G is also implicated in chronic hepatits B pathogenesis [101, 102] and we reported the importance of 14 bp ins/del polymorphism influence also concerning Hepatitis B virus (HBV) infection. Our study revealed an association between the 14 bp ins/del polymorphism and an enhanced HBV activity in presence of high HBV DNA levels [103 paper attached].

Reference 93

TISSUE ANTIGENS
IMMUNE RESPONSE GENETICS

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BRIEF COMMUNICATION

Implication of HLA-G 3′ untranslated region polymorphisms in human papillomavirus infection

D. Bortolotti, V. Gentili, A. Rotola, D. Di Luca & R. Rizzo

Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Ferrara, Italy

Key words
human leukocyte antigen-G; human papillomavirus; invasive cervical cancer

Correspondence
Roberta Rizzo
Department of Medical Sciences
Section of Microbiology
University of Ferrara
Via Luigi Borsari 46
Ferrara 44122
Italy
Tel: +39 0532 455 382
Fax: +39 0532 974 470
e-mail: rbr@unife.it

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Abstract

Human papillomavirus (HPV) infection is involved in cervical lesion development. It interferes with host immune response and modifies the expression of human leukocyte antigen-G (HLA-G), a nonclassical HLA-I antigen with immune-inhibitory functions. We analyzed the frequencies of two HLA-G 3′ untranslated region polymorphisms (14 bp *ins/del*, +3142*C*>*G*), involved in HLA-G modulation, in 33 condyloma acuminatum, 14 low grade squamous intraepithelial lesion and 100 invasive cervical cancer (ICC) HPV infected patients. We showed the involvement of HLA-G polymorphisms in HPV infection and lesion development, and suggested that 14 bp *del* allele promotes high-risk HPV infection, with *del/C* haplotype associated with ICC development. On the basis of these evidences, HLA-G polymorphisms could represent a risk factor in HPV positive subjects.

Introduction

Human papillomaviruses (HPV) are small unenveloped double-stranded DNA viruses with strict tissue and species specificity. Many different papillomaviruses infect animals, and over 150 genotypes have been so far identified in humans. Papillomaviruses infect squamous epithelia of skin and mucosae. The mucosal types of HPV fall in two groups: low-risk types (mainly HPV-6 and -11), which induce genital warts, and the high-risk types, which lead to squamous intraepithelial lesions (SILs), invasive cervical cancer (ICC) and several other malignancies, such as anal cancer and oropharyngeal carcinomas. The most prevalent high-risk HPV types are HPV-16 and HPV-18, being responsible for 50% and 20%, respectively, of cervical cancer cases globally (1).

HPV infection is necessary for cancer development but it is not sufficient (2). In fact, HPV infection is often transient and the host immune system could counteract viral invasion leading to lesion regression (3). On the other hand, HPV is able to down-regulate host immune system (4), blocking interferon response and antigen processing and presentation (5) and modifying human leukocyte antigen (HLA)-G expression (6).

HLA-G is a nonclassical HLA class I molecule with a physiological tissue-restricted distribution in cytotrophoblast (7), amniotic cells (8), thymus (9) and endothelial cells of chorionic blood vessels (10). HLA-G molecules are generated by an alternative splicing of the primary transcript of the gene: HLA-G exists as four membrane bound (HLA-G1, -G2, -G3 and -G4) and three soluble isoforms (HLA-G5, -G6 and -G7) (11, 12). HLA-G exhibits low allelic polymorphisms in comparison with classical HLA class I genes, with only 50 alleles (IMGT HLA database, April 2013) and 16 proteins. HLA-G is characterized by tolerogenic functions, inducing apoptosis of activated CD8+ T cells (13), promoting T regulatory cells (14), modulating the activity of natural killer cells (15) and of dendritic cells (16) and blocking allo-cytotoxic T lymphocyte response (17). These immuno-regulatory functions are mediated by the interaction of HLA-G molecules with specific inhibitory receptors: ILT-2 (LILRB1/CD85j), ILT-4 (LILRB2/CD85d), CD8 and KIR2DL4 (CD158d) expressed by immune cells (18). HLA-G expression is differently modulated during pathological conditions as viral infections and tumors, where HLA-G acts as immune escape mechanism (19) and is controlled by several polymorphisms both in the promoter and in the 3′ untranslated region (UTR) modifying the affinity of gene-targeted sequences for transcriptional or post-transcriptional factors, respectively (20). In particular, the rs66554220 14 base pairs (14 bp) insertion/deletion (*ins/del*) polymorphism in the

3′ UTR of HLA-G gene is associated with the stability and splicing of the HLA-G mRNA: *ins* and *del* alleles are associated with a decreased or increased mRNA stability, respectively. The 14 bp *ins/del* polymorphism is associated with several disorders (21, 22). In particular, the *del/del* genotype is recognized as a risk factor for viral infection (23) and tumor progression (24). The single-nucleotide polymorphism (SNP) *C>G* at the +3142 bp position (rs1063320) of the 3′UTR of HLA-G gene controls HLA-G expression. The presence of a *G* at the +3142 position influences the expression of the HLA-G locus by increasing the affinity of this region for the microRNAs, miR-148a, miR-148b and miR-152, therefore decreasing the mRNA availability by mRNA degradation and translation suppression (25). The +3142G allele presenting an increased frequency among systemic lupus erythematosus patients (26), is associated with a lower protection against hepatitis C virus infection (27) and protects from asthma development (28). These two polymorphisms are in linkage disequilibrium (29) and their combination into haplotypes results into HLA-G expression control (22). The *del/C* haplotype is linked to the highest while *ins/G* to the lowest HLA-G mRNA and protein production (22, 30).

Previous studies reported an increased HLA-G expression in ICC, associated with disease progression (31–33) and HPV infection (6). HLA-G expression was up-modulated in the plasma of ICC patients (34), with an increase in HLA-G5 isoform in ICC patients without HPV infection (35). The analysis of HLA-G genetic polymorphisms reported a correlation between 14 bp *ins/del* polymorphism and ICC (36, 37) and high grade squamous intraepithelial lesions (HSIL) (38). However, no literature data are available on the implication of HLA-G genetic background with HPV infection and the differences in lesion development.

The interest of this study is to evaluate the role of HLA-G 14 bp *ins/del* and +3142*C>G* polymorphisms in the formation of HPV-associated lesions.

We performed HPV high risk (HPV-16, -18, -31, -33, -35, -39, -45, -52, -53, -56, -58, -59, -66 and -70) screen with the Sacace Biotechnologies kit (Sacace Biotechnologies, Como, Italy) and Nested polymerase chain reaction (PCR) for HPV low risk (HPV-6 and -11) detection (39) in 100 healthy women without lesions, 33 women affected by condyloma acuminatum, 14 with low grade squamous intraepithelial lesion (LGSIL, CIN 1) and 100 ICC patients. All the subjects were recruited at the University Hospital of Ferrara, selected for a documented Caucasian and consistent geographical origin. DNA for HPV detection was extracted from biopsies by protease/sodium dodecylsulfate (SDS) digestion, purified by phenol–chloroform followed by an extraction in ethyl ether. The nucleic acids were precipitated by addition of ethanol and suspended in Tris–HCl added with Na_2EDTA (39). HLA-G 3′UTR polymorphisms were analyzed by real-time PCR as previously reported on DNA extracted from peripheral blood cells (40, 41). For the +3142*C>G* polymorphism the forward primer, 3142-for, was 5′-CCTTTAATTAACCCATCAATCTCTCTTG-3′ and the reverse primer, 3142-rev, was 5′-TGTCTCCGTCTCTGTCT CAAATTT-3′. The minor groove binder (MGB) probe used for detection of the 3142*C* allele was 5′-VIC-TAAGTTATA GCTCAGTGGAC-3′ (3142CVIC) and the MGB probe for the 3142G allele was 5′-FAM-TAAGTTATAGCTCAGTGCAC-3′ (3142GFAM) (40). For the rs66554220 polymorphism the forward primer, HLAG14-for, was 5′-GTG ATG GGC TGT TTA AAG TGT CAC C-3′, and the reverse primer, HLAG14-rev, was 5′-GGA AGG AAT GCA GTT CAG CAT GA-3′ (41). The probe used for detection of *del* allele was 5′-VIC-GAG TGG CAAGTC CCT TTG TG-BHQ-3-3′ (HLAG14VIC) and the probe for the *ins* allele was 5′-Fam-CAA GAT TTGTTC ATG CCT TCC C-BHQ-1-3′ (HLAG14FAM). We analyzed HLA-G 3′UTR polymorphisms alleles, genotypes and haplotypes by ARLEQUIN 3.5.1.2 software. Statistical analysis was performed using STATVIEW software package (SAS Institute Inc., Cary, NC) and GRAPHPAD PRISM 6.0 software (Graphpad Software, San Diego, CA). Significance was assumed for $P < 0.05$. Bonferroni correction for multiple comparisons was applied when P value was significant.

The HPV analysis reported a 100% ICC, 79% LGSIL and 18% condyloma patients with high-risk HPV infection and a 100% condyloma patients and 21% LGSIL subjects with low-risk HPV (HPV-6 and -11) positivity (Table 1). These data sustain the significant association between high-risk HPV positivity and LGSIL and ICC development and the strict correlation between condyloma and low-risk HPV infection (condyloma low risk *vs* LGSIL high risk + ICC = OR: 166.5, 95% CI: 39.1–708.6). Six condyloma patients (18%) presented both high- and low-risk HPV infections. This condition was previously demonstrated in other studies, where most condyloma lesions contained multiple HPV types, including types associated with dysplastic epithelial abnormalities (42). The presence of multiple HPV types in a large percentage of condyloma lesions suggests that many individuals acquire additional HPV types at the time of infection with HPV types

Table 1 Distribution of HPV types in the test group stratified for lesion type

HPV types	Condyloma (33) *n* (%)	LGSIL (14) *n* (%)	ICC (100) *n* (%)
High/intermediate risk (16, 18, 31, 45, 33, 35, 39, 51, 52, 56, 58, 59, 68)	6 (18)	11 (79)	100 (100)
Low risk (6, 11)	33 (100)	3 (21)	0 (0)
P^a		<0.0001	<0.0001

HPV, human papillomavirus; ICC, invasive cervical cancer; LGSIL, low grade squamous intraepithelial lesion.

[a]*P* values were obtained by chi-squared test and comparing LGSIL and ICC groups with condyloma group subdivided according with HPV types. Only subjects with one type HPV infection were included in the analysis.

Table 2 Allelic, genotypic and haplotypic frequencies of 3′UTR 14 bp *ins/del* and +3142*C/G* in the control and test groups

	Control group HPV-(100)	Test group HPV+ (147)	*P*	Test group HPV+ low risk (30)	Test group HPV+ high risk (111)	*P*
Alleles *n* (%)						
14 bp *ins/del*						
ins	84 (42)	99 (31)	0.07[a]	26 (43)	73 (33)	0.17[a]
del	116 (58)	195 (69)		34 (57)	149 (67)	
Total	200	294		60	222	
+3142*C/G*						
C	83 (42)	164 (56)	0.008[a]	35 (56)	118 (53)	0.14[a]
G	117 (58)	130 (44)		25 (42)	104 (47)	
Total	200	294		60	222	
Genotypes *n* (%)						
14 bp *ins/del*						
ins/ins	22 (22)	13 (9)		0 (0)	13 (12)	
ins/del	40 (40)	73 (50)	0.05[b]	26 (87)	47 (42)	$3.110^{-4[b]}$
del/del	38 (38)	61 (41)		4 (13)	51 (46)	
Total	100	147		30	111	
+3142*C/G*						
C/C	21 (21)	47 (32)		12 (40)	30 (27)	
C/G	41 (41)	70 (48)	0.028[b]	11 (37)	58 (52)	0.27[b]
G/G	38 (38)	30 (20)		7 (23)	23 (21)	
Total	100	147		30	111	
Haplotypes *n* (%)						
del/G	60 (30)	43 (15)		4 (7)	32 (14)	
del/C	72 (36)	152 (52)	$4 \times 10^{-4[b]}$	30 (50)	117 (53)	<0.0001[b]
ins/G	56 (28)	87 (28)		15 (25)	73 (33)	
ins/C	10 (5)	12 (4)		11 (18)	—	

HPV, human papillomavirus; UTR, untranslated region.
[a]Fisher exact test.
[b]Chi-squared test.

6 or 11. Three patients (21%) with LGSIL presented low-risk HPV infection. These data are consistent with the different HPV genotype distribution in LGSIL in comparison with ICC, highlighting the importance of HPV genotype in the risk of progression from LGSIL to malignancy (43). Healthy controls presented no positivity for both high- and low-grade HPV infection.

Firstly, we considered the possible implication of HLA-G polymorphisms in modifying the risk of HPV infection. Both HLA-G polymorphisms were in Hardy–Weinberg equilibrium in both control and HPV infected populations (14 bp *ins/del*: controls X^2: 3.2, $P = 0.2$; HPV patients X^2: 1.83, $P = 0.4$; +3142*C*>*G* controls X^2: 2.42, $P = 0.3$; HPV patients X^2: 0.18, $P = 0.9$). We compared HPV negative controls with all HPV positive patients (condyloma + LGSIL + ICC, Table 2). We observed a decrease in *G* allele (P_c: 0.008) and *ins/ins* and *G/G* genotypes in HPV positive patients in comparison with HPV negative controls (P_c: 0.05; P_c: 0.028, respectively). As increased levels of HLA-G production were observed in the presence of HPV infection (6), these two genotypes, that are characterized by low HLA-G production (22), could interfere with virus immune escape and decrease the risk of HPV infection [odds ratio (OR): 0.434; 95% confidence interval (CI): 0.2–0.7; OR: 0.4; 95% CI: 0.2–0.7, respectively].

The analysis of haplotypes revealed a different distribution between controls and test group ($P = 4 \times 10^{-4}$). We observed an increase in *del/C* haplotype in HPV positive patients in comparison with HPV negative controls (Table 2). These data suggest that the presence of a high HLA-G producing haplotype *del/C* (22) could facilitate HPV infection. We then evaluated the effect of these HLA-G polymorphisms in high- and low-risk HPV infection. We considered only the patients with one type (high or low risk) of HPV infection. We observed an increase in *del/del* genotype (46%; 13%; $P_c = 3.1 \times 10^{-4}$) and of the *del/G* haplotype (14%; 7%; $P_c < 0.0001$) in high-risk HPV infected patients in comparison with low-risk HPV infected patients. These data suggest that the presence of a high HLA-*G* producing 14 bp *del* allele (22) could facilitate high-risk HPV in comparison with low-risk HPV infection (OR: 5.5; 95% CI: 1.8–16.9).

Because HLA-G polymorphisms seem to influence high-risk HPV infection, we then evaluated the role of HLA-G polymorphisms in the development of HPV high-risk infection into preneoplastic and neoplastic lesions (Table 3). The +3142*C* allele resulted increased in ICC (58%) patients in comparison with LGSIL (14%) (P_c: 3.2×10^{-4}). Similarly, the *C/C* genotype was over-represented in ICC (30%) patients

Table 3 Allelic, genotypic and haplotypic frequencies of 3′UTR 14 bp *ins/del* and +3142*C/G* in the LGSIL, ICC and condyloma groups

	LGSIL HPV + high risk (11)	ICC (100)	*P* (LGSIL *vs* ICC)	Condyloma (33)	*P* (LGSIL + ICC *vs* condyloma)
Alleles *n* (%)					
14 bp *ins/del*					
ins	8 (36)	62 (31)	0.63[a]	26 (39)	0.2[a]
del	14 (64)	138 (69)		40 (61)	
Total	22	200		66	
+3142*C/G*					
C	3 (14)	115 (58)	3.2×10^{-4}[a]	46 (70)	0.08[a]
G	19 (86)	85 (42)		20 (30)	
Total	22	200		66	
Genotypes *n* (%)					
14 bp *ins/del*					
ins/ins	2 (18)	11 (11)		0(0)	
ins/del	4 (36)	40 (40)	0.8[a]	26 (79)	1.1×10^{-3}[b]
del/del	5 (46)	49 (49)		7 (21)	
Total	11	100		33	
+3142*C/G*					
C/C	0 (0)	30 (30)		17 (52)	
C/G	3 (27)	55 (55)	8×10^{-4}[a]	12 (36)	0.12[b]
G/G	8 (73)	15 (15)		4 (12)	
Total	11	100		33	
Haplotypes *n* (%)					
del/G	11 (50)	23 (11)		5 (8)	
del/C	3 (14)	115 (58)		35 (53)	
ins/G	8 (36)	62 (31)	8×10^{-6}[b]	15 (22)	<0.00001[b]
ins/C	—	—		11 (17)	

HPV, human papillomavirus; ICC, invasive cervical cancer; UTR, untranslated region; LGSIL, low grade squamous intraepithelial lesion.
[a]Fisher exact test.
[b]Chi-squared test.

in comparison with LGSIL (0%) (P_c: 8×10^{-4}), where no subjects with *C/C* genotype were observed. The analysis of combined haplotypes revealed an increase of *del/C* haplotype in ICC patients (58%) in comparison with LGSIL patients (14%) (P_c: 8×10^{-6}), resulting in an increased risk to develop an invasive lesion (OR: 8.6; 95% CI: 2.5–29.9). On the contrary, the *del/G* haplotype was down-represented in ICC patients (11%) in comparison with LGSIL (50%), with a protective effect against neoplastic evolution (OR: 0.12; 95% CI: 0.05–0.33). The logistic regression analysis excluded the influence of confounding variables (age, smoke habits and familial history of cancer). These data are in agreement with the results obtained by Silva et al. (37) in a Brazilian cohort, where they observed a similar decrease in *del/G* haplotype in ICC patient group, suggesting a role in lesion progression. Meanwhile, Silva et al. (37) and Simões et al. (38) suggested an implication of the 14 bp *ins* allele with an increased risk of lesion development. We confirmed these results comparing the patients with preneoplastic and neoplastic lesions (LGSIL + ICC) with condyloma patients. We observed no condyloma patients with *ins/ins* genotype (0% *vs* 11%; P_c: 1.1×10^{-3}), supporting its role in lesion follow-up. Interestingly, condyloma patients presented the *ins/C* haplotype, that is totally absent in LGSIL and ICC patients (17% *vs* 0%; $P_c < 0.00001$). As this haplotype is commonly present at low frequency (26, 27), this result is suggestive of a specific effect in condyloma patients.

Taken together, these results support an implication of HLA-G polymorphisms in HPV infection and lesion development. The decreased frequency of low HLA-G producer genotypes (*ins/ins*, *G/G*) and the increase in high HLA-G producer haplotype (*del/C*) in HPV infected subjects sustain the role of HLA-G genetic background in HPV infection. In particular, we could hypothesize that a low HLA-G production could interfere with virus immune escape and decrease the risk of HPV infection, while a high HLA-G production acts as an immune-escape mechanism. However, when the HPV infection is present, 14 bp *ins* allele, a low HLA-G producer, allows lesion progression. We can speculate that the lower expression of HLA-G could facilitate the creation of an inflammatory environment, that facilitates lesion progression. The role of HLA-G expression is peculiar in high-risk HPV infected patients. The presence of 14 bp *del* and +3142*C* alleles, both high HLA-G producers, increases the risk of high-risk HPV infected patients to evolve into ICC. We hypothesize that the *del/C* haplotype could favor both viral and tumor immune escape, inhibiting immune cell response via HLA-G interaction with immune-inhibitory receptors. In particular, HPV infection and ICC development are characterized by interleukin-10 (IL-10) release (5, 32, 44). IL-10 is one of

the main HLA-G inducers and this cytokine could create an environment promoting HLA-G expression and immune suppression. On the contrary, the *del/G* haplotype was down-represented in ICC patients in comparison with LGSIL subjects. We could suggest that the presence of the +3142*G* allele reduces partly mRNA stability, leading to immune control of lesion progression.

In conclusion, this is the first study that demonstrates a primary association between HPV infection development and HLA-G 3′UTR polymorphisms, where the simultaneous presence of high-risk HPV infection and HLA-G high production genetic background promotes the neoplastic progression. The confirmation of these results in a larger cohort with a prospective follow-up would suggest HLA-G polymorphism analysis as a risk marker to be considered in the evaluation of disease progression in HPV positive subjects.

Conflicts of Interest

The authors have declared no conflicting interests.

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Association of an HLA-G 14-bp Insertion/Deletion polymorphism with high HBV replication in chronic hepatitis

A. B. Laaribi,[1,2,*] I. Zidi,[1,*] N. Hannachi,[2] H. Ben Yahia,[1] H. Chaouch,[3] D. Bortolotti,[4] N. Zidi,[5] A. Letaief,[3] S. Yacoub,[6] A. Boudabous,[1] R. Rizzo[4] and J. Boukadida[2] [1]*Laboratory Microorganisms and Active Biomolecules, Sciences Faculty of Tunis, University of Tunis El Manar, Tunis, Tunisia;* [2]*Laboratory of Microbiology-Immunology, URO2SP13, University Hospital Farhat Hached, Sousse, Tunisia;* [3]*Department of Internal Medicine and Infectious Diseases, University Hospital Farhat Hached, Sousse, Tunisia;* [4]*Department of Experimental and Diagnostic Medicine, Section Microbiology, University of Ferrara, Ferrara, Italy;* [5]*Faculty of Medicine Ibn Al Jazzar, University of Sousse, Sousse, Tunisia; and* [6]*Regional Center of Blood Transfusion, University Hospital Farhat Hached, Sousse, Tunisia*

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SUMMARY. Identification of an HLA-G 14-bp Insertion/Deletion (Ins/Del) polymorphism at the 3′ untranslated region of HLA-G revealed its importance in HLA-G mRNA stability and HLA-G protein level variation. We evaluated the association between the HLA-G 14-bp Ins/Del polymorphism in patients with chronic Hepatitis B virus (HBV) infection in a case–control study. Genomic DNA was extracted from 263 patients with chronic HBV hepatitis and 246 control subjects and was examined for the HLA-G 14-bp Ins/Del polymorphism by PCR. The polymorphic variants were genotyped in chronic HBV seropositive cases stratified according to HBV DNA levels, fibrosis stages and in a control population. There was no statistical significant association between the 14-bp Ins/Del polymorphism and increased susceptibility to HBV infection neither for alleles ($P = 0.09$) nor for genotypes ($P = 0.18$). The stratification of HBV patients based on HBV DNA levels revealed an association between the 14-bp Ins/Del polymorphism and an enhanced HBV activity with high HBV DNA levels. In particular, the Ins allele was significantly associated with high HBV DNA levels ($P = 0.0024$, OR = 1.71, 95% CI 1.2–2.4). The genotype Ins/Ins was associated with a 2.5-fold (95% CI, 1.29–4.88) increased risk of susceptibility to high HBV replication compared with the Del/Del and Ins/Del genotypes. This susceptibility is linked to the presence of two Ins alleles. No association was observed between the 14-bp Ins/Del polymorphism and fibrosis stage of HBV infection. We observed an association between the 14-bp Ins/Del polymorphism and high HBV replication characterized by high HBV DNA levels in chronic HBV patients. These results suggest a potential prognostic value for disease outcome evaluation.

Keywords: HLA-G 14-bp Insertion/Deletion, association study, chronic hepatitis, fibrosis, hepatitis B virus, HLA-G.

INTRODUCTION

Hepatitis B virus (HBV) infection is responsible for the frequent morbidity and mortality in patients with hepatitis disease. Indeed, more than 240 million people worldwide are infected by HBV, and it causes about 600 000 deaths each year all over the world [1]. HBV infection outcomes range from spontaneous infection clearance to infection persistence that may progress into cirrhosis or hepatocellular carcinoma (HCC) [2]. The HBV infection course is thought to involve a complex array of host immune responses that are in part genetically determined [3,4]. Several candidate genes are suggested to influence the outcome of HBV infection as human leucocyte antigen (HLA) class-I and class-II genes. Indeed, the association between the HLA-DRB1*1302 allele and HBV clearance has been well documented for Gambians [5] and German Caucasian subjects [6]. For the Qatarian population, HLA-DRB1*07 was found to be associated with persistent HBV infection [7].

HLA-G is a nonclassical class-I molecule. It plays a major role in the immune regulation inhibiting natural killer cells (NK), as well as CD8+ T cell cytotoxicity and dendritic cell maturation, and inducing Th2 cytokine

Abbreviations: Del, Deletion; HLA, Human leucocyte antigen; HLA-G, Human leucocyte antigen-G; Ins, Insertion.

Correspondence: Ines Zidi, Laboratoire Microorganismes et Biomolécules Actives, Département de Biologie, Faculté des Sciences de Tunis, University of Tunis El Manar, 2092 Tunis, Tunisia.
E-mail: ines.zidi@techemail.com
*These authors contributed equally to this work.

secretion [8,9]. HLA-G has been implicated in pathological viral infections as its tolerogenic function contributes to the virus escape from host immune defences [10–12]. Importantly, HLA-G is characterized by low allelic polymorphisms in comparison with classical HLA class-I genes. However, a 14-base pairs (14-bp) Insertion/Deletion (Ins/Del) polymorphism (rs66554220) in the 3′ untranslated region of the HLA-G gene has been associated with HLA-G mRNA stability as well as protein levels [13,14]. The presence of the 14-bp sequence generates the deletion of 92 bases in the primary transcript of HLA-G [15]. The Ins allele is associated with lower mRNA and protein levels [16,17]. Elsewhere, it was reported that the Del/Del and Ins/Del Genotypes are the high producers of soluble HLA-G molecules [18].

Several studies demonstrated that the 14-bp Ins/Del polymorphism is associated with recurrent spontaneous abortion [19], pemphigus vulgaris [20], inflammatory bowel disease [21] and sarcoidosis [22]. Taking into account the role of HLA-G in viral infection and the control of HLA-G expression by the 14-bp Ins/Del polymorphism, we evaluated the association between the HLA-G 14-bp Ins/Del polymorphism and with chronic HBV infection.

SUBJECTS AND METHODS

Patients

This study included 263 Tunisian chronic hepatitis B subjects [with persistent hepatitis B surface antigen (HBsAg) for longer than 6 months]. Patients included 132 females and 131 males with a mean age 36.75 ± 0.66 years (SEM; age range: 14–65 years). All patients were recruited from the infectious diseases service of Farhat Hached University Hospital (November 2011–June 2012).

Hepatitis B virus markers including HBsAg, HBe antigen (HBeAg), HBe antibodies (anti-HBe) and anticore antibodies (anti-HBc) were detected by Microparticle Enzyme Immunoassay technology (AxSYM; Abbott Laboratories, Abbott Park, IL, USA). Patients with other liver diseases (including autoimmune liver disease and alcoholic liver disease) or other viral diseases [including the hepatitis C virus (HCV), hepatitis delta virus and HIV] were excluded from the study. In addition, patients who have access to antiviral treatment were excluded.

Several liver biochemistry measurements were regularly taken including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBil), γ-glutamyltransferase (GGT) and alkaline phosphatase (ALP).

Patient were divided into two groups according to their HBV DNA loads [23]. Group 1 includes patients with low serum HBV DNA levels (<2000 IU/mL; $n = 147$), and group 2 includes patients with high titres of HBV DNA (≥2000 IU/mL; $n = 116$) [24] [24–26]. The viral levels were expressed as log of measured HBV copy numbers.

Liver biopsy was performed for 59 HBV patients. The stage of hepatic fibrosis was assessed using the METAVIR system [27]. Based on fibrosis score, HBV patients were divided in two subgroups: the first includes 36 patients with mild to moderate fibrosis (METAVIR F0, F1 or F2), and the second one includes 23 patients with severe fibrosis (METAVIR F3 or F4). All patients gave their consent for the performed study.

Controls

The control population consisted of 246 ethnically and geographically matched healthy blood donors that were seronegative for all HBV markers, HCV and HIV infection. Patients included 125 females and 121 males with a mean age 34.03 ± 0.67 years (SEM; age range: 19–64 years). They were recruited from the Regional Center of Blood Transfusion of Farhat Hached University Hospital. All controls gave their consent for the performed study.

DNA extraction and genotyping assays

Genomic DNA was extracted from whole blood EDTA using the QIAamp DNA blood kit according to the manufacturer's instructions (Qiagen, Chatsworth, CA, USA). Genotyping of the 14-bp Ins/Del polymorphism was performed by polymerase chain reaction (PCR) with the following primers: the forward primer (5′-GTGATGGGCTGTTTAAAGTGTCACC-3′) and the reverse primer (5′-GGAAGGAATGCAGTTCAGCATGA-3′). PCR amplification was performed with initial denaturation at 94° C for 2 min, followed by 35 cycles at 94° C for 30 s, 64° C for 60 s and 72° C for 60 s, and with a final extension at 72° C for 10 min. The HLA-G 14-bp Ins/Del polymorphism was analysed by electrophoresis on a 3% agarose gel stained with ethidium bromide. PCR products of 224 bp and 210 bp in length correspond to the insertion and deletion alleles, respectively.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 for windows (SPSS, Inc., Chicago, IL, USA). Allele and genotype frequencies were calculated by direct gene counting. Comparisons between genotype and allele frequencies according to the Hardy–Weinberg equilibrium in patient and control groups were examined by chi-square test. The odds ratio (OR) and 95% confidence interval (CI) were calculated to estimate the relative risk. For the statistical tests, the level of significance of P-value was set at 0.05. Moreover, a logistic regression including the significantly associated 14-bp Ins/Del polymorphism with age as covariate was also performed.

Table 1 Distribution of allelic and genotypic frequencies of HLA-G 14-bp Ins/Del polymorphism in HBV-infected patients and healthy controls

14-bp Ins/Del	Patients (N = 263) n (%)	Controls (N = 246) n (%)	χ^{2}*	P-value*	OR* [95% CI]
Alleles					
Insertion	283 (53.8)	239 (48.6)	2.77	0.09	1.23 [0.96–1.57]
Deletion	243 (46.2)	253 (51.4)			
Genotypes‡					
Ins/Ins	87 (33.1)	63 (25.6)	2.38	0.12	1.44 [0.9–2.29]
Ins/Del	109 (41.4)	113 (45.9)	0.001	0.97	1 [0.65–1.54]
Del/Del	67 (25.5)	70 (28.5)	1	–	–
Ins/Ins+Ins/Del	196 (74.5)	176 (71.5)	0.57	0.44	1.16 [0.78–1.72]
Del/Del+Ins/Del	176 (66.9)	183 (74.4)	3.41†	0.064†	1.43 [0.47–2.1]

95% CI, 95% confidence interval; Del, Deletion; Ins, Insertion; OR, odds ratio. *χ^{2}, P-values and ORs were calculated using the Del allele and the Del/Del genotype as reference. †χ^{2}, P-values and ORs were calculated using the Ins/Ins genotype as reference. ‡2 × 3 contingency table: χ^{2} = 3.414, P = 0.181.

RESULTS

Allelic and genotypic distribution of HLA-G 14-pb Ins/Del polymorphism among patients and controls

The distribution of allele/genotype frequencies of the HLA-G 14-bp Ins/Del in HBV cases and controls is shown in Table 1. The genotype frequencies in controls were consistent with the Hardy–Weinberg equilibrium (χ^{2} = 3.41; P = 0.18). The Ins allele frequency was more prevalent in patients (53.8%) than among control subjects (48.6%) but without reaching statistical significance (χ^{2} = 2.77; P = 0.09). Furthermore, there were no significant differences in the genotype frequencies of the HLA-G 14-bp Ins/Del between these two groups. According to these results, we did not observe any statistically significant association between the HLA-G 14-bp Ins/Del allele/genotype distribution and the susceptibility to chronic HBV infection.

HLA-G 14-bp Ins/Del in patients with high HBV DNA levels and controls

The frequency of the 14-bp Ins allele is enhanced in patients with high HBV replication (see Subjects and Methods Section) when compared with controls (respectively, 61.2% *vs* 48.6%) as shown in Table 2. This difference is statistically significant [P = 0.001; OR=1.60 (95% CI 1.2–2.2)]. We report also a significant increase in the 14-bp Ins/Ins homozygous HLA-G genotype in patients with high HBV DNA [41.4% *vs* 25.6%; P = 0.003; OR= 2.42 (95%

Table 2 Distribution of allelic and genotypic frequencies of HLA-G 14-bp Ins/Del polymorphism in patients' subgroups with high HBV levels HBV-infected patients and healthy controls

14-bp Ins/Del	Patients with high HBV DNA Levels (N = 116) n (%)	Controls (N = 246) n (%)	χ^{2}*	P-value*	OR* [95% CI]
Alleles					
Insertion	142 (61.2)	239 (48.6)	10.08	**0.001**	1.6 [1.2–2.2]
Deletion	90 (38.8)	253 (51.4)			
Genotypes†					
Ins/Ins	48 (41.4)	63 (25.6)	8.32	**0.003**	2.42 [1.3–4.4]
Ins/Del	46 (39.6)	113 (45.9)	0.74	0.38	1.2 [0.7–2.3]
Del/Del	22 (19)	70 (28.5)	1	–	–
Ins/Ins+Ins/Del	94 (81)	176 (71.5)	3.74	0.052	1.6 [0.9–2.9]
Del/Del+Ins/Del	68 (58.6)	183 (74.4)	9.22	**0.002**	2.05 [1.2–3.2]

95% CI, 95% confidence interval; Del, Deletion; Ins, Insertion; OR, odds ratio. * χ^{2}, P-values and ORs were calculated using the Del allele and the Del/Del genotype as reference. †2 × 3 contingency table: χ^{2} = 9.89, P = 0.0071. Significant P-values are highlighted in bold.

Table 3 Demographic and clinical characteristics of HBV patients' subgroups with low- or high HBV DNA levels

Parameter	Patients with high HBV DNA levels (N = 116) Mean (SEM)	Patients with low HBV DNA levels (N = 147) Mean (SEM)	P-value
Gender (male/female)	68/48	63/84	–
Age (years)	35.35 (0.926)	37.87 (0.926)	**0.060**
ALT (IU/L)	36.32 (3.105)	23.29 (0.86)	**<0.001**
AST (IU/L)	30.38 (2.16)	22.28 (0.743)	**<0.001**
GGT (IU/L)	31.1 (3.939)	23.25 (1.379)	**0.032**
ALP (IU/L)	87.09 (7.21)	90.15 (5.128)	0.720
HBV DNA (IU/mL log)	4.19 (0.119)	2.15 (0.065)	**<0.001**
Liver biopsy specimen available during study period	59		
Stage of fibrosis			
F0-F2	36	0	0
F3-F4	23	0	0

Significant P-values are highlighted in bold.

CI 1.3–4.4)] and a decrease of the Del/Del homozygous genotype in these patients (19% *vs* 28.5%).

HLA-G 14-bp Ins/Del in patients with low or high HBV DNA levels

The demographic and clinical features of HBV patients with low- or high HBV DNA levels ($P < 0.001$) are summarized in Table 3. The ALT, AST and GGT levels were significantly enhanced in the subgroup with high HBV DNA levels compared to the subgroup with low HBV DNA levels.

The distribution of the HLA-G 14-pb Ins/Del allele/genotype frequencies in the patient subgroups is reported in Table 4. The HLA-G 14-bp Ins allele frequency was significantly higher in the subgroup with high HBV DNA levels (61.2%) than in the subgroup with low HBV DNA (48%) [P = 0.0024; OR=1.71 (95% CI 1.2–2.4)]. This significance was maintained after adjustment for age (P = 0.009).

This difference was due to the statistically significant over-representation of the 14-bp Ins/Ins homozygous HLA-G genotype, which was significantly increased in the subgroup with high HBV DNA [41.4% *vs* 26.5%; P = 0.005; OR= 2.5 (95% CI 1.29–4.88)], and the decrease of the HLA-G 14-bp Ins/Del heterozygous genotype in this subgroup (39.6% *vs* 42.9%).

Table 4 Distribution of allelic and genotypic frequencies of HLA-G 14-bp polymorphism in patients' subgroups with low- or high HBV levels

14-bp Ins/Del	Patients with high HBV DNA levels (N = 116) n (%)	Patients with low HBVDNA levels (N = 147) n (%)	χ^{2}*	P-value*	OR* [95% CI]
Alleles					
Insertion	142 (61.2)	141 (48)	9.15	**0.0024**	1.71 [1.2–2.4]
Deletion	90 (38.8)	153 (52)			
Genotypes‡					
Ins/Ins	48 (41.4)	39 (26.5)	7.61	**0.005**	2.5 [1.29–4.88]
Ins/Del	46 (39.6)	63 (42.9)	1.5	0.21	1.49 [0.79–2.82]
Del/Del	22 (19)	45 (30.6)	1	–	–
Ins/Ins+Ins/Del	94 (81)	102 (69.4)	4.63	**0.031**	1.88 [1.05–3.37]
Del/Del+Ins/Del	68 (58.6)	108 (73.5)	6.45†	**0.011†**	1.9 [1.16–3.2]

95% CI, 95% confidence interval; 14 bp, 14 base pairs; Del, Deletion; HBV, Hepatitis B Virus; Ins, Insertion; OR, odds ratio. *χ^{2}, P-values and ORs were calculated using the Del allele and the Del/Del genotype as reference. †χ^{2}, P-values and ORs were calculated using the Ins/Ins genotype as reference. ‡2 × 3 contingency table: χ^{2} = 7.934, P = 0.019. Significant P-values are highlighted in bold.

Table 5 Distribution of ALT, AST and GGT levels among HBV patients with low- or high HBV DNA levels

	Patients with high HBV DNA levels (N = 116) Mean (SEM)		Patients with low HBV DNA levels (N = 147) Mean (SEM)		*P*-value			
Parameter (IU/L)	Del/Del and Del/Ins (N = 68) (a)	Ins/Ins (N = 48) (b)	Del/Del and Del/Ins (N = 108) (c)	Ins/Ins (N = 39) (d)	(a)/(b)	(c)/(d)	(a)/(c)	(b)/(d)
ALT	27.17 (1.88)	35.19 (4.53)	22.81 (1.01)	24.66 (1.66)	0.068	0.346	**0.026**	**0.029**
AST	31.18 (2.72)	43.27 (6.22)	22.56 (0.94)	21.5 (1.06)	0.054	0.533	**0.001**	**0.005**
GGT	32.98 (5.36)	27.67 (5.37)	23.84 (1.65)	21.71 (2.54)	0.523	0.494	0.052	0.299

Significant P-values are highlighted in bold.

We found that the 14-bp Ins/Ins genotype was associated with high HBV DNA levels (55.2%), whereas the genotypes 14-bp Del/Del and 14-bp Ins/Del were associated with low HBV DNA levels (66.3%) (P = 0.011). We observed also that 73.5% of patients with low HBV DNA had the genotypes 14-bp Del/Del and 14-bp Ins/Del.

Patients with high HBV replication and with the genotype 14-bp Ins/Ins present had significantly enhanced levels of transaminases compared with patients with low HBV replication (P = 0.029 for ALT, P = 0.005 for AST, Table 5). On the other hand, patients with low HBV DNA loads and genotypes 14-bp Del/Del and 14-bp Ins/Del had low serum levels of ALT (P = 0.0264), AST (P = 0.0008) and also GGT (P = 0.052) compared with patients with high HBV DNA loads (Table 5).

HLA-G 14-bp Ins/Del in patients stratified by fibrosis staging

To determine the possible implication of the HLA-G 14-bp Ins/Del in the fibrosis stage, we categorized 59 patients who had liver biopsy into two subgroups, based on the severity of fibrosis: patients with mild to moderate fibrosis (METAVIR scores: F0, F1 or F2; n = 36) and patients with severe fibrosis (METAVIR scores: F3 or F4; n = 23). There was no statistically significant difference between the patient subgroups concerning either the HLA-G 14-bp alleles (χ^2 = 0.02; P = 0.89), or the HLA-G 14-bp genotypes (χ^2 = 0.014; P = 0.99) and fibrosis stage (Table 6).

DISCUSSION

The outcome of HBV infection ranges from spontaneous recovery to a chronic persistent infection which may progress into cirrhosis or HCC [2]. Several factors may influence the course of infection. These factors include find, virological, environmental, immunological and host genetic factors, but the interaction between all of these elements is not yet elucidated [28,29]. Many studies focused on the importance of the immunogenetic background in HBV infection. The vast majority of published studies about HBV persistence point out the role of the major histocompatibility complex

Table 6 Genotypic and allelic distribution in patients with high HBV DNA levels according to stage of fibrosis

14-bp Ins/Del	F ≤ 2 (N = 36) n (%)	F > 2 (N = 23) n (%)	χ^{2*}	*P*-value*	OR* [95% CI]
Alleles					
Insertion	40 (55.6)	25 (54.4)	0.016	0.89	1.05 [0.49–2.2]
Deletion	32 (44.4)	21 (45.6)			
Genotypes‡					
Ins/Ins	13 (36.1)	8 (34.8)	0.013	0.9	1.08 [0.27–4.2]
Ins/Del	14 (38.9)	9 (39.1)	0.005	0.94	1.04 [0.3–3.5]
Del/Del	9 (25)	6 (26.1)	1	–	–
Ins/Ins+Ins/Del	27 (75)	17 (73.9)	0.008	0.92	1.05 [0.31–3.5]
Del/Del+Ind/Del	23 (63.9)	15 (65.2)	0.01†	0.91†	1.05 [0.35–3.16]

95% CI, 95% confidence interval; 14 bp, 14 base pairs; Del, Deletion; F, stage of fibrosis; HBV, Hepatitis B virus; Ins, Insertion; OR, odds ratio. * χ^2, *P*-values and ORs were calculated using the Del allele and the Del/Del genotype as reference. † χ^2, *P*-values and ORs were calculated using the Ins/Ins genotype as reference. ‡2 × 3 contingency table: χ^2 = 0.014, P = 0.99.

(MHC) in determining the infection outcome. Immunological factors, including the innate and adaptive immune responses against viral infection, play important roles in modulating both the antiviral immune response and host susceptibility to HBV [29]. The most convincing evidence refers to associations between HBV infection and MHC class-II molecules [7,30,31]. The relationship between various clinical manifestations and HLA molecules has been the subject of several studies, but no definitive relationship has as yet been found, primarily because of inconsistent results. Therefore, in this study, we were interested in the potential association between HBV infection and the HLA-G 14-bp Ins/Del polymorphism.

In the present study, we suggest that the HLA-G 14-bp Ins/Del was associated with high HBV replication. At first, the HLA-G 14-bp Ins/Del was compared between the 116 patients with high HBV DNA levels and 246 healthy controls. There was a significant increase in Ins allele distribution between the two groups ($P = 0.001$). This significance was maintained when we compared patients with high HBV DNA levels *vs* patients with low HBV DNA levels ($P = 0.0024$). Our data suggest a possible role of HLA-G in the immune regulation during HBV infection. One study concerning other viral infections [27] obtained similar results supporting our findings. The authors found an increased frequency of the 14-bp Ins/Ins genotype and HIV infection in an African population [32]. Low sHLA-G expression alleles may be more susceptible to HBV infection and lead to a worse outcome. This hypothesis could be sustained by the finding of Jiang *et al.* [33] that reported an association between the 14-bp Ins/Ins genotype and an increased risk to HCC in a Chinese population.

Our data point out to the protective role of the 14-bp Del allele against HBV replication. Indeed, 73.5% of patients with low HBV DNA had the 14-bp Del allele (genotypes Del/Del and Ins/Del). These genotypes correspond to high sHLA-G producers [18]. However, patients with the 14-bp Ins/Ins genotype, and probably low sHLA-G production [18], have higher HBV levels (55.2%), as well as significantly enhanced levels of transaminases (ALT, AST) and GGT (Table 5). sHLA-G molecules may be associated with low HBV activity (low HBV DNA levels). The potential local tolerance of HBV induced by sHLA-G could be associated with the persistence of HBV infection and enhanced liver damage. This hypothesis is sustained by our data on cytolysis of infected liver cells by immune cells. Indeed, patients with low HBV DNA levels have low AST, ALT and GGT levels compared to advanced stages of HBV infection with high HBV DNA levels; however, both groups had normal values for these molecules (Table 5). This condition probably sustains advanced stages of fibrosis. Our hypothesis may explain the higher sHLA-G expression in the liver with worse follow-up [25]. However, further studies are necessary to validate our hypotheses.

HLA-G plays an important role in HBV infection. Based on the findings of Souto *et al.* [34] and other studies about the implication of sHLA-G in HBV infection [33,25], we suggest that HLA-G, either in its membranous or soluble forms, is implicated in the persistence and in the advancement of HBV infection. On the contrary, the HLA-G 14-bp Ins/Del polymorphism seems not to be involved in the severity of liver fibrosis in patients with chronic HBV infection (Table 6). In line with this finding, no association was found between HLA-G expression and severity of liver fibrosis in patients with chronic HBV infection [34].

In conclusion, we report for the first time the association between the HLA-G 3′UTR 14-bp Ins/Del polymorphism and HBV infection in Tunisian patients with chronic HBV. The 14-bp Ins allele seems to be associated with high HBV replication. Our results suggest that HLA-G has a possible role in modulating immunity against HBV. Further studies in other populations are necessary to validate our findings.

CONFLICT OF INTEREST

The authors declare that there are no conflict of interests.

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In general, viral proteins are able to decrease HLA class I expression, but their effect on HLA-G expression at the cell surface is more ambiguous. Indeed, they may have no effect [104-106] or an inhibitory effect [107-110], or as reported by Onno et al. [111], even induce HLA-G after viral reactivation in activated macrophages infected by hCMV. By contrast, another study reported that HLA-G1 expressed on cell surface resulted downregulated and that this decrese was due to hCMV short viral US glycoproteins [109]. In fact, hCMV produces different US proteins that act on MHC expression at different levels (**Figure 2**) and show differential effects on the expression of classical HLA class I and HLA-G molecules at the cell surface. This different effect is due to the shorter cytoplasmic tail of HLA-G [112] and other structural characteristics.

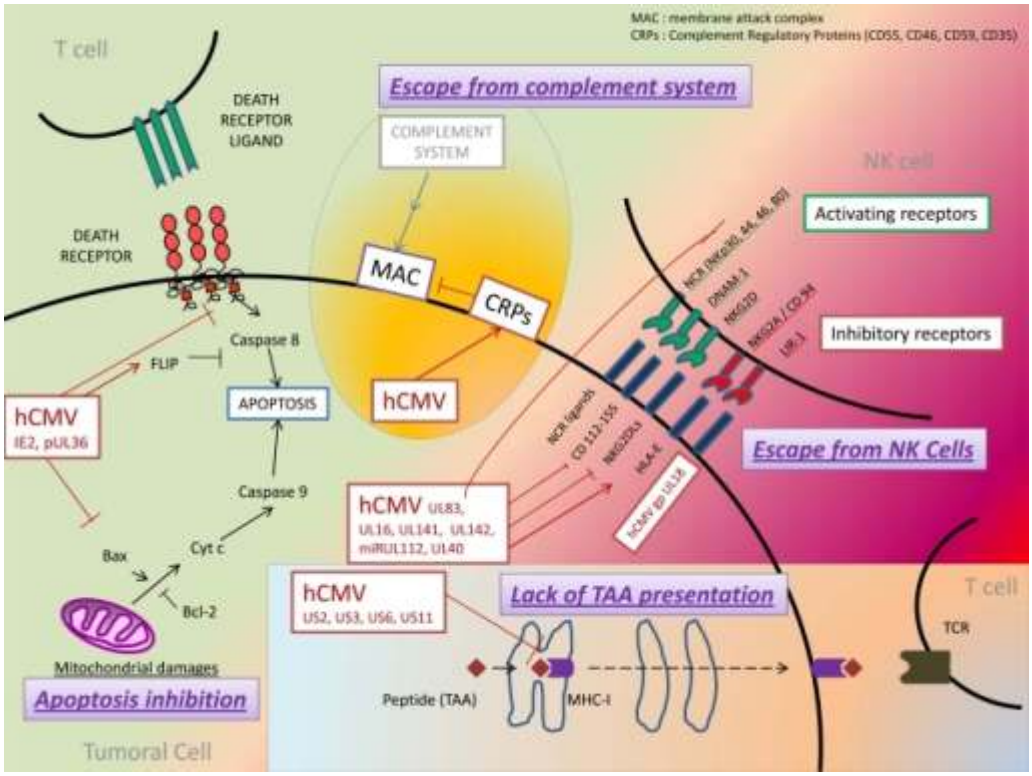

Figure 2. Model for immune escape in hCMV infection.

These conflicting results for hCMV may depend on different cell types studied (monocytes, trophoblasts, or the U373-MG astrocytoma cell line). In fact, the viral proteins exhibit different effects depending on the infected cell target, the type (classical or otherwise) of HLA class I molecules, and the membrane-bound or soluble nature of the HLA-G protein. For example, US10 recognizes the short cytoplasmic tail of HLA-G (RKKSSD) as a substrate and downregulates the cell surface expression of HLA-G but not that of classical class I MHC

molecules [109]. On the other hand, the US2 protein affects levels of HLA class I molecules by increasing proteasome-mediated degradation, unlike HLA-G1, which lacks the residues essential for interaction with US2 [104]. Furthermore, HLA-G1 could also be targeted for degradation, independently of the cytoplasmic tail 105]. In the case of HIV infections, the short cytoplasmic tail of HLAG avoids Nef-induced downregulation, whereas Nef downregulates MHC class I molecules [105, 113].

Basing on this peculiarity of hCMV in modulating HLA-G expression, we evaluate the possible role of HLA-G in pregnant women ondergoing primary hCMV infection. In fact, about 1-7% of pregnant women present a primary hCMV infection, with a transmission rate to the fetus of 30–40%. Plasma samples from pregnant women (divided according to their infectous status in: uninfected, with primary hCMV infection and with non primary hCMV infection), from non-pregnant women and amniotic fluids corresponding to primary infected women were analyzed for hCMV presence and soluble HLA-G (sHLA-G) levels. We observed an increased expression of sHLA-G molecules in plasma samples from primary infected women. Moreover, amniotic fluids from fetuses symptomatic for hCMV infection presented higher sHLA-G levels, were mainly characterized by sHLA-G1 isoform, presence of HLA-G heavy chain and a concentration gradient to the maternal plasma. Our result supported the hypothesis that the determination of sHLA-G molecules in both maternal plasma and amniotic fluid represents a predictive marker for perinatal outcome in fetuses with HCMV infection [114 paper attached].

Reference 114

Soluble HLA-G1 as a novel prognostic biomarker of symptomatic congenital cytomegalovirus infection.

Running title: HLA-G in congenital CMV infection.

Roberta Rizzo[1], Liliana Gabrielli[2], Daria Bortolotti[1], Valentina Gentili[1], Brunella Guerra[3], Francesca Cervi[3], Maria Grazia Capretti[4], Dario Di Luca[1], Tiziana Lazzarotto[5]

1. Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Ferrara, Italy
2. Operative Unit of Clinical Microbiology, Laboratory of Virology, St. Orsola-Malpighi General Hospital, Bologna, Italy
3. Operative Unit of Obstetrics and Prenatal Medicine, St. Orsola-Malpighi General Hospital, Bologna, Italy
4. Operative Unit of Neonatology, St. Orsola-Malpighi General Hospital, Bologna, Italy
5. DIMES, Operative Unit of Clinical Microbiology, Laboratory of Virology, St. Orsola-Malpighi General Hospital, University of Bologna, Bologna, Italy

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Corresponding author: Roberta Rizzo,Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Via Luigi Borsari, 46 44121 Ferrara, Italy; Tel: 00390532455382; Fax: 00390532974470; e-mail: rbr@unife.it

Abstract

Background: Human leukocyte antigen (HLA)-G molecules are non classical HLA class I antigens expressed as membrane bound and soluble isoforms (sHLA-G) with a restricted tissue distribution and immune-regulatory functions. They are expressed at the fetal-maternal interface and have an important role in the maintenance of a tolerogenic condition. Previous researches reported an influence of HCMV infection in HLA-G molecule expression. The objective of this research was to identify maternal and fetal characteristics as prognostic markers of congenital human cytomegalovirus (HCMV) infection.

Methods: Plasma samples from 130 pregnant women (30 uninfected, 56 with primary HCMV infection, 44 with non primary HCMV infection), 52 non-pregnant women and 56 amniotic fluids corresponding to primary infected women were analyzed for HCMV presence, soluble HLA-G (sHLA-G), classical sHLA-I, beta-2microglobulin levels.

Results: We observed an increased expression of sHLA-G molecules in plasma samples from primary infected women. Amniotic fluids from fetuses symptomatic for HCMV infection presented higher sHLA-G levels, were mainly characterized by sHLA-G1 isoform, presence of HLA-G heavy chain and a concentration gradient to the maternal plasma.

Conclusions: Our results suggest the determination of sHLA-G molecules in both maternal plasma and amniotic fluid as a predictive marker for perinatal outcome in fetuses with HCMV infection.

Key words: HLA-G; cytomegalovirus; pregnancy

Introduction

Human cytomegalovirus (HCMV) is the most common cause of intrauterine infection, occurring in 0.3% to 2.3% of births [1]. HCMV intrauterine transmission is more common after primary infection (30-40% of probability) than after secondary/recurrent infection (1%) [2, 3]. Ten to fifteen percent of congenitally infected infants will have symptoms at birth and around 10% of them will die. 70-80% of surviving babies will suffer delayed sequelae such as sensorineural hearing loss, delay of psychomotor development, and visual impairment [4]. Most congenital infected infants (85–90%) have no symptoms at birth, but 8% to 15% of them will develop delayed injury [3, 4].

Within 20-21 weeks of gestation prenatal diagnosis is offered to women with proven primary HCMV infection occurring before 14-16 weeks of gestation. The fetal compartment can be studied by invasive (amniocentesis) and non-invasive (ultrasound examination) techniques [5]. Ultrasonographic findings are helpful but not diagnostic because HCMV has features in common with other intrauterine infections and its sensitivity is poor [6]. HCMV detection in amniotic fluid with virus isolation and/or Real Time PCR is useful for prenatal diagnosis of fetal infection, due to its high sensitivity and specificity [7]. Low viral loads in amniotic fluids with negative ultrasound findings are associated with asymptomatic congenital HCMV infection. However, high viral loads with negative ultrasound findings may be associated with symptomatic or asymptomatic congenital HCMV infections [8-10].

Therefore, there is still a need for reliable prognostic factors for the outcome of HCMV fetal infection.

HCMV can modulate the expression and/or function of human leukocyte antigens (HLA), by encoding proteins to detain and destroy the expression of HLA molecules on the surface of infected cells or selectively up-regulate specific HLA class I molecules binding to immune cell inhibitory receptors [11]. In this scenario, there is an interesting non-classical HLA class I

antigen, HLA-G, characterized by low allelic polymorphism, restricted tissue distribution and alternative mRNA splicing which creates different isoforms, 4 membrane-bound (HLA-G1–G4) and three soluble (HLA-G5–G7) [12]. HLA-G is expressed at the maternal-fetal interface, on surface of trophoblasts [13] and soluble HLA-G in plasma (sHLA-G) increases during the first trimester of pregnancy [14]. HLA-G is responsible for reprogramming the local maternal immune response towards tolerance and remodelling uterine vascularization [15]. The importance of HLA-G production during pregnancy is evident in pre-eclampsia and in unexplained recurrent spontaneous abortions, where HLA-G expression in placenta is reduced [16]. HLA-G has tolerogenic functions, inducing apoptosis of activated CD8+ T cells [17], acting on T regulatory cells [18], modulating the activity of natural killer cells [19] and dendritic cells [20] and blocking allo-cytotoxic T lymphocyte response [21]. HCMV infection modifies HLA-G expression in tissues and immune cells, with a downmodulation in infected cytotrophoblasts [22] and upregulation in infected peripheral blood cells [23]. Specific HCMV proteins increase HLA-G expression interacting with the HLA-G promoter, and affecting mRNA stability, protein translation and the secretory pathway [22, 24-27]. The increase in HLA-G expression is suggested as a mechanism for virus immune escape, because of the immune-inhibitory functions of HLA-G.

Since HLA-G has an important role during pregnancy and HCMV modifies its expression, we analyse the role of HLA-G in congenital infections. Moreover, it has been observed that one important component of HLA-I molecules, beta-2 microglobulin (b2M), has diagnostic efficacy for differentiating symptomatic from asymptomatic HCMV congenital infection [28]. Since free-b2M mainly derives from the shedding of HLA-I-b2M associated molecules and HLA-G molecules generate free-heavy chains releasing b2M during specific conditions [29, 30], we supposed a role for HLA-G antigens in generating b2M-free molecules.

To explore the possible role of HLA-G molecules in congenital HCMV infection, we analyzed maternal and fetal classical HLA-I, HLA-G and beta-2microglobulin expression in correlation with the risk of HCMV congenital infection.

Materials and Methods

Subjects

The study analyzed a cohort of 130 pregnant women who were referred between 2006 and 2011 to the Maternal-Fetal Medicine Unit, St. Orsola-Malpighi University Hospital, Bologna, for suspected primary HCMV infection. Maternal primary HCMV infection was assessed at the Virology Unit of the same University Hospital. Fifty-six amniotic fluid samples from primary HCMV infected women were collected during amniocentesis. Written informed consent was obtained from each woman before amniocentesis.

The women, aged between 18 and 40 years, were in the first and second trimester of pregnancy. They presented no previous autoimmune and inflammatory diseases and they were no assuming anti-inflammatory or immune-modulatory drugs.

Primary infection was diagnosed on clinical history as well as HCMV IgM-positive and low/moderate HCMV IgG avidity results, positive DNAemia and/or seroconversion for HCMV. Non primary maternal HCMV infection was diagnosed, within the first 16 weeks gestation, according to blot-confirmed IgM-positivity with high avidity anti HCMV IgG and presence of DNA-HCMV in blood and/or urine and or/saliva. HCMV-seronegative women (for both IgG and IgM) were defined as uninfected.

Detection of HCMV fetal infection was performed on amniotic fluid samples with virus isolation and real-time PCR. The results of viral tests were confirmed by histological examination on terminated fetuses or testing urine of neonates within the first 2 weeks of life.

Fifty-two non-pregnant women were recruited as healthy controls.

Diagnosis of symptomatic HCMV infection in fetuses and infants

Fetal symptomatic infection was defined as the presence of ultrasound abnormalities and histological and immunoistochemical findings in fetal organs with particular attention to the brain of aborted or dead *in utero* fetuses [31]. Congenitally infected newborns were assessed by clinical, instrumental and laboratory findings for disease in the neonatal period and subsequent monitoring up to 6 years [32].

Anti-HCMV IgM and IgG detection and IgG-avidity

Maternal serum samples were tested using the Enzygnost® HCMV IgM and IgG assays (Siemens Healthcare Diagnostics) and an in-house immunoblot for detection of HCMV-specific IgM [33]. HCMV IgG avidity was tested with the Radim® Cytomegalovirus IgG Avidity EIA WELL assay (Radim).

Virological examinations

HCMV isolation from amniotic fluid was performed by shell-vial procedure as described elsewhere [34]. DNA was extracted from amniotic fluid and saliva with the NucliSens easyMAG System (bioMerieux) and from blood and urine with the QIAsymphony SP/AS System (QIAGEN).

HCMV-DNA was quantified with a real-time PCR assay (HCMV ELITe MGB kit, ELITechGroup) using the ELITe-MGB technology. Amplification, detection, and analysis were performed with the ABI PRISM 7300 platform (PE Applied Biosystems). The detection limit was 11 copies/reaction and viral load was reported as number of copies/mL for all body fluids examined.

Enzyme-Linked Immunosorbent Assay (ELISA) for soluble HLA-G

sHLA-G levels in plasma and amniotic fluid samples were assayed in triplicate as previously reported [35] using the monoclonal antibody (MoAb) MEM-G9 (Exbio), which recognizes the HLA-G molecules, in β2-microglobulin associated form. The intra-assay coefficient of variation (CV) was 1.4% and the inter-assay CV was 4.0%. The limit of sensitivity was 1.0 ng/ml.

Plasma and amniotic fluid concentrations of HLA-G5/G6 were quantified as previously reported [36], using the 5A6G7 MoAb (Exbio). The intra-assay coefficient of variation (CV) was 2.0%, the inter-assay CV was 3.5%. The limit of sensitivity was 1.0 ng/ml. The amount of sHLA-G1 was expressed by the difference between sHLA-G and HLA-G5/-G6 concentrations [36].

ELISA for soluble HLA-I

Plasma and amniotic fluid concentrations of sHLA- I were measured as previously reported [37] using an anti-HLA class I monoclonal antibody (W6/32, ATCC). The amount of sHLA-Ia was expressed as the difference between sHLA-I and sHLA-G concentrations since monoclonal antibody W6/32 used in our ELISA procedure detects both classical sHLA-I (A, B, C) and non-classical sHLA-G.

ELISA for soluble Beta-2microglobulin and Albumin

Beta-2microglobulin (b2M) concentration was determined in triplicate using a commercial Human beta-2microglobulin ELISA Kit (Abcam) with a detection limit <6 pg/ml.

Albumin concentration was determined in triplicate with a 1:200 dilution using the commercial Human albumin ELISA Kit (Alpha Diagnostic International) with intra-assay CV of 6.8 to 11.4 % and inter-assay CV of 3.5 to 6.4%.

Determination of sHLA-G and sHLA-I Indexes

Fetal production of HLA-G was calculated with the following formula [38]:

sHLA-G Index = amniotic fluid/plasma sHLA-G : amniotic fluid/plasma albumin

where the ratio between amniotic fluid and plasma albumin concentrations represents the status of placental barrier.

sHLA-G immunoprecipitation

Plasma and amniotic fluids were biotynilated with 0.2 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce)[27]. Samples were immunoprecipitated for 2 hrs at RT with anti-HLA-G MoAb (MEMG1, specific for HLA-G free heavy chain, or MEMG9, specific for beta2-microglobulin conjugated HLA-G, Exbio), washed twice and incubated overnight with protein G-Sepharose (Santa Cruz,) at 4°C. Samples were washed twice and resuspended in 20ul Laemli Buffer (BioRad).

Western Blot analysis

We quantified protein concentration in immunoprecipitates by the Bradford assay (Bio-Rad Laboratories). Total proteins, denatured at 100 °C for 5 min, were loaded with native or reducing buffers in 10% TGX-Pre-cast gel (Biorad), and electroblotted onto a PVDF membrane (Millipore) [39]. The membrane was incubated with a horseradish peroxidase (HRP)-conjugated antimouse antibody (1:5000; Amersham Biosciences,) and developed with ECL kit (Amersham Biosciences). Images were acquired with Geliance 600 (Perkin

Elmer).Human choriocarcinoma trophoblastic cells (JEG-3) were used as positive control for constitutive HLA-G expression [40].

Statistics

Statistical analysis was performed with Stat View software package (SAS Institute Inc).

Since data, screened by Kolmogorov-Smirnov test, presented a normal distribution, statistical analyses were performed using Student's t-test when normally distributed or non-parametric Mann-Whitney *U* test when distribution was non-normal. Frequencies of positive samples for a specific variable were compared by Fisher exact test. A logistic regression analysis was performed to evaluate the effect of different variables. The relationship between sHLA-G presence and HCMV infection status was investigated by the Receiver Operating Characteristic (ROC) curve (Zweig and Campbell, 1993) analysis (JROCFIT software, John Hopkins University, Baltimore, MD, USA).

Ethics

The study was carried out according to the policies of the Ethical Committee of St. Orsola-Malpighi University Hospital, Bologna, Italy (n. 135/2013/O/Tess), of the University of Ferrara, Ferrara, Italy (n. 06/2013) and the rules of the Italian Ministry of Health, Rome, Italy.

Results

sHLA-I, b2M and sHLA-G levels in maternal serum samples

We evaluated sHLA-I, b2M and sHLA-G levels in sera of 130 pregnant (30 uninfected, 56 with primary HCMV infection and 44 with non primary HCMV infection) and 52 non-pregnant women.

Detectable serum levels of classical sHLA-I (sHLA-Ia) and sHLA-G were significantly more frequent in pregnant women (130/130; 100%) than in non-pregnant subjects (31/52; 59.6%) ($p < 0.0001$, Fisher exact test). b2M molecules presented a trend for fewer positive samples in uninfected pregnant (11/30; 36.7%) and non-pregnant women (20/52; 38.5%) than in infected pregnant women (54/100; 54.0%) ($p=0.14$, $p=0.087$, respectively).

Pregnant women showed higher levels of sHLA-Ia and sHLA-G in comparison with non-pregnant women (sHLA-Ia: $p=0.022$, $p=0.014$; sHLA-G: $p<0.001$, $p<0.001$; Student's t test) irrespective to HCMV infection (**Figure 1a, b**). We observed no statistical differences in sHLA-Ia and sHLA-G serum levels between infected and uninfected pregnant women (**Figure 1a, b**). b2M levels were slightly higher in infected pregnant women than in uninfected pregnant women ($p=0.05$) (**Figure 1c**). Interestingly, subdividing the subjects according to the maternal HCMV infection status, primary infected women presented higher sHLA-G concentrations than in uninfected and non primary infected women (sHLA-G: $p<0.001$, $p<0.001$; respectively) (**Figure 1e**), with no difference in sHLA-Ia and b2M levels (**Figure 1d, f**). sHLA-Ia, sHLA-G and b2M levels were similar in non-pregnant women subdivided on the basis HCMV seropositivity (data non shown).

Fetal and neonatal HCMV infection status

Fifty-six pregnant women with primary HCMV infection underwent invasive prenatal diagnosis. PCR and virus isolation gave no positive result in 39 amniotic fluid specimens and no congenitally infected newborns were found in this group. Two of 17 amniotic fluids from mothers who transmitted the virus to their fetuses/babies, were negative both for virus isolation and PCR. These 2 congenitally infected babies were asymptomatic at birth and during subsequent monitoring confirmed normal development and the absence of late-onset sequelae, in agreement with literature [8-10]. The remaining 15 amniotic fluids were positive for both virological tests (6x10^5 copies/mL median viral load) except one case where only HCVMV-DNA was detected (10^3 copies/mL).

Overall, out of the 17 fetuses/babies congenitally HCMV infected, 5 newborns were asymptomatic, 11 fetuses and 1 newborn symptomatic.

All symptomatic fetuses had HCMV positive brain with severe histological brain damage and cerebral necrosis. 4 of them also showed pathological neurosonographic findings (periventricular hyperecogenicity and ventriculomegaly).

The only symptomatic newborn had hepatosplenomegaly, thrombocytopenia (platelet count: <100.000/mm^3), alanine amniotransferase (ALT) elevation (>80U/L) at birth and developed sequelae with sensorineural hearing loss and mild psychomotor retardation.

sHLA-I, b2M and sHLA-G levels in amniotic fluid

We evaluated sHLA-Ia, sHLA-G and b2M levels in the amniotic fluid samples from the 56 primary infected pregnancies: 5 from infected asymptomatic fetuses, 12 from symptomatic infected fetuses and 39 from uninfected fetuses. All amniotic fluid samples were positive for sHLA-Ia, sHLA-G and b2M molecules. Detectable levels of sHLA-G were significantly

higher in amniotic fluids from infected symptomatic fetuses than in infected asymptomatic fetuses ($p<0.001$) and in uninfected fetuses ($p<0.001$) (**Figure 2b**). b2M presented slightly higher levels in infected symptomatic fetuses than in uninfected fetuses ($p=0.042$) (**Figure 2c**). We observed no statistical difference in sHLA-Ia and b2M levels in amniotic fluids from infected symptomatic, infected asymptomatic and uninfected fetuses (**Figure 2a, c**).

sHLA-G concentration gradient between maternal serum and amniotic fluid

The sHLA-G increase in infected symptomatic fetus prompted the question whether sHLA-G was produced locally in amniotic compartment or derived from maternal blood. Fetal and maternal compartments are mutually interconnected and several molecules are exchanged through the amniotic and chorionic membrane. This molecular interchange could be hypothesized also for HLA molecules. Therefore we evaluated the concentration gradient between serum samples from primary infected women and the corresponding amniotic fluids. A sHLA-G concentration gradient from the amniotic fluid to the maternal serum was observed only in infected symptomatic fetuses, while uninfected and infected asymptomatic fetuses presented an inverse sHLA-G gradient (**Figure 3a**). These results suggest a local fetal production of sHLA-G, increased only in fetuses with symptomatic HCMV infection.

sHLA-G index

The association between fetal HCMV infection and increased fetal sHLA-G expression was confirmed calculating the sHLA-G index in comparison with albumin. Albumin is the most prevalent serum protein, it surrounds the embryo's and it is detected in amniotic fluids [41]. Fainardi et al. [38] reported the use of cerebrospinal fluid and serum albumin content to

evaluate sHLA-G brain production. Since both blood-brain interface and placenta are considered selective barriers, we applied the same concept to quantify the fetal compartment production of HLA-G, evaluating the relative amount of amniotic sHLA-G and albumin compared with maternal serum. Any increase in the index could be ascribed to sHLA-G production in the fetal compartment. Albumin levels presented a decreasing trend both in maternal serum and in amniotic fluid when the fetus was infected (**Figure 4a, b**). Higher sHLA-G indexes were detected in infected symptomatic fetuses (19.45%) than in uninfected and infected asymptomatic fetuses ($p<0.001$, $p<0.001$ respectively) (**Figure 4c**).

sHLA-G isoforms: HLA-G5, HLA-G6 and sHLA-G1

To discriminate between sHLA-G1 and soluble isoforms HLA-G5 and -G6, we analysed isoform distribution in maternal sera and amniotic fluid samples. Interestingly, amniotic fluid is mainly characterized by sHLA-G1 isoform (**Figure 5b, d**), while maternal serum samples showed higher levels of HLA-G5/-G6 isoforms (**Figure 6a, c**). When the samples were subdivided according to fetal HCMV infection, sHLA-G1 isoform increased in the presence of symptomatic fetal infection, both in sera (**Figure 5c**) and in amniotic fluids (**Figure 5d**) with a positive concentration gradient from the amniotic fluid to the serum.

HLA-G free heavy chain analysis

HLA-G can be expressed as b2M associated or free heavy chain (HC). Previous studies documented a different distribution of these two conformation at the maternal-fetus interface. In particular, HLA-G free HC is present on distal trophoblasts, while b2M associated conformation is expressed by proximal trophoblasts [42]. We evaluated the presence of HLA-

G free-HC both in both sera and in amniotic fluids from primary HCMV infected pregnant women with asymptomatic or symptomatic fetus. HLA-G free-HC was detected more frequently in amniotic fluids from symptomatic fetuses (**Figure 6c, d**). On the contrary, maternal sera did not present HLA-G free-HC (**Figure 6a, b**).

sHLA-G predictive efficacy

We analyzed serum and amniotic fluid samples for total sHLA-G, sHLA-G1, HLA-G5 levels and HLA-G free HC frequencies and selected different cut-offs to be used as differentiation values. ROC analysis showed that serum values above 50ng/ml and amniotic values above 30ng/ml with the highest sensitivity and specificity (**Table 1**), in differentiating symptomatic from asymptomatic congenital infections. Logistic regression analysis confirmed the prognostic role of serum and amniotic sHLA-G levels independently from virological parameters ($p=0.02$). The isoform with the highest predictive value was sHLA-G1 for both serum and amniotic samples.

Discussion

Virological markers in fetal blood (DNAemia, IgM ratio) cannot predict the prognosis of HCMV congenital infection [43].

Our results show that sHLA-G levels in maternal sera and amniotic fluids are significantly related to symptomatic fetal HCMV infection and suggest a predictive role for sHLA-G in HCMV congenital infection, as supported by ROC analysis (**Table 1**). In fact, sHLA-G levels increased only in symptomatic fetuses. Several evidences support a specific fetal production of sHLA-G: i) the concentration gradient in symptomatic infection is clearly directed from amniotic fluid to maternal serum; ii) The sHLA-G indexes were significantly higher in infected symptomatic fetuses; iii) the different distribution of sHLA-G isoforms: shedded sHLA-G1 was predominant in amniotic fluids while spliced HLA-G5/-G6 isoforms were over-represented in the maternal sera. Interestingly, sHLA-G1 isoform increased in both compartments when fetal infection was symptomatic suggesting that the sHLA-G increase in maternal serum had a fetal origin. These data explain why HLA-G secretion does not increase when mothers are infected but fetuses are not (**Figure 2**). Likewise, HLA-G free HC increases only in amniotic fluids but not in maternal sera. These results suggest the opportunity for further studies, necessary to understand the mechanisms behind the transfer of sHLA-G molecules from the fetus to the maternal blood, possibly involving trophoblasts [44]. HCMV may regulate HLA-G production by post-transcriptional events such as increasing mRNA stability or protein translation and controlling the secretory pathway [25-28]. Since HCMV transmission rate is limited to 40% of cases, it is possible that placental mechanisms might limit viral transmission to the fetus. Different patterns of HLA-G production could affect the ability to counteract HCMV infection, increasing the risk of symptomatic sequelae, and increased HLA-G expression could facilitate fetal transmission without inducing an adequate immune cell response.

Our results suggest that sHLA-G, mainly sHLA-G1 isoform, might be a reliable marker for congenital infection. However, as the results of prenatal diagnosis might lead to irrevocable decisions, it is highly desirable not to base these decisions on a single marker. A combination of viral and biological markers may provide the best key to the reliable identification of fetuses at risk of congenital disease as well as fetuses with a favorable outcome. A limitation of our study is the small number of symptomatic cases and these data must be considered only investigational. However, to the best of our knowledge, this is the first observation on the possible use of sHLA-G as a marker to discriminate between symptomatic and asymptomatic HCMV congenital infection. Future studies in a larger number of fetuses should be performed to verify whether the combination of sHLA-G and virologic markers may have a better diagnostic accuracy.

The prospective use of defined cutoffs might help to integrate the routinely used HCMV infection markers with sHLA-G detection in the definition of at risk fetus in clinical counselling. Predicting prenatal outcome has undoubted advantages: i) it will decrease the mother's anxiety if the predictive factors of good outcome are present; and ii) it may significantly reduce the rate of unnecessary abortions.

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Figure legends

Figure 1. Maternal serum samples expression of a, d) sHLA-Ia; b, e) sHLA-G; and c, f) b2M molecules, (a-c): in non-pregnant and pregnant women, without infection and with HCMV actively infection, (d-f): in non-pregnant and pregnant women, without infection and with primary and non primary HCMV infection. p values obtained by Student's T test and mean ± standard deviation are reported.

Figure 2. Amniotc fluid samples expression of a) sHLA-Ia, b) sHLA-G and c) b2M molecules according to the fetal and neonatal outcome, HCMV infection with and without symptoms. p values obtained by Student's T test and mean ± standard deviation are reported.

Figure 3. sHLA-G concentration gradients calculated by: (serum concentration-amniotic fluid concentration) and subdivided according to the fetal/neonatal outcome, HCMV infection with and without symptoms. Mean ± standard deviation are reported.

Figure 4. Albumin expression in a) maternal serum samples and b) amniotic fluids from HCMV primary infected pregnancy subdivided according to the fetal/neonatal outcome, HCMV infection with and without symptoms. c) sHLA-G indexes obtained as reported in the Materials and Methods section. p values obtained by Student's T test and mean ± standard deviation are reported.

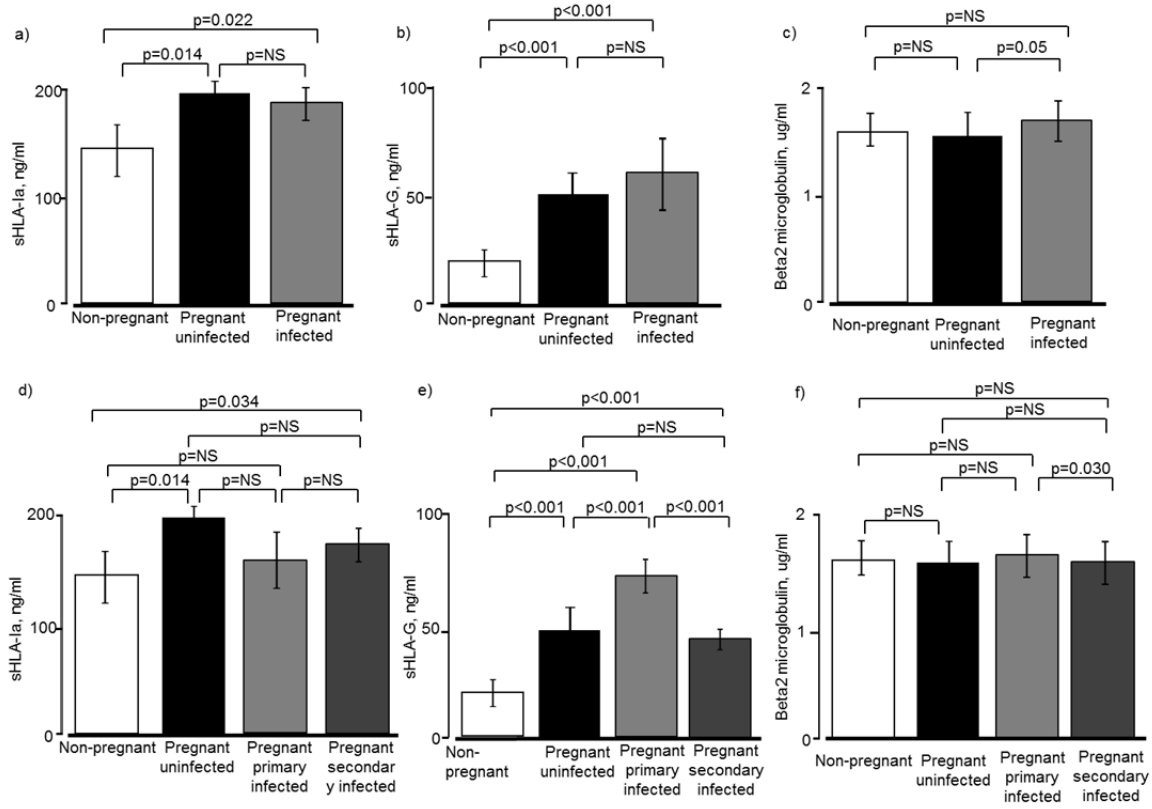
Figure 5. Maternal serum samples expression of a) HLA-G5/-G6 and c) sHLA-G1 molecules. Amniotc fluid samples expression of b) HLA-G5/-G6 and d) sHLA-G1 b2M molecules. Samples were subdivided according to the fetal/neonatal outcome, HCMV infection with and without symptoms. p values obtained by Student's T test and mean ± standard deviation are reported.

Figure 6. Western Blot analysis. a, b) Maternal serum samples and c, d) amniotic fluids from HCMV primary infected pregnancy subdivided according to the fetal/neonatal outcome, HCMV infection with and without symptoms, were analyzed after immunoprecipitation with anti-free HLA-G HC moAb (MEMG1, Exbio) or anti-b2M associated HLA-G moAb (MEM-G9, Exbio). The positivity for HLA-G molecule was evidenced at 39kD. JEG3 cell line supernatants were used as positive control (M). The densitometry results are reported as arbitrary units.

Table 1. Prognostic HLA-G marker of symptomatic congenital HCMV infection (receiver operator characteristics analysis)

Parameters	Cutoff		Fetuses		Diagnostic accuracy (%, 95%CI)			
			Sympt.	Asympt.	Sens.	Spec.	PPV	NPV
Maternal serum samples								
sHLA-G	50ng/ml	Above	10	0	83.3	100	100	71.4
		Below	2	5	51.6-97.4	47.9-100	68.9-100	29.3-95.5
sHLA-G1	50ng/ml	Above	10	0	83.3	100	100	71.4
		Below	2	5	51.6-97.4	47.9-100	68.9-100	29.3-95.5
HLA-G5	50ng/ml	Above	0	3	0	40.0	0	14.3
		Below	12	2	0-26.7	6.5-84.6	0-69.5	2.2-42.8
HLA-G free heavy chain		Presence	0	0	0	100	0	29.4
		Absence	12	5	0-26.7	47.9-100		10.4-55.9
Amniotic fluid								
sHLA-G	30ng/ml	Above	11	0	91.7	100	100	83.3
		Below	1	5	61.5-98.6	47.9-100	71.3-100	36.1-97.2
sHLA-G1	50ng/ml	Above	11	0	91.7	100	100	83.3
		Below	1	5	61.5-98.6	47.9-100	71.3-100	36.1-97.2
HLA-G5	50ng/ml	Above	0	0	0	100	0	29.4
		Below	12	5	0-26.7	47.9-100		10.4-55.9
HLA-G free heavy chain		Presence	12	3	100	40	80	100
		Absence	0	2	73.3-100	6.4-84.6	51.9-95.4	19.2-100

Figure 1.



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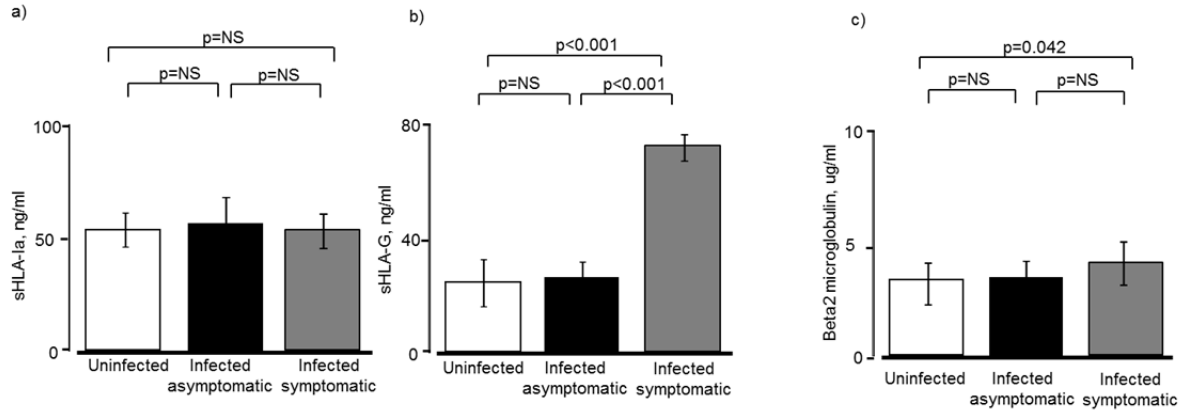

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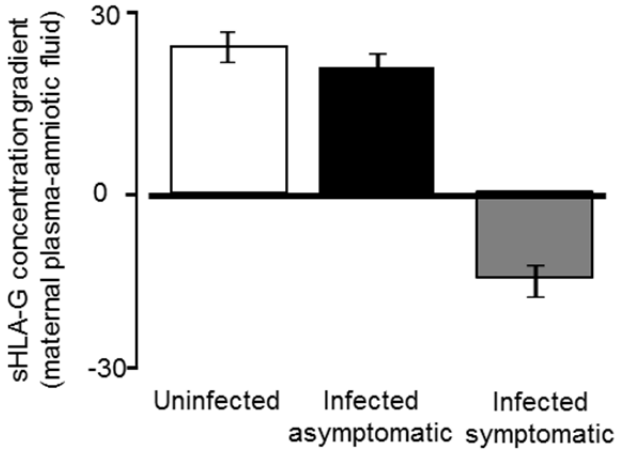

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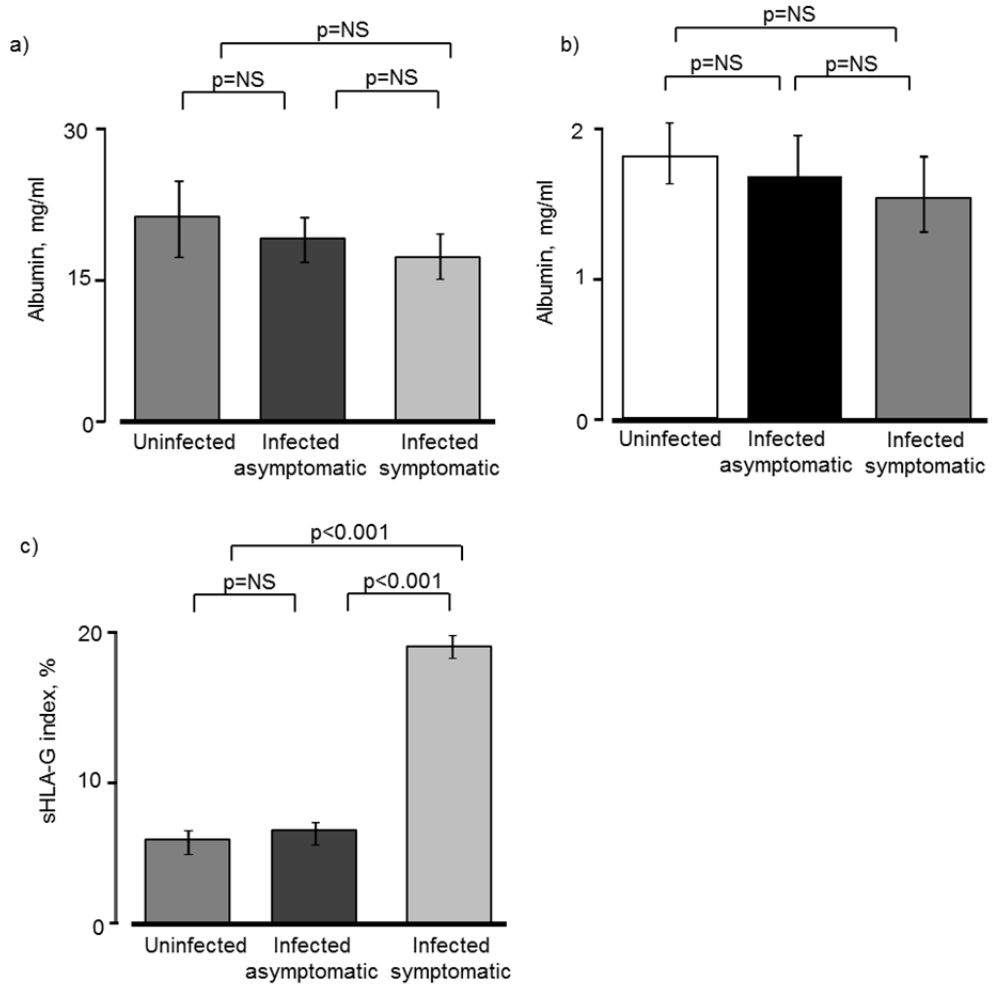


Figure 5.

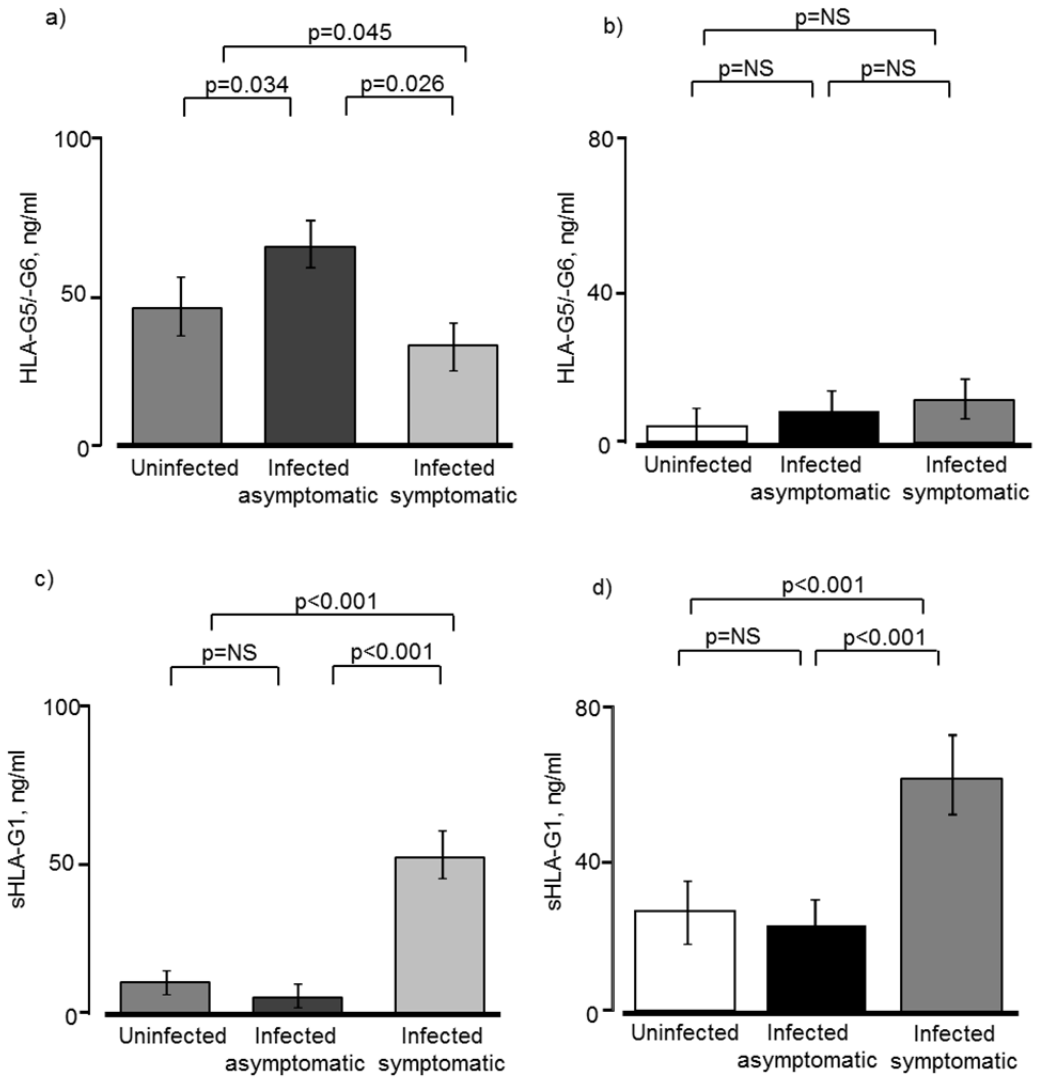
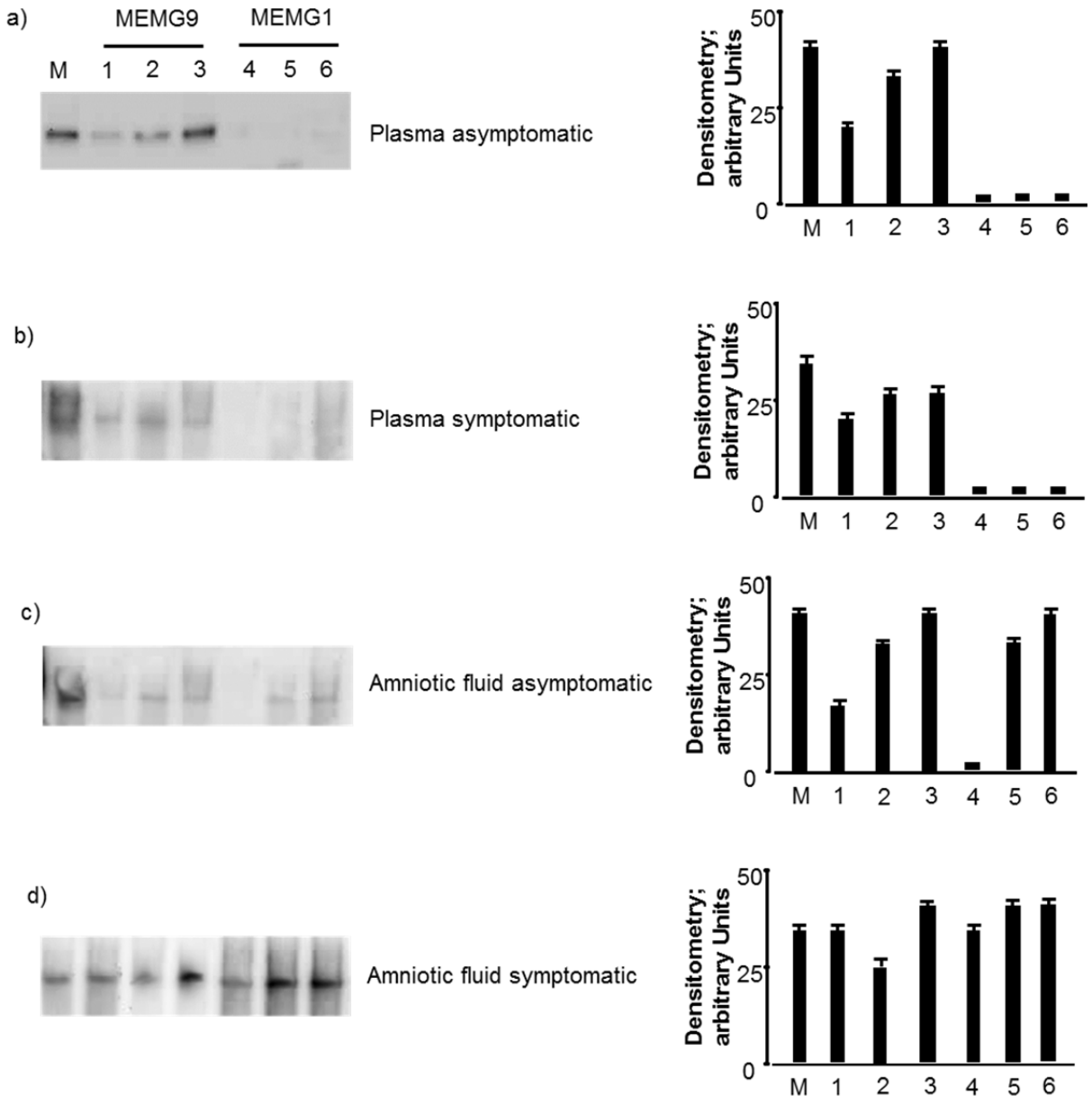

a)
p=0.045
p=0.034
p=0.026
HLA-G5/-G6, ng/ml
100
50
0
Uninfected
Infected asymptomatic
Infected symptomatic
b)
p=NS
p=NS
p=NS
HLA-G5/-G6, ng/ml
80
40
0
Uninfected
Infected asymptomatic
Infected symptomatic
c)
p<0.001
p=NS
p<0.001
sHLA-G1, ng/ml
100
50
0
Uninfected
Infected asymptomatic
Infected symptomatic
d)
p<0.001
p=NS
p<0.001
sHLA-G1, ng/ml
80
40
0
Uninfected
Infected asymptomatic
Infected symptomatic

Figure 6.


a)
MEMG9
MEMG1
M 1 2 3 4 5 6
Plasma asymptomatic
Densitometry; arbitrary Units
50
25
0
M 1 2 3 4 5 6
b)
Plasma symptomatic
Densitometry; arbitrary Units
50
25
0
M 1 2 3 4 5 6
c)
Amniotic fluid asymptomatic
Densitometry; arbitrary Units
50
25
0
M 1 2 3 4 5 6
d)
Amniotic fluid symptomatic
Densitometry; arbitrary Units
50
25
0
M 1 2 3 4 5 6

HLA-G induction by viruses represents an important immune-escape strategy [86]. For this reason, the comprehension of viral mechanisms that lead to HLA-G expression are crucial to counteract the infection itself. The important role played by HLA-G induction during viral infection was reported by many studies in different viral infections [82, 86] and also confirmed by our study on HLA-G expression in nasal polyposis. Sinonasal polyposis (SNP) is a chronic inflammatory pathology that could develop after HPV infection. Effectively, HPV infection is one candidate for the development of the disease for its epithelial cell trophism, hyperproliferative effect, and the induction of immune-modulatory molecules as HLA-G. In our study, we reported that HPV-11 positive SNP without concomitant allergic diseases (SNP-WoAD) patients presented with mHLA-G and IL-10R on epithelial cells from nasal polyps and showed secretion of sHLA-G and IL-10 in culture supernatants. On the contrary, no HLA-G expression was observed in HPV negative polyps. Our data support the crucial role of HLA-G expression induced by HPV in polyposis aetiopathogenesis [115 paper attached].

Reference 115

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Research Article

Infection and HLA-G Molecules in Nasal Polyposis

Roberta Rizzo,[1] Nicola Malagutti,[2] Daria Bortolotti,[1] Valentina Gentili,[1] Antonella Rotola,[2] Enrico Fainardi,[3] Teresa Pezzolo,[2] Claudia Aimoni,[2] Stefano Pelucchi,[2] Dario Di Luca,[1] and Antonio Pastore[2]

[1] *Section of Microbiology and Medical Genetics, Department of Medical Sciences, University of Ferrara, Via Luigi Borsari, 46, 44121 Ferrara, Italy*
[2] *Operative Unit of Otolaryngology, St. Anna Hospital, Via A. Moro, 844124 Ferrara, Italy*
[3] *Operative Unit of Neuroradiology, St. Anna Hospital, Via A. Moro, 844124 Ferrara, Italy*

Correspondence should be addressed to Roberta Rizzo; roberta.rizzo@unife.it

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Sinonasal polyposis (SNP) is a chronic inflammatory pathology with an unclear aetiopathogenesis. Human papillomavirus (HPV) infection is one candidate for the development of SNP for its epithelial cell trophism, hyperproliferative effect, and the induction of immune-modulatory molecules as HLA-G. We enrolled 10 patients with SNP without concomitant allergic diseases (SNP-WoAD), 10 patients with SNP and suffering from allergic diseases (SNP-WAD), and 10 control subjects who underwent rhinoplasty. We analyzed the presence of high- and low-risk HPV DNA and the expression of membrane HLA-G (mHLA-G) and IL-10 receptor (IL-10R) and of soluble HLA-G (sHLA-G) and IL-10 by polyp epithelial cells. The results showed the presence of HPV-11 in 50% of SNP-WoAD patients (OR:5.5), all characterized by a relapsing disease. HPV-11 infection was absent in nonrelapsing SNP-WoAD patients, in SNP-WAD patients and in controls, supporting the hypothesis that HPV-11 increases risk of relapsing disease. HPV-11 positive SNP-WoAD patients presented with mHLA-G and IL-10R on epithelial cells from nasal polyps and showed secretion of sHLA-G and IL-10 in culture supernatants. No HLA-G expression was observed in HPV negative polyps. These data highlight new aspects of polyposis aetiopathogenesis and suggest HPV-11 and HLA-G/IL-10 presence as prognostic markers in the follow-up of SNP-WoAD.

1. Introduction

Sinonasal polyposis (SNP) is a chronic inflammatory pathology characterized by the formation of nasal polyps at the level of the nasal cavity and paranasal sinuses, resulting from an edematous multifocal degeneration of the mucosa. These benign lesions affect approximately 1–4% of the general population, with a slight preference towards elderly men [1]. They are most often treated with steroids or surgery, although nasal polyps removed by surgery have a 70% chance of recurrence. The mechanisms for polyps development are not clear, even though allergies, asthma, aspirin-sensitive individuals, and chronic sinus infections are frequently associated [2, 3]. Viral infection has been postulated to be one important aetiological factor in the pathogenesis, progression, and recurrence of nasal polyps [4], with human papillomavirus (HPV) infection as a candidate for the development of nasal polyps [5, 6].

HPV is a small unenveloped double-stranded DNA virus with strict tissue and species specificity. Many different papillomaviruses infect animals, and over 150 genotypes have been so far identified in humans. Papillomaviruses infect squamous epithelia as skin and mucosae. The mucosal types of HPV fall in two groups: low-risk types (LR-HPV) (mainly HPV-6 and -11), which induce benign cell hyperproliferation, and the high-risk types (HR-HPV), which lead to malignancies as invasive cervical carcinoma, anal cancer, and oropharyngeal carcinomas.

Previous studies have shown that HPV infection may be associated with human nasal polyposis, such as inverted papilloma [5, 6]. However, the role of HPV infection and type in SNP has not been clearly demonstrated. Moreover, HPV

infection is often transient and the host immune system could counteract viral invasion leading to lesion regression [7], as the host immune system is able to counteract the infection. On the other hand, HPV is able to downregulate host immune system [8], blocking interferon response, antigen processing, and presentation [9] and modifying human leukocyte antigen (HLA)-G expression [10, 11].

HLA-G is a nonclassical HLA class I molecule with a physiological tissue-restricted distribution in cytotrophoblast [12], amniotic cells [13], thymus [14], and endothelial cells of chorionic blood vessels [15]. HLA-G molecules are generated by an alternative splicing of the primary transcript of the gene; HLA-G exists as four-membrane bound (HLA-G1, -G2, -G3, and -G4) and three soluble isoforms (HLA-G5, -G6, and -G7) [16, 17]. HLA-G exhibits low allelic polymorphisms in comparison with classical HLA class I genes, with only 50 alleles (IMGT HLA database, December 2013) and 16 proteins. HLA-G is characterized by tolerogenic functions, inducing apoptosis of activated CD8+ T cells [18], promoting T regulatory cells [19], modulating the activity of natural killer cells [20] and of dendritic cells [21], and blocking allocytotoxic T lymphocyte response [22]. These immunoregulatory functions are mediated by the interaction of HLA-G molecules with specific inhibitory receptors: ILT-2 (LILRB1/CD85j), ILT-4 (LILRB2/CD85d), CD8, and KIR2DL4 (CD158d) expressed by immune cells [23]. We previously demonstrated a generalized defect in sHLA-G production by peripheral blood mononuclear cells of SNP patients [23] that seems to be mainly related to the interleukin (IL)-10/HLA-G pathway. IL-10 is one of the main HLA-G inducers [24] but it does not seem to be able to upmodulate sHLA-G production in SNP patients despite the elevated/normal production of IL-10. Since previous studies reported an involvement of HLA-G molecules in HPV-associated tumours [10, 11, 25–27], we determined the presence of HPV infection and HPV types in the nasal polyps of patients affected by SNP and the possible effect on HLA-G expression.

2. Materials and Methods

2.1. Population. A total of 20 subjects who met the diagnostic criteria of nasal polyps were recruited from the Operative Unit of Otolaryngology, St. Anna Hospital, Ferrara, between the years 2010 and 2013. Among these patients, 15 were male and 5 were female. The median age of these patients was 52.8 years (range: 37–84 years, SD 15.2). All of the 20 patients underwent surgery for the removal of nasal polyps at least once, and in 11 cases such surgery was performed twice. Ten patients presented with allergic diseases (SNP-WAD) while 10 patients did not present with any allergic diseases (SNP-WoAD). All the patients were followed for almost two years from the first surgery, in order to identify the occurring relapses. All the polyps resulted to be of edematous type.

In addition, 10 healthy individuals surgically treated for rhinoplasty (6 males and 4 females) with a median age of 47.5 years (range: 34–79 years, SD 10.2) were included.

Nasal polyps from patients or middle turbinate mucosa from healthy subjects were removed and used for experiments.

All subjects agreed to participate in this study by providing written informed consent (University of Ferrara Ethical Committee Protocol N° 140194).

2.2. Nasal Biopsies. Nasal biopsies were collected from inferior turbinates in controls or from nasal polyps in SNP patients. The nasal biopsies were freshly processed to isolate nasal epithelial cells as previously described [28]. Polyps were washed in DMEM and incubated with 0.1% collagenase in DMEM-F12 supplemented with 100 IU/mL of penicillin/streptomycin at 4°C overnight. After incubation, the nasal epithelial cells were isolated by gentle agitation. The residual polyp tissue was discarded. After centrifugation at 1200 rev/min for 5 min, the supernatant was removed and 1 mL of pure FCS was added to the cell pellet to neutralize the enzyme. After further centrifugation, the cells were suspended in DMEM-F12 supplemented with antibiotics and 10% FCS. The dissociated cells were used to separate epithelial cells.

2.3. Epithelial Cell Purification. Epithelial cells were isolated from polyps dissociated cells by anti-EpCAM (Ber-EP4) coated immune-magnetic beads (Dynal CELLection Epithelial Enrich, Dynal AS, Oslo, Norway).

Epithelial and residual cells were grown for 5 days in DMEM-F12 medium added with penicillin/streptomycin, Hepes buffer, L-glutamine, and 10% FBS.

2.4. DNA Extraction and HPV-PCR. Genomic DNA was extracted from epithelial and residual cells using DNA sorb-B extraction kit (Sacace Biotechnologies, Como, Italy), according to the manufacturer's instructions. The presence of HR-HPV (HPV-16, -18, -31, -33, -35, -39, -45, -52, -53, -56, -58, -59, -66, and -70) and LR-HPV (HPV-6 and HPV-11) was investigated by PCR using the HPV high-risk screen amplification kit and the HPV6/11 screen amplification kit (Sacace Biotechnologies).

2.5. Flow Cytometry. HLA-G and IL-10R expression on polyp cells was monitored by immunofluorescence assay with anti-HLA-G (87G-Alexa Fluor 488, Exbio, Praha, CZ) and anti-IL-10R (anti-IL-10RPE, Chemicon, Millipore, MA, USA) moAbs. Anti-isotype controls (Exbio, Praha, Czech Republic) were performed. Cells viability was assessed by propidium iodide staining. Cells were analyzed by a flow cytofluorimetric approach with FACSCount flow cytometer (Becton Dickinson, San Jose, CA, USA) using standard settings and CellQuest software (Becton Dickinson, San Jose, CA, USA) for data analysis.

2.6. Immunofluorescence. Adherent epithelial cells and adherent residual cells were analyzed by immunofluorescence with anti-Mucin8-FITC or fibroblast marker-FITC (Santa Cruz Biotechnology, TX, USA) and anti-HLA-G 87G-PE

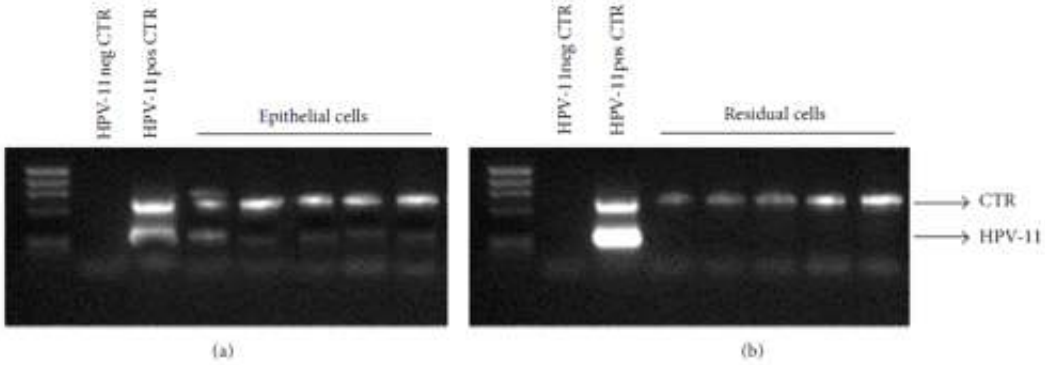

FIGURE 1: PCR products obtained from HPV-11 DNA analysis by HPV6/11 screen amplification kit (Sacace Biotechnologies) of (a) epithelial cells and (b) residual cells from polyps of the 5 relapsing SNP-WoAD patients. Internal control at 723 bp, HPV-11 at 425 bp. The kit has a sensitivity of 500 copies/mL.

(Exbio). All samples were observed under a UV light microscope (Nikon Eclipse TE2000S, Nikon, Italy).

2.7. sHLA-G Enzyme-Linked Immunosorbent Assay (ELISA). sHLA-G levels in epithelial cell culture supernatants were assayed in triplicate as previously reported [29–31] using, as capture antibody, the monoclonal antibody (MoAb) MEM-G9 (Exbio), which recognizes the HLA-G molecule, in β2-microglobulin associated form. The intra-assay coefficient of variation (CV) was 1.4% and the interassay CV was 4.0%. The limit of sensitivity was 1.0 ng/mL.

2.8. IL-10 ELISA. IL-10 concentrations were determined in triplicate using the commercial Human IL-10 BioSource immunoassay kit (Human IL-10 US, BioSource, Camarillo, CA, USA) with a detection limit of 0.2 pg/mL.

2.9. Statistical Analysis. The samples were analyzed with odds ratio, logistic regression, and Student's t-test. Significant P values were considered <0.05.

3. Results

3.1. Presence of HPV-11 Infection in Polyp Biopsies from Relapsing SNP-WoAD Patients. We detected the presence of LR-HPV-11 in 50% (5/10) of samples obtained from patients with SNP-WoAD. Interestingly, these 5 patients presented with a relapsing SNP-WoAD. The other 5 SNP-WoAD did not preset LR-HPV infection and were not characterized by a relapsing disease. None of the SNP-WoAD patients presented with HR-HPV infection. We found no LR-HPV and HR-HPV infection neither in biopsies obtained from SNP-WAD patients nor in healthy controls. These results sustain a role for HPV-11 in the development of relapsing SNP-WoAD (OR: 5.5, 95%, CI: 0.8–38.7). The logistic regression analysis sustains the role of HPV-11 infection as a risk factor for the development of relapsing SNP-WoAD, regardless of gender, age, disease course, and number of relapses (P = NS).

3.2. Presence of HPV-11 Infection in the Epithelial Fraction of Polyps from Relapsing SNP-WoAD Patients. In order to assess the polyp cell fraction infected by HPV-11, we processed the HPV-11 positive biopsies and purified the epithelial cells. The positivity for the presence of HPV-11 was maintained only in the fraction containing epithelial cells (Figure 1(a)). Conversely, the residual fraction was not positive for the presence of HPV-11 DNA (Figure 1(b)).

3.3. HLA-G Expression in the Epithelial Fraction of Polyps from Relapsing SNP-WoAD Patients. Since HPV infection modifies HLA-G expression [10, 11, 25–27], we analyzed the different fraction of cells extracted from nasal polyps for HLA-G expression. Only epithelial cells (Mucin 8^{positive}) arising from HPV-11 positive relapsing SNP-WoAD patients showed HLA-G membrane expression (Figure 2(a)). Conversely, the epithelial cells from biopsies of SNP-WoAD patients without HPV infection, of SNP-WAD patients and of healthy controls, presented with no HLA-G expression (Figure 2(a)). In the residual fraction obtained after the purification of epithelial cells, which consisted in fibroblast cells (fibroblast $\text{marker}^{\text{positive}}$), no HLA-G expression was found in all the three groups of subjects (Figure 2(b)).

3.4. sHLA-G and IL-0 Expression in the Epithelial Fraction of Polyps from Relapsing SNP-WoAD Patients. Since HLA-G molecules are present in both membrane and soluble isoforms, we analyzed the epithelial cell culture supernatants from SNP-WoAD for sHLA-G presence. We found the presence of sHLA-G molecules only in HPV-11 positive samples (Figure 3(a)), while no sHLA-G was observed in HPV negative samples (Figure 3(a)) and in all the fibroblasts cultures (data not shown). Interestingly, the secretion of sHLA-G decreased over 5 days of culture. Since IL-10 is known to be an inducer of HLA-G expression and we proposed an implication in SNP [32], we analyzed the levels of IL-10 in epithelial cell culture supernatants. We documented the secretion of IL-10 in both HPV-11 positive and HPV negative epithelial cell cultures (Figure 3(b)). In HPV negative samples, IL-10

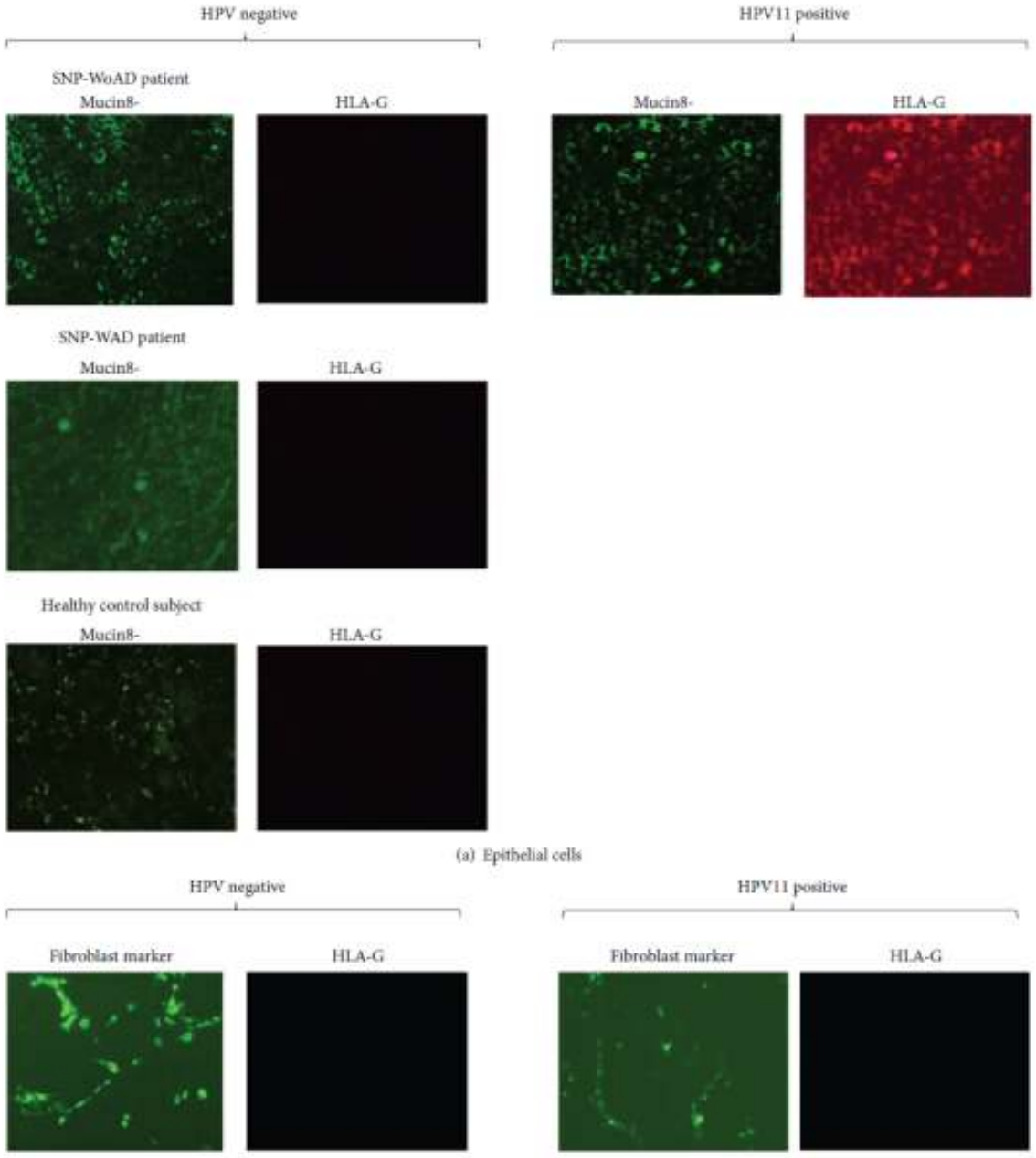

FIGURE 2: Immunofluorescence analysis of (a) epithelial cells and (b) residual cells (fibroblasts) from representative SNP-WoAD and SNP-WAD patients and controls. The cells were stained with anti-Mucin8-FITC or fibroblast marker-FITC (Santa Cruz Biotechnology) and 87G-PE (Exbio).

decreased after 48 hrs while it lasted for 5 days of culture in HPV-11 positive samples (Figure 3(b)).

Since IL-10 interacts with a specific receptor (IL-10R) at the cell surface, we analyzed its expression on the cells extracted from polyp biopsies. We observed IL-10R on the 27% (median) of epithelial cells from SNP-WoAD patients with HPV-11 infection (Figure 4), while only the 1.4% of epithelial cells from SNP-WoAD patients without HPV infection expressed this receptor (Figure 4) ($P = 0.0022$, Student's t-test).

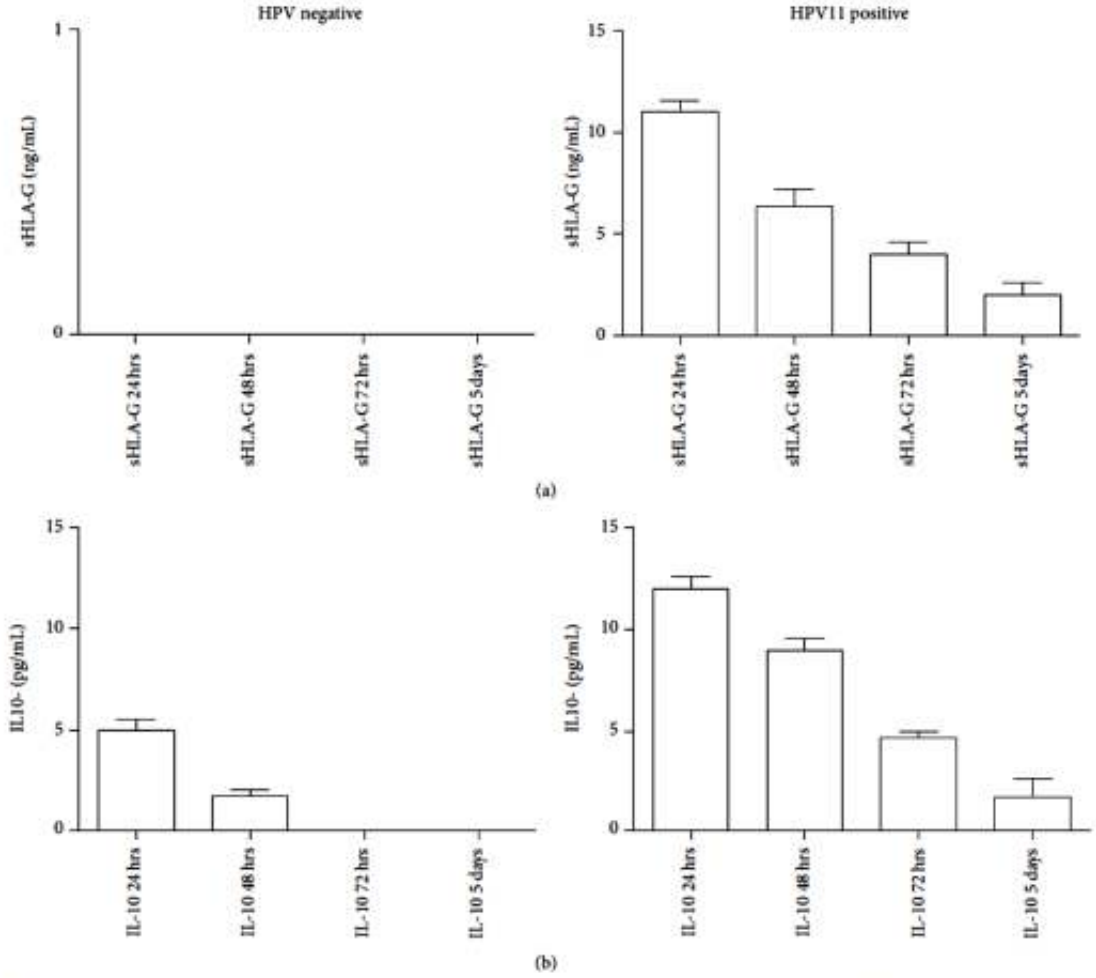

FIGURE 3: (a) sHLA-G and (b) IL-10 levels in epithelial cell culture supernatants from HPV negative and HPV-11 positive SNP-WoAD patients during a 5-day in vitro culture.

4. Discussion

Previous studies in literature have analyzed the role of HPV infection in SNP, demonstrating different correlation ranges [5, 6, 33]. We hypothesize that these differences may be due to the absence of a preselection of patients according to the presence or absence of allergic diseases that could be a confounder for the results observed. In our study, we enrolled the patients on the basis of the characteristics of SNP. Interestingly, the 50% of the SNP-WoAD patients showed positivity for HPV-11 infection and a relapsing disease. On the contrary, HPV infection was absent in SNP-WAD patients and in SNP-WoAD patients without a relapsing disease. These results confirm the importance of the methodological approach in the selection of patients for the study of this pathology and support previous results [33], where HPV-11 was observed as the prevalent infection SNP. Interestingly, HPV infection was restricted to epithelial cells, as it is known to be highly tropic for epithelial cells, while no infection sign was evidenced in fibroblast cells.

The presence of allergic diseases could increase the risk of SNP because of the creation of an inflammatory nasal environment. On the contrary, in the absence of an allergic background, the causes could be found in different environmental factors. In particular, we observed the presence of HPV-11 infection only in relapsing SNP patients, where the persistence of a viral infection could worsen the disease follow-up.

We then evaluated the immunological mechanisms which can be the basis of the virus effect on SNP-WoAD aetiopathogenesis. Interestingly, we found HLA-G expression in HPV-11 positive SNP-WoAD patients, with a secretion of HLA-G molecules that lasted for 5 days of culture. The same behavior was followed by IL-10 that was mainly present in epithelial cell

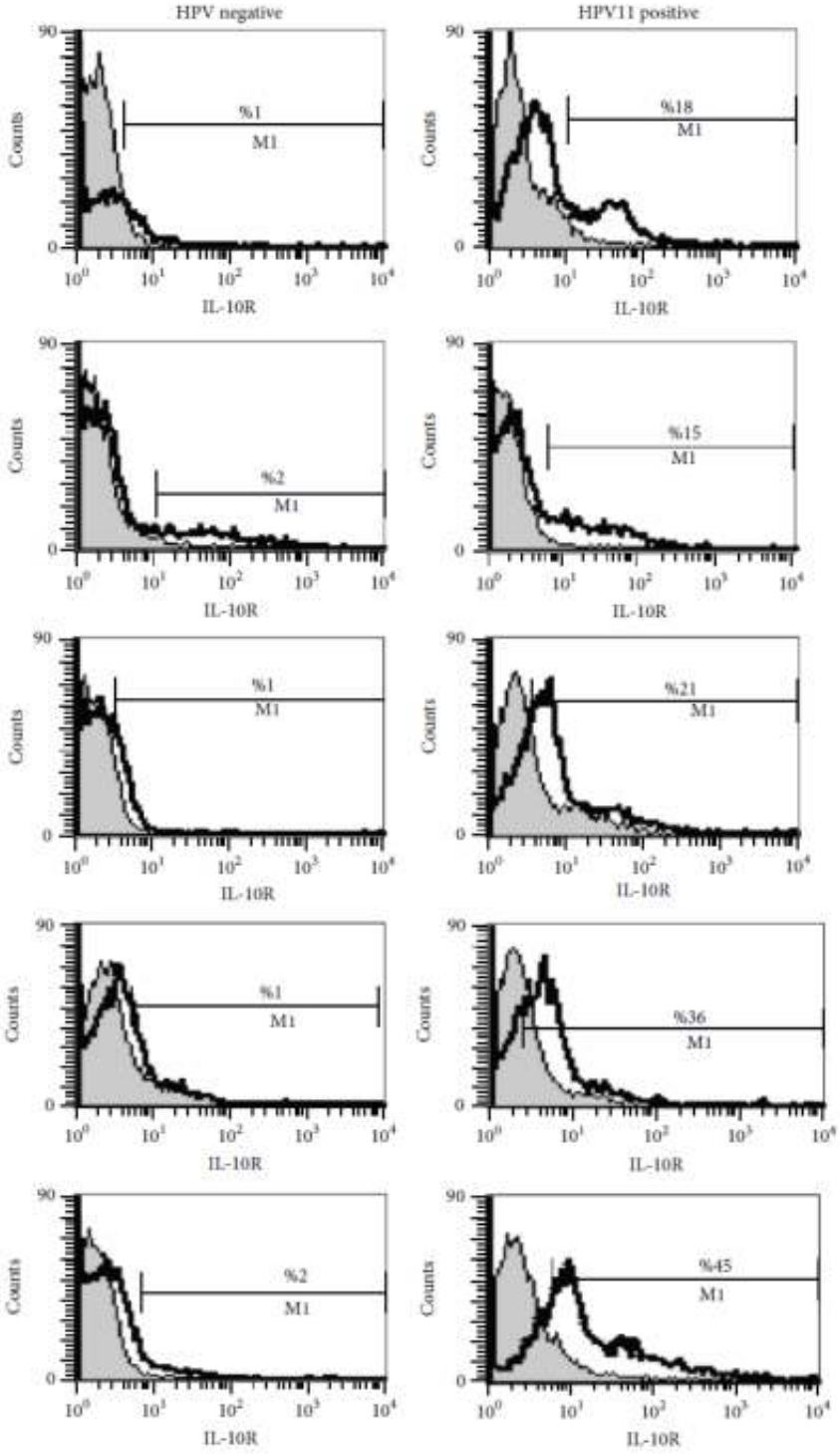

FIGURE 4: IL-10R expression on epithelial cells from HPV negative and HPV-11 positive SNP-WoAD patients. Grey histogram: anti-isotype controls (Exbio) and white histogram: IL-10R (anti-IL-10RPE) (Chemicon).

culture supernatants of SNP-WoAD patients. The presence of HPV-11 infection upmodulated also IL-10R on epithelial cells, suggesting a direct role of IL-10 in controlling HLA-G expression. On the contrary, the low IL-10R expression in HPV negative samples could explain the absence of HLA-G expression, even in the presence of IL-10 secretion. Interestingly, we previously reported the absence of sHLA-G production by peripheral blood mononuclear cells (PBMCs) from SNP-WoAD patients after lipopolysaccharide activation and reported IL-10 secretion after PBMCs lipopolysaccharide activation but no induction of HLA-G expression that was restored only after exogenous IL-10 addition [32]. These differences in HLA-G/IL-10 expression pattern between PMBCs and polyp epithelial cells from SNP-WoAD patients support the involvement of HPV-11 infection in local HLA-G and IL-10 induction. In fact, previous results documented the ability of HPV-11 E6 in inducing IL-10 expression [34]. HPV E6 gene is one of the most relevant viral gene products that contribute to the immortalization and transformation of HPV-infected cells [35]. The activation of E6 appears to be critical for in vivo induction of epithelial hyperplasia [36] and thus may lead to the recurrence of nasal polyps. On the basis of our results, we can hypothesize that HPV infection can modify the immune control, possibly via E6, creating a Th2 environment that maintains the chronicization of the infection. We suggest a role for IL-10 and HLA-G molecules, as component of the immune-modulatory cell mechanism that is exploited by the virus to escape the host immune response.

In conclusion, this is the first study elucidating the possible involvement of IL-10/HLA-G feedback loop in maintaining HPV infection in nasal polyps from SNP-WoAD patients. This modification in immune regulation could be at the basis of SNP-WoAD relapsing follow-up. Further studies will be necessary to evaluate the possible role of HPV-11 E6 in inducing IL-10 and consequently HLA-G in this specific pathology. The confirmation of our results could suggest HPV-11 and HLA-G/IL-10 presence as prognostic markers in the follow-up of SNP-WoAD.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Many viruses have also developed other strategies for escaping host immune surveillance, or example through the secretion of cytokines that affect the host cytokine network. Cytokines are also important in bacterial infections. The interleukin- (IL-) 10 family of cytokines and the related interferon (IFN) family form the larger class II cytokine family [116]. There are three subgroups into the IL-10 family, defined on the basis of their biological functions: IL-10, the IL-20 subfamily cytokines (including IL-19, IL-20, IL-22, IL-24, and IL-26), and the type III IFN group (IFNλs).

Several viruses are able to induce the expression of cellular IL-10, produced mainly by monocytes and, to a latter extent, by lymphocytes and, possibly, mast cells. Other viruses, such as the Epstein-Barr virus and hCMV, produce functional orthologs of IL-10 [114].

1.1.2 HLA-G and bacterial infections

To date, the role of HLA-G in maintenance of bacterial infections has been poorly investigate.

As previously shown in Figure 1, during bacterial infections the main immune escape mechanisms are involved in downregulate the innate immunity and it is in this scenario that HLA-G is used by bacteria as an immune escape mechanism [86].

Septic shock is a condition associated to high mortality (40–50%). initially characterized by an important systemic inflammatory response immediately followed by an anti-inflammatory process that acts as negative feedback. This compensatory inhibitory response could subsequently worsen the condition, as nearly all immune functions are compromised [118]. It has been reported that the persistent HLA-G5 expression in septic shock was predictive of survival [119]. During sepsis, the exocytosis-mediated upregulation of ILT4 expression on neutrophils is inhibited, so the huge amounts of HLA-G5 found in the plasma samples of patients surviving sepsis may have allowed them to control neutrophil inflammatory activity [120]. However, soluble HLA-G concentration was not found to be predictive of the detection of bacteremia and sepsis in pediatric oncology patients with chemotherapy-induced febrile neutropenia [121].

Conserning bacterial infection, I focused on the analysis of the possible role of HLA-G during Pseudomonas aeruginosa (P. aeruginosa) infections. P.aeruginosa is a gram negative bacteria, often associated with respiratory diseases, such as Cystic Fibrosis (CF) [122]. In this work, we performed ELISA assays and qualitative and quantitative mRNA analysis of cell lines, exhaled breath condensate (EBC) and plasma from CF patients and healthy controls (CTRLs) in order to identify if P.aeruginosa infection regulated HLA-G expression. We found lower HLA-G levels in plasma of CF patients that, after treatment, became undistinguishable from those found in CTRLs. HLA-G was higher in the EBC of CF patients and was normalized after antibiotic therapy only in CF patients that were free of P. aeruginosa infection. These data demonstered that HLA-G levels can monitor the efficacy of IV antibiotic treatment in CF patients and that in the CF lung microenvironment, higher expression of HLA-G is associated with P. aeruginosa infection suggesting that this molecule could play a role in bacterial immune-escape mechanisms [123 paper attached].

Reference 123

HLA-G expression and regulation in cystic fibrosis during *Pseudomonas aeruginosa* infection

Roberta Rizzo[1]*, Gabriella Bergamini[1-2]*, Daria Bortolotti[1], Teresinha Leal[3], Ciro D'Orazio[4], Emily Pintani[4], Loredana Melchiorri[1], Eleonora Zavatti[5], Baroukh Maurice Assael[4], Claudio Sorio[2°], Paola Melotti[4].

[1]Medical Sciences Department, Microbiology and Medical Genetics, Ferrara University, Italy

[2]Cystic Fibrosis Translational Research Laboratory "Daniele Lissandrini", Department of Pathology and Diagnostics, University of Verona, Italy

[3]Louvain Centre for Toxicology and Applied Pharmacology, UniversitéCatholique de Louvain, Belgium

[4]Cystic Fibrosis Center, Azienda Ospedaliera Universitaria Integrata di Verona, Italy

[5]Azienda Ospedaliero-Universitaria S. Anna, Ferrara, Italy

* Equal contribution to this study, °Corresponding author

Running title: HLA-G modulation in cystic fibrosis *P. aeruginosa* infection.

Corresponding author

Claudio Sorio MD, PhD

Phone number: +39 0458027668

Fax number: +39 0458027127

e-mail: claudio.sorio@univr.it

Abstract

Background. Chronic *P. aeruginosa* infection is a major cause of death in cystic fibrosis (CF). Non-classical Human Leukocyte Antigen (HLA)-G are immunomodulatory molecules playing a role in respiratory diseases.

Methods. ELISA assays and qualitative and quantitative mRNA analysis of cell lines, exaled breath condensate (EBC) and plasma from CF patients hospitalized for intravenous antibiotic treatment and healthy controls (CTRLs).

Results. Lower HLA-G levels were found in the plasma of CF patients that, after treatment, became undistinguishable from those found in CTRLs. HLA-G was higher in the EBC of CF patients and was normalized after antibiotic therapy only in CF patients that were free of *P. aeruginosa* infection.

Conclusions. HLA-G levels can monitor the efficacy of IV antibiotic treatment in CF patients. In the CF lung microenvironment, higher expression of HLA-G is associated with *P. aeruginosa* infection suggesting that it could play a role in bacterial immune-escape mechanisms.

Introduction

Chronic *P. aeruginosa* infection in the bronchopulmonary tract and the conversion of *P. aeruginosa* to mucoid strains represents an adverse prognostic factor in Cystic fibrosis (CF) [1]. In spite of aggressive antibiotics treatment, the eradication of bacterial infection is difficult to achieve in CF patients often leading to chronic airway infection that could be related to an imbalanced local immune defenses [2]. However the underlying mechanisms of persistence of pulmonary infection are largely unclear.

Recent studies have shown that Human Leukocyte Antigen (HLA)-G molecules play a role in airway immune responses [3-5]. Compared to Class Ia HLA, the non-classical Class I HLA-G antigen has low allelic polymorphism, highly restricted distribution in tissue and alternative mRNA splicing. The latter creates distinct membrane-bound (HLA-G1 to G4) and soluble (HLA-G5 to G7) variant isoforms [6]. In addition, a soluble HLA-G1 isoform (sHLA-G1) can be generated by membrane HLA-G1 proteolytic cleavage [7]. In healthy tissues, HLA-G1, HLA-G5, and sHLA-G1 are the most frequently reported isoforms. Their structure are similar to those of classical HLA Class I molecules. A tolerogenic function for HLA-G has been suggested, based on its ability to inhibit activated CD8+ T, natural killer and dendritic cells, to stimulate T regulatory cells and to block T lymphocyte allo-response [6]. These functions are mediated by interactions with specific, inhibitory immune cell receptors ILT-2, ILT-4, CD8 and KIR2DL4. HLA-G synthesis is controlled by several polymorphisms that modify the affinity of gene-targeted sequences of transcriptional or post-transcriptional factors [8]. A 14 base pair (14 bp) insertion/deletion (ins/del) polymorphism (rs66554220) in exon 8 affects mRNA stability and protein expression while the ins allele is characterized by mRNA destabilization and lower protein production [9] and associates with pathological events such as pregnancy failure [10], autoimmune diseases [7], organ transplant failure [11], increased susceptibility to viral infection [12] and tumor progression [13].

HLA-G is regarded as a potential asthma and bronchial hyper-responsiveness susceptibility gene [4-6], is expressed by airway epithelium [14], is detectable in bronchoalveolar lavage (BAL) samples from asthmatic patients [15] and is involved in lung development [16].

This study was designed to investigate the role of HLA-G as a potential marker of antibiotic therapy efficacy and of pulmonary (primarily *P. aeruginosa*-associated) infections in CF patients.

Materials and Methods

Human subjects

Plasma (n=49) and Exhaled Breath Condensate (EBC) samples (n=28) were collected from CF patients with pulmonary infection and treated with intravenous (IV) antibiotics (beta-lactamic, aminoglycosides) for 14 + 2 days at recommended doses [17] (**Table I**). CF patients were considered infected by *P. aeruginosa* when it was isolated in three consecutive sputum cultures or when mucoid colonies were present in the sample [17]. A cohort of 195 patients with CF and of 230 sex and age-matched non-CF individuals was tested for HLA-G ins/del 14bp polymorphism. Seventy-six healthy control individuals (CTRLs) were tested for HLA-G protein levels in plasma and 7 for Exhaled Breath Condensate (EBC). Written informed consent was obtained from all subjects enrolled in the study approved by the Institutional Review Board of AOUI Verona as project 1849.

To assess pulmonary function the forced expiratory volume in one second (FEV1) was assessed and expressed as percentage of the predictive value for age, sex and height [18]. The inflammatory biomarker C-reactive protein (CRP) was measured in CF patients by enzyme immunosorbent assay (Cell Biolabs, San Diego, CA, USA). The kit has detection sensitivity limit of 1 ng/mL human CRP.

Cell lines

HLA-G expression was studied in CF IB3-1 cells and in the corresponding isogenic controls (C38 cells), a kind gift from Pamela Zeitlin, Johns Hopkins University, Baltimore, USA, cultured as previously described[19]. Replicates of $5x10^5$ cells were exposed for 6, 12, and 24 hours to 5 or 10 ng/ml lipopolysaccharide from *P. aeruginosa* (LPS; Sigma-Aldrich, St. Louis, MO, USA) or to 10% conditioned medium (CM)[19]. JEG-3 cells (ATCC, HTB-36)

cultured in RPMI medium (Sigma-Aldrich) containing 10% fetal calf serum were used as a positive control since they constitutively express HLA-G.

CF mouse model

Sex- and weight-matched 129/FVB mice, homozygous for the F508del-CFTR mutation, and wild-type littermates were housed at the animal facility of the Université Catholique de Louvain (Brussels) and anesthetized with an intra-peritoneal mixture of 100 mg/kg ketamine (Pfizer, NY, USA) and 15 mg/kg xylazine (Bayer, Leverkusen, Germany). Bronchoalveolar lavage (BAL) was collected using a laryngoscope and a fine pipette tip, in the presence or in the absence of induction of inflammatory reaction by LPS (50 µl volume, 100 µg/25g body weight) instilled in the trachea. The local Animal Care and Use Committee approved the experiments (2013/UCL/MD/012).

HLA-G assay

HLA-G levels were measured in 100µl of cell CM, and in EBC samples respectively. EBCs were collected using a condenser (TURBO-DECCS, Medivac, Parma; Italy) and concentrated by evaporation with a SpeedVac Concentrator SVC100H (Savant™ Universal SpeedVac™ Vacuum System; Thermo Scientific, Waltham, MA, USA). A bead array Bio-Plex system (BioRad, Hercules, CA, USA) was used to assay sHLA-G with anti-HLA-G MoAbs conjugated beads: MEM-G9 for sHLA-G1 and HLA-G5 isoform, and 5A6G7 for HLA-G5 isoform (Exbio, Vestec, Czech Republic), respectively. sHLA-G1 levels were calculated as the difference between total sHLA-G and HLA-G5 [20]. The sensitivity of the method is 1 pg/mL. Plasma samples were analyzed for sHLA-G levels by enzyme immunosorbent assay[20]. The limit of sensitivity was 1.0 ng/ml.

Immunoblotting. JEG-3, IB3-1 and C38 cell samples of conditioned medium, murine splenocytes (C57B1/6 mice) and BALs were biotinylated with 0.2 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) and immunoprecipitated for 2 hours with anti-HLA-G MoAb (MEMG9) or anti-Qa2 MoAb (e-Bioscience, San Diego, CA, USA). Immunoprecipitates, previously normalized for the total protein content to 1 mg/mL were loaded and separated in 10% TGX-Pre-cast gel (BioRad), transferred onto a PVDF membrane (Merck Millipore), incubated with horseradish peroxidase (HRP)-conjugated anti-mouse MoAb (GE Healthcare Europe GmbH, Milan, Italy) and developed by enhanced chemi-luminescence (ECL kit, GE Healthcare). Densitometric analysis was then performed with a Geliance Imaging System (Perkin Elmer, Waltham, MA, USA).

Flow cytometry

The expression of HLA-G was analyzed in cells by direct immunofluorescence with anti-HLA-G AlexaFluor-conjugated MoAb (87G) (Exbio) and flow cytometry performed with FacsVantage (Becton Dickinson).

Quantification of HLA-G transcripts and HLA-G 14bp ins/del polymorphism typing by PCR

Total RNA (RNeasy Mini Kit; Qiagen, Valencia, CA, USA) was reverse transcribed (SuperScript™ System; Life Technologies) and amplified with β-actin and HLA-G primers [21]. Quantitative PCR was performed using the endogenous control RNaseP eukaryotic gene (MGB, Applied Biosystems®, Milan, Italy).

Genomic DNA, extracted from EDTA blood (Nucleon; GE Healthcare) was genotyped for HLA-G 14bp ins/del polymorphism by Real Time-PCR [22].

Statistical analysis

According to data distribution, parametric or non-parametric statistics (StatView software, SAS Institute Inc, USA) were applied to compare means or medians, respectively. A significance level (P-value) ≤ 0.05 was considered significant.

Results

Influence of *P. aeruginosa* and i.v. therapy on the expression of sHLA-G

We first examined how the presence of *P. aeruginosa* could influence the expression of sHLA-G in a group of 49 CF patients hospitalized for respiratory exacerbation. At the time of admission, 49% patient sputum cultures resulted positive for *P. aeruginosa* and 45% were positive for *Staphylococcus aureus*. 9 patients (18%) scored positive for both strains (Table II). Prior to treatment, sHLA-G levels in the plasma of CF patients (median 2.5 ng/mL, n = 49) resulted significantly lower than in the plasma of 76 healthy CTRLs (median 17.05 ng/mL, P <0.001, Mann-Whitney Rank Sum Test). After therapy, HLA-G significantly increased in the plasma of CF patients (median 13.77 ng/mL, P < 0.001) reaching levels similar to those observed in the plasma of healthy controls (CTRLs), (**Figure 1A**).

Clinical and functional laboratory data (FEV1 and CRP variations) at discharge indicated that all patients had benefited from antibiotic therapy. The i.v. treatment improved the values of FEV1 significantly (≥ 5% positive increase of FEV1, **Table** 2). The widely utilized inflammation-related biomarker, acute phase reaction pentraxin C-reactive protein (CRP) [23], was drastically reduced at the end of antibiotic therapy (**Table 2**). Interestingly, variations of plasmatic CRP levels were inversely and significantly correlated to sHLA-G in 30% of CF patients (Spearman Correlation r=-0.307; P=0.032). However, no significant relationship was observed between EBC levels of sHLA-G and plasmatic levels of both sHLA-G and CRP (data not shown).

The therapeutic association of corticosteroids to antibiotics did not affect the levels of HLA-G in plasma and in EBC (data not shown).

HLA-G 14bp ins/del polymorphism distribution in CF patients

A possible relationship between *P. aeruginosa* infection and HLA-G genetic background in CF individuals was hypothesized as a14bp ins/del polymorphism (rs66554220) in the *HLA-G* gene is known to affect its expression. The 14bp del allele is known to stabilize its mRNA and to increase HLA-G protein levels [9]. Based on the presence of the HLA-G ins/del 14bp polymorphism, three ins/del, ins/ins, del/del genotypes were categorized but their distribution did not differ between CF patients and controls (p=0.062) in cohorts composed of 195 CF patients and of 230 sex and age-matched non-CF individuals (**Table 3**). However an increased risk of chronic *P. aeruginosa* infection was noticed in the presence of the del/del genotype (OR: 3.3; 95% CI: 1.8-6.1) (**Table 4**), a genotype associated to an increased HLA-G production [22]. However, when measured in patients where a plasma sample was available, sHLA-G levels were not significantly different among the three genotypes either before or after IV antibiotic therapy (**Figure 2**). A possibility still exists that the difference could be found in specific cell types/microenvironments and we focused on lung being this organ a major target of CF-associated morbidity.

Bacterial infection affects HLA-G expression in lung microenvironment

EBC was used as a non-invasive matrix to monitor sHLA-G molecules in the lung microenvironment [24]. sHLA-G levels in CF patients before treatment (median 6.31 pg/mL, n=28) were higher than in healthy CTRLs (median 1.7 pg/mL, n=7, P <0.05) and were reduced in EBC following i.v. therapy (median 1.75 pg/mL, n=28, <0.05) (**Figure 1B**). As we noticed a bimodal distribution of sHLAG values within the 28 EBCs of CF individuals, we tested the hypotheses that *P. aeruginosa* infection might affect sHLA levels in EBC. Indeed, in **Figure 1C** we show that in CF patients positive for *P. aeruginosa* infection the sHLA-G levels inEBC were higher (median 17.3 pg/mL, n = 10) than in CF patients negative for the

bacterium (median 2.1 pg/mL, n=16, $P < 0.05$) as well as in healthy CTRLs (median 1.7 pg/mL, n = 7, $P < 0.05$).

In order to identify its source in the lungs of CF patients, we measured HLA-G levels in CF (IB3-1) and isogenic, corrected (C38) broncoepithelial cells. *P. aeruginosa* CM was able to induce the release of larger amounts (10-fold) of sHLA-G in IB3-1 CF cells as compared to the non-CF C38 cell line where only a 4-fold sHLA-G increase was recorded (**Figure 3A**). Similar results were obtained when the TLR4 receptor agonist LPS was utilized.

HLA-G undergoes alternative mRNA splicing, producing both membrane (HLA-G1) and soluble (HLA-G5) isoforms. It is known that LPS acts also as a powerful agonist for HLA-G expression in peripheral blood mononuclear cells [9] from which it is released abundantly into the airways in response to actively growing *P aeruginosa*. Indeed HLA-G was up regulated at the mRNA (**Figure 3B, C**) and intracellular protein levels levels (**Figure 3D, E**) in both C38 and IB3-1 cells after 12 hours of co-culture with LPS. IB3 releases higher amounts of HLA-G than C38 cell line when exposed to LPS (**Figure 3D, right panel**). MRNA analysis indicate that only the HLA-G5 isoform is produced by bronchial epithelial cells, thus excluding membrane shedding as the source of the protein found in the CM (**Figure 4 A**).

To confirm that bronchial epithelial cells are the source of the HLA-G detected in EBCs, we characterized HLA-G isoforms present in EBC samples. Both sHLA-G1 and HLA-G5 isoforms were detected in EBCs, with HLA-G5 isoform representing the only detectable isoform after IV therapy in CF patients (**Figure 4B**). Altogether, these data indicate that bronchial epithelial cells and not leukocytes are the source of HLA-G present in the EBCs of CF patients suffering from persistent *P. aeruginosa* infection.

Bacterial infection affects HLA-G expression in the lung of CF murine model

To further confirm the differential regulation of HLA-G in CF, we compared the levels of HLA-G murine ortholog Qa2 molecule in the broncho alveolar lavages (BAL) of wild type (CTRL) and CF mice following exposed to vehicle or to LPS, a condition that mimics bacterial infection [9]. Lower Qa2 levels were found in BAL samples from naïve, non-LPS-stimulated CF mice as compared to wild-type mice ($p=0.001$). LPS treatment was able to increase Qa2 levels only in the BAL of CF mice ($p=0.001$) (**Figure 5**).

Conclusions

How the airways counteract bacterial infections and why those responses are so altered in CF disease, which is the specific link between CFTR mutations and persistent airway infection and inflammation still remain matter of debate. This is a crucial point to understand in order to develop new therapeutic strategies aimed at restoring airway defenses against microbial infections in CF as well as in other pulmonary diseases. Moreover, focusing on molecules implicated in those mechanisms could lead to identification of specific therapeutic targets and/or biomarkers for therapeutic outcomes.

The expression of HLA-G molecules during pathogen infection is an important component of microbial immune escape mechanisms and influences disease severity during viral infections. Indeed, alveolar macrophages collected from patients suffering from acute cytomegalovirus pneumonitis usually express high levels of HLA-G molecules [25]. HLA-G expression could also be a predisposing factor in asthma and bronchial hyper-responsiveness [6] and might have a role in chronic lung inflammation and infections.

Our data indicate that plasma sHLA-G levels are lower in hospitalized CF patients in comparison to healthy control but increase following i.v. antibacterial therapy to levels that are indistinguishable from healthy controls. This increase recorded in CF patients is inversely related to levels of CRP, a biomarker of systemic inflammation [23], in a relevant number of them. The importance of the role of HLA-G molecules in immune-regulation, for example in the creation of a tolerogenic environment at the maternal-fetal interface [10] and in transplanted patients [11, 26], has been already established. On the other hand, the presence of HLA-G molecules facilitates tumor growth [13, 27], viral immune-escape [12, 25] and is implicated in the pathogenesis of several diseases such as multiple sclerosis [28], rheumatoid arthritis [29] and psoriasis [3]. Data from literature thus suggest that the role of HLA-G is rather complex and disease-dependent. In the contest of CF disease, sHLA-G levels increase

following the improvement of the clinical condition of CF patient in a pathogen-driven proinflammatory status, and as such represent a potential circulating biomarker of effective drug treatment in addition to and independently on acute phase protein CRP.

HLA-G expression levels are influenced by genetic polymorphisms. We could therefore assume that different combinations of these traits might affect some of the parameters we considered in this study. No significant correlation was observed between persistent *P. aeruginosa* infection and plasma HLA-G levels. Nevertheless, an increased frequency of the 14bp del/del genotype was observed in CF patients with persistent *P. aeruginosa* infection. Since this genotype is characterized by increased HLA-G expression with respect to the other genotypes [22], it is tempting to speculate that HLA-G expression could facilitate bacterial immune-escape and the consequent increased risk of chronic infections in selected body microenvironments, especially the lung.

We indeed observed an association between decreased sHLA-G levels in EBCs and recovery from *P. aeruginosa* infection after i.v. therapy in CF patients. This result supports a potential role for HLA-G in promoting persistent bacterial infection in the lung microenvironment of CF individuals.

Experiments in mice confirmed that the CF condition associated with the increased susceptibility to respond to *P. aeruginosa* LPS challenge by up-regulating HLA-G expression in the lung nicroenvironment. In fact, BALs from untreated CF mice had significant lower HLA-G murine ortholog Qa2 levels than wild type mice, while Qa2 concentrations reached those of the wild type mice after *P. aeruginosa* LPS challenge. Taken together these findings indicate that homozygous CFTR mutations influence the regulation of Qa2/HLA-G molecules.

The candidate sources of HLA-G found in the lung microenvironment are epithelial cells and leukocytes. We found that HLA-G5 is the isoform present in the EBC of CF patients, and that

this is relased by CF bronchial epithelial cells at higher amounts in comparison to isogenic CFTR-corrected cell lines upon challenge with *P. aeruginosa* CM or LPS. Interestingly, LPS seems to be the candidate factor for HLA-G induction during bacterial infection. How the presence of a higher HLA-G concentration in the lung microenvironment might contribute to the development of chronic *P. aeruginosa* infection in CF remains still unknown. We speculate that the failure to activate both the innate and the adaptive immune systems is likely to be involved, both in the early stages of bacterial clearance and during bacterial persistence. Bacteria may inhibit immune cell responses by inducing HLA-G that in turn interacts with immune inhibitory receptors, including ILT2, ILT4 and KIR2DL4, to counteract the host immune system. The overall effect would contribute to an impaired activation of both innate and adaptive immune systems towards the bacteria. The importance of the local microenvironment in the modulation of immune response is supported by the notion that in the site of inflammation activated neutrophils can cleave CXCR1 and impair the capability of adjacent neutrophils to properly respond to pro-inflammatory stimuli [30].

HLA-G differential regulation in the plasma and lung microenvironment might suggest a role as an anti-inflammatory molecule at systemic level, whereas in the lung, HLA-G seems to impair bacterial clearance mechanisms and to increase the probability of developing chronic infection. This study drive for the first time attention to this important modulator of immune response, however additional independent studies are necessary to fully validate HLA-G as a new biomarker of the effectiveness of systemic treatment in CF patients and its role as a potential therapeutic target of airway immune system regulation in CF.

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Footnotes

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Abbreviations:

CF: cystic fibrosis

HLA: Human leukocyte antigen

CRP: C reactive protein

BAL: bronco-alveolar lavage

LPS: lipopolysaccharide

CM: culture medium

EBC: exhaled breath condensate

Figure legends

Figure 1

Panel A: Levels of sHLA-G in plasma samples from 49 CF patients before and after I.V. antibiotic therapy and in 76 control subjects (CTRL). Panel B: Levels of sHLA-G in EBC samples from CF patients (n=28) before and after I.V. antibiotic therapy and control subjects (CTRL, n=7). Median values are reported. P values were obtained by Mann Whitney rank sum test. Panel C: Levels of sHLA-G in EBC from 26 CF patients before and after I.V. antibiotic therapy and in control subjects (CTRL, n=7). CF patients were categorized according to the presence (Pa+) (n=10) or absence (Pa-) (n=16) of P. aeruginosa infection. Median values are reported. P values were obtained by Mann Whitney rank sum test

Figure 2. sHLA-G plasma levels in 33 CF patients before (pre) and after (post) I.V. antibiotic therapy subdivided accordingly with 14bp ins/del polymorphism (rs66554220) genotypes (13 for genotype E., 8 for genotype I, 12 for genotype D)

Figure 3. Panel A: Western Blot analysis of IB3-1 and C38 CM untreated or after *P. aeruginosa* CM exposure. Panel B: Q-PCR for total HLA-G mRNA expression in IB3-1 and C38 cell lines after 10ng/ml *P. aeruginosa* LPS treatment for 12 hrs. Results of Q-PCR analysis shown as relative quantities (RQ) of HLA-G transcripts in IB3-1 and C38 cell lines treated or not with LPS compared to those of JEG-3 positive control cell line (assigned an arbitrary value of 10); *P* values were obtained by Mann Whitney U test. Means ± standard deviations (SD) are reported. Panel C: Representative Q-PCR result for total HLA-G mRNA expression in IB3-1 and C38 cell lines after *P. aeruginosa* LPS treatment. Beta-actin (b-actin) was used as an internal control in RT-PCR analysis. JEG-3 cell line was used as positive control. M: DNA marker fiX174 DNA HaeIII digest (Biolabs, Ipswich, MA, USA). The

densitometry results are reported as arbitrary units. Panel D: Flow cytometric analysis HLA-G expression on a representative experiment on IB3-1 and C38 cell lines after LPS exposure. Cells were stained with 87G-Alexa Fluor 488 (Exbio, Praha, CZ) for membrane and intracellular HLA-G expression. Panel E:sHLA-G levels in IB3-1 and C38 left untreated or after treatment for 6, 12 or 24 hrs with 5ng/ml 10ng/ml of LPS; p values were obtained by Mann Whitney U test. Means ± SD are reported.

Figure 4. Panel A: Representative Q-PCR analysis of HLA-G isoforms mRNA expression in the indicated cell lines treated for 12 hrs with 10ng/ml LPS. Isoforms analyzed were the following: HLA-G1, -G5 (left panel), -G2, -G3, -G4, -G6 (right panel). Panel B: sHLA-G1 and HLA-G5 protein levels in EBC samples before (pre) and after (post) I.V. therapy. Median values are reported. P values were obtained by Wilcoxon matched-pairs signed rank test.

Figure 5. Western Blot analysis of BALs from CF (CF) and wild type (WT) mice before and after treatmentwithLPS from *P. aeruginosa*. SP: murine splenocytes, used as positive control for Qa2 expression. One representative of four experiments. Means ± SD are reported for the densitometric analysis of four experiments.

Table 1. Demographic and Clinical Characteristics of CF patients enrolled during exacerbation.

Patients N	49
Male/Female	22/27
Age years	18.0±8.9
Weight Z score	-1.03±1.32
Height Z score	-0.65±1.19
BMI Z score	-0.61±1.56
Diabetes n (%)	17 (35%)

BMI: body mass index

Table 2. Clinical conditions of CF Subjects before and after I.V. antibiotic therapy.

	Before	After	p value
Lung function			
FEV1 (%)	51.38 ± 19.68	57.16 ± 22.30	<0.001*
Serum values			
CRP (mg/L)	15.56 ± 7.73	2.06 ± 1.15	<0.001*
Sputum Microbiology			
P. aeruginosa	45/49 (92%)	18/49 (37%)	<0.0001†
Staphylococcus aureus	18/49 (37%)	15/49 (31%)	0.68†

*Wilcoxon signed rank test

†Fisher exact test

FEV1: forced expiratory volume in 1 sec; CRP: C-reactive protein

Table 3. CF patients and control subjects subdivided according to HLA-G 14bp ins/del polymorphism.

Genotype 14bp	del/del n (%)	ins/del n (%)	ins/ins n (%)	p value
CF (192)	93 (47)	72 (38)	27 (15)	0.062*
CTRL (213)	80 (38)	90 (42)	43 (20)	

*Chi squared test (3x2 Table)

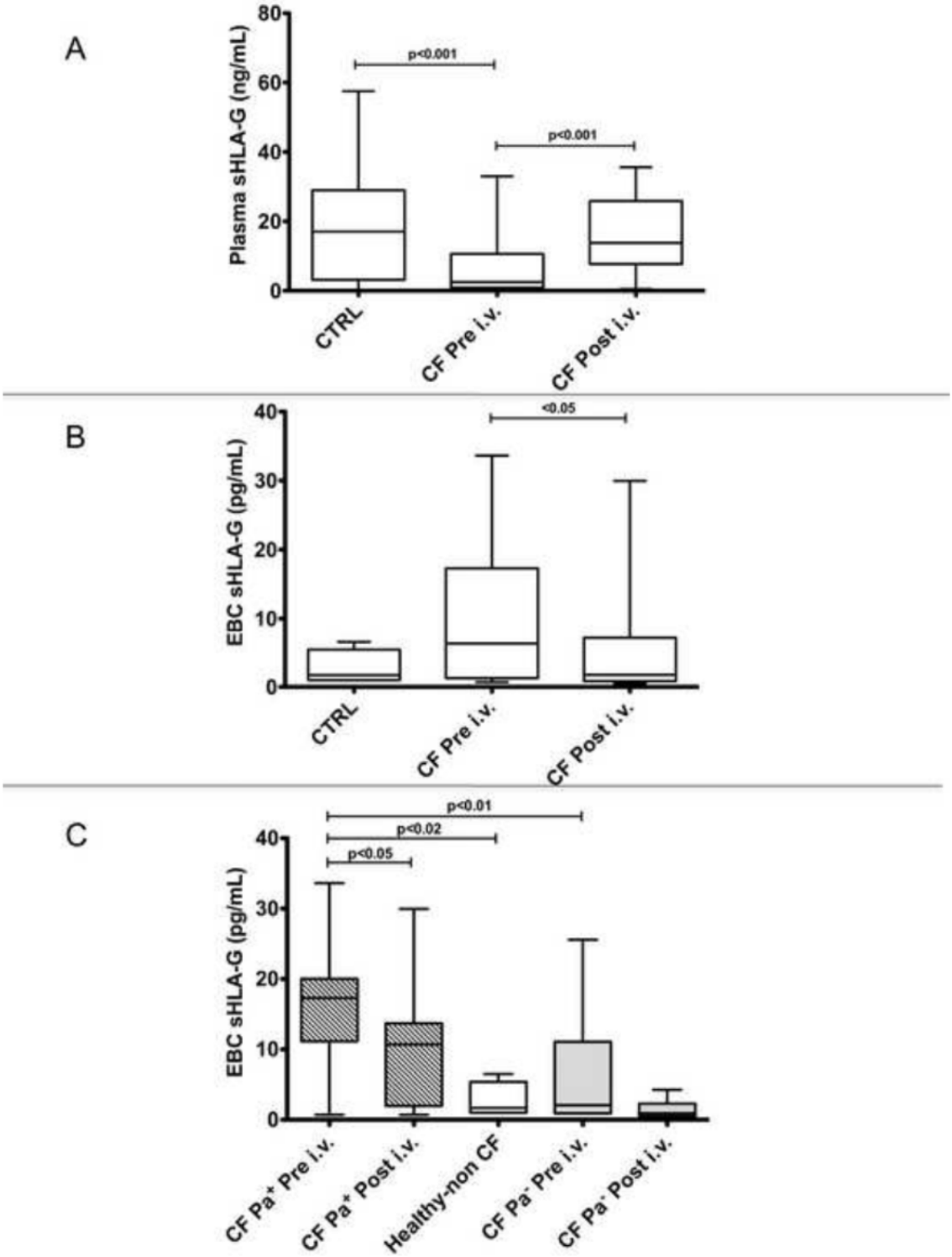
25

Table 4. CF patients subdivided according to HLA-G 14bp ins/del polymorphism.

Genotype	14bp del/del	14bp ins/del	14bp ins/ins	p value
Age years	25.5±11.0	25.5±10.1	24.0±10.2	0.34*
Height Z score	-0.5±2.5	-0.5±1.2	-0.4±1.2	0.25*
Weight Z score	-0.7±1.3	-0.7±1.3	-0.5±1.4	0.18*
BMI Z score	0.2±1.0	0.1±0.9	0.2±1.0	0.80*
FEV1 (% predicted)	73.4±22.8	65.5±29.6	70.8±24.0	0.35*
Chronic P. aeruginosa infection, n (%)	49 (53)	19 (26)	6 (22)	0.0005†
Diabetes n (%)	14 (15)	11 (15)	3 (11)	0.85†

* Kruskal Wallis test; †Chi squared test

Figure 1.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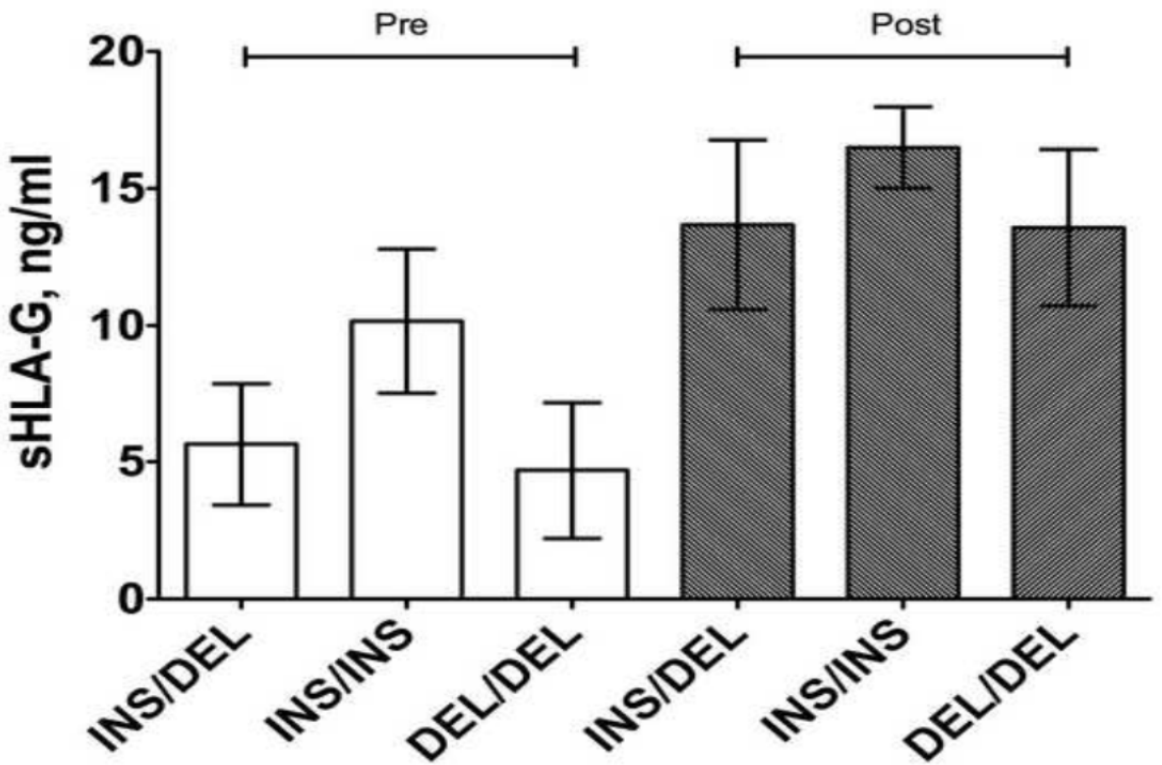
60
40
20
0
p<0.001
p<0.001
CTRL
CF Pre i.v.
CF Post i.v.
B
EBC sHLA-G (pg/mL)
40
30
20
10
0
<0.05
CTRL
CF Pre i.v.
CF Post i.v.
C
EBC sHLA-G (pg/mL)
40
30
20
10
0
p<0.01
p<0.02
p<0.05
CF Pa+ Pre i.v.
CF Pa+ Post i.v.
Healthy-non CF
CF Pa- Pre i.v.
CF Pa- Post i.v.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sHLA-G, ng/ml
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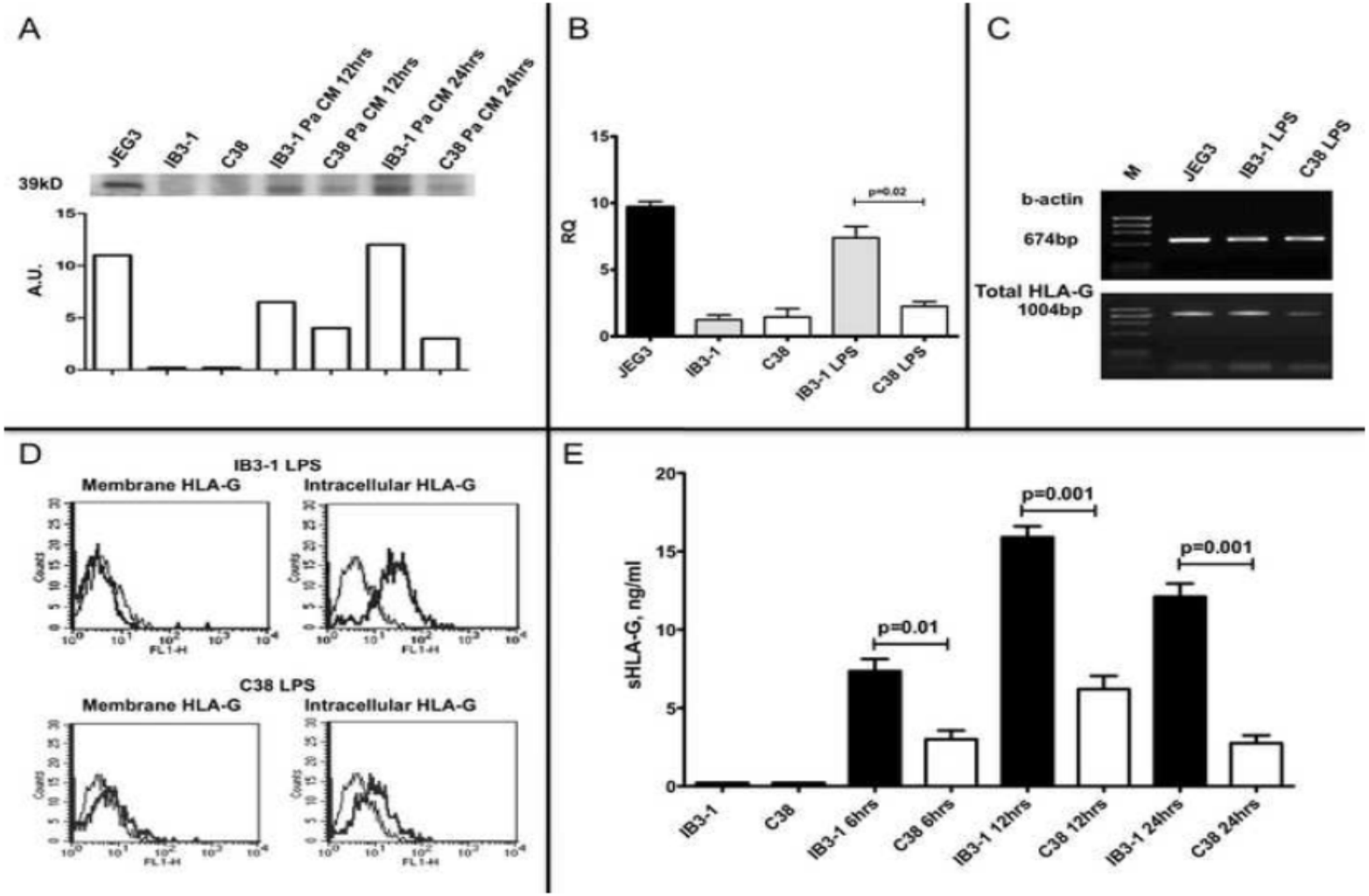
DEL/DEL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JEG3
IB3-1
C38
IB3-1 Pa CM 12hrs
C38 Pa CM 12hrs
IB3-1 Pa CM 24hrs
C38 Pa CM 24hrs
39kD
A.U.
B
RQ
p=0.02
JEG3
IB3-1
C38
IB3-1 LPS
C38 LPS
C
M
JEG3
IB3-1 LPS
C38 LPS
b-actin
674bp
Total HLA-G
1004bp
D
IB3-1 LPS
Membrane HLA-G
Intracellular HLA-G
C38 LPS
Membrane HLA-G
Intracellular HLA-G
Counts
FL1-H
E
sHLA-G, ng/ml
p=0.01
p=0.001
p=0.001
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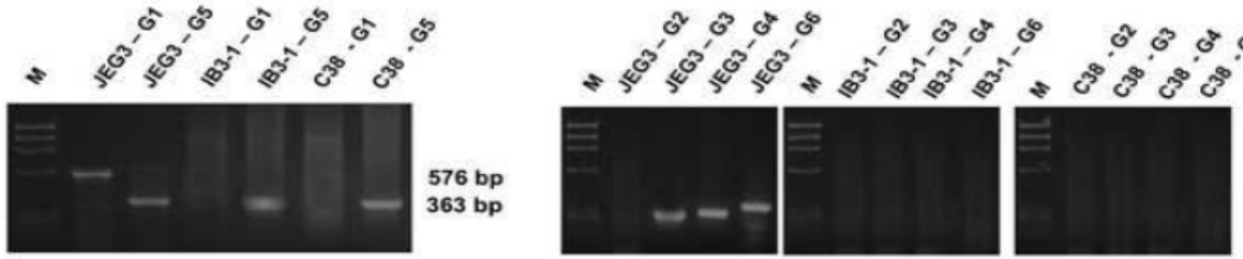
C38 24hrs

A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JEG3 – G1
JEG3 – G5
IB3-1 – G1
IB3-1 – G5
C38 - G1
C38 - G5
576 bp
363 bp
M
JEG3 – G2
JEG3 – G3
JEG3 – G4
JEG3 – G6
M
IB3-1 – G2
IB3-1 – G3
IB3-1 – G4
IB3-1 – G6
M
C38 - G2
C38 - G3
C38 - G4
C38 - G6

B

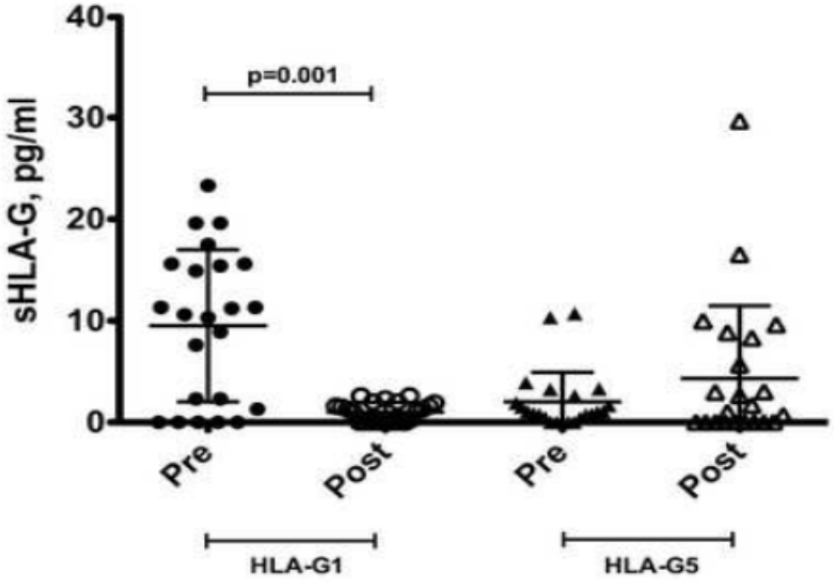
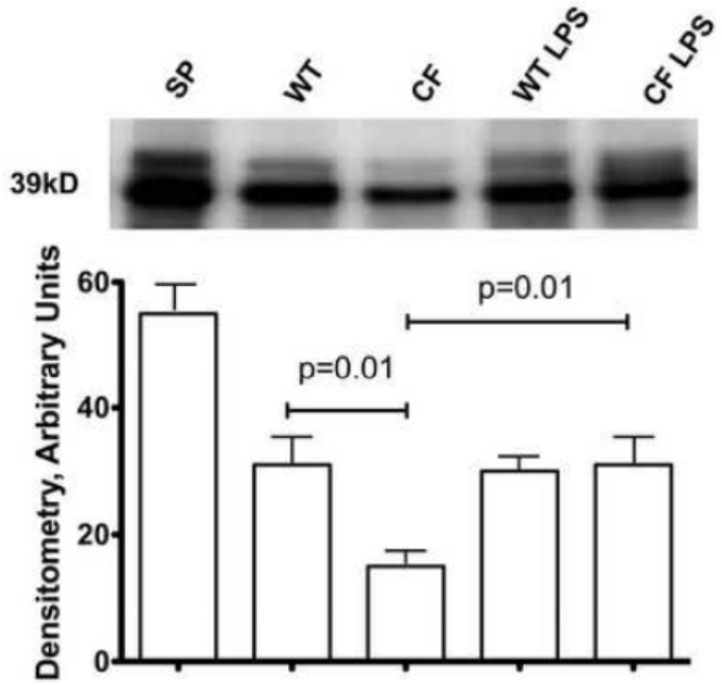

sHLA-G, pg/ml
40
30
20
10
0
p=0.001
Pre
Post
Pre
Post
HLA-G1
HLA-G5

Figure 5.


SP
WT
CF
WT LPS
CF LPS
39kD
Densitometry, Arbitrary Units
60
40
20
0
p=0.01
p=0.01

In order to confirm the results obstained in CF patients, we investigate how P.aeruginosa could affect HLA-G expression. P.aeruginosa is able to produce a series of soluble molecule that altogether constitute the system of "Quorum sensing" (QS) [124] (**Figure 3**). The QS system is composed by two main kind of autoinducers that are crucial for the bacterial virulence and biofilm formation. As shown in Figure 3, the main molecules employed in the QS system are 3-o-C12-HLS and C4- HLS, which interact respectively with the Lasr/LasI and RhlR/RhlI elements [125]. By literature, it is known that QS molecules influence the host immune system, as for example the 3-o-C12-HSL [126-130]. The analysis of HLA-G membrane expression in PBMCs after exposition to biofilm forming *P. aeruginosa* culture supernatant evidenced an increase in membrane-bound HLA-G expression in monocyte ($CD14^+$) and T ($CD3^+$) cells [131 paper attached]. These data are in agreement with the hypothesis that quorum sensing molecule 3-o-C12-HLS production could up-regulate HLA-G expression (Patent n. FE2014A000005 filed on 6th November 2014). Basing on these results, we demonstrated that 3-o-C12-HSL was an HLA-G inducer by treating different cell lines [131 paper attached]. The data confirmed that 3-o-C12-HSL was able to induce HLA-G expression particularly in monocyte U937 and Jurkat T cell lines through the CREB\p38 phosphorylation, confirming in this way our hypotesis that P.aeruginosa can induce HLA-G expression during infection.

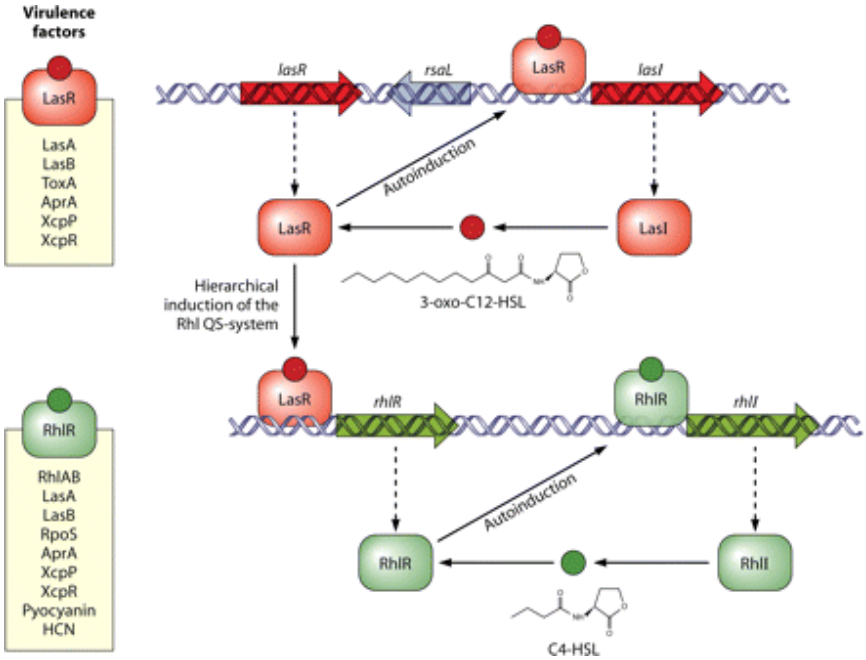


Figure 3. **Virulence regulation of and interactions between the two AHL quorum-sensing systems in *P. aeruginosa*.** After a threshold concentration of 3-oxo-C_{12}-HSL is produced, the 3-oxo-C_{12}-HSL–LasR complex binds the promoter regions of multiple genes, activating or repressing their transcription. Among the genes upregulated by this complex are *lasI*, which enhances the production of 3-oxo-C_{12}-HSL (autoinduction effect), and *rhlR*, which increases the production of the *rhl* response regulator RhlR, activating the second AHL pathway at an earlier stage. Virulence factors regulated by each respective receptor-ligand complex are detailed on the left.

Reference 131

Pseudomonas aeruginosa quorum sensing molecule N-(3-oxododecanoyl)-homoserine-L-lactone (3-o-C12-HSL) induces HLA-G expression in human immune cells.

Introduction

Quorum sensing (QS) is the process through which bacterial cells communicate enabling unicellular populations to coordinate their response to an external stimulus as a function of population density, [1]. Gram negative bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) employ N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) QS signal molecules. *P. aeruginosa* is an opportunistic human pathogen responsible for causing infection in immune compromised individuals [2]. *P. aeruginosa* employs an OdDHL-dependent QS system that via las/rhl plays a key role in controlling virulence factor production, biofilm maturation, swarming motility and the expression of antibiotic efflux pumps [3]. There is an increasing body of evidence that OdDHLs are able to interact with a range of mammalian cell types. In particular, they can modify function of respiratory epithelial cells and fibroblasts [4,5] and they interact with immune cells [6-9]. In particular, N-3-(oxododecanoyl)-L-homoserine lactone (3-o-C12-HSL) has the potential to modulate the immune system of the host. 3-o-C12-HLS has a powerful inhibitory effect on professional immune cells, inhibiting dendritic cell and T-cell activation [10], promoting apoptosis [11 – 13] and inhibiting the ability of macrophage and monocytes to respond to a range of Toll-like receptor (TLR) agonists through disruption of NF-κB signalling [14]. Taken together, these

data suggest that 3-o-C12-HLS suppresses the function of key immune networks responsible for bacterial clearance.

HLA-G is a non-classical Human Leukocyte Antigen (HLA) class I characterized by the presence of membrane bound and soluble isoforms [15]. HLA-G is involved in mechanisms of immune tolerance in several conditions including pregnancy, organ transplantation, autoimmune and inflammatory diseases by inhibiting cytolytic functions of natural killer cells, cytotoxic T-lymphocytes, and T-cell allo-proliferative responses [15]. Both soluble and membrane-bound HLA-G isoforms have similar functions and interact with specific inhibitory receptors (ILT-2 ILT-4) expressed by immune cells [15]. During viral infections, HLA-G molecules are up-regulated by the virus as a mechanism of immune-escape [16], inhibiting the host immune response.

A recent work by Glucksam-Galnoy et al. evidenced that 3-o-C12-HLS is able to modulate IL-10 production in macrophages [17] involving the transcriptional factors NFkB and p38. Since IL-10 is one of the major HLA-G inducers [18], it seems reasonable to speculate that also HLA-G expression could be regulated in turn by 3-o-C12-HLS.

Moreover, a marked elevation of soluble human leukocyte antigen-G protein was observed during septic shock [19] and polymorphisms in exon 8 at the 3 'UTR of HLA-G gene (+2960IN_+3142G_+3187A haplotype) recognized septic patients with an increased risk for septic shock [20]. For this, it seems reasonable a possible implication of HLA-G during bacterial infections, with a possible dual explanation: i) immune-inhibitory mechanism induced by the bacteria to prevent host immune response; ii) HLA-G expression or secretion may reflect an appropriate and efficient response to the inflammatory process occurring during septic shock.

The objective of the present study was to determine whether 3-o-C12-HLS could modify HLA-G expression by immune cells, supporting the hypothesis of a direct involvement of bacteria in the induction of HLA-G molecules.

Materials and Methods

Cell lines and Bacteria strain

U937 (ATCC CRL1593.2) and THP-1 (ATCC TIB202) monocyte cell lines, Jurkat (ATCC TIB152) T-cell line and 721.221 (ATCC CRL1885) B-cell line were grown in RPMI 1640 (Gibco) added with 10% FBS, 10% Hepes Buffer, 5% peniciline\strepatamicin at 37°C with 5% of CO_2.

P.aeruginosa (ATCC BAA-47) PAO1 strain was grown in Tryptic Soy broth (Sigma) at 37°C for supernatant collection.

Peripheral blood mononuclear cell purification

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of 10 control subjects by Ficoll gradient (Cederlane, Hornby, Ontario, Canada) and resuspended in RPMI medium (EuroClone, Milano, Italy) with 10% FCS, 100U/ml penicillin and 100 U/ml streptomycin (Sigma-Aldrich, S.Louis, MO, USA).

Cell treatments

The cells were treated with 3-o-C12-HSL (Sigma-Aldrich) at the concentrations of 10, 25 and 50uM, suggested by literature [17], at different time points and then analyzed.

For the inhibitory experiments, Fc receptors were first blocked using human serum, and cells were incubated for 30 min at 37°C with a final concentration of 10 μg/ml isotype control

monoclonal antibody (moAb) or blocking anti-IL-10 (Biolegend) or anti-ILT2 (Becton Dickinson) monoclonal antibodies (moAbs) prior to use.

To induce monocyte differentiation and activation, $5x10^4$ cells were seeded in 500µl complete medium containing 160 nM PMA (Sigma Chemical). Cells were then analyzed by flow cytometry.

Cytometric analysis

For flow cytometric analysis, cells (10^6) were washed and incubated for 30 minutes on ice in 100 µl of Phosphate Buffered Saline (PBS) containing 1% FBS, 10 mM sodium azide plus appropriately diluted fluorescent moAb. After two washes with cold washing buffer, cells were then washed, fixed in 2% formaldehyde, and analyzed by flow cytometry with a FACSVantage flow cytometer (Becton Dickinson, San Jose, CA, USA) using standard settings and CellQuest software (Becton Dickinson, San Jose, CA, USA) for data analysis. The membrane bound HLA-G antigens were detected by anti-HLA-G FITC moAb (87GExbio, Praha, Czech Republic), HLA class I molecules were stained by HC10-FITC moAb (kind gift of Prof. Fabio Malavasi). Anti-isotype controls (Exbio, Praha, Czech Republic) were performed. PBMCs were analyzed using anti-CD3-PerCP, anti-CD14-PE, anti-CD45-PE, anti-CD56-PE (BD) moAbs.

Cell cycle was analyzed by Propidium Iodide (PI) staining. Cells were resuspended cells in 500 µl PI/Triton X-100 staining solution [(0. 1 % (v/v) Triton X-100 (Sigma) in PBS, 2 mg DNAse-free RNAse A (Sigma), 0.40 ml of 500 µg/ml PI (Roche)], incubated for 30 minutes at RT and analyzed by flow cytometry.

Annexin-V staining (Annexin V-FITC apoptosis detection kit; Bender MedSystem) was used to measure the apoptosis. The cells were resuspended in 1x binding buffer [10 mmol/L

HEPES (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl2] for analysis. Cells were stained with Annexin V-FITC to reveal apoptosis and with propidium iodide to detect dead cells.

Monocyte differentiation was evaluated with anti CD11b-FITC and anti-CD14-PE (BD) moAbs.

Immunofluorescence microscopy

For immunofluorescence microscopy, cells (10^6) were washed and incubated for 30 minutes on ice in 100 μl of Phosphate Buffered Saline (PBS) containing 1% FBS, 10 mM sodium azide plus appropriately diluted fluorescent mAb. After two washes with cold washing buffer, cells were then washed, fixed in 2% formaldehyde, and analyzed by fluorescence microscopy. Membrane bounded HLA-G (mHLA-G) was detected by direct immunofluorescence using the anti-HLA-G FITC moAb (MEM-G9, Exbio, Praha, Czech Republic), while CREB\pCREB (Immulogical Sciences) and p38\pp38 (SantaCruz) were detected by indirect immunofluorescence with the secondary antibody Goat anti-Mouse FITC (Dako).

ELISA

Soluble HLA-G (sHLA-G) levels in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using as capture antibody the MoAb MEM-G9 (Exbio, Praha, Czech Republic), which recognizes the HLA-G molecule in β_2-microglobulin associated form. The intra-assay coefficient of variation (CV) was 1.4% and the inter-assay CV was 4.0%. The limit of sensitivity was 1.0 ng/ml [21].

IL-10 levels were analyzed using Human IL10 ELISA detection kit (EBioscience).

MTT cell viability assay

100ul of cells at the density of $1x10^{6}$\ml were seaded into 96-well plates and treated with 3o-C12-HSL (10uM and 25uM) for 6, 12 and 24 hours (hrs). After incubation, 10ul of MTT (Sigma-Aldrich) for 4 hours at 37°C. Cells were then lisate by adding 100ul of MTT solvent. Plates were then read at 570nm for viability evaluation using ELISA-reader (Victor, Perkin-Elmer).

mRNA preparation

Total cellular RNA was prepared from each cell cultures with TRIzol reagent (Life Technologies, NY, USA). The RNA samples were digested with DNase. The quality and quantity of RNA samples were assessed by a 1% agarose gel electrophoresis, followed by ethidium bromide staining. These mRNA samples were immediately used for cDNA synthesis or stored frozen at -80° C until use.

RealTime PCR

To analyze the presence of HLA-G mRNA, 2 ug mRNA were reverse transcribed for each sample using SuperScript™ First-Strand Synthesis System (Invitrogen, San Giuliano Milanese, MI, Italy) according to manufacturer's instructions. The primers and the detection probe for HLA-G gene expression analysis were: forward primer HLAG-F (5'-CCCACCATCCCCATCATG-3'); reverse primer HLA-G-R (5'-CCAGTGACTACAGCTGCAAGGA-3') and the MGB probe (5'-TATCGTTGCTGGCCTGG-FAM-3') (Applied Biosystem) [22]. Fold expression changes were determined by the $2^{-\Delta\Delta CT}$ method. Amplification was performed with 100ng of RNA converted into cDNA, TaqMan 2X Universal PCR Master Mix in a final volume of 50ul (Applied Biosystem) following this protocol: 2 minutes at 50°C for AmpErase UNG

activation, 20 seconds at 95°C for initial denaturation followed by 40 cycles 20 second at 95°C and 60 seconds at 60°C for amplification. All reactions were performed in triplicate

Reporter Constructs and Expression Vectors

Luciferase reporter plasmids were generated by cloning genomic promoter fragments into pGL3-Basic (Promega, Madison, WI). These constructs contain, respectively, a 1438-bp promoter fragment of*HLA-G* (pGL3-G1500), and a 269-bp *Asp*I-*Aha*II*HLA-B7* promoter fragment (pGL3-HLA-B) (kind gift of Prof. Sam JP Gobin) [23]. All inserts were verified by sequence analysis.

The *Renilla* luciferase control plasmid pRL-actin was used as transfection efficiency control.

Transient Transfection

721.221 cells were transfected by Amaxa Nucleofector technology (Lonza) with a DNA precipitate of 1 μg of pGL3 reporter plasmid, 1 or 0.5 μg of expression vector, and 0.1 μg of *Renilla* luciferase control plasmid (pRL-actin) per well. Luciferase activity was determined using a luminometer (Victor, Perkin Elmer) and corrected for transfection efficiency with the *Renilla* luciferase activity values.

Statistical analysis

Since the values presented a normal distribution (Kolmogorov-Smirnov test) , the differences were evaluated by the Student T test using Stat View software (SAS Institute Inc, Cary, NC, USA). p value was considered to be statistically significant when <0.05.

Results

P. aeruginosa culture supernatant induces HLA-G$^+$CD3$^+$ and HLA-G$^+$CD14$^+$ cells.

To evaluate the induction of HLA-G molecules by *P. aeruginosa* in human immune cells, we exposed peripheral blood mononuclear cells (PBMCs) from 10 control subjects to the culture supernatants (SN) of biofilm-forming *P. aeruginosa*. PBMCs were negative for HLA-G staining before the treatment. After 6 hrs of incubation with 25ul of *P. aeruginosa* SN, we observed a 1.5±0.5% (mean±SD) of CD14$^+$HLA-G$^+$ cells ($p=0.002$, Student T test) (Figure1a, black histogram), that increased to the 8.0±0.4% after 12hrs ($p<0.0001$) and returned to 1.8±0.2% ($p=0.001$) after 24hrs of treatment. 10uL and 50uL *P. aeruginosa* SN were able to induce CD14$^+$HLA-G$^+$ cells after 12hrs (3.4±0.5% and 3.9±0.6% respectively) ($p<0.001$), that decreased to 1.9±0.2% and 1.4±0.3% after 24hrs ($p=0.001$). The induction of CD3$^+$HLA-G$^+$ cells was observed after 12hrs incubation with 25uL (2.5±0.3%) ($p<0.001$) and 50uL (2.3±0.3%) of *P. aeruginosa* SN (Figure 1a, white histogram) and decreased to 1.2±0.1% and 1.75±0.2% after 24hrs. Both CD3$^+$ and CD14$^+$ cells presented the highest mHLA-G induction after 12hrs incubation with 25ul *P. aeruginosa* SN (2.5±0.3% CD3$^+$HLA-G$^+$ and 8.0±0.4% CD14$^+$HLA-G$^+$ cells) (Figure 1a). The decrease in HLA-G expression after 24hrs incubation sustains a time-dependent induction of HLA-G expression. As a confirm, we performed a RealTime PCR quantification of HLA-G mRNA in PBMCs after *P. aeruginosa* SN treatment. We observed a 4 folds increase in HLA-G mRNA transcription 6hrs after the incubation with *P. aeruginosa* SN (Figure 1b) ($p<0.0001$), that reached a 6 folds increase after 12hrs treatment ($p<0.001$) but was lost after 24hrs. The expression of HLA-G molecules was evaluated also on the surface of B and Natural killer cells, however we found no HLA-G induction (data non shown).

We are aware that *P. aeruginosa* SN could contain several molecules able to modify HLA-G expression. For this, we performed a literature meta-analysis to identify the molecule with the

highest probability to be an immune-modifier, We selected 3-o-C12-HSL, that has the potential to modulate the immune response of monocytes and T cells [13].

***P. aeruginosa* 3-o-C12-HSL effect on monocyte and T cell viability.**

First of all, we verified if 3-o-C12-HSL treatment could affect monocyte and T cell viability. We used monocyte THP-1 and U937 and T- (Jurkat) cell lines to standardize the experiments. We treated the cells with 10, 25 or 50 uM of 3-o-C12-HSL [17] for 6, 12 and 24 hours. After the treatments, cell viability was evaluated by MTT assay. The major effect on cell viability was observed in U937 cells with all the concentrations of 3-o-C12-HSL. In fact, we observed a 40% decrease in cell viability after 12hrs of incubation with all the concentrations of 3-o-C12-HSL (Figure 2a). Interestingly, U937 cells reconstituted their viability after 24hrs of incubation. On the contrary, neither the monocyte cell line THP-1 (Figure 2b), nor Jurkat (Figure 2c) cell line showed any significant decrease in cell viability.

On the basis of this results, we selected to work with the lowest 3-o-C12-HSL concentrations (10 and 25uM) and 24 hrs of incubation.

***P. aeruginosa* 3-o-C12-HSL induces HLA-G expression in human monocyte and T cells.**

To be sure that 3-o-C12-HSL is able to induce HLA-G transcription and transduction, we treated U937 monocyte cell line with 3-o-C12-HSL for 24hrs and evaluated HLA-G mRNA and protein expression. We observed an increased production of HLA-G specific mRNA (Figure 3a) that followed a dose-dependent effect, with 10uM 3-o-C12-HSL inducing a 3 folds increase of HLA-G mRNA and 25uM inducing a 6 folds increase in comparison with untreated cells ($p<0.001$; Student t test). Membrane HLA-G expression acts similarly, with an increase of 10% after 10uM and 30% after 25uM 3-o-C12-HSL treatment (Figure 3b, c). Jurkat cells presented a 5 and 7 folds increase of HLA-G^+ cells with 10uM and 25uM

respectively (Figure 3d). On the contrary, THP1 cells presented a lower increase of HLA-G expression after 3-o-C12-HSL treatment (Figure 3e). These results suggest the presence of a specific pathway of HLA-G induction following 3-o-C12-HSL treatment that presents differences between cells.

P. aeruginosa **3-o-C12-HSL did not induce classical HLA-I expression in human monocyte U937 cell line.**

To be sure that the effect of 3-o-C12-HSL treatment is HLA-G specific, we treated U937 cell line with 3-o-C12-HSL for 24hrs and evaluated classical HLA-I expression, by means of HC-10 moAb, that is recognized not to bind HLA-G molecules [24]. We observed no modifications in classical HLA-I molecule expression in U937 cells (Figure 4a, left panel), while HLA-G was up-modulated (Figure 4a, right panel). On the basis of these results, we sustain that 3-o-C12-HSL effect is specific for HLA-G. To account the direct effect of 3-o-C12-HSL on HLA-G gene promoter, we transfected 721.221 cell line (classical and non classical HLA-I negative cells) with HLA-G or HLA-B promoters [23]. These cells were treated with 3-o-C12-HSL and luciferase assay was performed. We observed an increased activation of HLA-G promoter after 3-o-C12-HSL treatment. In particular, the promoter activation was dose-dependent with an increased activation of 21 and 39 folds with 10uM and 25uM respectively (Figure 4b, black histograms). On the contrary, HLA-B promoter was not affected by 3-o-C12-HSL treatment (Figure 4b, gray histograms). These data sustain a specific effect of 3-o-C12-HSL on HLA-G promoter.

Because of the characteristics of 3-o-C12-HSL, that can act via transcription factors [14, 25] and IL-10 expression [17], we hypothesized two possible pathways for HLA-G induction: i) via IL-10, as one of the main modulator of HLA-G expression [18] or ii) direct HLA-G induction via transcriptional factors.

***P. aeruginosa* 3-o-C12-HSL enhances HLA-G expression via IL-10 induction.**

Since IL-10 is one of the major HLA-G inducers [18], and it is known that 3-o-C12-HSL is able to induce IL-10 expression in macrophages [17], we evaluated the possible implication of this cytokine in 3-o-C12-HSL/HLA-G pathway. We analyzed the levels of IL-10 and sHLA-G expression in Jurkat, U937 and THP1cell culture supernatants after 3-o-C12-HSL treatment. We observed an increased secretion of IL-10 already 6hrs after 3-o-C12-HSL addition in both Jurkat and U937 cells (Figure 5a, white histograms) that could account for the sHLA-G increase that appeared to be significant after 12hrs treatment (Figure 5a, gray histograms). On the contrary, THP1 presented no IL-10 induction after 3-o-C12-HSL treatment (Figure 5a), that could account for the absence of HLA-G up-regulation in THP1 cells. To evaluate the role of IL-10, we pre-treated U937 cells with anti-IL-10 moAb and observed the reduction of HLA-G secretion in 3-o-C12-HSL treated U937 cell culture supernatants (Figure 6, gray histograms). It is to note that this blocking treatment was not able to completely inhibit HLA-G expression, even increasing anti-IL-10 moAb concentrations (data non shown), demonstrating that IL-10 is only one of the mechanisms used by 3-o-C12-HSL to induce HLA-G expression.

***P. aeruginosa* 3-o-C12-HSL enhances HLA-G expression via p38-CREB transcription factors.**

Since 3-o-C12-HSL is known to modify transduction pathways [25], in particular inducing p38, a mitogen-activated protein kinase (MAPK) that phosporylates and activates the cAMP-response-element-binding protein (CREB) [26], a transcription factor that regulates HLA-G gene expression [23],we evaluated the phosphorylation status of p38 and CREB after 3 -o-C12-HSL treatment in U937 cells. We observed a clear increase in total p38 and phosphorylated p38 after 15 minutes (Figure 7a) and an increased phosphorylation of CREB

after 30 minutes of 10 uM 3-o-C12-HSL treatment (Figure 7b). On the contrary, THP1 cells did not present p38/CREB phosphorylation (Figure 7c, d). These data suggest the induction of p38 and CREB phosphorylation by 3-o-C12-HSL as responsible for the direct effect of this QS molecule on HLA-G expression [23].

***P. aeruginosa* 3-o-C12-HSL enhanced HLA-G expression affects U937 cell viability via ILT2 receptor.**

If we take into consideration the results on cell viability (Figure 2a, b, c), we observe an intriguing result. In particular, we observed a decrease in cell viability only in U937 cells (Figure 2a) and not in THP1 and Jurkat cells (Figure 2b, c) after 6 and 12hrs 3-o-C12-HSL treatment. We can speculate that MTT assay could reveal cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of 3-o-C12-HSL treatment. For this, we analysed U937 cells with Anexin-V staining to account cell apoptosis and necrosis and PI staining for cell cycle evaluation. Anexin-V staining failed to reveal cell apoptosis/necrosis after 12hrs 3-o-C12-HSL treatments (Figure 8a). On the contrary, the analysis of cell cycle showed that the treatment with 3-o-C12-HSL for 12hrs blocked a 62% of cells in a G0/G1 stage in comparison with the 20% of G0/G1 cells before the treatment (Figure 8a). Since decreased cell division could be associated with differentiation, we evaluated U937 phenotype. Flow cytometry was carried out to monitor two surface proteins characteristic of U937: 1) CD14, marker for both monocyte and macrophage, involved in LPS recognition and contact with TLR4 receptor; 2) CD11b, macrophage marker, involved in phagocytosis of bacteria. CD14 expression was maintained after 12hrs of 3-o-C12-HSL treatment (Figure 9a), yielding the characteristics of monocyte-like cell type. CD11b expression was increased only after PMA treatment, indicating macrophage-like differentiation, meanwhile no CD11b induction was observed after 12hrs 3-o-C12-HSL

treatment (Figure 9b). Since we observed that 3-o-C12-HSL induces HLA-G expression, we could speculate that the interaction with its ligands could affect cell viability. We tested U937, THP1 and Jurkat cells for ILT2 expression and we observed the presence of this receptor only in U937 cells (Figure 9c, d, e). ILT2 is a surface receptor expressed by different immune system cells, including monocytes, that is able to bind HLA-G and modify monocyte status [27]. We performed again the experiments on U937 cells in the presence of anti-ILT2 moAb. Interesting, we observed no decrease in U937 viability when the experiments were performed with anti-ILT2 antibody (Figure 9f). These data suggest that the induction of HLA-G by 3-o-C12-HSL could act on ILT2 explaining the cytostatic activity on U937 cells.

Proposed pathways

Figure 10 illustrates a potential network that might operate in the presence of 3-o-C12-HS. It incorporates previous findings and the observations made in this study. In this network, 3-o-C12-HS activates HLA-G expression via p38 and CREB phosphorylation [23] and IL-10 induction, that acts in a feed-back loop pathway of HLA-G induction [18]. In the presence of ILT2 expression, the interaction with HLA-G molecules could modify cell activity, promoting a resting status.

Discussion

3-o-C12-HLS is a bacterial quorum sensing molecule that coordinates bacteria growth and virulence in a cell density-dependent manner [1]. Furthermore, different studies have already reported the ability of 3-o-C12-HLS in regulating both pro- and anti-inflammatory cytokines production by host cells [4-14; 27].

Even more evidences suggested that 3-o-C12-HLS plays a critical role not only in inter-bacterial communication, but also in inter-kingdom signaling. In fact, 3-o-C12-HLS can interact with mammalian cells, including immune cells [4-14; 25]. In particular, 3-o-C12-HLS exhibit modulatory effect on monocytes [28] inducing changes in mRNA transcription. Since HLA-G is a key molecule in the immune system switch-off [15] a the aim of our study was to investigate whether 3-o-C12-HLS induced HLA-G modification in immune cells.

In this research we report that 3-o-C12-HLS affects HLA-G production in monocyte and T cells, involving the CREB\p38 and IL-10 pathways.

The analysis of HLA-G membrane expression in PBMCs after exposition to biofilm forming *P. aeruginosa* culture supernatant evidenced an increase in membrane-bound HLA-G expression in monocyte ($CD14^+$) and T ($CD3^+$) cells. These data are in agreement with the hypothesis that quorum sensing molecule 3-o-C12-HLS production could up-regulate HLA-G expression.

The induction of HLA-G by 3-o-C12-HLS was confirmed in monocyte U937 and Jurkat T cell lines. As a proof of concept, THP1 cell line did not present HLA-G expression nor IL-10 induction and CREB\p38 phosphorylation after 3-o-C12-HLS treatment, supporting the existence of a network between IL-10\CREB\p38 phosphorylation and HLA-G. The implication of ILT2 receptor expression on the cytostatic effect of 3-o-C12-HLS on U937 cells is a direct proof of the functional activity of the HLA-G molecules induced by 3-o-C12-HLS. In particular, they sustain the immunosuppressive properties of HLA-G molecules and

could explain the effect of in vivo 3-o-C12-HLS production by bacteria, as an efficient mechanism to modulate host immune response via HLA-G. On the other hand, HLA-G expression could be a response to the inflammatory process occurring during microbial infection, reflecting an appropriate and effective feedback control of inflammatory process. On the basis of our knowledge, we propose a dual role for HLA-G induction during bacterial infections, as an immune escape factor for the bacteria and a rescue mechanism for the host. In particular, bacteria could induce cell release of sHLA-G in peripheral blood leading to the down-regulation of chemokine receptors on T lymphocytes and NK cells, that can affect the migration of these cell populations toward inflamed tissues, leading to immune escape of bacteria [29]. The loss of balanced sHLA-G levels may cause a the activation of immune effector cells and their migration to the inflamed tissues, contributing to the exacerbation of bacterial infection.

In conclusion, we presented for the first time the effect of 3-o-C12-HSL treatment on HLA-G induction. As a future perspective, it will be of importance to recognize the cell types responsive to 3-o-C12-HSL treatment. Using 3-o-C12-HSL to induce HLA-G expression could also be useful for clinical purposes where a natural tolerance is beneficial instead of the use of immune-suppressive drugs, as in transplantation and pregnancy

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Figure legends

Figure 1. a) Membrane HLA-G expression in PBMCs from 10 healthy subjects. Cells were treated with 10, 25, 50 uL *P. aeruginosa* culture supernatnats (SN) for 6, 12 and 24hrs. $CD3^+$ (white hystogram) and $CD14^+$ (black histogram) cell results are reported. b) HLA-G mRNA expression analysis in PBMCs after 6, 12 and 24hrs treatment with 25 uL *P. aeruginosa* culture supernatnats (SN). Mean ± standrd deviation (SD) is reported. ** Significant p value <0.05 obtained by Student T test.

Figure 2. MTT cell viability assay. Cells were treated with 10, 25 and 50uM 3-o-C12-HSL for 6 (white histogram), 12 (gray histogram) or 24 (black histogram) hrs. a) U937 cell line; b) THP-1 cell line; c) Jurkat cell line. Mean ± standrd deviation (SD) is reported. ** Significant p value <0.05 obtained by Student T test.

Figure 3. a) HLA-G mRNA and membrane HLA-G expression, evaluated by b) flow cytometry (87G-FITC moAb) and c) immunofluorescence (MEM-G9 moAb) in U937 cell lines. Membrane HLA-G expression, evaluated by b) flow cytometry (87G-FITC moAb) in d) Jurkat and e) THP1 cell lines. Cells were treated with 10 and 25uM 3-o-C12-HSL for 24hrs. Mean ± standrd deviation (SD) is reported. ** Significant p value <0.05 obtained by Student T test.

Figure 4. a) Membrane HLA-G (left panels) and classical HLA-I (right panels) expression in U937 cell line. Cells were treated with 10 and 25uM 3-o-C12-HSL for 24hrs. The percentage of HLA-G^+ and HLA-I^+ cells are reported. b) Luciferase assay: HLA-B (gray histogram) and HLA-G (black histogram) promoter activation analysis in 721.221 transfected cell lines after treatment with 10 and 25uM 3-o-C12-HSL for 24 hrs. Mean ± standrd deviation (SD) is reported. ** Significant p value <0.05 obtained by Student T test.

Figure 5. a) IL-10 and b) sHLA-G levels in Jurkat, U937 and THP1 cell culture supernatants. Cells were treated with 10 and 25uM 3-o-C12-HSL for 6 (white histogram), 12 (gray histogram), 24 (black histogram) hrs. Mean ± standrd deviation (SD) is reported. ** Significant p value <0.05 obtained by Student T test.

Figure 6. sHLA-G levels in cell culture supernatants of U937 cells with anti-IL-10 or anti-isotype moAb treatment and addition of 10 or 25uM 3-o-C12-HSL for 24hrs. Mean ± SD is reported. ** Significant p value <0.05 obtained by Student T test.

Figure 7. Intracellular pathway activation analysis in U937 and THP1 cell lines. U93 and THP1 cells were treated with 25uM 3-o-C12-HSL . Protein analysis and phosphorilation status was performed by Immunofluorescence for: a, c) total and phoshorilated p38,b, d) total and phosphorilated CREB Here are reported the most representative results for p38\pp38 and CREB\pCREB at 0 and 30 seconds of 25uM 3-o-C12-HSL treatment.

Figure 8. a) Annexin-V staining of U937 cells treated with 10, 25 and 50uM 3-o-C12-HSL for 24hrs. The percentage of Annexin V positive cells is reported. b) and c) Propidium Iodide (PI) staining. For cell cycle analysis in U937 cells treated with 25 M 3-o-C12-HSL for 24hrs. The percentage of cell in each cell cycle stage are reported. Here are reported the most representative results.

Figure 9. a) Membrane CD14 and CD11b expression was evaluated by flow cytometry in U937 cells treated with PMA or with 10 and 25uM 3-o-C12-HSL for 12hrs. Fold increase in comparison with basal expression is reported. ILT2 expression on b) U937, c) THP1, d) Jurkat cells. Here are reported the most representative results. e) MTT cell viability assay on U937 cells after 10 or25uM 3-o-C12-HSL treatment for 12hrs in presence of anti-ILT2 moAb (gray histogram) or anti-isotype moAb (black histogram). Mean ± SD is reported. ** Significant p value <0.05 obtained by Student T test.

Figure 10. Hypothesis of 3-o-C12-HSL effects on HLA-G induction. We suggest the implication of p38 and CREB phosphorylation and IL-10 induction, that acts in a feed-back loop pathway of HLA-G induction. In the presence of ILT2 expression, the interaction with HLA-G molecules could modify cell activity, promoting a resting status (G0/G1 cell cycle stage).

Figure 1.

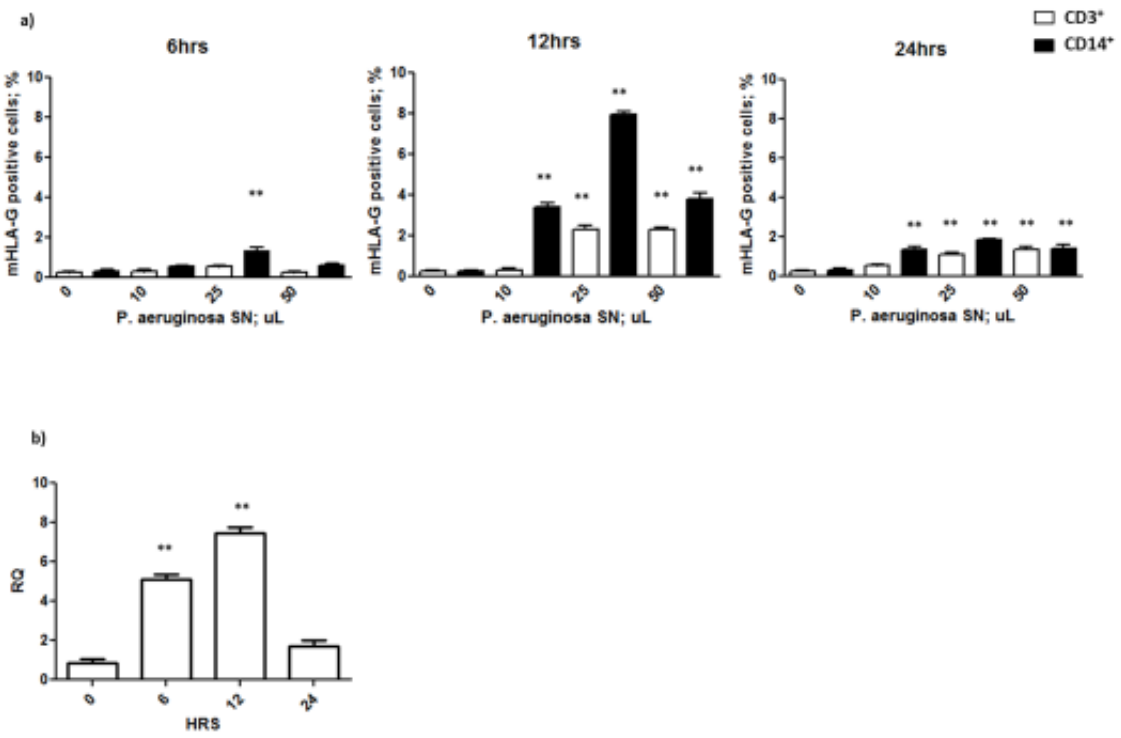


Figure 2.

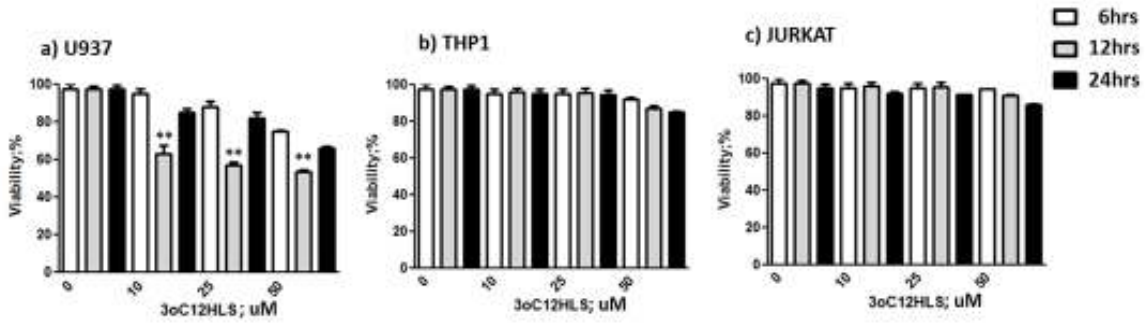


Figure 3.

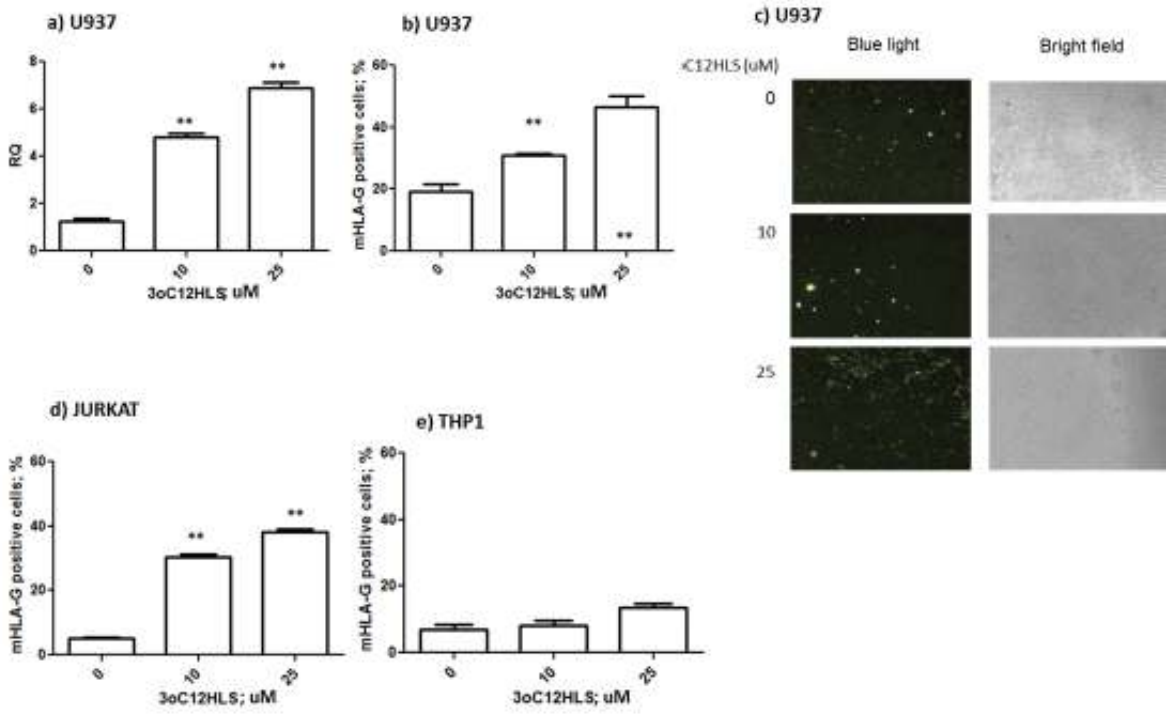

a) U937
RQ
3oC12HLS; uM
b) U937
mHLA-G positive cells; %
3oC12HLS; uM
c) U937
Blue light
Bright field
3oC12HLS (uM)
0
10
25
d) JURKAT
mHLA-G positive cells; %
3oC12HLS; uM
e) THP1
mHLA-G positive cells; %
3oC12HLS; uM

Figure 4.

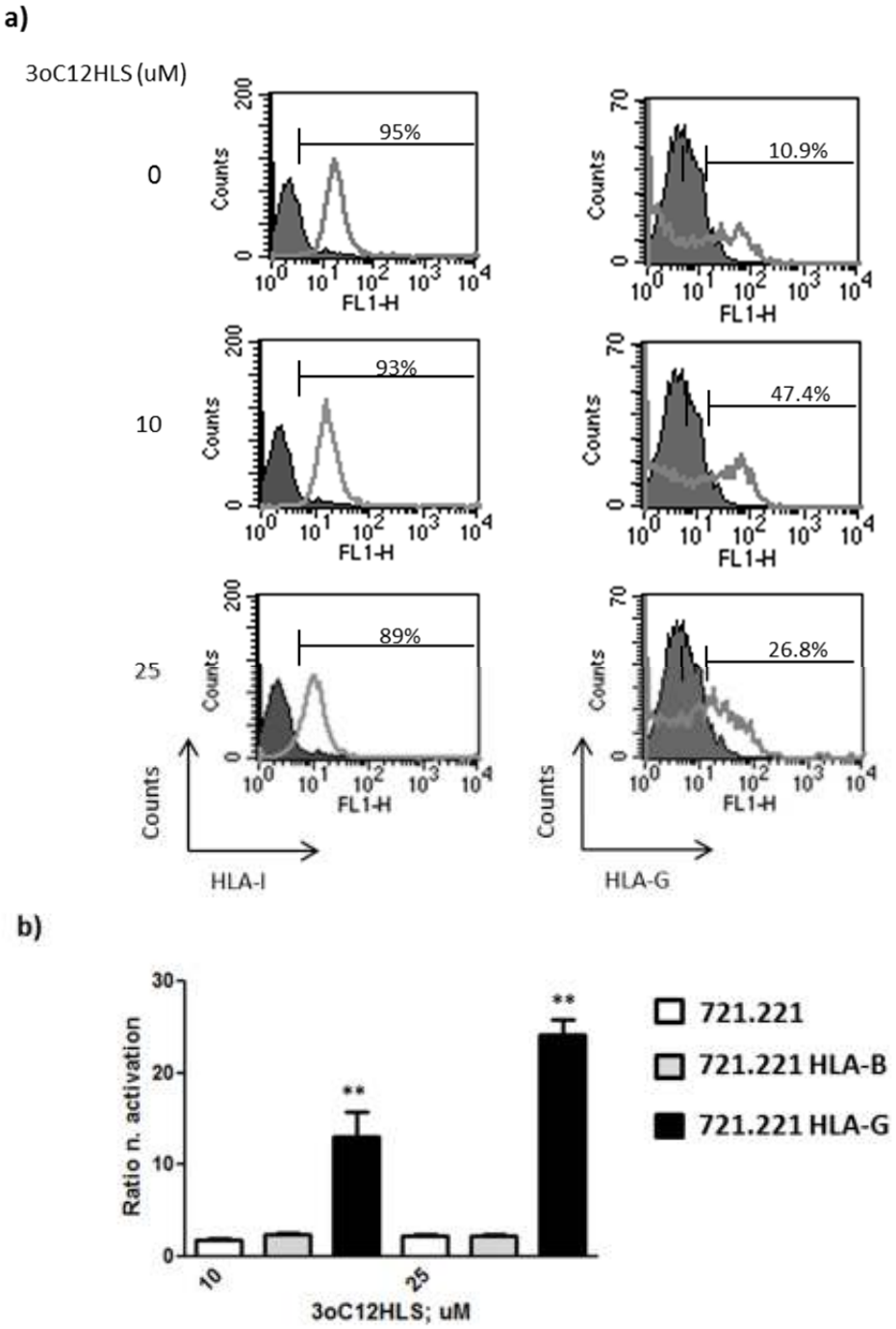

a)
3oC12HLS (uM)
0
10
25
95%
10.9%
93%
47.4%
89%
26.8%
Counts
FL1-H
HLA-I
HLA-G
b)
Ratio n. activation
3oC12HLS; uM
721.221
721.221 HLA-B
721.221 HLA-G

Figure 5.

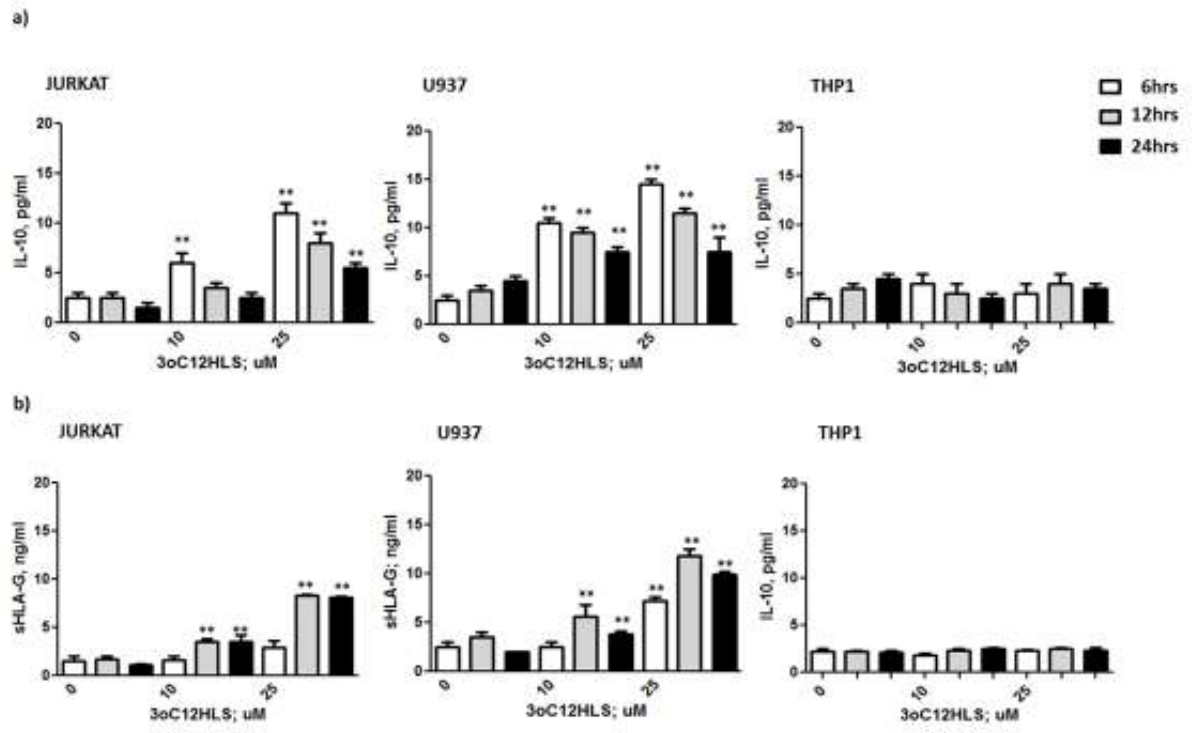


Figure 6.

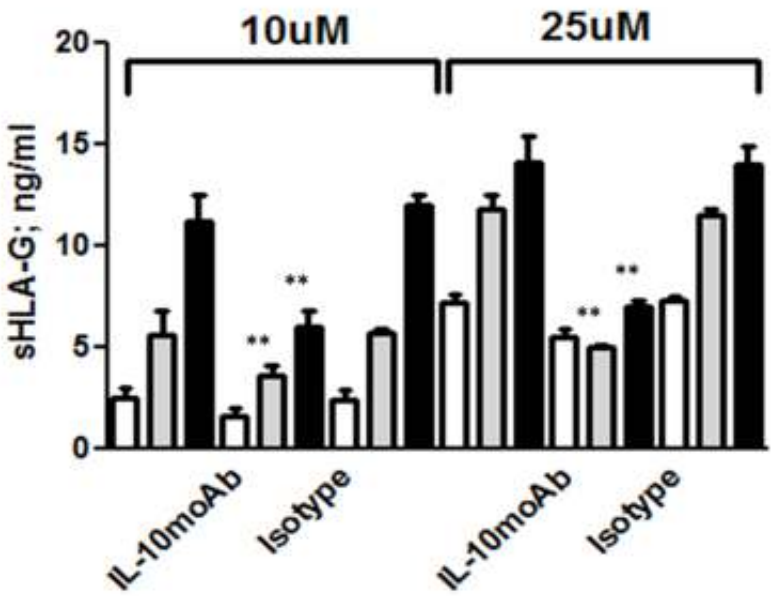


Figure 7.

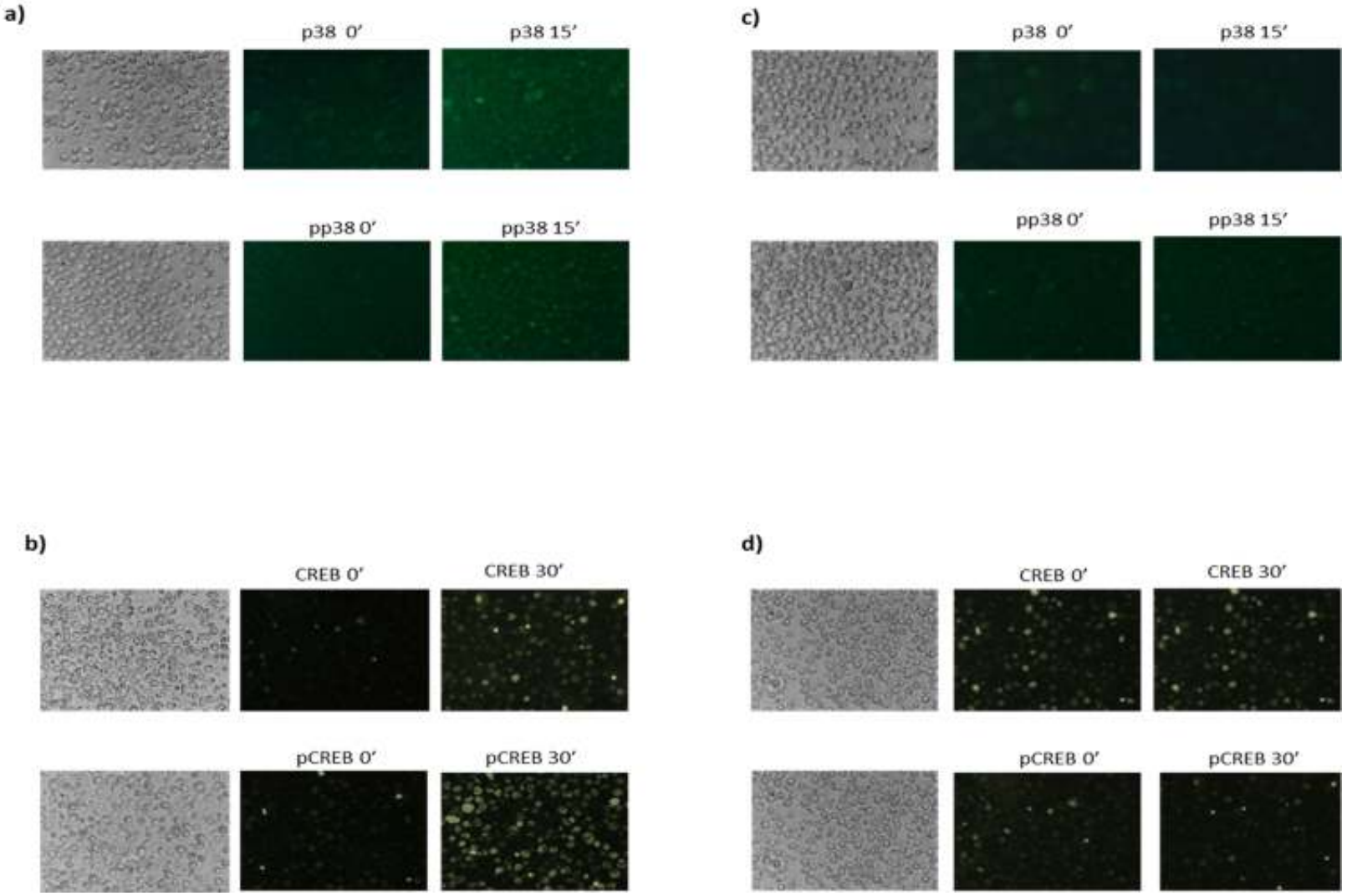
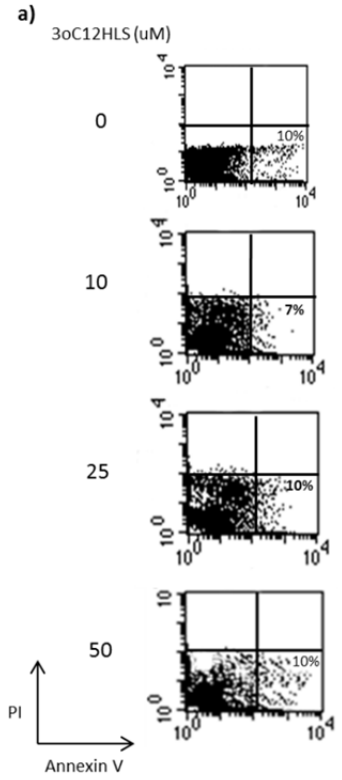
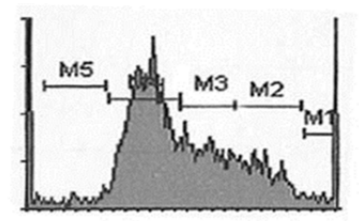
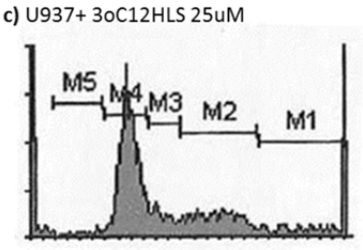

a)
p38 0′
p38 15′
pp38 0′
pp38 15′
c)
p38 0′
p38 15′
pp38 0′
pp38 15′
b)
CREB 0′
CREB 30′
pCREB 0′
pCREB 30′
d)
CREB 0′
CREB 30′
pCREB 0′
pCREB 30′

Figure 8.





	Cell cycle	Cell %
M5	Apoptosis	2.1
M4	G0/G1	20.3
M3	S	40.3
M2	G2/M	35.2
M1	Doublets	2.2



	Cell cycle	Cell %
M5	Apoptosis	2.5
M4	G0/G1	61.8
M3	S	9.9
M2	G2/M	19.7
M1	Doublets	6.1

Figure 9.

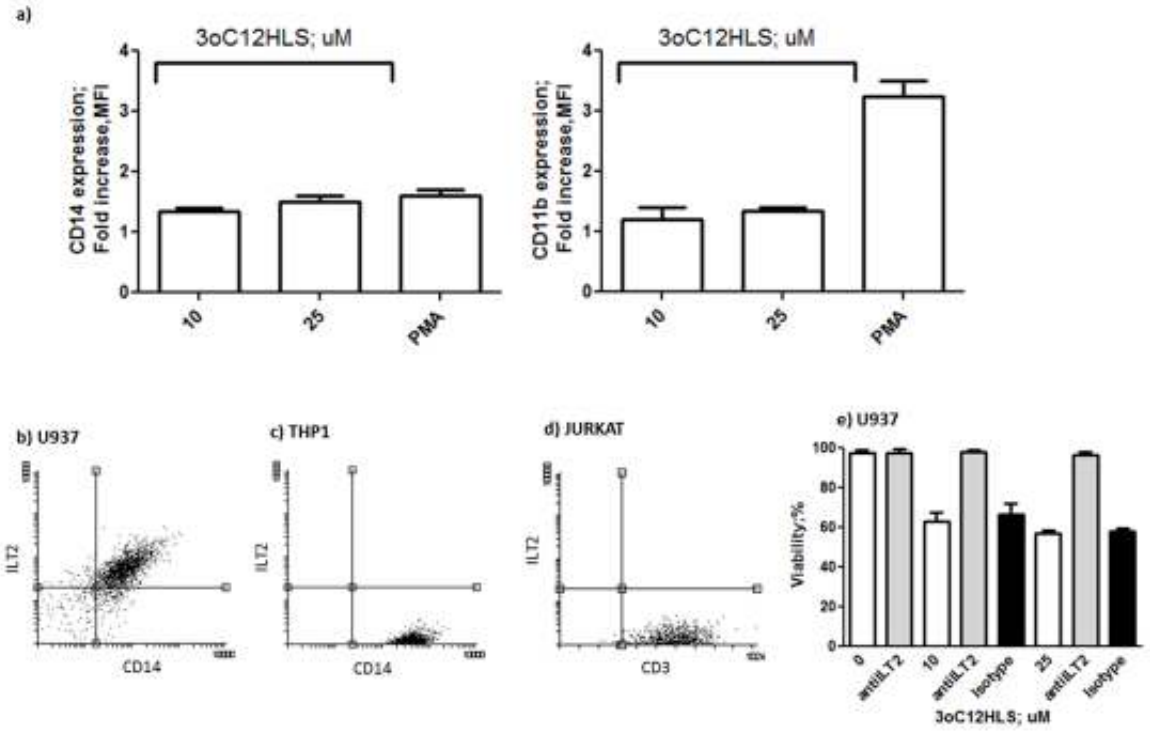
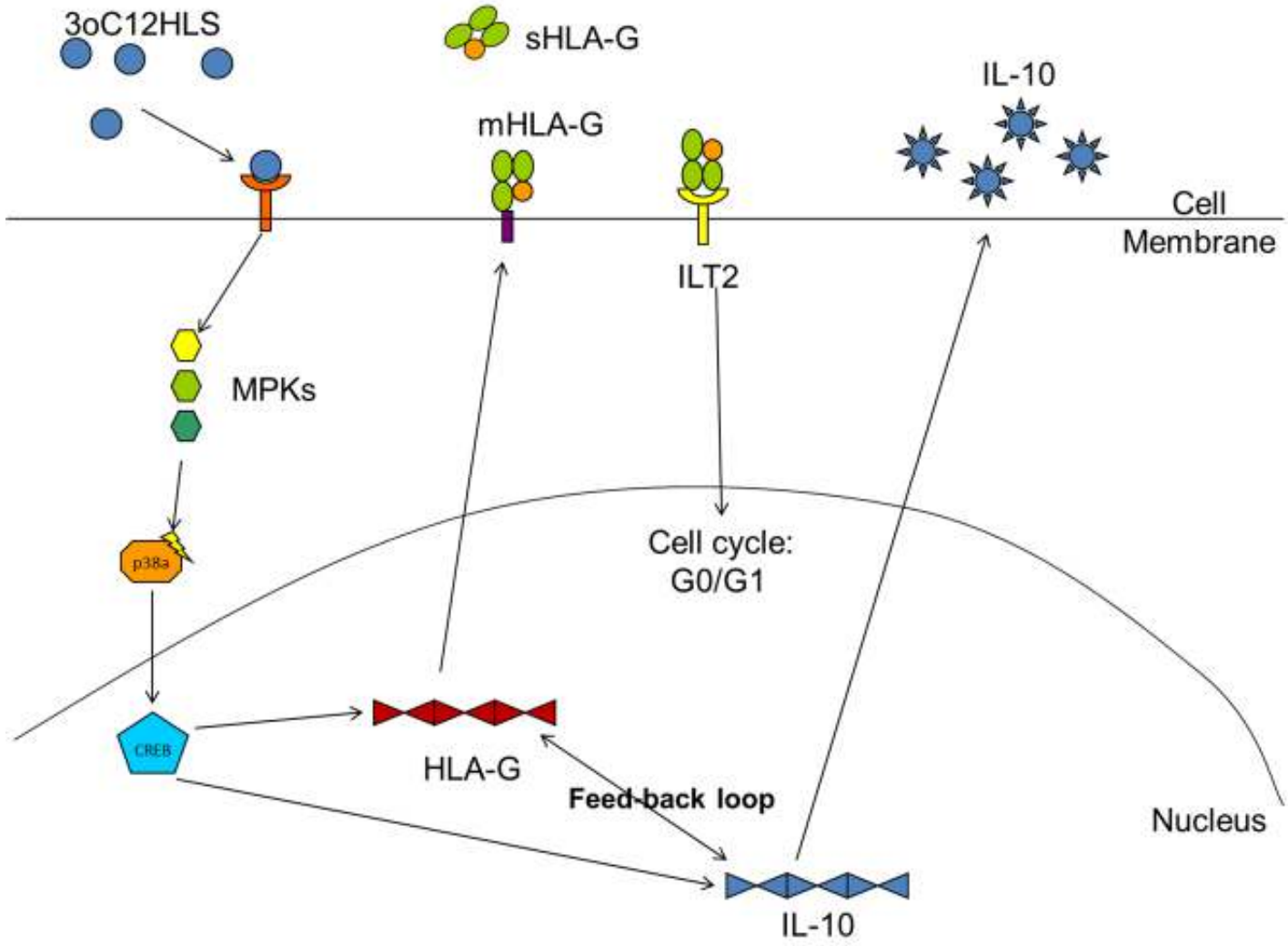

a)
3oC12HLS; uM
CD14 expression; Fold increase,MFI
10
25
PMA
3oC12HLS; uM
CD11b expression; Fold increase,MFI
10
25
PMA
b) U937
ILT2
CD14
c) THP1
ILT2
CD14
d) JURKAT
ILT2
CD3
e) U937
Viability;%
0
antiILT2
10
antiILT2
isotype
25
antiILT2
isotype
3oC12HLS; uM

Figure 10.


3oC12HLS
sHLA-G
IL-10
mHLA-G
Cell
Membrane
ILT2
MPKs
p38a
Cell cycle:
G0/G1
CREB
HLA-G
Feed-back loop
Nucleus
IL-10

1.2 HLA-G in autoimmune and inflammatory diseases

Inflammation is a localized protective reaction to different kind of injury and/or infections. The characteristic signs of inflammation include pain, heat, redness, swelling and loss of function that result from dilation of the blood vessels leading to an increased blood supply and intercellular spaces, movement of leukocytes, protein and fluids into the inflamed regions. Inflammatory response could be subdivided into: i) **acute inflammation**, with a rapid onset and a short duration. It is considered the main defense mechanism against bacteria, virus and parasites. It is characterized by the exudation of fluids and plasma proteins and the migration of leukocytes, most notably neutrophils into the injured area; ii) **chronic inflammation**, with a more prolonged duration and the presence of fibrosis and tissue necrosis. This persistent chronic inflammation facilitates the development of degenerative and autoimmune diseases such as rheumatological diseases, atherosclerosis, heart diseases, asthma, multiple sclerosis (MS), diabetes, inflammatory bowel diseases (IBD) [132,133], because of the persistent immune cell reactivity and cytokine secretion. The immune cell modulation due to the interacion between anti-inflammatory factors and immune cells and trascription elements plays a crucial role in the regulation of a chronic inflammation. One of these anti-inflammatory components is suggested to be HLA-G.

Given the immunomodulatory nature of HLA-G molecule, it could be considered a good reference parameter for prevention, diagnosis and treatment in autoimmune and inflammatory diseases.During my PhD period I investigated HLA-G in different pathologies characterized by a dysregulation in host immune system in which HLA-G plays a central role [79-82 papers attached, 83].

1.2.1 HLA-G in rheumatic diseases

More than 100 pathologies are generally described as Rheumatic disease referring to conditions that affect the joints (rheumatoid arthritis), connective tissues (scleroderma, systemic lupus erythematosus) and vessels (vasculitis). Rheumatic diseases are inflammatory and autoimmune diseases, that are the second most common cause of disability after musculoskeletal injuries. Rheumatoid arthritis (RA) (OMIM, #180300) is an autoimmune

disease caused by the attack of the immune system to synovial cells. RA is characterized by inflammation, pain and stiffness in the joints that can lead to a loss of function. Both genetic and environmental factors may contribute to develop the disease. Gene expression profiles (GEPs) in bone marrow-derived RA mononuclear cells [134] reported 1,910 down-regulated and 764 up-regulated gene, which include the *HLA-G* gene. In fact, many groups investigated the role of HLA-G in RA, underlining the importance of both sHLA-G and HLA-G polymorphisms in the disease course and treatment response [45, 135]. The role of HLA-G polymorphisms, and in particular the HLA-G 14bpINS/DEL polymorphism, has also been evaluated for RA susceptibility and for its use as marker for RA therapy. The most common therapy used in the treatment of RA patient is based on Methotrexate (MTX), a disease-modifying anti-rheumaticdrug (DMARD). MTX incresed IL-10 production in RA patients that exhibit a better therapeutic response [136] and is able to enhance HLA-G secretion by peripheral blood mononuclear cells [12]. Interestingly, RA patient with good response to MTX also showed an increased frequency of the 14bpDEL/DEL [45], with a possible implication in the control of immune activation. It must be underlined, however, that contrasting results have been obtained [137, 138], possibly due to a different dosage of MTX, a different cut-off value for RA therapy response assessment.

Concernig sHLA-G levels, protein concentration in serum is significantly lower in RA patients [137] than in controls. This low sHLA-G concentration could contribute to develope the disease leading to a chronic activation of inflammatory cells. The evaluation of sHLA-G molecules at the specific inflammation site of the synovia reported higher levels of sHLA-G in RA patients [139]. The release of HLA-G in the inflamed synovium may be due to the recruitment of activated HLA-G positive immune cells and the local production by activated synovial fibroblasts [140] that could interact with immune inhibitory receptors and maintain a chronic inflammatory response. These data suggest that there is a different production of HLA-G molecules on the basis of the local and systemic environments, characterized by different molecular factors and cell types. Interestingly, the role of HLA-G molecules in RA was confirmed by a recent work. The authors used an intracutaneous treatment of HLA-G monomer or dimer molecules in collagen induced arthritis model mice. The treatment with these molecules produced excellent anti-inflammatory effects with a single, local administration [141]. Notably, the dimer exhibited higher immunosuppressive effects than the monomer due to the higher dimer affinity for PIR-B, the mouse homolog of the LILRBs.

Basing on these data, we investigated the possible role of HLA-G molecules as biomarkers for RA treatment in a follow-up study [142 paper attached]. Twenty-three early RA (ERA) patients were analyzed during a 12mo follow-up disease treatment for sHLA-G levels in plasma samples, membrane HLA-G (mHLA-G) and ILT2 expression on peripheral blood CD14 positive cells, and typed for HLA-G 14bp DEL/INS polymorphism. Interestingly, the results showed that ERA patients with low sHLA-G and membrane HLA-G expression suffered a more severe disease. In fact, sHLA-G levels inversely correlated with DAS28 and ultrasonographic power Doppler scores, used to define the severity and progression of the disease. Surprisingly, sHLA-G up-modulation is already evident after 3 mo of DMARDs therapy, while a significant reduction in tumor necrosis factor-α levels is evident after 9 mo therapy. At this point of therapy a clear amelioration of the disease is evident, with a high specificity for HLA-G detection in EA condition. Moreover, the implication of the HLA-G 14bp INS/DEL polymorphism is confirmed as patients that showed a significantly improved disease status were characterized by the presence of the DEL allele.

This result corrobores the prognostic and therapeutic value of HLA-G in RA. In fact, considering al the different studies on HLA-G in RA, it seems that HLA-G molecule evaluation can help to predict the course of the disease [143 paper attached] .

Reference 142

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HLA-G may predict the disease course in patients with early rheumatoid arthritis

Roberta Rizzo[a,*], Ilaria Farina[b], Daria Bortolotti[a], Elisa Galuppi[b], Antonella Rotola[a], Loredana Melchiorri[c], Giovanni Ciancio[b], Dario Di Luca[a], Marcello Govoni[b]

[a] Department of Medical Sciences, Section of Microbiology, University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy
[b] Department of Medical Sciences, Section of Rheumatology, University of Ferrara, Via Aldo Moro 8, Cona (FE), 44121 Ferrara, Italy
[c] Department of Medical Sciences, Section of Medical Genetics, University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy

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ABSTRACT

The current management of early rheumatoid arthritis (ERA) is to start an intensive treatment as soon as possible. To avoid under/overtreatment, it is important to identify reliable ERA evolution biomarkers.

HLA-G molecules has been associated with rheumatoid arthritis, suggesting a role in disease regulation. HLA-G antigens are expressed as membrane bound and soluble isoforms (mHLA-G, sHLA-G) that act as ligand for immune-inhibitory receptors (ILT2, ILT4, KIR2DL4). Expression of HLA-G is influenced by a 14 bp insertion/deletion polymorphism in exon 8 of the gene, where the deletion is associated with mRNA stability.

We analyzed 23 ERA patients during a 12 months follow-up disease treatment for sHLA-G, IL-1beta, IL-6, IL-10 and TNF-alpha levels in plasma samples by ELISA, mHLA-G and ILT2 expression on peripheral blood CD14 positive cells by flow cytometry and typed HLA-G 14 bp deletion/insertion polymorphism by Real-Time PCR. Disease status (DAS28), ultrasonography with power Doppler and laboratory data were checked.

Cytokine levels confirmed the anti-inflammatory effect of the treatment. sHLA-G, mHLA-G and ILT2 expression inversely correlated with DAS28 disease scores. The frequency of 14 bp deletion allele increased in patients with disease remission.

Based on these results, HLA-G may be a candidate biomarker to evaluate early prognosis and disease activity in ERA patients.

1. Introduction

The term Early Arthritis (EA) is a generic definition which does apply to all arthritis with recent onset and includes a number of different diseases, among which early rheumatoid arthritis (ERA) should be carefully checked for, being one of the most crippling inflammatory disease. Rheumatoid arthritis (RA) (OMIM ID 180300) is a chronic systemic autoimmune inflammatory disease that mainly involves the joints and, if not adequately treated or even incompletely controlled by therapy, can lead to joint destruction, severe functional impairment and disability [1]. Joint damage occurs early in the course of the disease, and around 75% of patients with ERA develop erosive changes within the first 2 years of disease [2–4]. Controlled clinical trials have shown that an early aggressive therapeutic approach with disease-modifying anti-rheumatic drugs can slow or even stop the progression of damage in RA, so an early diagnosis is of capital importance [5,6]. Besides physical examination, which still remains the gold standard in identifying the presence of arthritis, musculoskeletal ultrasonography (US) and power Doppler (PD) have been proved to be helpful in detecting joint inflammation, especially at an early and subclinical stage [7] and to distinguish active from inactive synovitis [8]. The identification of significant biomarkers aimed to monitor patient therapy and to avoid under/overtreatment is of outstanding importance to assess and predict EA evolution. Rheumatoid factor (RF) and anti-cyclic citrullinated peptide (ACPA), when present, are well recognized prognostic factors for joint damage progression but their predictive value is still limited [1,2,5,6]. For these reasons new prognostic and reliable biomarkers are urgently needed.

A growing body of evidence has indicated a possible involvement of HLA (Human Leukocyte Antigen)-G antigens in RA where

Abbreviations: ACPA, anti-cyclic citrullinated peptide; ACR, American College of Rheumatology; CI, confidence interval; CV, coefficient of variation; DAS28, disease activity score-28; DMARDs, disease-modifying anti-rheumatic drugs; EA, early arthritis; ERA, early rheumatoid arthritis; EAC, early arthitis clinic; ESR, erythrocyte sedimentation rate; GH, global health; HLA-G, human leukocyte antigens-G; IL-1\6\10, Interleukin-1\6\10; ILT24, immunoglobuline-like transcript 2; KIR2DL4, killer cell immunoglobulin-like receptor 2DL4; MoAb, monoclonal antibody; OArth, other arthropathies; OR, odds ratio; PD, power Doppler; PCR, polymerase chain reaction; RA, rheumatoid arthritis; RF, rheumatoid factor; TNF-alpha, tumor necrosis factor-alpha; US-PD, ultrasonographic power Doppler.

* Corresponding author. Fax: +39 0532974470.
E-mail address: rbr@unife.it (R. Rizzo).

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this molecule seems to exhibit anti-inflammatory properties. HLA-G is a HLA-Ib molecule with a physiological tissue-restricted distribution in cytotrophoblast [9], amniotic cells, thymus, and endothelial cells of chorionic blood vessels [10]. HLA-G molecules are generated by an alternative splicing of the primary transcript of the gene [11]: HLA-G exists as four membrane-bound (HLA-G1, -G2, -G3 and -G4) and three secreted soluble isoforms (HLA-G5, -G6, -G7). HLA-G is characterized by tolerogenic functions, inducing apoptosis of activated $CD8^+$ T cells [12], acting on T regulatory cells [13], modulating the activity of natural killer cells [14] and of dendritic cells [15] and blocking allo-cytotoxic T lymphocyte response [16]. These immuno-regulatory functions are mediated by the interaction of HLA-G molecules with specific inhibitory receptors: ILT2 (LILRB1/CD85j), ILT4 (LILRB2/CD85d), CD8 and KIR2DL4 (CD158d) expressed by immune cells [17].

The HLA-G production is controlled by several polymorphisms both in the promoter and in the 3′ untranslated region (3′ UTR) modifying the affinity of gene targeted sequences for transcriptional or post-transcriptional factors, respectively. A 14 base pair (14 bp) insertion/deletion (INS/DEL) polymorphism in exon 8 involves mRNA stability and expression (rs1704). In particular, the INS allele is characterized by a mRNA destabilization and a consequent lower protein production [18].

Serum sHLA-G concentration is significantly lower in RA [19] and JIA patients than in controls [19] while higher sHLA-G is present in JIA synovial fluids (SF) [20]. Decreased sHLA-G serum concentrations may lead to a chronic activation of inflammatory cells and contribute to the development of autoimmune diseases. The high release of HLA-G in the inflamed synovium may be related to the recruitment of activated HLA-G positive immune cells that could interact with immune inhibitory ILT2 receptors, up-regulated in SF, and maintain a chronic inflammatory response. In fact, it has been reported that the production of HLA-G molecules is enhanced in synovial fibroblasts from inflamed joints, as a tentative compensatory mechanism to counteract the inflammation [21].

On the basis of these previous data we evaluated the role of HLA-G molecules both in disease immunopathology and as a biomarker of disease course and treatment response in early RA patients.

2. Materials and methods

2.1. Subjects

Thirty six consecutive patients attending the Early Arthritis Clinic (EAC) of the Rheumatology Section of the University of Ferrara underwent DMARD therapy and were prospectively followed for 1 year follow-up. 23 patients (17 women and 6 men; mean age 61.8 ± 12.5 years) met the criteria of the American College of Rheumatology (ACR) for the diagnosis of RA after 1 year follow-up from symptom onset [22]. The other 13 subjects presented other arthropathies (OArth), such as microcrystalline and undifferentiated polyarthritis. All patients were evaluated between May 2010 and May 2012 with a complete physical examination, including evaluation of the number of swollen and tender joints, calculation of the disease activity 28 joint score (DAS28), a composite index that includes erythrocyte sedimentation rate (ESR), patient self-reported global health (GH) status, the number of swollen and tender joints. Clinical and demographic data were also collected, as well as smoking habit, family history of rheumatic and/or cardiovascular diseases, comorbidities, extra-articular involvement, routine haematochemical parameters, RF and ACPA.

To identify the presence of active synovitis, all patients underwent an US-PD joint assessment at the same time as the clinical evaluation. US-PD examination was performed with the same scanner (MyLab 70, ESAOTE) using a multifrequency linear transducer (12–18 MHz). Bilaterally, eight joint regions were assessed: namely, the second and third metacarpophalangeal (MCP) joints and the wrist (inter-carpal and radiocarpal joints). All joints were analyzed according to the standard European League Against Rheumatism (EULAR) reference scans [23]. Each joint was graded from 0 to 3 based upon a semiquantitative scale (Grade 0: no synovial flow; Grade 1: isolated signals; Grade 2: confluent signal in less than half of the synovial area; Grade 3: confluent signals in more than half of the synovial area) [24]. Based on US-PD score, synovitis was considered severe (US-PD score: 3 in almost one joint), moderate (US-PD score > 1 < 3 in almost one joint) or mild (US-PD score: 1 in almost one joint). For each patient, the overall US-PD score was calculated by adding US-PD scores obtained from each joint (range 0–24) [25].

All patients were examined, for all parameters, at baseline before starting the DMARD therapy, and after 3, 6, 9 and 12 months of treatment. Clinical assessment and blood sampling were performed during routine clinics, with written informed consent and local ethical board approval.

Thirty healthy subjects (22 women and 8 men; mean age 52.8 ± 10.38 years) were recruited as control group .

2.2. Biological samples

Whole blood samples were collected from control subjects and before the DMARD therapy, after 3, 6, 9 and 12 months of therapy from EA patients. Plasma samples were obtained by centrifugation of blood specimens withdrawn by puncture of an anterocubital vein at the same time points. They were collected under sterile conditions, coded and stored in aliquots at −80 °C until use.

2.3. HLA-G polymorphism typing

EDTA blood (7–10 ml) was obtained from ERA patients. Genomic DNA was extracted from the EDTA blood using a Nucleon Bacc 3 Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. The HLA-G 14 bp polymorphism (14 bp INS/DEL) was genotyped by PCR performed as previously described [26]. Briefly 100 ng of genomic DNA were amplified in a 25 ul reaction performing the analysis using the 7300 Real-Time PCR System (Applied Biosystems). The forward primer, HLAG14-forw, was 5′-GTG ATG GGC TGT TTA AAG TGT CAC C-3′, and the reverse primer, HLAG14-rev, was 5′-GGA AGG AAT GCA GTT CAG CAT GA-3′. The probe used for detection of the 14 bp DEL allele was 5′-VIC-GAG TGG CAAGTC CCT TTG TG-BHQ-3-3′ (HLAG14VIC) and the probe for the 14 bp INS allele was 5′-Fam-CAA GAT TTGTTC ATG CCT TCC C-BHQ-1-3′ (HLAG14FAM). Amplification was performed with 0.625 µl Assay mix 40X (Applied Biosystem) and 12.5 µl PCR master mix 2X.

2.4. sHLA-G enzyme-linked immunosorbent assay (ELISA)

sHLA-G levels in plasma samples were assayed in triplicate as previously reported [27] using, as capture antibody, the monoclonal antibody (MoAb) MEM-G9 (Exbio, Praha, Czech Republic), which recognizes the HLA-G molecule, in β2-microglobulin associated form. The intra-assay coefficient of variation (CV) was 1.4% and the inter-assay CV was 4.0%. The limit of sensitivity was 1.0 ng/ml.

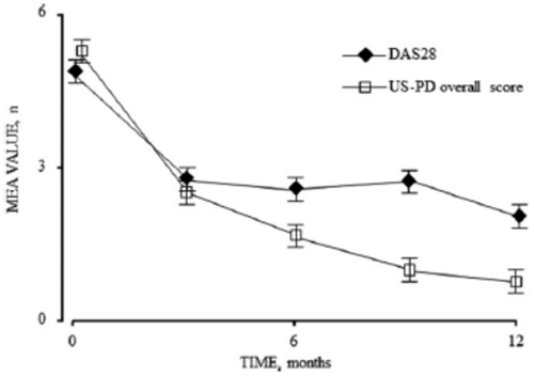

Fig. 1. ERA disease follow-up during DMARDs treatment. DAS28 score and US-PD overall score are reported during the 12 months follow-up.

2.5. Cytokine plasma levels

Interleukin (IL)-1 beta, IL-6, IL-10 and Tumor necrosis factor-alpha (TNF-alpha) [28] concentrations were determined in triplicate in 1:10 diluted plasma samples using the commercially available Human BioSource Immunoassay Kit (BioSource, Camarillo, CA, USA).

2.6. Cytometric analysis of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll gradient (Cederlane, Hornby, Ontario, Canada) and resuspended in RPMI medium (EuroClone, Milano, Italy) with 10% FCS, 100 U/ml penicillin and 100 U/ml streptomycin (Sigma–Aldrich, S. Louis, MO, USA). 10^6 PBMCs were resuspended in 1x PBS for analysis. Cells were analyzed by a flow cytofluorimetric approach with FACSCount flow cytometer (Becton Dickinson, San Jose, CA, USA) using standard settings and CellQuest software (Becton Dickinson, San Jose, CA, USA) for data analysis. Cells viability was assessed by propidium iodide staining. The membrane bound HLA-G antigens were detected by anti-HLA-G FITC MoAb (MEM-G/9; Exbio, Praha, Czech Republic) and ILT2 expression by anti-ILT2-FITC MoAb (ReD Systems, Minneapolis, MN, USA) in CD14 positive peripheral blood cells, recognized by anti-CD14-PE MoAb (Sigma–Aldrich, St. Louis, MO, USA). Anti-isotype controls (Exbio, Praha, Czech Republic) were performed.

2.7. Statistical analysis

The normality of each variable was checked using the Kolmogorov–Smirnov test. As normality of data distribution was rejected in several variables, continuous variables were compared using Kruskal–Wallis and Mann Whitney *U* tests and correlations were assessed by the Spearman rank correlation coefficient test. A logistic regression analysis was performed to evaluate the effect of confounding variables. HLA-G 14 bp INS/DEL frequencies were compared by Chi squared test. p-Value < 0.05 was considered to be statistically significant. The relative risk was estimated by calculating the odds ratio (OR) with confidence interval of 95%.

3. Results

3.1. sHLA-G plasma levels

Nineteen out of the 23 ERA patients presented a progressive improvement in their disease status during the 12 months therapy, as evidenced by the DAS28 and the overall US-PD score mean values at the different time points (Fig. 1). Four out of the 23 ERA patients did not present an improvement in the disease status.

When the sHLA-G plasma levels before the DMARD therapy was taken into account, specificity for ERA condition appears to be almost absolute. In fact, we observed the presence of sHLA-G in the 100% of ERA patients, in the 8% of the 13 subjects with other arthropathies (OArth) and in the 23% of controls (Table 1). The ROC curve evaluation reported an accuracy of 87.9%, a sensitivity of 100% and specificity of 81.4%. We compared the predictive value of sHLA-G in comparison with ACPA and RF plasma levels. As shown in Table 1, the ACPA was observed in the 74% ERA patients, 31% OArth subjects and 0% controls. The RF was detected in the 61% ERA patients, 0% OArth subjects and 3% controls. The ROC curve reported an accuracy of 84.8%, a sensitivity of 73.9% and a specificity of 90.7% for ACPA; an accuracy of 84.8%, a sensitivity of 60.9% and a specificity of 97.7% for RF. The combination of the three parameters decreased the test value (accuracy of 66.7%, sensitivity of 60.9%, specificity of 69.8%), while the co-evaluation of sHLA-G and ACPA positivities provided a test with an accuracy of 84.8%, a sensitivity of 73.9% and a specificity of 91.7%.

The sHLA-G levels before DMARD therapy were lower in ERA patients in comparison with control subjects ($p = 0.001$, Mann Whitney *U* test), while no differences were observed in comparison with OArth patients (Fig. 2).

The levels of sHLA-G molecules were also evaluated in the plasma samples collected after the initiation of DMARD therapy, and after 3, 6, 9 and 12 months of treatment. sHLA-G levels were correlated with different parameters as DAS28 and US-PD scores. We divided DAS28 scores into four subgroups (<2.6: remission; 2.6–3.2: low activity; >3.2 <5.1: moderate activity; >5.1: severe activity). We observed the highest sHLA-G levels in the remission group and the lowest concentrations in the severe group at the different time points (Fig. 3a). Similarly, patients with a US-PD mild synovitis (1) presented higher plasma sHLA-G levels than those with US-PD moderate (2) and US-PD severe (3) synovitis at all the different time points (Fig. 3b). The disappearance of severe synovitis after 3 months of therapy supports the beneficial effect of the treatment.

To evaluate the predictive role of sHLA-G levels in the therapy outcome, we analyzed the correlation between sHLA-G before the treatment and the decrease in DAS28 value after 6 and 12 months of therapy. We evidenced a positive correlation between sHLA-G levels and DAS28 decrease after 6 and 12 months of therapy (6 months: $p = 0.024$, rho: 0.569; 12 months:

Table 1
Positivity for ACPA, RF, sHLA-G, IL-1, TNF-alpha, IL-6 and IL-10 in control subjects (HC), early RA patients (ERA) and subjects with other arthrophaties (OArth).

	ACPA positive[a] *N* (%)	RF positive[a] *N* (%)	sHLA-G positive[a] *N* (%)	IL-1 positive[a] *N* (%)	TNF-alpha positive[a] *N* (%)	IL-6 positive[a] *N* (%)	IL-10 positive[a] *N* (%)
HC (30)	0 (0)	1 (3.3)	7 (23.3)	16 (53.3)	21 (70)	30 (100)	19 (63.3)
ERA (23)	17 (73.9)	14 (60.9)	23 (100.0)	14 (60.9)	17 (73.9)	23 (100)	15 (65.2)
OArth(13)	4 (30.8)	0 (0)	1 (7.7)	11 (84.6)	10 (76.9)	13 (100)	8 (61.5)

[a] Positive: detectable levels in plasma samples

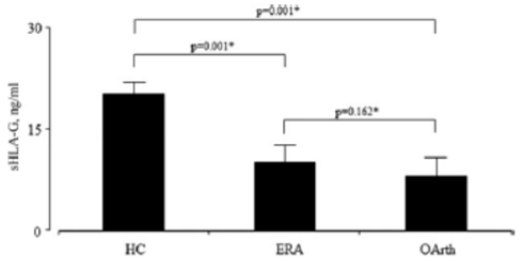

Fig. 2. sHLA-G plasma levels. sHLA-G plasma levels in control subjects (HC), ERA patients (ERA) and OArth subjects (OArth) before the therapy.*p values obtained by Mann Whitney *U* test.

$p = 0.0083$, rho: 0.603; Spearman correlation test). The logistic regression for confounding variables confirmed the predictive value of sHLA-G plasma levels ($p = 0.0008$).

3.2. Cytokine plasma levels

We evaluated the levels of IL-1beta, TNF-alpha, IL-6 and IL-10 cytokines [28]. IL-1beta was detected at baseline in 14 patients, TNF-alpha in 17 patients, IL-6 in 23 patients and IL-10 in 15 patients. The baseline levels of IL-1beta, TNF-alpha, IL-6 were higher in ERA patients than control subjects ($p = 0.012$, $p = 0.002$, $p = 0.001$ respectively, Mann Whitney *U* test) (Fig. 4a). No difference was reported in comparison with OArth subjects concentrations. IL-10 levels were higher in control subjects than in ERA patients ($p = 0.014$).

We evaluated the effect of DMARD on inflammatory condition by cytokine quantification. We observed a decrease in IL-1beta ($p = 0.013$, Mann Whitney *U* test), TNF-alpha ($p = 0.011$) and IL-6 ($p = 0.005$) levels while IL-10 increased ($p = 0.006$) during 12 months therapy (Fig. 4b). These modification in cytokine levels support an anti-inflammatory effect of DMARDs, by decreasing Th1 cytokines (IL-1beta, TNF-alpha, IL-6) and increasing Th2 cytokine (IL-10).

3.3. Membrane HLA-G and ILT-2 expression

Since CD14 positive monocytes were shown to be the main peripheral blood HLA-G producers in physiological conditions [29], we analyzed their membrane HLA-G expression in ERA patients. In Fig. 5 two representative results are reported: (a) patient 6 characterized by a reduction of 32% in the DAS28 score after the therapy; (b) patient 3 with an unmodified DAS28 score after DMARD treatment.

CD14 positive cell HLA-G expression increased of 50% in patient 6 after 12 months therapy (Fig. 5a upper panel), while no modification was shown in patient 3 (Fig. 5b, upper panel).

Since ILT2 is the main HLA-G receptor [17], we analyzed the receptor expression during DMARD therapy. ILT-2 expression followed the same trend as HLA-G. In particular, patient 6 presented a 56% increase after the therapy (Fig. 5a, low panel), while a slight increase of 12% was evidenced in patient 3 (Fig. 5b, low panel).

3.4. HLA-G 14bp INS/DEL polymorphism frequency

Since HLA-G 14bp INS/DEL polymorphism is implicated in HLA-G expression control [18], we evaluated the influence of the HLA-G genetic background in the DMARD treatment follow-up. We observed a prevalence of the 14 bp DEL allele, high HLA-G producer, in patients which reached a remission after 12 months of therapy, as documented by DAS28 score (<2.6) (OR: 1.9; CI 95%: 0.49–5.73) (Fig. 6).

4. Discussion

In ERA, sustained high disease activity results in a poor outcome [5,6]. Successful management aimed to suppress inflammation to

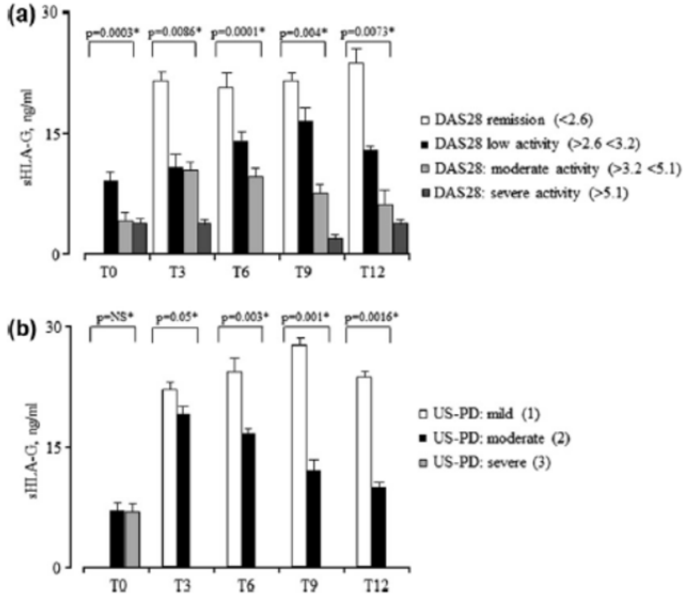

Fig. 3. sHLA-G plasma levels during DMARDs treatment. sHLA-G levels are reported during the 12 months (time 0, 3, 6, 9, 12 months) follow-up subdividing the ERA patients on the basis of (a) DAS28 and (b) US-PD scores. *p values obtained by Kruskal-Wallis *U* test.

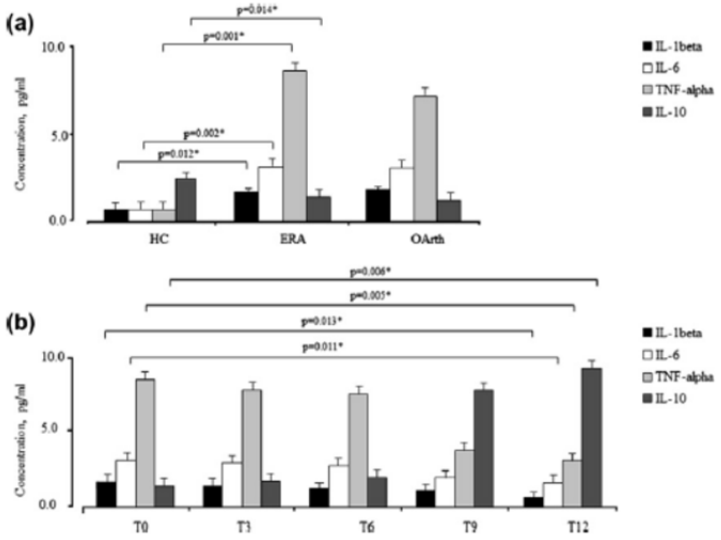

Fig. 4. Cytokine plasma levels during DMARDs treatment. (a) IL-1beta, IL-6, TNF-alpha, IL-10 plasma levels in control subjects (HC), ERA patients (ERA) and OArth subjects (OArth) before the therapy. (b) IL-1beta, IL-6, TNF-alpha, IL-10 levels are reported during the 12 months follow-up disease treatment (time 0, 3, 6, 9, 12 months). *p values obtained by Mann–Whitney U test.

reach a remission or low disease activity status, needs regular assessments of disease activity and a coherent appropriate treatment adjustment. This approach is now recognized as the "treat to target" and "tight control" strategies which represent the recommended management in ERA [30,31]. For this purpose, rheumatologists have now widely adopted clinimetric instruments, such as DAS28 and advanced imaging techniques, such as US-PD, to assess and monitor disease activity not only in clinical trials but also in daily practice. These parameters are validated instruments in determining and evaluating disease status in RA, ERA and EA. Nevertheless, the scores need to be interpreted with some caution, given the subjectivity and the limited sensitivity of the clinical assessments. It is necessary to determine new biomarkers that could be evaluate with objective assays. In this study we analyzed the role of HLA-G molecules both in disease immunopathology and as a biomarker of disease course and treatment response in ERA patients.

We observed that ERA patients with both low sHLA-G and membrane HLA-G expression suffered a more severe disease. In fact, HLA-G levels inversely correlated with DAS28 and US-PD scores. Given the immune-modulating effect of HLA-G these data also sustain a possible implication of HLA-G molecules in the disease physiopathology. It was previously reported that serum sHLA-G concentration is significantly lower in ERA patients than in controls [20,21] and that DMARD thearpy is able to modify HLA-G secretion [32]. As mentioned above, HLA-G antigens are characterized by anti-inflammatory and immuno-inhibitory functions, thus the presence of these molecules could affect disease activity. We confirmed the anti-inflammatory effect of DMARD by cytokine evaluation [33]. The levels of Th1 cytokines (IL-1beta, IL-6 and TNF-alpha) decreased during the 12 months therapy, while Th2 cytokine (IL-10) increased the levels [34]. These results suggest that DMARD therapy may exert its disease modifying effect partly by suppressing Th1 cytokine production and inducing an anti-inflammatory environment. Indeed, RA is characterized by a marked increase in Th1 mediated immunology [35], where Th1 cytokines are important in inflammation perpetuation. Interestingly, CD4 positive T cells from ERA patients manifest a profound inability to mount Th2 responses [36]. It is known that HLA-G production maintains a feed-back loop-interaction with IL-10 secretion [37], creating an anti-inflammatory network, that could improve RA patient condition. We hypothesize a direct role of HLA-G in early therapy outcome, since HLA-G up-modulation was consistent after 3 months therapy (Fig. 3a and b) while significant modifications in TNF-alpha and IL-10 were evident after 9 months therapy and IL-1beta and IL-6 decreased after 12 months (Fig. 4a and b).

The novelty of this prospective study consists in the analysis of ERA patients for both genetics and HLA-G expression before and during the therapy. The implication of the HLA-G 14 bp INS/DEL polymorphism is confirmed, as the presence of the DEL allele characterized the patients with a significant improvement in disease status. These data are in agreement with the previous results on 156 DMARD-treated RA patients [32], which showed an increase in HLA-G 14 bp DEL/DEL genotype in patients with a reduction in disease activity after six months of treatment. In contrast to this study, there are two researches with negative results: (i) 130 RA patients presented no significant difference in 14 bp DEL/INS allelic and genotypic distribution in patients responsive to DMARDs [38]; (ii) 186 RA patients, prospectively followed during six months of treatment [39] presented no significant association between HLA-G 14 bp INS/DEL and DMARD efficacy. Comparing these studies, the opposite results may reflect population differences in gene expression, that could influence the power of association studies and lead to different levels of association. In addition, the different doses of MTX and the different cut-off used for RA therapy response definition could affect the results obtained.

Taking into account these considerations, another strength of this study is the evaluation of early RA patients naives from previous treatment. This approach, ruling out possible confounding factors, allows the correct evaluation of the role of HLA-G as a true and reliable biomarker for ERA disease status and treatment. Moreover, the specificity for ERA condition is almost absolute. In fact, we observed the presence of sHLA-G in the 100% of ERA patients, in comparison with the 8% of subjects with other arthropathies and the 23% of controls. The ROC curve analysis reported an accuracy

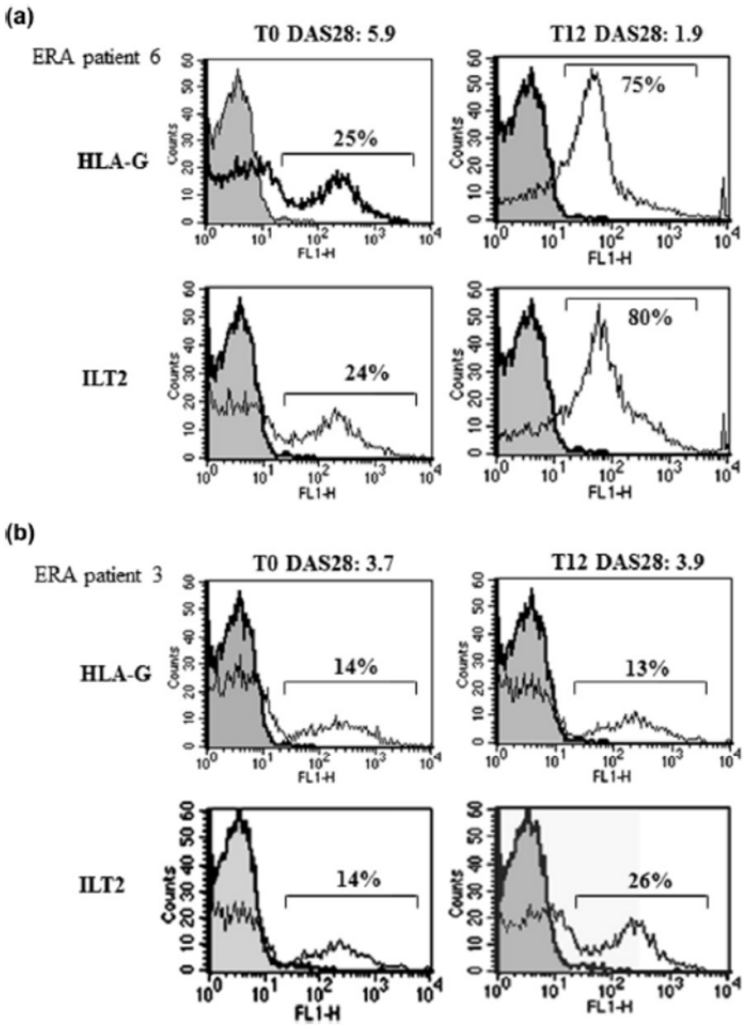

Fig. 5. Membrane HLA-G and ILT2 analysis by flow cytometry. Two representative ERA patients are reported: (a) patients 6 characterized by a reduction of 32% in the DAS28 score after the therapy; (b) patient 3 with an unmodified DAS28 score after DMARD treatment. The HLA-G and ILT2 expression was evaluated on CD14 positive peripheral blood cells.

of 87.9%, a sensitivity of 100% and specificity of 81.4%. These values are comparable with ACPA and RF positivities, which are well known plasma markers for ERA disease. In particular the co-evaluation of sHLA-G and ACPA positivities provides a test with an accuracy of 84.8%, a sensitivity of 73.9% and a specificity of 91.7%. These results suggest the potential use of both these molecules in the prognostic evaluation of EA patients and treatment follow-up. The combination of these two biomarkers increases the specificity of the evaluation, that is the main target in biomarker identification. HLA-G could be an important biomarker not only for EA patient identification at risk to develop RA (i.e. ERA) but also for treatment follow-up. In this context, the availability of new, reproducible and standardized biomarkers could be of interest in ERA clinical management. We showed a clear association between sHLA-G levels before the therapy and the disease follow-up after 12 months of DMARD. In particular, there was an increase in sHLA-G levels in patients with an improvement in disease status during the therapy. The membrane HLA-G expression by CD14 positive peripheral blood monocytes followed the same trend, with a clear modification during the therapy. We observed a consistent up-modulation of ILT2 receptor too. ILT2 is the main HLA-G receptor, implicated in the immunoinhibitory and anti-inflammatory HLA-G functions. It is known that its expression is up-regulated by HLA-G in immune cells [17]. Indeed, we revealed a significant increase in ILT2 expression during the DMARD therapy in patients with an improvement in disease status. These results suggest the implication of HLA-G also in RA pathogenesis. In fact, the presence of ILT2 receptor is essential for HLA-G activity. The ILT2/HLA-G co-expression in ERA patients could sustain an immunoregulatory environment, that is fundamental for disease activity control. A disequilibrium in this setting would maintain an inflammatory and immune-disregulated condition, typical for RA disease.

Patients with recent-onset RA develop joint damage very early and when the disease becomes clinically evident, it is already in the chronic phase. The time the joints are exposed to the pathological process therefore plays a decisive role so that making an early

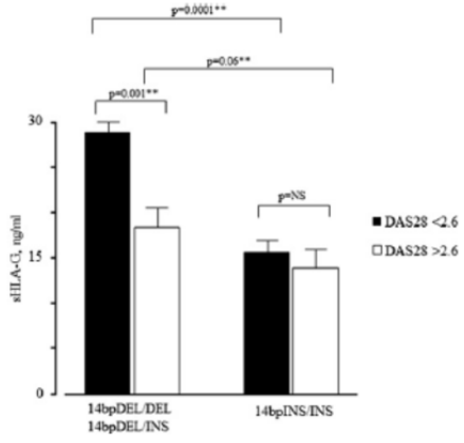

Fig. 6. Distribution of HLA-G 14 bp INS/DEL polymorphism in ERA patients. ERA patients were subdivided on the basis of DAS28 score in remitting patients (<2.6) and active disease patients (>2.6). HLA-G 14 bp INS/DEL frequencies are reported. **Chi squared test.

diagnosis, a concrete prognosis and undertaking appropriate treatment is of outstanding importance. The results obtained in this study, if confirmed in a larger number of patients, may be extremely important, given the ability of HLA-G to provide useful information for the clinical management of the RA patient in early phases of the disease, in that it seems able to evaluate the real status of patients and to predict the possible evolution.

Conflict of interests

None to be declared

Submission declaration

The manuscript has not previously been published, it is not under consideration for publication elsewhere and its publication is approved by all the authors.

Contributors

Roberta Rizzo, Daria Bortolotti, Antonella Rotola, Loredana Melchiorri: performed the analysis.

Ilaria Farina, Elisa Galuppi, Giovanni Ciancio: recruited the patients, performed the clinical evaluations.

Dario Di Luca, Marcello Govoni, Roberta Rizzo: planned the research, wrote the paper.

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Can HLA-G predict disease course in rheumatoid arthritis patients?

Rheumatoid arthritis remains a major clinical problem, with many patients having persistent systemic inflammatory disease, resulting in progressive erosive joint damage and high levels of disability. The goal of rheumatoid arthritis therapy has shifted to initiate treatment early and aggressively to achieve remission, or low disease activity as quickly as possible. To achieve this 'treat-to-target' concept, it is necessary to identify new biomarkers for disease progression and treatment follow-up, and to identify new therapeutic targets. The focus of this review is to summarize current research regarding the expression of HLA-G molecules in rheumatoid arthritis and the possible implications for the future management of the disease.

KEYWORDS: biomarker disease-modifying antirheumatic drugs early arthritis HLA-G rheumatoid arthritis

Roberta Rizzo*[1], Daria Bortolotti[1], Olavio R Baricordi[1], Ilaria Farina[2], Melissa Padovan[2] & Marcello Govoni[2]

[1]Department of Medical Sciences, Section of Microbiology & Medical Genetics, University of Ferrara, Ferrara, Luigi Borsari, 46-44121 Ferrara, Italy
[2]Department of Medical Sciences, Section of Rheumatology, University of Ferrara, Ferrara, Italy
*Author for correspondence:
Tel.: +39 053 245 5332
Fax: +39 053 297 4470
rbr@unife.it

Rheumatoid arthritis (RA; Online Mendelian Inheritance in Man [OMIM], #180300 [201]) is a chronic systemic inflammatory autoimmune disease causing symmetrical polyarthritis of the large and small joints. It affects 0.5–1.0% of the general population in the developed world, mainly between the ages of 30–50 years, and it is 2.5-times more common in women than men. It is clinically characterized by joint pain, stiffness and swelling due to synovial inflammation, and effusion [1]. Patients may develop extra-articular symptoms including fever, fatigue, anemia, interstitial lung involvement, vasculitis, nodules and osteoporosis, and show an elevation of acute-phase reactants such as C-reactive protein and erythrocyte sedimentation rate [2]. The disease can be a fluctuating or progressive course, which can result in joint destruction, deformity, disability and premature death.

The etiology of RA is not fully understood but is believed to result from interactions between genetic, infectious and environmental factors, where a triggering event, possibly an autoimmune or infectious response, initiates joint inflammation, causing a complex immune response that eventually leads to RA complications. Proinflammatory cytokines such as TNF-α, IL-1 and IL-6 play an important role in the pathogenesis of RA.

Managing RA

RA treatments include disease-modifying antirheumatic drugs (DMARDs) and more recently, biological agents. Traditionally, first-line treatment incorporates conventional DMARDs that counteract inflammatory processes and slow disease progression [3]. These include methotrexate (MTX) as an 'anchor drug', leflunomide, sulfasalazine and hydroxychloroquine. Nowadays, for patients who do not respond to conventional DMARDs, biological agents are available and include TNF-α inhibitors (infliximab, adalimumab, golimumab, certolizumab and etanercept), IL-1 inhibitors (anakinra), selective modulators of T-cell activation (abatacept), CD20 B-cell depleting agent (rituximab) and IL-6 inhibitors (tocilizumab), as well as small-molecules inhibitors (tofacitinib) [4].

The past decade has seen a tremendous amount of change in the field of rheumatology. The treatment of RA is undergoing steady change as new medications are approved and new regimens are attempted. Patients can now be diagnosed, treated and expect a high quality of life, with less joint damage and fewer disabilities. An important goal of RA therapy has shifted to initiate treatment early and aggressively to achieve remission or low disease activity as quickly as possible. This 'treat-to-target' concept has shown to maximize long-term healthy life [5].

The recent revision in RA classification criteria and updated recommendations for treatments are useful to diagnose RA patients at an earlier point in the disease course [6]. The concept of achieving tight control of RA and treating-to-target has been well established and utilizes early diagnosis and aggressive treatment. Regular monitoring requires the availability of new markers for disease progression and treatment follow-up, in order to improve the efficacy of the RA management, thus reducing the clinical and social costs. This review summarizes current research regarding the

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expression of HLA-G molecules in RA and their possible implications for the future management of the disease (Table 1).

HLA-G antigen

HLA-G is a MHC class I antigen encoded by a gene on chromosome 6p21. It differs from classical HLA class I molecules due to limited polymorphisms in the coding region, 50 alleles (IMGT HLA database [202]) and 16 proteins and a restricted tissue distribution. Seven HLA-G isoforms exist owing to mRNA alternative splicing and differential association with β2-microglobulin. Two isoforms are found on cell surfaces and in biological fluids; membrane-bound G1 and soluble G5, which lacks the transmembrane and intracellular domains of membrane-bound G1 (Figure 1) [7]. *HLA-G* possesses an unpaired cysteine residue at position 42 on an external loop of the peptide binding groove that enables the dimerisation [8,9]. HLA-G monomers are recognized by the inhibitory receptors LILRB1 and LILRB2 and by KIR2DL4, but leukocyte Ig-like receptors present a greater affinity for the dimeric form [9]. The interaction of HLA-G molecules with inhibitory receptors induces apoptosis of activated $CD8^+$ T cells [10], modulates the activity of NK cells [11,12] and dendritic cells (DC) [13,14], blocks allocytotoxic T-lymphocyte responses [8,15] and induces expansion of suppressor T-cell populations, such as $CD4^+$, $CD25^+$, FOX-$P3^+$ and Treg cells [16,17]. Moreover, HLA-G is expressed at high levels on DC-10 cells, human DCs with tolerogenic activity and an outstanding ability to produce IL-10 [14]. Interestingly, the expression of membrane-bound HLA-G1 and that of its receptors is upregulated by IL-10 on DC-10 and the expression of high levels of membrane-bound HLA-G1, ILT4 and IL-10 by DC-10 is critical to the generation of allergen-specific Tr1 cells by DC-10 [14].

The HLA-G production is controlled by several polymorphisms both in the promoter and in the 3′ untranslated region modifying the affinity of gene-targeted sequences for transcriptional or post-transcriptional factors, respectively [18].

In total, 29 single nucleotide polymorphisms (SNPs) have been identified in the *HLA-G* promoter region, which may be involved in the regulation of *HLA-G* expression, considering that many of these polymorphisms are within or close to known or putative regulatory elements. The *HLA-G* 5′ upstream regulatory region is unique

Table 1. Updated literature on the role of HLA-G in rheumatoid arthritis.

Study (year)	Samples	Technique	Results	Ref.
Lee *et al.* (2011)	Bone marrow-derived mononuclear cells from nine RA and ten osteoarthritis patients	Gene-expression profiles by DNA microarray	*HLA-G* gene is upregulated in RA patients	[66]
Veit *et al.* (2008)	Blood samples from 106 juvenile idiopathic arthritis patients, 265 RA patients, 356 healthy adults and 85 healthy children	PCR	Increased frequency of the 14 bp DEL allele in juvenile idiopathic arthritis patients	[68]
Verbruggen *et al.* (2006)	Plasma samples from 106 RA patients (80 women and 26 men) and control plasma samples were obtained from 56 healthy men and 48 women	ELISA	sHLA-G levels are lower in RA patients in comparison with controls. sHLA-G levels increase in correlation with disease activity and are affected by the presence of disease-predisposing HLA	[79]
Rizzo *et al.* (2006)	Peripheral blood mononuclear cells from healthy individuals and non-MTX-treated RA patients	ELISA and PCR	MTX induces the sHLA-G molecules. A significant association is observed between the highest levels of sHLA-G and the 14 bp DEL/DEL genotype	[39]
Bakir-Gungor and Serzerman (2011)	Genomic DNA	GWAS	*HLA-G* is a gene associated with RA	[67]
Rizzo *et al.* (2012)	Plasma sample and peripheral blood mononuclear cells from 23 MTX-treated ERA patients	ELISA, Flow cytometry, PCR	sHLA-G upregulation is evident after a 3 month course of disease-modifying antirheumatic drugs, with a positive follow-up	[77]
Kuroki *et al.* (2013)	Collagen-induced arthritis model mice	Intracutaneous treatment with HLA-G monomer or dimer molecules	HLA-G monomer or dimer molecules have produced excellent anti-inflammatory effects with a single, local administration	[74]

DEL: Deletion; ERA: Early rheumatoid arthritis; GWAS: Genome-wide association studies; MTX: Methotrexate; RA: Rheumatoid arthritis; sHLA-G: Serum HLA-G.

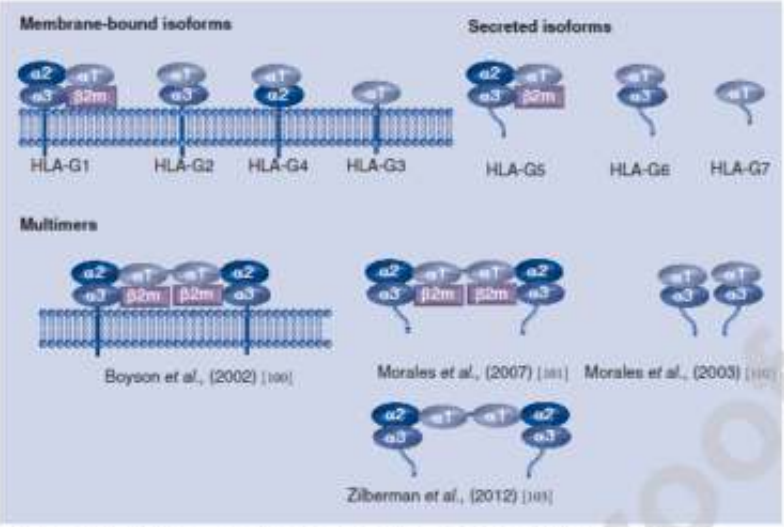

Figure 1. HLA-G isoforms and conformations. Membrane and soluble HLA-G isoforms are reported as monomeric and dimeric conformation.
Data taken from [100–103].

among the HLA genes [19] and is unresponsive to NF-κB [20] and IFN-γ [21], owing to the presence of a modified enhancer A and a deleted interferon sequence responsive element. A locus control region located -1.2 kb from exon 1 exhibits a binding site for CREB1 factor, which also binds to two additional cyclic AMP response elements at -934 and -770 positions from the initiation codon ATG. In addition, a binding site interferon sequence responsive element for IRF-1 is located at the -744 Base pair (bp) position [22] and is involved in *HLA-G* transactivation following IFN-β treatment [23]. The *HLA-G* promoter also contains a heat shock element at the -459/-454 position that binds HSF-1 [25] and a progesterone receptor binding site at -37 bp from ATG [24]. Several promoter region polymorphisms coincide with, or are close to, known or putative regulatory elements and thus may affect the binding of *HLA-G* regulatory factors [25]. The -725C>G/T SNP is very close to interferon sequence responsive element, in which the -725G allele is associated with a significantly higher expression level compared with the other alleles [26]. The polymorphic sites at the 5′ upstream regulatory region are frequently in linkage disequilibrium with the polymorphic sites identified at the 3′ untranslated region, some of which influencing alternative splicing and mRNA stability.

A 14 bp insertion/deletion (INS/DEL) polymorphism (rs66554220) in exon 8 involves mRNA stability and expression [27,28]. In particular the DEL alleles stabilize the mRNA with a consequent higher HLA-G expression [28,29]. The presence of adenine at position +3187, modifies an AU-rich motif in the *HLA-G* mRNA; decreasing its stability [30]. One SNP C>G at the +3142 bp position (rs1063320) has been explored by Tan and coauthors [31]. The presence of a guanine at the +3142 position may influence the expression of the *HLA-G* locus by increasing the affinity of this region for the miRNAs: miR-148a, miR-148b and miR-152; therefore, decreasing the mRNA availability by mRNA degradation and translation suppression. The influence of the +3142G allele has been demonstrated by a functional study in which *HLA-G* high-expressing JEG-3 choriocarcinoma-derived cells have been transfected with miR-148a, decreasing soluble HLA-G levels. The contrasting results obtained by Manaster and coauthors [32], who have reported the absence of +3142C>G effect on the miRNA control of membrane *HLA-G* expression, prompt further considerations on the relationship between this polymorphism and membrane *HLA-G* expression. Other SNPs are identified as implicated in miRNA interaction. In particular, +3003, +3010, +3027 and +3035 SNPs are influenced by miR-513a-5p, miR-518c*, miR-1262 and miR-92a-1*, miR-92a-2*, miR-661, miR-1224-5p and miR-433 miRNAs [33]. The miR-2110, miR-93,

miR-508–5p, miR-331–5p, miR-616, miR-513b and miR-589* miRNAs target the 14 bp INS/DEL fragment region, and miR-148a, miR-19a*, miR-152, miR-148b and miR-218-2 also influence the +3142 C/G polymorphism.

HLA-G is a stress-inducible gene: heat shock, hypoxia and arsenite increase different *HLA-G* alternative transcripts [34,35]. The indolamine 2,3-dioxygenase, an enzyme which metabolizes tryptophan, induces *HLA-G* expression during monocyte differentiation into dendritic cells [36]. The anti-inflammatory and immunosuppressive IL-10 has been correlated with concomitant *HLA-G* expression [28,37]. Transactivation of *HLA-G* transcription has also been demonstrated by leukemia inhibitory factor [38], progesterone [24] and MTX [39] cell exposure. Furthermore, IFN-α, IFN-β and IFN-γ enhance HLA-G cell-surface expression by tumors or monocytes [40,41]. HLA-G expression could be acquired by trogocytosis, where a 'donor' cell that expresses membrane HLA-G exchanges membrane parts containing *HLA-G* with a 'recipient' cell, that is not expressing HLA-G molecules. In this particular situation, 'recipient' cells will acquire and make use of membrane HLA-G molecules from a 'donor' HLA-G-positive cell without the activation of *HLA-G* gene transduction into protein. Trogocytosis of HLA-G1 molecules expressed by antigen-presenting cells and transferred to T cells in humans, makes T cells unresponsive [42]. It has been demonstrated that HLA-G1 can be acquired by NK cells from tumor cells. NK cells that acquire HLA-G1 stop proliferating, are no longer cytotoxic and therefore behave like suppressor cells capable of inhibiting other NK-cell functions [43].

The role of *HLA-G* in immune-tolerance has been discovered by studying its expression in trophoblast cells at the fetus–maternal interface [44]. The importance of HLA-G production by placental trophoblasts is evident in pre-eclampsia and unexplained recurrent spontaneous abortion. Several studies have found an aberrant or reduced transcription and protein expression of HLA-G in pathological placentas in comparison with control samples [45–47] with a possible implication in fetal protection and vascular events.

HLA-G expression has been documented in a number of tissues, such as cornea, thymus, erythroid and endothelial precursors, during physiological processes [48–50] and in a variable percentage of serum/plasma samples from healthy subjects [51], where the main producers are activated $CD14^+$ monocytes [52]. A modified expression of HLA-G molecules have been observed during 'nophysiological' conditions, such as viral infection [53–56], cancer [57–58], transplantation [59–63], and inflammatory and autoimmune diseases [64,65]. Recently, the role of HLA-G molecules in inflammation has gained a scientific and clinical interest as a proposed molecular biomarker and a possible therapeutic target.

HLA-G in RA

Bone marrow mononuclear cells from RA patients present an abnormal regulatory networks in the immune response [66]. Gene-expression profiles in bone marrow-derived RA mononuclear cells have shown 1910 downregulated and 764 upregulated genes, which include the *HLA-G* gene. A confirmation was obtained by Bakir-Gungor and Sezerman [67] using genome-wide association studies (GWAS), where they identified *HLA-G* as a gene associated with RA. To understand the role of *HLA-G* in RA, several studies on both gene polymorphisms and protein expression have been conducted. The genetic analysis of the *HLA-G* 14 bp INS/DEL polymorphism and two-promoter SNPs (rs1736936, -1202T/C and rs2735022, -586C/T) in *HLA-G* gene reported no allelic and genotypic differences in RA patients subdivided according to disease characteristics and development [68,69]. On the contrary, the analysis of patients with juvenile idiopathic arthritis (JIA; OMIM, #604302 [201]), the most common form of persistent arthritis in children, have shown a significant correlation between the 14 bp DEL allele and JIA susceptibility in females when compared with controls of the same gender [68]. These data exclude any implication of *HLA-G* genetic background in RA, although they do support a possible role in JIA. These observations support the presence of different physiopathogenic pathways between RA and JIA. Moreover, RA and JIA present different HLA associations, demonstrating that the immunogenetic factors involved in susceptibility to these two diseases are different. At a protein level, serum HLA-G (sHLA-G) concentration is significantly lower in both RA [70] and JIA [71] patients than in controls. The similar decrease in sHLA-G concentrations may lead to a chronic activation of inflammatory cells and contribute to the development of these two diseases. We can hypothesize that low levels of sHLA-G are not able to maintain an anti-inflammatory and immune-regulated systemic environment, which could worsen the disease development. Interestingly, even though JIA susceptibility in females correlates with the high producer 14 bp DEL allele, the secretion of HLA-G is limited in the serum. These data suggest a stronger implication of

protein transduction mechanisms compared with a genetic background. The evaluation of sHLA-G molecules at the specific inflammation site of the synovia, reported higher levels of sHLA-G in both RA and JIA patients [71,72]. The release of HLA-G into the inflamed synovium may be related to the recruitment of activated HLA-G-positive immune cells and the local production by activated synovial fibroblasts [73], which could interact with immune-inhibitory receptors and maintain a chronic inflammatory response. Interestingly, in a group of RA patients with HLA specificities associated with the disease (*HLA-DRB1*01*, *HLA-DRB1*04* and *HLA-DRB1*10*), the sHLA-G levels are higher and positively correlated with disease activity parameters, such as C-reactive protein and the number of swollen joints [70]. The highest sHLA-G concentrations may represent the inability to control inflammation and confirm the upregulation of *HLA-G* gene transcription observed by gene-expression profile analysis [66]. These data suggest that there is a different production of HLA-G molecules on the basis of the local and systemic environments, characterized by different molecular factors and cell types. Notably, further research is needed to evaluate the role of HLA-G molecules in these different compartments and in RA development.

Interestingly, a recent work has confirmed the role of HLA-G molecules in RA. The authors made an intracutaneous treatment of collagen-induced arthritis mouse model with HLA-G monomer or dimer molecules. These molecules were able to produce excellent anti-inflammatory effects with a single, local administration [74]. Notably, the dimer has exhibited higher immunosuppressive effects in comparison with the monomer conformation owing to the higher affinity of the dimers for paired immunoglobulin-like receptor B, the mouse homolog of the LILRBs. These data should be confirmed by further experiments, but support the use of HLA-G dimers as a useful anti-RA agent, in small amounts with minimal side effects.

HLA-G as a marker of RA treatment

HLA-G expression in inflammatory and autoimmune diseases is a relatively new area of investigation. The specific role of HLA-G molecules in the control of inflammation and immune response suggest an implication in both risk and disease chronicization, where this antigen is characterized by an impaired expression, depending on the different disease environment.

The *HLA-G* 14 bp INS/DEL polymorphism has been evaluated as pharmacogenetic marker of RA therapy. MTX, the major DMARD, is implicated in the increased production of IL-10 in patients with RA, which correlates with better therapeutic response [39]. IL-10 is one of the most efficient inducers of HLA-G secretion by peripheral blood monocytes, activated by lipopolysaccharides and with the highest levels in the 14 bp DEL/DEL genotype [37]. The analysis of the *HLA-G* 14 bp INS/DEL polymorphism in 156 MTX-treated RA patients has demonstrated an increase of the 14 bp DEL/DEL genotype in the responder group, characterized by a reduction in disease activity score in 28 joints (DAS28) measured before and after 6 months of treatment with MTX [38]. In contrast to this study, there are two studies with negative results: in one study, 130 RA patients have presented no significant difference in 14 bp DEL/INS allelic and genotypic distribution in patients responsive to MTX (DAS28: <3.2) [75]; in the second study, 186 RA patients, who had never been treated with MTX, were prospectively followed and were considered to be responders, with a DAS28 of up to 2.4 after 6 months of treatment [76]. No significant association between *HLA-G* 14 bp INS/DEL and MTX efficacy has been observed. Comparing these studies, the opposite results may reflect population differences in gene expression, which could influence the power-of-association studies and lead to different levels of association. In addition, the different doses of MTX and the different cutoffs used for RA therapy response definition could affect the results obtained.

Interestingly, Rizzo and coauthors [77] have evaluated the possible role of HLA-G molecules as biomarkers for RA treatment in a follow-up study. In total, 23 early RA (ERA) patients were analyzed during a 12-month follow-up of disease treatment for sHLA-G levels in plasma samples, mHLA-G and ILT2 expression on peripheral blood $CD14^+$ cells and typed for *HLA-G* 14 bp DEL/INS polymorphism. The authors have observed that ERA patients with low sHLA-G and membrane HLA-G expression suffered a more severe disease. In fact, sHLA-G levels inversely correlated with DAS28 and ultrasonographic power Doppler scores, used to define the severity and progression of the disease. Interestingly, sHLA-G upregulation is evident after 3 months of DMARD therapy, while a significant reduction in TNF-α levels is evident after 9 months of therapy, when a clear amelioration of the disease is evident, with a high specificity for HLA-G detection in EA condition. In fact, the presence of sHLA-G has been observed in 100% of ERA patients, in comparison with 8% of subjects with other

arthropathies and 23% of controls. The receiver operating characteristic curve analysis reports an accuracy of 87.9%, sensitivity of 100% and specificity of 81.4%. In particular, the coevaluation of sHLA-G and ACPA positivities provides a test with an accuracy of 84.8%, a sensitivity of 73.9% and specificity of 91.7%, suggesting the use of both these molecules in the definition of ERA patients and treatment follow-up. Moreover, the implication of the *HLA-G* 14 bp INS/DEL polymorphism is confirmed, as the presence of the DEL allele characterizes the patients with a significant improvement in disease status.

HLA-G impact in other rheumatic diseases

The expression of HLA-G molecules has also been evaluated in other rheumatic diseases, such as scleroderma, systemic lupus erythematosus (SLE), Kawasaki disease, Behcet's disease and sarcoidosis.

Scleroderma (SSc; OMIM, #18175 [201]) is an autoimmune rheumatic disease of the connective tissue. Based on the extent of cutaneous involvement SSc can be classified as limited SSc, involving acral skin, or diffuse SSc if skin involvement extends more proximally including abdomen, trunk and face. SSc is characterized by alterations of the microvasculature, disturbances of the immune system and by massive deposits of collagen and other matrix substances in the connective tissue. The course, and even the initial events in the pathogenesis of SSc, are still poorly understood. The presence of inflammatory infiltrates, mainly $CD4^+$ T cells, around blood vessels and at sites of active connective tissue formation suggests their pathogenetic role, together with an increased secretion of Th1 cytokines [78]. The skin biopsies from patients with SSc with a longer survival, lower frequency of vascular cutaneous ulcers, telangiectasias and inflammatory polyarthralgia, present HLA-G molecule expression, suggesting a role in immune control [79].

SLE (OMIM, #601744 [201]) is a systemic autoimmune disease of the connective tissue that can affect any part of the body. The immune response is mainly characterized by Th2-cell predominance. Rosaso and coauthors [80] and Chen and coauthors [81] have demonstrated higher levels of sHLA-G and IL-10 in SLE patients in comparison with healthy controls, while Rizzo and coauthors [82] have observed lower sHLA-G concentrations in SLE patients. The differences in sHLA-G levels in these two papers could be due to the difference in the analyzed samples (serum or plasma) [83]. As proof, Monsiváis-Urenda and coauthors [84] have provided evidence of a diminished expression of HLA-G in monocytes and in mature $CD83^+$ dendritic cells from SLE patients compared with healthy controls. In addition, monocytes from SLE patients have shown a diminished induction of *HLA-G* expression in response to IL-10. Finally, lymphocytes from SLE patients have displayed a lower acquisition of HLA-G (by trogocytosis) from autologous monocytes compared with controls. Interestingly, ILT-2 receptor expression is increased on lymphocytes from SLE patients, in particular in $CD3^+$, $CD19^+$, $CD56^+$ cells and related to IL-10 and anti-DNA antibodies [81]. These results confirm the presence of a *HLA-G* impaired expression in patients with SLE and a possible role in pathogenesis. Using SNP mapping approach, the *HLA-G* gene is reported as a novel independent locus with SLE interaction [85]. In particular, the *HLA-G* 14 bp INS/DEL polymorphism and the *HLA-G* +3142C>G SNP have been analyzed in the SLE population. SLE patients have shown a higher frequency of 14 bp INS allele and 14 bp INS/INS genotype [82]. Moreover, 14 bp INS/INS patients have presented the highest disease activity [86]. On the contrary, the evaluation of *HLA-G* 14 bp INS/DEL polymorphism in a SLE Brazilian population has failed to present an association [87]. The +3142G allele and the +3142GG genotype frequencies are increased among SLE patients as compared with controls [88]. These data sustain the role of HLA-G molecules in the control of SLEs; in particular several results sustain lower *HLA-G* expression as a risk factor for SLE development.

Behçet's syndrome (OMIM, # 109650 [201]) is a rare, immune-mediated, systemic vasculitis with mucous membrane ulceration and ocular involvements. The *HLA-G*010101* allele is associated with a reduced risk of Behçet's syndrome while *HLA-G*010102*, *G*0105N* alleles and the 14 bp INS/DEL polymorphism are associated with an increased risk of Behçet's syndrome [89,90].

Kawasaki disease (OMIM, # 611775 [201]) is an acute, self-limited vasculitis that effects infants and children. Without treatment, approximately 15–25% of patients with Kawasaki disease will develop coronary artery aneurysms, making this disease the leading cause of acquired heart disease among children in developed countries. Although an infectious agent is highly suspected, the etiology of the disease is largely unknown. However, it has been established that inflammation is a central feature of Kawasaki disease. Several lines of evidence suggest that genetic and immunological factors play important roles in disease susceptibility and outcomes.

Interestingly, nonsynonymous SNP (C\A) of the *HLA-G* gene (rs12722477, Leu134Ile) is significantly associated with Kawasaki disease [91].

Sarcoidosis togliere is a systemic inflammatory granulomatous disease associated with an accumulation of $CD4^+$ T cells and a Th1 immune response. The etiology is unknown but at the molecular level several studies have shown HLA associations (e.g., *HLA-DRB1*1101*) [92]. Overall, 47 patients with sarcoidosis have been analyzed for different *HLA-G* alleles/polymorphisms [93]. The 14 bp INS allele has been observed more often in sarcoidosis patients than in controls. Only rare and weak expression of *HLA-G* has been observed in granulomas from sarcoidosis patients, supporting the genetic results.

Conclusion

Joint damage occurs early in the course of RA and approximately 75% of patients with ERA develop erosive changes within the first 2 years of disease. Controlled clinical trials have shown that an early aggressive therapeutic approach with DMARDs can slow or even stop the progression of damage in RA, therefore an early diagnosis is of extreme importance. Besides physical examination, which still remains the gold standard in identifying the presence of arthritis, musculoskeletal ultrasonography has been proven to be helpful in detecting joint inflammation, especially at an early and subclinical stage [94]. Moreover, power Doppler technique helps today to distinguish active from inactive synovitis [95].

The reviewed literature seems to sustain a direct role of HLA-G molecules in RA and other rheumatic diseases. In particular, HLA-G could create an immune-regulatory environment that is fundamental for disease activity control. A disequilibrium in this setting would maintain an inflammatory and immune-dysregulated condition, typical for RA disease. Although several studies have indicated the association between HLA-G and RA as previously revised by Brenol *et al.* [96], these results should be validated by further scientific investigations, before a clinical use of HLA-G as a reliable biomarker for RA treatment is proposed.

Indeed, the use of sHLA-G as a biomarker to evaluate early prognosis and disease activity in ERA patients is of extreme interest. The identification of significant biomarkers aimed to monitor patient therapy and to avoid under/overtreatment is of outstanding importance to assess and predict ERA evolution. Rheumatoid factor and ACPA, when present, are well recognized negative prognostic factors for joint damage progression but their predictive value is still limited [97]. For this reason new prognostic and reliable biomarkers are urgently needed. The evaluation of HLA-G levels in plasma samples from ERA patients, in combination with other biomarkers, such as ACPA, increases the specificity of DMARD treatment follow-up and disease progression, which is the main target in biomarker identification. The use of accessible plasma samples for sHLA-G quantitation would be an important factor in order to obtain a ready-to-use system for clinical protocols. It will be important to identify a cutoff value of sHLA-G levels to monitor treatment follow-up and disease progression, and bring back the considerations made on average concentrations obtained from a cohort of patients to the single individual. In this context, the availability of new, reproducible and standardized biomarkers is of interest in ERA clinical management. Moreover, since HLA-G molecules have a role in other rheumatic diseases, we could hypothesize to transfer the knowledge acquired with RA studies in other clinical contexts.

Future perspective

The understanding of the specific role and mechanisms of action of HLA-G molecules in the development and progression of RA could justify the use of HLA-G molecules as a marker of inflammation and drug treatment, and open up new therapeutic perspectives for RA patients. A possible interaction in the complex RA molecular pathways is reported in Figure 2. HLA-G could be hypothesized to act as an immune-modulator balancing B-cell auto-antibody production and Treg dysregulation. The identification of pharmacological strategies, with the aim of controlling HLA-G production, could be a possible method in improving the control of inflammation, guiding a therapeutic approach. In fact, the possible use of HLA-G as a therapeutic target is of extreme interest. These considerations are sustained by the recent results on HLA-G dimer use to control collagen-induced arthritis in murine models [74] and the role of HLA-G molecules in the control of the immune-regulatory functions of mesenchymal stem cells derived from human umbilical cord (HUCSC) [98]. Recently, human umbilical cord stem cells have been shown to have a therapeutic potential for cartilage repair in RA [99]. The efficacy of HUCSCs is connected with the expression of HLA-G molecules that guarantee the immune regulatory functions of the treatment. Interestingly, TNF-α is able to downmodulate *HLA-G* expression and

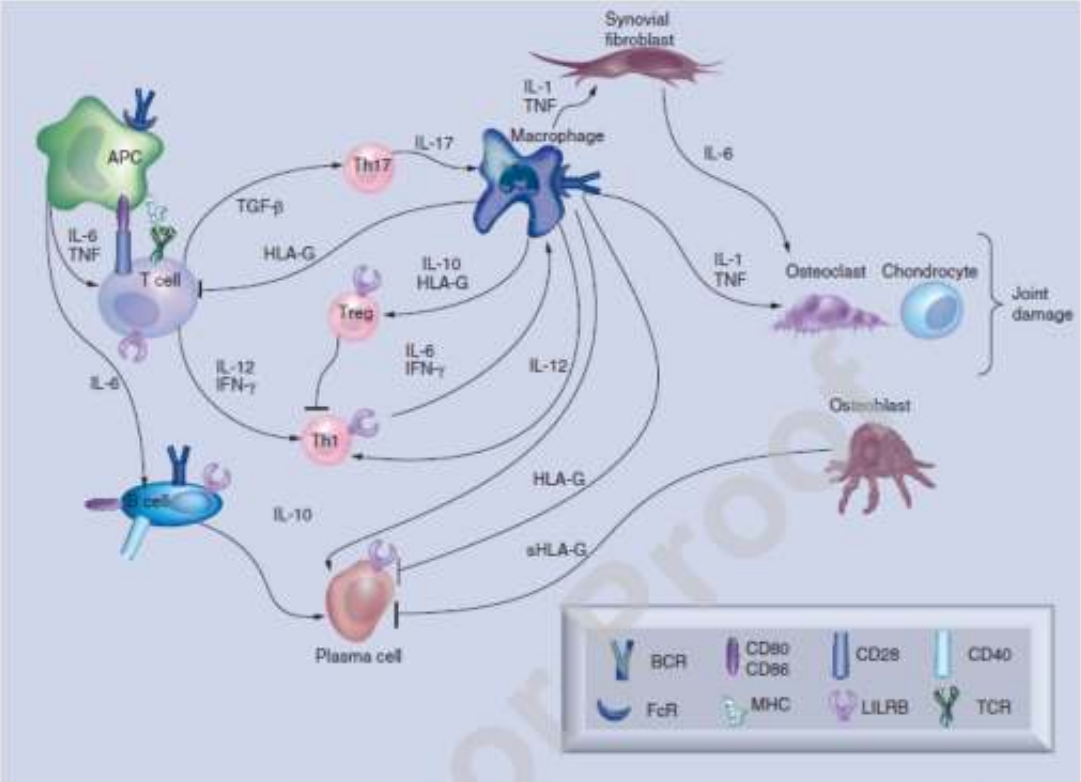

Figure 2. Schematic representation of the possible implication of HLA-G molecules in the complex rheumatoid arthritis molecular pathways. The autoimmune response in rheumatoid arthritis is triggered by antigen presenting cells recognized by T cells. This interaction induces Th1 and Th17 cell shift and the production of IL-6 and TNF by APCs that activate B-cell differentiation in plasma cells, secreting auto-antibodies. Th1-cell secretion of IL-6 and IFN-γ and Th17-cell production of IL-17 activate macrophage secretion of IL-12 that maintains the Th1-cell activation, and IL-1 and TNF production. These two cytokines activate synovial fibroblasts secretion of IL-6 that promote osteoclast and condrocyte survival, as well as differentiation and activation with consequent joint damage. The production of IL-10 and HLA-G molecules by macrophages and osteblasts interferes with plasma cell activation, Th1 differentiation and induces the formation of Tregs.
APC: Antigen-presenting cell; BCR: B-cell receptor; FcR: Fc receptor; sHLA: Serum HLA-G; TCR: T-cell receptor.

HUCSC-dependent immune suppression. The administration of a TNF inhibitor seems to be a potential effective therapy for ameliorating HUCSC efficacy in RA control, maintaining HLA-G expression. For this, the ability to modulate HLA-G molecules on cell surface seems to be at the basis of these cell therapies, suggesting the importance of further study on HLA-G role in RA and the possibility to have a controlled modification of the HLA-G level according to disease status.

Moreover, the confirmation of the role of HLA-G molecules and genetic polymorphisms as risk and pharmacogenetic markers in RA disease could improve the laboratory routine analysis for RA management. In particular, the possibility to use simple, noninvasive and standardized [78] tools for HLA-G analysis makes it quickly transferable to the healthcare system practice. These could help in RA outcome prediction and support the clinicians in treatment decisions.

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Executive summary

Managing rheumatoid arthritis

- Rheumatoid arthritis (RA) treatments include disease-modifying antirheumatic drugs and, more recently, biological agents.
- An important goal of RA therapy has shifted to initiate treatment early and aggressively to achieve remission as quickly as possible.
- Regular monitoring requires the availability of new markers for disease progression and treatment follow-up, in order to improve the efficacy of the RA management, thus reducing the clinical and social costs.

HLA-G antigen

- HLA-G are immune regulatory molecules.
- HLA-G expression is controlled by specific gene polymorphisms.
- HLA-G expression is induced by different molecules such as methotrexate.

HLA-G in RA

- Serum HLA-G concentration is significantly lower in RA than in controls.
- The intracutaneous treatment of collagen-induced arthritis model mice with HLA-G monomer or dimer molecules has produced excellent anti-inflammatory effects with a single, local administration.

HLA-G as a marker of RA treatment

- An increase of the 14 bp DEL/DEL genotype has been observed in the responder group, characterized by a reduction in disease activity score in 28 joints measured before and after 6 months of treatment with methotrexate.
- HLA-G upregulation is evident after 3 months of disease-modifying antirheumatic drug therapy when a clear amelioration of the disease is evident.
- The presence of the 14 bp DEL allele characterizes the patients with a significant improvement in disease status.

Conclusion

- The reviewed literature seems to corroborate direct role of HLA-G molecules in RA.
- HLA-G could create an immune-regulatory environment that is fundamental for disease activity control. A disequilibrium in this setting would maintain an inflammatory and immune-dysregulated condition, typical of RA disease.
- The evaluation of HLA-G levels in plasma samples from EA patients, in combination with other biomarkers as anticyclic citrullinated peptide, increases the specificity of disease modifying antirheumatic drug treatment follow-up and disease progression.
- The confirmation of the role of HLA-G molecules and genetic polymorphisms, as risk and pharmacogenetic markers in RA could improve the laboratory routine analysis for RA management.
- The identification of a cutoff value of serum HLA-G levels to monitor treatment follow-up and disease progression will bring back the considerations made on average concentrations obtained from a cohort of patients to the single individual.
- The ability to modulate HLA-G molecules according to disease status seems to be a potential new therapeutic target in RA.

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1.2.2 HLA-G in central nervous system diseases

The central nervous system (CNS) represent an immune-privileged compartment that maintains an adaptable immune surveillance system. Dysregulated immune function within the CNS may lead to the development of brain tumor growth, and consitent immune activation results in excessive inflammation.

Multiple sclerosis (MS) is the prototypic autoimmune disease of the CNS characterized by chronic inflammatory demyelination and neurodegeneration of unidentified origin [144]. MS typically occurs in young adults and manifests in women twice as frequently as in men with neurological symptoms and signs, called relapses, which are usually disseminated in space and time [145]. MS onset could be distinguished in: i) the relapsing–remitting (RR) form, that affects about the 80% of MS patients followed by ii) a secondary progressive (SP) course that arises after years and iii) a primary progressive (PP) forma in approximately the 20% of MS subjects [146]. However, the recent proposed criteria [147] suggest that the coexistence of multifocal lesions in the periventricular white matter on T2-weighted Magnetic Resonance Imaging (MRI) scans with or without Gadolinium (Gd) enhancement on T1-weighted MRI scans are needed for the diagnosis of MS. Different epidemiological studies affirm that exposure to an environmental factor, e.g., an infectious agent, in genetically predisposed individuals could be crucial for MS pathogenesis [148] above all for the effect of the traffic into the CNS of activated auto-reactive $CD4^+$ T helper1 (Th1) [135, 149, 150]. In fact, infiltrating $CD4^+$ T cells support the initiation of brain inflammation through the activation of microglia leading to the generation of Th1-mediated immuneresponses (IL-12/IFN-g and IL-23/IL-17). On the contrary, the resolution of neuroinflammation is triggered by astrocytes, which promote anti-inflammatory Th2-polarized responses (IL-10 and TGF-b) and the elimination of infiltrating immune cells through Fas/FasL-dependent apoptosis [144, 149] (**Figure4**).

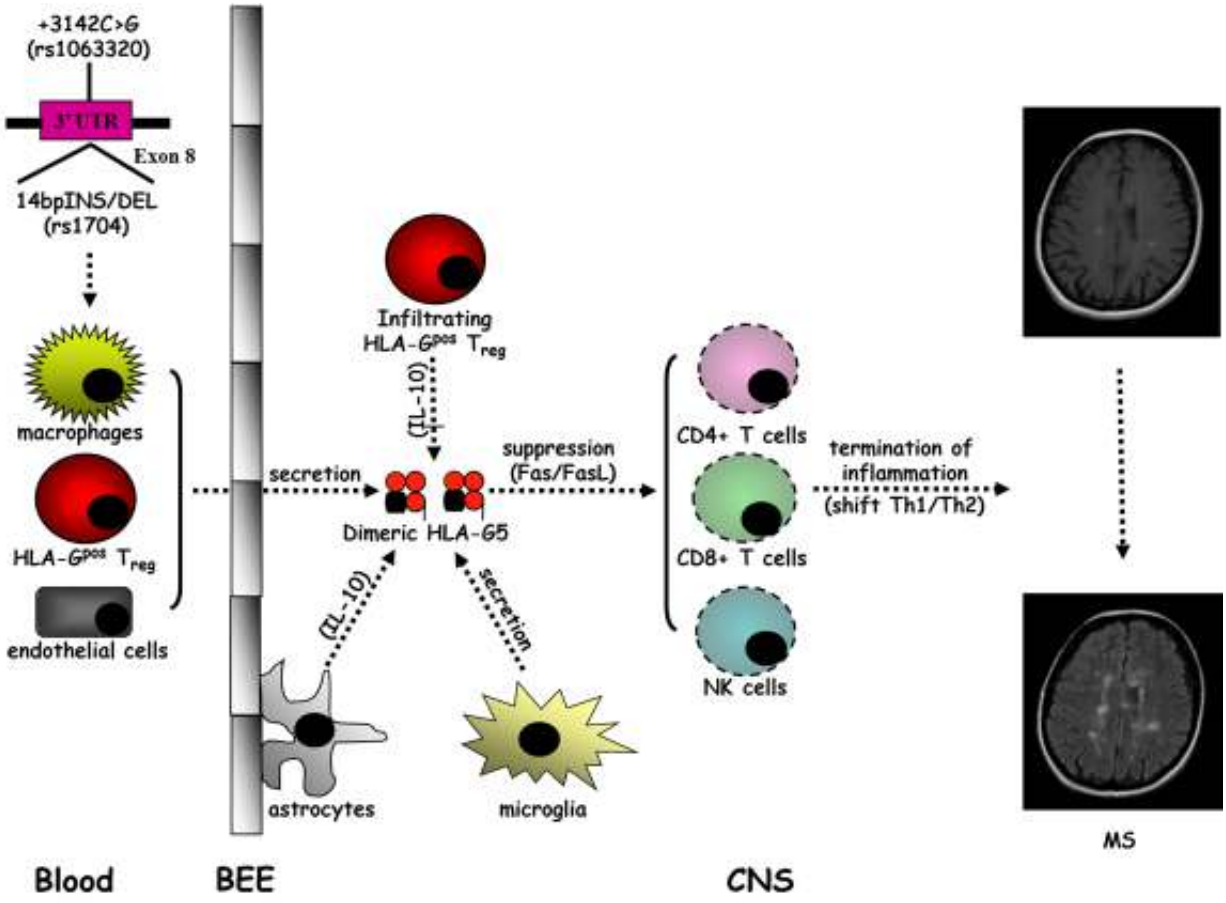

Figure 4. Intrathecal immune milieu in MS

The presence in CSF of detectable sHLA-G levels in relapsing-remitting MS (RRMS) patients and, occasionally, in other inflammatory neurological disorders and non-inflammatory neurological disorders was reported for the first time by Fainardi and coauthors [151].

In addition, RRMS patients present higher sHLA-G levels in CSF than controls that was more increased, in association with IL-10 values, in RRMS patients without than in those with magnetic resonance imaging (MRI) evidence of disease activity [152]. The importance of sHLA-G level evaluation as a biomarker for MS is confirmed [153]. In fact, CSF concentrations of sHLA-G and IL-10 are positively correlated in patients with inactive MRI disease and CSF IL-10 titers are more elevated in patients with than in those without CSF measurable levels of sHLA-G.. HLA-G acts as anti-inflammatory molecules under the control of IL-10 CSF levels which, together with the influence of HLA-G polymorphysms, may

enhance sHLA-G production [154 paper attached]. In particular, we analysed the association of *HLA-G* 14bp DEL/INS and +3142 C>G polymorphisms with CSF and serum levels of sHLA-G molecules in a group of relapsing-remitting (RR)-MS patients, categorized according to MRI disease activity [154 paper attached]. The aim of our study was to better understand the actual role of these genetic polymorphisms in sHLA-G production occurring in MS, and, furthermore, to clarify whether serum and CSF sHLA-G concentrations measured in MS depend only on local inflammatory microenvironment, or are also influenced by the individual genetic background. We found that serum and CSF sHLA-G levels were more elevated in high than in low DEL/INS 14bp and +3142C>G sHLA-G producers and were different among the various combined *HLA-G* genotypes in both MRI inactive and active diseases. The highest and the lowest sHLA-G values were identified in MS patients with C/C,DEL/DEL and G/G,INS/INS genotypes, respectively. Our findings suggest that serum and CSF sHLA-G levels in MS could be influenced by *HLA-G* polymorphisms irrespective of the inflammatory microenvironment.

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Role of *HLA-G* 14bp deletion/insertion and +3142C>G polymorphisms in the production of sHLA-G molecules in relapsing-remitting multiple sclerosis

Roberta Rizzo [a,*], Daria Bortolotti [a], Nadia Ben Fredj [b], Antonella Rotola [a], Francesca Cura [a], Massimiliano Castellazzi [c], Carmine Tamborino [c], Silva Seraceni [d], Eleonora Baldi [e], Loredana Melchiorri [f], Maria Rosaria Tola [e], Enrico Granieri [c], Olavio R. Baricordi [f], Enrico Fainardi [g]

[a] Department of Experimental and Diagnostic Medicine, Section of Microbiology, University of Ferrara, Ferrara 44121, Italy
[b] Laboratory of Transmissible Diseases and Biological Active substances, LR99-ES27, Faculty of Pharmacy, University of Monastir, Monastir 5000, Tunisia
[c] Multiple Sclerosis Centre, Section of Neurology, Department of Medical and Surgical Sciences of the Communication and Behaviour, University of Ferrara, Arcispedale S. Anna, Ferrara 44121, Italy
[d] Section of Infectious Diseases, Department of Clinical & Experimental Medicine, University of Ferrara, Ferrara 44121, Italy
[e] Multiple Sclerosis Centre, Neurology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliera-Universitaria, Arcispedale S. Anna, Ferrara 4421, Italy
[f] Department of Experimental and Diagnostic Medicine, Section of Medical Genetics, University of Ferrara, Ferrara 44121, Italy
[g] Neuroradiology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliera-Universitaria, Arcispedale S. Anna, Ferrara 44121, Italy

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ABSTRACT

HLA-G is believed to act as an anti-inflammatory molecule in Multiple Sclerosis (MS). The 3′ untranslated region of the *HLA-G* gene is characterized by two polymorphisms, DEL/INS14bp and +3142C>G, which control soluble HLA-G (sHLA-G) production. The influence of these two *HLA-G* variants on sHLA-G serum and cerebrospinal fluid (CSF) levels was investigated in 69 Relapsing-Remitting MS patients grouped in magnetic resonance imaging (MRI) inactive and active disease. Serum and CSF sHLA-G levels were more elevated in high than in low DEL/INS 14bp and +3142C>G sHLA-G producers and were different among the various combined *HLA-G* genotypes in both MRI inactive and active diseases. The highest and the lowest sHLA-G values were identified in MS patients with C/C,DEL/DEL and G/G,INS/INS genotypes, respectively. Our preliminary findings suggest that serum and CSF sHLA-G levels in MS could be influenced by *HLA-G* polymorphisms irrespective of the inflammatory microenvironment.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) with an uncertain etiology that is commonly considered to be autoimmune in nature [1].

A growing body of evidence has indicated a possible involvement of HLA (Human Leukocyte Antigen)-G antigens in MS where this molecule seems to exhibit anti-inflammatory properties. HLA-G is a HLA-Ib molecule with a physiological tissue-restricted distribution in cytotrophoblast [2], amniotic cells, thymus, and endothelial cells of chorionic blood vessels [3]. HLA-G molecules are generated by an alternative splicing of the primary transcript of the gene [4]: HLA-G exists as four membrane-bound (HLA-G1, -G2, -G3 and -G4) and three secreted soluble isoforms (HLA-G5, -G6, -G7). HLA-G is characterized by tolerogenic functions, inducing apoptosis of activated CD8+ T cells [5], acting on T regulatory cells [6], modulating the activity of natural killer cells [7] and of dendritic cells [8] and blocking allo-cytotoxic T lymphocyte response [9]. These immuno-regulatory functions are mediated by the interaction of HLA-G molecules with specific inhibitory receptors: ILT-2 (LILRB1/CD85j), ILT-4 (LILRB2/CD85d), CD8 and KIR2DL4 (CD158d) expressed by immune cells [10]. Cerebrospinal fluid (CSF) levels and the intrathecal synthesis of soluble HLA-G (sHLA-G) were demonstrated to be more elevated in MS than in controls, and in MS patients without magnetic resonance imaging (MRI) evidence of disease activity [11,12]. In these patients, CSF sHLA-G concentrations were positively correlated with CSF levels of IL-10 [11], inversely associated with CSF titers of anti-apoptotic sFas molecules [13], and predominantly represented by HLA-G5 isoform [14]. On the other hand, immunohistochemical expression of HLA-G and its inhibitory receptors (ILT-2 and ILT-4) was strongly upregulated within and around MS lesions, whereas protein HLA-G expression on monocytes was high in CSF of MS patients [15]. Finally, a novel subpopulation of naturally occurring

* Corresponding author. Address: Department of Experimental and Diagnostic Medicine, Section of Microbiology, University of Ferrara, Via Luigi Borsari, 46, 44121 Ferrara, Italy. Fax: +39 0532974470.
E-mail address: rbr@unife.it (R. Rizzo).

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$CD4^+$ and $CD8^+$ regulatory T (T_{reg}) cells expressing HLA-G (HLA-G^{pos} T_{reg}) was increased in CSF and inflammatory brain lesions of MS patients [16].

Cree et al. [17] confirmed the contribution of the MHC locus to MS susceptibility, not only through the well recognized effect of HLA-DRB1*15:01, but also through the rs4959039 single nucleotide polymorphism (SNP) in the 3′ untranslated region (UTR) of the HLA-G gene. Interestingly, it has been suggested that HLA-G production is controlled by polymorphisms at the 5′ upstream regulatory region and at the 3′ UTR of the *HLA-G gene* [18]. Two polymorphisms at the 3′ UTR are clearly implicated: a deletion/insertion (DEL/INS) of 14 base pairs (14bp) polymorphism (rs1704) and a C>G SNP at the +3142bp position (rs1063320) [19]. In particular, the presence of the 14bpINS affects mRNA stability, protein production [20] and is associated with pregnancy pathologies and autoimmune diseases [21,22]. The +3142G allele binding to three microRNAs (miRNAs) *miR-148a*, *miR-148b*, and *miR-152* is predicted to be more stable than binding to the +3142C allele, resulting in a lower soluble protein production [23].

Therefore, we analysed the association of *HLA-G* 14bpDEL/INS and +3142 C>G polymorphisms with CSF and serum levels of sHLA-G molecules in a group of relapsing-remitting (RR)-MS patients, categorized according to MRI disease activity, in order to gain a better understanding of the actual role of these genetic polymorphisms in sHLA-G production occurring in MS, and, furthermore, to clarify whether serum and CSF sHLA-G concentrations measured in MS depend only on local inflammatory microenvironment, or are also influenced by the individual genetic background.

2. Materials and methods

2.1. Subjects

Sixty-nine Italian (45 females, 24 males; mean age 37.3 ± 10 years) unrelated patients affected by definite MS according to the classification of McDonald [24] and followed at the MS Centre of the Department of Neurology, University of Ferrara, Italy during the period from 2001 to 2010, were prospectively included in the study. All patients had RR course in agreement with the criteria of Lublin [25]. The occurrence of relapses was not recorded since it is well-known that, as several MRI active lesions are asymptomatic [26], MRI studies are more sensitive in measuring disease activity than clinical examination. In addition, our previous studies repeatedly demonstrated an association between sHLA-G levels and MRI, but not clinical disease activity [11–14]. For these reasons we considered only the presence of lesions with Gadolinium (Gd)-DTPA-enhancement on MRI T1-weighted scans as disease activity. Disease disability was assessed in all MS patients at the time of sample collection using Kurtzke's Expanded Disability Status Scale (EDSS) [27] (mean at entry: 2.0 ± 1.1, range from 0 to 5.5). The duration of the disease was expressed in months (mean at entry: 35.6 ± 46.7, range from 1 to 240). At entry none of the patients had fever or other symptoms or signs of acute infections. Moreover, none of the patients had received any potential disease-modifying therapies (e.g. azathioprine or methylprednisolone, interferon-beta or glatiramer acetate) during the 6 months before the study. Clinical assessment and blood sampling were performed during routine clinics, with written informed consent and local ethical board approval. None of the female MS patients was pregnant before entering the study.

2.2. Handling of serum and CSF samples

Serum and CSF samples from 69 Italian RR-MS patients were collected under sterile conditions, coded and stored in aliquots at −80 °C until use. Serum samples were obtained by centrifugation of blood specimens withdrawn by puncture of an anterocubital vein at the same time as CSF extraction. CSF samples were obtained by atraumatic lumbar puncture performed for purposes of diagnosis in the absence of contraindications. Serum and CSF samples were measured under exactly the same conditions.

2.3. MRI evaluation

Brain MRI scans were performed at entry using a standard head coil in all patients with a 1.5-Tesla MRI unit (GE Signa Horizon, General Electric Medical Systems, Milwaukee, Wisconsin) within 48 h after CSF sampling. Routinely used T1-weighted axial spin echo images were obtained approximately 10 min after intravenous injection of 0.1 mmol/kg of Gd in each patient. As described above, lesions showing Gd-enhancement on T1-weighted scans were defined as indicative of MRI activity. Accordingly, 27 MS patients were classified as MRI active and 42 were considered as MRI inactive. All brain MRI scans were evaluated by one investigator (EF) blinded to clinical and sample data.

2.4. HLA-G polymorphism typing

EDTA blood (7–10 ml) was obtained from RR-MS patients. Genomic DNA was extracted from the EDTA blood using a Nucleon Bacc 3 Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. The HLA-G 14bp polymorphism (14bp INS/DEL) was genotyped by PCR performed as previously described [28]. Briefly 100 ng of genomic DNA were amplified in a 25 μl reaction with 10 pmol of each primer (GE14H-LAG, RHG4). +3142 polymorphism was analyzed performing the analysis using the 7300 Real-Time PCR System (Applied Biosystems) [29]. The forward primer, 3142for, was 5′-CCTTTAATTAACC-CATCAATCTCTCTTG-3′, and the reverse primer, 3142rev, was 5′-TGTCTCCGTCTCTGTCTCAAATTT-3′. The MGB probe used for detection of the 3142C allele was 5′-VIC-TAAGTTATAGCTCAGTGGAC-3′ (3142CPVIC) and the MGB probe for the 3142G allele was 5′-FAM-TAAGTTATAGCTCAGTGCAC-3′ (3142GFAM).

2.5. sHLA-G Enzyme-Linked Immunosorbent Assay (ELISA)

sHLA-G levels in serum and cerebrospinal fluid samples were assayed in triplicate as previously reported [11–14] using, as capture antibody, the monoclonal antibody (MoAb) MEM-G9 (Exbio, Praha, Czech Republic), which recognizes the HLA-G molecule, in β2-microglobulin associated form. The intra-assay coefficient of variation (CV) was 1.4% and the inter-assay CV was 4.0%. The limit of sensitivity was 1.0 ng/ml.

2.6. sHLA-G immunoprecipitation

CSF and Jeg-3, human choriocarcinoma trophoblastic cells HLA-G positive control, culture supernatants were biotynilated with 0.2 mg/ml. EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA). Samples were then immunoprecipitated for 90 min at 4 °C with MEM-G9 MoAb (Exbio, Praha, CZ), and incubated overnight with protein G-Sepharose beads (Santa Cruz, CA,USA) at 4 °C. The samples were resuspended in 20 μl Laemli Buffer (BioRad, Segrate, MI, Italy).

2.7. Western blot analysis

Protein concentration was quantified in immunoprecipitates by means of the Bradford assay (Bio-Rad Laboratories) using bovine albumin (Sigma-Aldrich, St. Louis, MO, USA) as standard. Total protein was denatured at 100 °C for 5 min. Proteins were loaded in

10% TGX-Pre-cast gel (Biorad, Segrate, MI, Italy), with subsequent electroblotting transfer onto a PVDF membrane (Millipore, MA, USA). The membrane was incubated with a horseradish peroxidase (HRP)-conjugated antimouse antibody (1:5000; Amersham Biosciences, NJ, USA) and developed with the ECL kit (Amersham Biosciences, NJ, USA). The images were acquired by Geliance 600 (Perkin Elmer, Massachussets, USA).

2.8. Statistical analysis

The normality of each variable was checked using the Kolmogorov-Smirnov test. As normality of data distribution was rejected in several variables, continuous variables were compared using Kruskal-Wallis and Mann Whitney U tests and correlations were assessed by the Spearman rank correlation coefficient test. *p*-value <0.05 was considered to be statistically significant.

3. Results

3.1. HLA-G genetic polymorphisms typing

Allelic and genotypic frequencies of *HLA-G* polymorphisms in RR-MS patients are listed in Table 1.

3.2. Influence of HLA-G genetic polymorphisms on serum and CSF sHLA-G in MS patients grouped according to MRI disease activity

While no difference was observed in serum sHLA-G concentrations between MRI inactive and MRI active RR-MS patients, a significant increase was found for sHLA-G levels detected in CSF samples from RR-MS patients with MRI inactivity compared with MRI active patients ($p < 0.01$; Mann Whitney) (Table 2). We next evaluated the effect of both 14bpDEL/INS and +3142C>G polymorphisms on serum and CSF sHLA-G concentrations in RR-MS patients divided according to MRI activity. In agreement with previous studies [20,23], RR-MS patients with one or both 14bpDEL alleles (14bpDEL/DEL and 14bpDEL/INS genotypes) and with one or both +3142C alleles (+3142C/C and +3142C/G genotypes) were considered as high HLA-G producers. Conversely, RR-MS patients without any 14bpDEL alleles (14bpINS/INS genotype) and those with no +3142C alleles (+3142G/G genotype) were classified as

Table 1
Allelic and genotypic frequencies in 69 RR-MS patients grouped according to MRI activity.

	Total MS (*n* = 69)	MRI active MS (*n* = 27)	MRI inactive MS (*n* = 42)
14bpINS/DEL			
Allele			
14bpDEL	52/69 (75.4%)	21/27 (77.7%)	31/42 (73.8%)
14bpINS	48/69 (69.6%)	17/27 (63%)	31/42 (73.8%)
Genotype			
14bpDEL/DEL	21/69 (30.4%)	10/27 (37%)	11/42 (26.2%)
14bpDEL/INS	31/69 (44.9%)	11/27 (40.7%)	20/42 (47.6%)
14bpINS/INS	17/69 (24.6%)	6/27 (22.2%)	11/42 (26.2%)
+3142C>G			
Allele			
C	39/69 (56.5%)	16/27 (59.3%)	23/42 (54.8%)
G	63/69 (91.3%)	24/27 (88.9%)	39/42 (92.9%)
Genotype			
C/C	6/69 (8.7%)	3/27 (11.1%)	3/42 (7.1%)
C/G	33/69 (47.8%)	13/27 (48.2%)	20/42 (47.6%)
G/G	30/69 (43.5%)	11/27 (40.7%)	19/42 (45.2%)

RR = Relapsing Remitting; MS = Multiple Sclerosis; MRI = Magnetic Resonance Imaging; 14bp = 14 base pairs; DEL = deletion; INS = insertion.

Table 2
Serum and cerebrospinal fluid (CSF) levels of sHLA-G in 69 patients with RR-MS grouped according to MRI activity.

	MRI active MS (*n* = 27)	MRI inactive MS (*n* = 42)
Serum sHLA-G levels (ng/ml)	7.6, 5.8–8.6	5.5, 4.3–6.9,
Median, IQR, range	2.1–9.6	1.3–9.4
CSF sHLA-G levels (ng/ml)	4.3, 3.3–5.5,	11.8, 6.4–18.9,
Median, IQR, range	2.1–13.0	2.3–26.5*

RR = Relapsing Remitting; MS = Multiple Sclerosis; MRI = Magnetic Resonance Imaging, IQR = Interquartile Range.
* $p < 0.01$.

low sHLA-G producers. As illustrated in Fig. 1, the analysis of 14bpDEL/INS polymorphism revealed that serum and CSF levels of sHLA-G were significantly more elevated in high than in low sHLA-G producers in presence of MRI inactive ($p < 0.01$ and $p < 0.00001$, respectively; Mann Whitney) and MRI active disease ($p < 0.001$; Mann Whitney) (panels a–d). Similarly, the examination of +3142 C>G polymorphism showed that serum and CSF sHLA-G values were significantly greater in high than in low sHLA-G producers in both MRI inactive ($p < 0.05$ and $p < 0.00001$, respectively; Mann Whitney) and active disease ($p < 0.01$ and $p < 0.05$, respectively; Mann Whitney) (panels e–h). We then assessed the influence of combined *HLA-G* genotypes on serum and CSF sHLA-G levels in RR-MS patient stratified according to MRI activity. The frequencies of this combined HLA-G genotypes are reported in Table 3. As depicted in Fig. 2, serum and CSF levels of sHLA-G were statistically different among the various groups analyzed. In particular, there was a significant difference among the distinct combined *HLA-G* genotypes for serum sHLA-G concentrations in both MRI inactive and active RR-MS patients ($p < 0.01$; Kruskal Wallis) (panels a and b) as well as for CSF sHLA-G values in RR-MS patients with MRI inactive and active disease ($p < 0.001$ and $p < 0.01$, respectively; Kruskal Wallis) (panels c and d). In this case, a post-hoc analysis was not performed due to the small number of patients included in some patient groups. However, as shown in Fig. 2, the highest serum and CSF sHLA-G levels predominated in MRI inactive and active RR-MS patients with C/C,DEL/DEL genotype, who can be judged as the "true" high HLA-G producers. In contrast, the lowest serum and CSF sHLA-G titers prevailed in MRI inactive and active RR-MS patients with G/G,INS/INS who can be accepted as the "true" low sHLA-G producers. Performing a Western Blot assay on CSF samples of MRI inactive RR-MS patients as confirmatory analysis, we reproduced ELISA results since patients with C/C,DEL/DEL genotype presented the highest sHLA-G levels, whereas those with G-G,INS/INS genotype had the lowest sHLA-G concentrations (Fig. 2e).

3.3. Correlation between HLA-G genetic polymorphisms and clinical features

No correlations were detected between *HLA-G* polymorphisms and disease duration and disability (data not shown; Spearman).

4. Discussion

In this study, we investigated whether 14bpDEL/INS and +3142C>G *HLA-G* polymorphisms are implicated in the modulation sHLA-G molecules in serum and CSF samples from MS patients. In fact, although HLA-G is currently believed to play a tolerogenic role in the regulation of MS autoimmunity, and the *HLA-G*-related SNP rs49559039 is independently associated with MS susceptibility [17], the possible link between *HLA-G* genetic polymorphisms and MS was not intensively investigated [30]. At present, only two works have analysed the relationship between HLA-G genotypes and

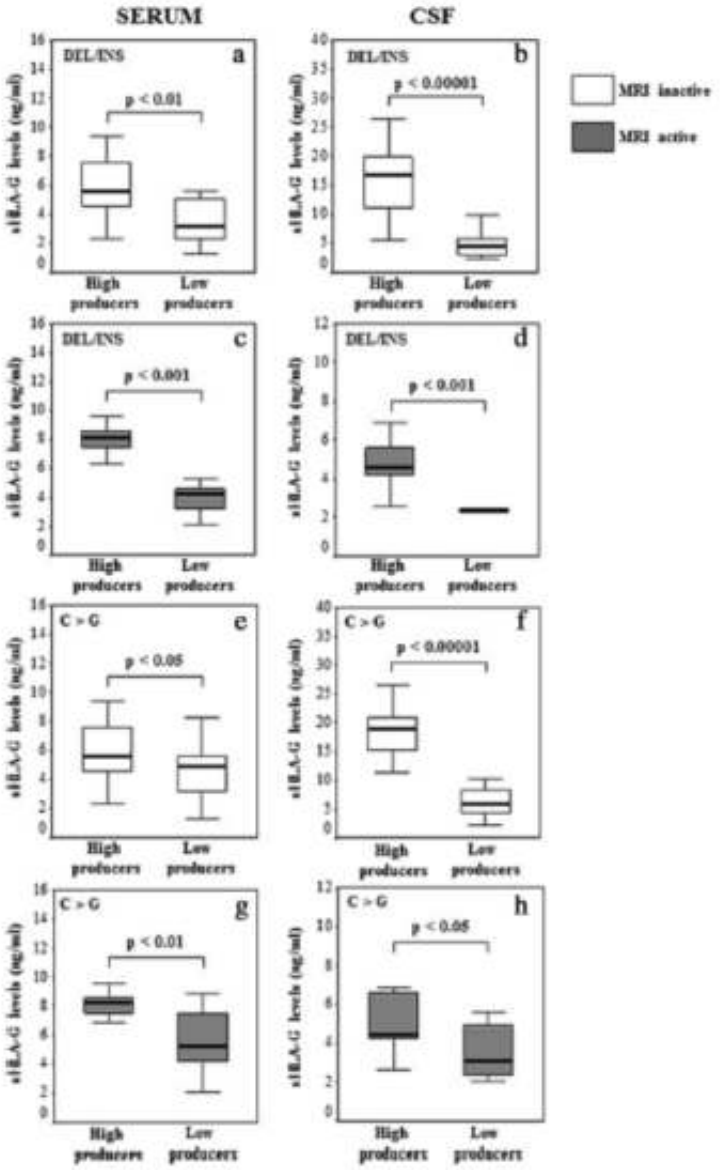

Fig. 1. Serum and CSF levels of sHLA-G in 69 relapsing-remitting (RR) multiple sclerosis (MS) patients categorized according to Magnetic Resonance Imaging (MRI) disease activity in MRI inactive ($n = 42$; white boxes) and MRI active ($n = 27$; grey boxes) and subdivided according to 14bpDEL/INS (panels a–d) and +3142C>G (panels e–h) genotypes. Regarding 14bpDEL/INS polymorphism, high HLA-G producers were RR-MS patients with 14bpDEL/DEL and 14bpDEL/INS genotypes (31 with MRI inactivity and 21 with MRI activity), whereas low HLA-G producers were those with 14bpINS/INS genotype (11 with MRI inactivity and 6 with MRI activity). Concerning +3142C>G polymorphism, while high HLA-G producers were RR-MS patients with +3142C-C and +3142C-G genotypes (23 with MRI inactivity and 16 with MRI activity), low HLA-G producers were those with +3142G-G genotype (19 with MRI inactivity and 11 with MRI activity). The samples were analysed by ELISA system with a specific anti-HLA-G mmAb (MEM-G/9). The boundaries of the box represent the 25th–75th quartile. The line within the box indicates the median. The whiskers above and below the box correspond to the highest and lowest values, excluding outliers. *p* Value was obtained with Mann Whitney U test.

MS with controversial results. While Kroner and coauthors failed to identify any association between *HLA-G* gene polymorphism and MS severity [31], Wisniewski and coauthors showed a relation between 14bpDEL/INS and −725C>G>T polymorphisms and MS [32]. Comparing these studies, the opposite results may reflect population differences in gene expression, that could influence the power of association studies and lead to different levels of association. In addition, the findings obtained by these two studies could be affected by the different disease activity status and the different patient selection protocol. Our approach was completely different since we selected two *HLA-G* polymorphisms with a confirmed effect on sHLA-G protein expression, 14bp INS/DEL and +3142C>G polymorphisms, and we evaluated the effect in the control of HLA-G expression in RR-MS patients.

Table 3
Combined genotype frequencies in 69 RR-MS patients grouped according to MRI activity.

	Total MS (n = 69)	MRI active MS (n = 27)	MRI inactive MS (n = 42)
Genotypes			
C/C DEL/DEL	6/69 (8.7%)	3/27 (11.1%)	3/42 (7.1%)
C/G DEL/DEL	11/69 (15.9%)	5/27 (18.5%)	6/42 (14.3%)
G/G DEL/DEL	4/69 (5.8%)	2/27 (7.4%)	2/42 (4.8%)
C/G DEL/INS	22/69 (31.9%)	8/27 (29.6%)	14/42 (33.3%)
G/G DEL/INS	9/69 (13%)	3/27 (11.1%)	6/42 (14.3%)
G/G INS/INS	17/69 (24.6%)	6/27 (22.2%)	11/42 (26.2%)

RR = Relapsing Remitting; MS = Multiple Sclerosis; MRI = Magnetic Resonance Imaging; DEL = deletion; INS = insertion.

Firstly, we replicated our previous data indicating that CSF levels of sHLA-G were higher in RR-MS patients with MRI active disease in comparison with MRI inactive disease patients [11–14]. Next, we tested the distribution of 14bpDEL/INS and +3142 C>G genotypes in these RR-MS patients showing more elevated serum and CSF sHLA-G concentrations in high than in low sHLA-G producers when the disease was both MRI inactive and active. In this setting, RR-MS patients with different combination of 14bpDEL/DEL, 14bpDEL/INS, +3142C/C and +3142C/G genotypes were classified as high sHLA-G producers, whereas those with 14bpINS/INS and +3142G/G genotypes were identified as low sHLA-G producers [20,23]. In addition, the analysis of combined 14bpDEL/INS and +3142 C>G genotypes revealed that the highest serum and CSF sHLA-G values were obtained in RR-MS patients who had C/

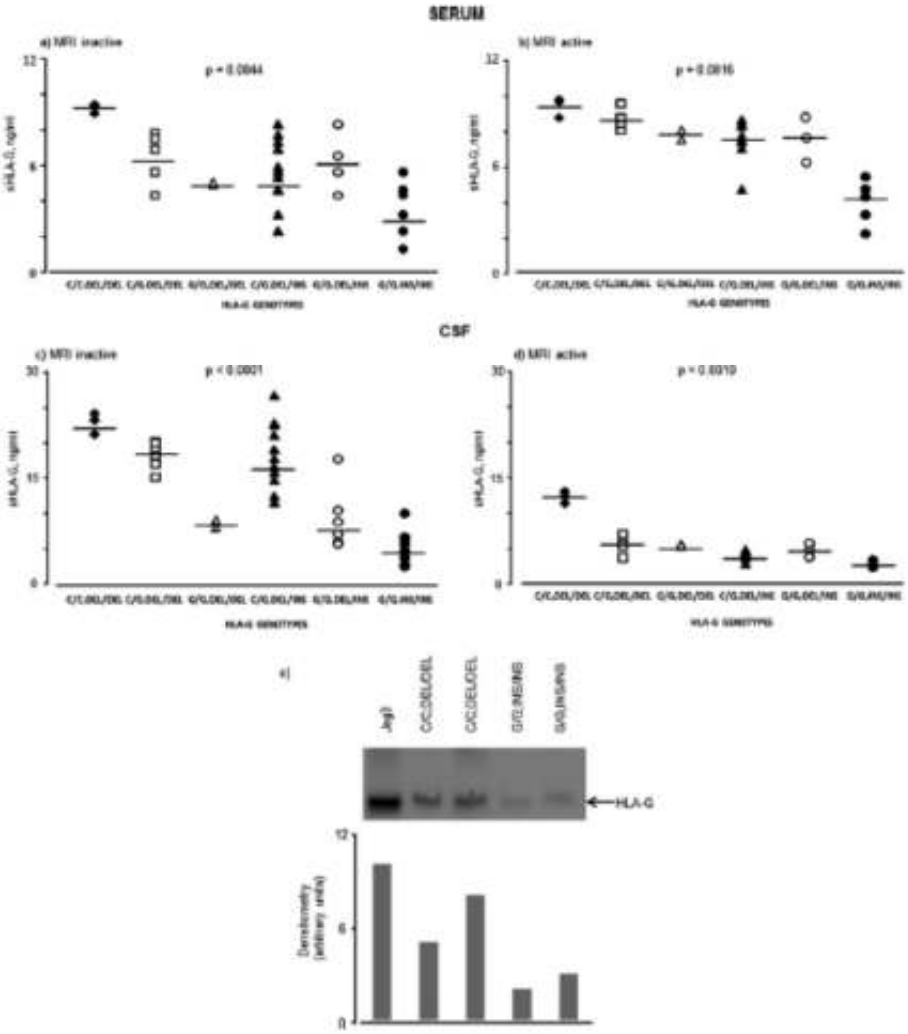

Fig. 2. Serum (panels a and b) and CSF (panels c and d) levels of sHLA-G in 69 relapsing-remitting (RR) multiple sclerosis (MS) patients categorized according to Magnetic Resonance Imaging (MRI) disease activity in MRI inactive (n = 42; white boxes) and MRI active (n = 27; grey boxes) and subdivided according to the combination of 14bpDEL/INS and +3142C>G genotypes. The samples were analysed by ELISA system with a specific anti-HLA-G mAb (MEM-G9). The horizontal bars indicate the median values. p Value was obtained with Kruskal-Wallis test. (e) CSF levels of sHLA-G in G/G_INS/INS and C/C_DEL/DEL relapsing-remitting (RR) multiple sclerosis (MS) patients without Magnetic Resonance Imaging (MRI) disease activity. Western Blot analysis in two representative G/G_INS/INS and two representative C/C_DEL/DEL MRI inactive RR-MS patients. We used a specific anti-HLA-G mAb (MEM-G9). The positivity for HLA-G molecule was evidenced at 39kD. Jeg3 cell line was used as positive control. The densitometry results are reported as arbitrary units.

C,DEL/DEL genotype and the lowest serum and CSF sHLA-G levels were observed in RR-MS patients who had G-G,INS/INS genotype. Of note, RR-MS patients with these two *HLA-G* genotypes can be considered as "true" high and low sHLA-G producers, respectively. These data, coming from ELISA measurements, were confirmed by those emerging from Western Blot determinations.

Our original results seems to suggest that, in MS, the release of sHLA-G molecules within blood and intrathecal compartments could depend not only on local microenvironment, represented by presence or absence of inflammatory activity, but may also be controlled by two main polymorphisms at the 3'UTR of the *HLA-G* gene. On the one hand, sHLA-G antigens could promote the resolution of MS autoimmunity by acting as anti-inflammatory molecules which lead to the development of a CNS immunosuppressive microenvironment at the sites of MS inflammation. These effects may be mediated by the apoptotic elimination of activated $CD8^+$ T and NK cells invading the brain through Fas/FasL-dependent pathway and a deviation of the Th1/Th2 balance towards Th2 directions by the inhibition of proliferation of $CD4^+$ Th1 cells and the stimulation of IL-10 synthesis [30]. On the other hand, serum and CSF levels of sHLA-G molecules could be influenced by *HLA-G* polymorphisms which determine a high or a low production of these molecules irrespective of the inflammatory status. In this context, HLA-G^{plus} T_{reg} was recently detected in CSF and, to a lesser extent, in peripheral blood of MS patients [16]. In addition, it has been reported that miRNAs are differentially expressed in MS $CD4^+CD25^{high}$ T_{reg} with an up-regulation of miR-148a in T_{reg} cells [33]. As this miRNA is implicated in the recognition of +3142G allele, its over-expression and the existence of a +3142G/G genotype could reduce the secretion and shedding of HLA-G molecules by T_{reg} cells. Thus, we are tempted to speculate that sHLA-G production by HLA-G^{plus} T_{reg} could decrease in MS patients with G/G,INS/INS genotype and increase in those with other combined 14bpDEL/INS and C>G genotypes and, in particular, in subjects with C/C,DEL/DEL genotype. These observations prompt us to hypothesize that, given the potential function of sHLA-G as anti-inflammatory molecules in MS, low sHLA-G producers may have a greater predisposition to develop inflammation compared to high sHLA-G producers irrespective to the phase of disease activity. In fact, MS patients with low HLA-G production would be characterized by low sHLA-G concentrations at intrathecal level also during MRI inactive disease, when an increase in CSF sHLA-G levels usually occur and most likely contribute to the resolution of inflammatory reaction. Therefore, this condition could increase the risk of disease reactivation due to the creation of a microenvironment in which anti-inflammatory response and immune-regulation are more difficult. However, these considerations are not supported by the current data since the absence of a longitudinal evaluation of MRI evolution in our MS patients did not allow us to verify whether low sHLA-G producers are really more prone to having Gd-enhancing lesions. Moreover, the small sample size and the lack of a Western Blot evaluation of sHLA-G isoforms represent other important limitations. A more extensive research in a larger number of these patients is, therefore, needed to better clarify the actual association between 14bpDEL/INS and +3142C>G polymorphisms and MS activity. It is to note that the contrasting results obtained by Manaster and coauthors [34], who reported the absence of +3142C>G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression in ms environment.

Taken together, our preliminary findings suggest that *HLA-G* polymorphisms should be taken into account when serum and CSF levels of sHLA-G are analyzed in MS, since, not only are they regulated by local inflammatory microenvironment, but they are also influenced by 14bpDEL/INS and +3142C>G individual polymorphisms.

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The existence of high CSF concentrations of sHLA-G in MS patients and their association with clinical and MRI stable disease have been repeatedly confirmed in subsequent investigations [152-156].

Our group also focused on the identification of the presence of HLA-G dimers in CSF of MS patients in comparison with inflammatory and non inflammatory controls [157 paper attached]. We found a higher frequency of HLA-G dimers in RRMS patients that suggests their implication in reducing the inflammatory status in MS.

Reference 157

Cerebrospinal fluid amounts of HLA-G in dimeric form are strongly associated to patients with MRI inactive multiple sclerosis

Enrico Fainardi,[1] Daria Bortolotti[2], Silvia Bolzani,[2] Massimiliano Castellazzi,[3,4] Carmine Tamborino,[3] Gloria Roversi,[3] Eleonora Baldi,[5] Maria Luisa Caniatti,[5] Ilaria Casetta,[3] Valentina Gentili,[2] Enrico Granieri,[3] Roberta Rizzo, and the ERMES study group[6]

[1]Neuroradiology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliero-Universitaria, Arcispedale S. Anna, Ferrara, Italy; [2]Section of Microbiology and Medical Genetics, Department of Medical Sciences, University of Ferrara, Ferrara, Italy; [3]Section of Neurology, Department of Biomedical and Specialist Surgical Sciences, University of Ferrara, Ferrara, Italy; [4]Section of Medical Biochemistry, Molecular Biology and Genetics, Department of Biomedical and Specialist Surgical Sciences, University of Ferrara, Ferrara, Italy; [5]Neurology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliero-Universitaria, Arcispedale S. Anna, Ferrara, Italy; [6]The Emilia-Romagna network for Multiple Sclerosis (ERMES) study group (Affiliations are indicated between brackets in Appendix)

Address correspondence to:

Enrico Fainardi MD, PhD

Unità Operativa di Neuroradiologia, Dipartimento di Neuroscienze, Azienda Ospedaliero-Universitaria, Arcispedale S. Anna, Via Aldo Moro 8, I-44124 Cona, Ferrara, Italy; Tel. ++39-532-236447; Fax. ++39-532-237639; E-mail: henryfai@tin.it

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Tables: 1

Figures: 1

Supplementary files: 2 (1 Supplementary Materials and 1 Supplementary Table)

ABSTRACT

Background-The relevance of HLA-G in dimeric form in MS is still unknown.

Objective-To investigate the contribution of CSF HLA-G dimers in MS pathogenesis.

Methods-CSF amounts of 78kDa HLA-G dimers were measured by western blot analysis in 80 MS relapsing-remitting MS (RRMS) patients and in 81 inflammatory and 70 non-inflammatory controls.

Results-CSF amounts of 78kDa HLA-G dimers were more frequent in RRMS than in inflammatory ($p<0.01$) and non-inflammatory controls ($p<0.001$) and in MRI inactive than in MRI active RRMS ($p<0.00001$).

Conclusion-Our findings suggest that HLA-G dimers may be implicated in termination of inflammatory response occurring in MS.

INTRODUCTION

Human Leukocyte Antigen-G (HLA-G) are immunonologically functional non-classical HLA-I proteins which have recently been indicated as potential immunomodulatory molecules in Multiple Sclerosis (MS). In fact, an overexpression of HLA-G and its inhibitory receptors (ILT-2 and ILT-4) was found in MS plaques and on cerebrospinal fluid (CSF) monocytes from MS patients.[1] In addition, high levels of intrathecally produced soluble HLA-G (sHLA-G) were observed in CSF of MS patients with magnetic resonance imaging (MRI) inactive disease,[2,3] in whom they were correlated positively with CSF concentrations of anti-inflammatory IL-10[2] and inversely with CSF titers of anti-apoptotic sFas molecules.[4] These findings argue for a tolerogenic role of sHLA-G in MS, favouring the remission of the disease. In this setting, the immunosuppressive function could be exerted by actively secreted HLA-G5 that was the preponderant sHLA-G isoform detectable in CSF of MS patients without evidence of MRI activity.[5] However, this hypothesis remains to be demonstrated since no data are currently available on the presence of HLA-G disulfide-linked dimers in CSF of MS patients. Indeed, HLA-G in dimeric form exhibits higher affinity for ILT2 and

ILT4 receptors and promotes a more efficient inhibitory receptor signaling compared to monomers[6,7] and, therefore, it is considered as the biologically active HLA-G form with the strongest immunosuppressive effects.[8] Based on these considerations, in this study we sought to investigate the distribution of CSF HLA-G dimers in MS and controls.

MATERIALS AND METHODS

CSF samples were prospectively collected in 80 consecutive newly diagnosed[9] definite relapsing-remitting (RRMS) patients (55 women and 25 men; mean age=38.7±10.9 years) and 151 age and sex matched neurological controls represented by 81 patients (55 women and 26 men; mean age=38.5±9.9 years) with other inflammatory neurological disorders (OIND) and 70 subjects (49 women and 21 men; mean age=39.9±10.3 years) with other non-inflammatory neurological disorders (NIND) (Supplementary Table). All patients were imaged with a 1.5-Tesla MRI unit within 48 hours after sampling. MS patients were considered as clinically and MRI disease active if they had evidence of a relapse at admission and lesions showing Gd-enhancement on T1-weighted scans, respectively. Median Expanded Disability Status Scale was 2.0 (interquartile range=1.0-3,5; mean=2.1±1.5). Median duration of the disease was 7.0 (interquartile range=1.0-39; mean=28±38.3). None of the patients had fever or other symptoms or signs of acute infections and had received any potential disease-modifying therapies during the 6 months before the study. None of the female MS patients was pregnant. All OIND and NIND patients were free of immunosuppressant drugs, including steroids. Informed consent was given by all patients before inclusion and the study design was approved by the Local Committee for Medical Ethics in Research. As previously reported,[2-5] CSF levels of sHLA-G were measured by enzyme-linked immunosorbent assay using the monoclonal antibody MEM-G9 as capture antibody. After biotynilation and immunoprecipitation of CSF samples, CSF HLA-G monomers and dimers were identified by western blot analysis under reducing and non-reducing conditions in all sHLA-G positive RRMS, OIND and NIND patients as described elsewhere[10] (Supplementary Material).

After checking data for normality by using the Kolmogorov-Smirnov test, continuous variables were compared using Kruskal-Wallis and Mann-Whitney U test, categorical variables were compared by means of Chi-square test. Bonferroni correction was utilized for multiple comparisons. A value of $p<0.05$ was assumed as statistically significant.

RESULTS

Detectable CSF levels of sHLA-G were more frequent ($p<0.00001$;Chi-square) in RRMS (58/80;72.5%) than in OIND (23/81;28.4%) and in NIND (12/70; 17.1%) patients. As shown in the Table, CSF concentrations of sHLA-G were different among RRMS and controls ($p<0.00001$;Kruskal-Wallis) since they were higher in RRMS than in OIND and NIND ($p<0.00001$) and equivalent between OIND and NIND. In sHLA-G positive patients, while a 39kDa monomeric band was present in 100% of RRMS and controls, a 78kDa dimeric HLA-G band was more represented in RRMS than in OIND ($p<0.001$) and NIND ($p<0.01$). When RRMS patients were stratified according to clinical and MRI activity, CSF titers of sHLA-G and CSF monomers and dimers distribution did not differ between clinically active and clinically stable RRMS. Conversely, CSF concentrations of sHLA-G were more elevated ($p<0.001$) in RRMS patients without than in those with Gd enhancing lesions, whereas CSF HLA-G dimers were more frequent in MRI inactive than in MRI active sHLA-G positive RRMS ($p<0.00001$). An additional HLA-G like band with a molecular weight of 53 kDa was detected in 6 clinically and MRI active RMMS, in 4 OIND and 3 NIND sHLA-G positive patients (Figure).

DISCUSSION

In this study, we confirmed previous data[2-5] showing that CSF levels of sHLA-G were higher in MS than in controls and predominated in MS patients without MRI evidence of active disease. These results further strengthen the possibility that sHLA-G can be implicated in immunomodulation of CNS inflammatory response operating in MS. However, whether sHLA-G molecules detected at

intrathecal level in MS are functionally active is still to be proven. Therefore, the main finding of our investigation was the demonstration, for the first time, that HLA-G in dimeric form was present in CSF of MS patients and its distribution reflected the fluctuations of sHLA-G antigens because HLA-G dimers were more frequent in MS than in controls and in MRI inactive than in MRI active MS. In fact, the unique characteristic of both membrane-bound and soluble isoforms of HLA-G is the ability to form disulfide-linked dimers which are created through the generation of disulfide bonds between two cysteine residues at position 42 of the HLA-G alpha-1 domain. As these structures interacts with high affinity with HLA-G specific receptors and, thus, are believed to represent the biologically active form of HLA-G generating immunosuppressive effects.[6-8] These data strongly support the involvement of HLA-G in MS autoimmunity as anti-inflammatory molecules mediating the termination of inflammation. Intriguingly, elevated amounts of a 53 kDa HLA-G like protein were found in CSF of a small proportion of clinically and MRI active RRMS patients and, with a lesser extent, OIND and NIND patients. However, the actual biological significance of this molecule is currently elusive.[10] Future studies are warranted to elucidate the actual significance of CSF HLA-G dimers in MS.

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CONFLICT OF INTEREST STATEMENT

The Authors declare that there is no conflict of interest.

APPENDIX

Steering Committee

Granieri E (Chair), Castellazzi M, Casetta I (Section of Neurology, Department of Biomedical and Specialist Surgical Sciences, University of Ferrara, Italy), Tola MR (Neurology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliera-Universitaria, Arcispedale S. Anna, Ferrara, Italy), Fainardi E (Neuroradiology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliera-Universitaria, Arcispedale S. Anna, Ferrara, Italy), Dallocchio F, Bellini T (Section of Medical Biochemistry, Molecular Biology and Genetics, Department of Biomedical and Specialist Surgical Sciences, University of Ferrara, Italy), Rizzo R, Rotola A, Di Luca D (Section of Microbiology and Medical Genetics, Department of Medical Sciences, University of Ferrara, Italy), Seraceni S, Contini C (Section of Infectious Diseases, Department of Medical Sciences, University of Ferrara, Italy), Sabbioni S (Section of Microbiology and Applies Pathology, Department of Life Sciences and Biotechnology, University of Ferrara, Italy), Negrini M (Section of Pathology, Oncology and Experimental Biology, Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Italy), Tognon M (Section of Pathology, Oncology and Experimental Biology, Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Italy), Antonelli T (Section of Pharmacology, Department of Medical Sciences, University of Ferrara, Italy).

Partecipants

Groppo E, Gentile M (Department of Biomedical and Specialist Surgical Sciences, University of Ferrara, Italy), Baldi E, Caniatti ML, Ceruti S (Department of Neurosciences and Rehabilitation, Azienda Ospedaliera-Universitaria, Arcispedale S. Anna, Ferrara, Italy), Manfrinato MR, Trentini A (Department of Biomedical and Specialist Surgical Sciences, University of Ferrara, Italy), Bortolotti D (Department of Medical Sciences, University of Ferrara, Italy), Miotto E, Ferracin M, Mazzoni E, Pietrobon S, Masini I, Rotondo JC, Martini F (Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Italy), Baruzzi A, Roberto D'Alessandro R,

Michelucci R, Salvi F, Stecchi S, Scandellari C (IRCCS Institute of Neurological Sciences, Bologna, Italy), Terzano G, Granella F (Department of Neurosciences, University of Parma, Parma, Italy), Nichelli P, Sola P, Ferraro D, Vitetta F, Simone AM, Bedin R (Department of Neurosciences Nuovo Ospedale Civile S. Agostino-Estense, Baggiovara, Modena, Italy), Marcello N, Motti L, Montepietra S (Department of Neurosciences, IRCCS S. Maria Nuova Hospital, Reggio Emilia, Italy), Guidetti D, Immovilli P (Department of Neurology, Guglielmo da Saliceto Hospital, Piacenza, Italy), Montanari E, Pesci I, Guareschi A (Department of Neurology, Civile Hospital, Fidenza, Parma Italy), Greco G, Santangelo M (Department of Neurosciences, Ramazzini Hospital, Carpi, Modena, Italy), Mauro AM, Malagù S (Neurology Unit, Bufalini Hospital, Cesena, Italy), Rasi F, Spadoni M, Galeotti M, Fiorani L (Neurology Unit, S. Maria delle Croci Hospital, Ravenna, per gli Infermi Hospital, Faenza, Umberto I Hospital, Lugo, Italy), Neri W (Department of Neurology, Morgagni-Pierantoni Hospital, Forlì, Italy), Ravasio A, Pasquinelli M (Neurology Unit, Infermi Hospital, Rimini, Italy), Gutman S, Monaldini C (Neurology Unit, Repubblica di S. Marino Hospital)

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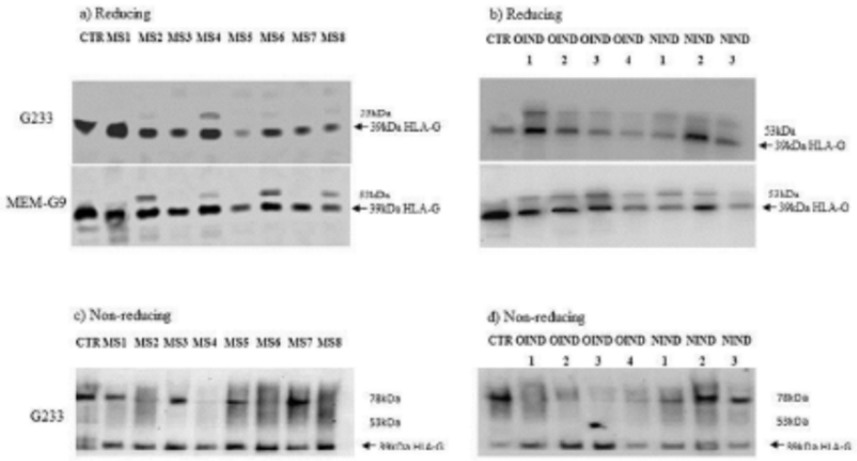
Table. CSF levels of sHLA-G in all patients with other non-inflammatory neurological disorders (NIND), other inflammatory neurological disorders (OIND) and relapsing-remitting (RRMS) categorized according to clinical and magnetic resonance imaging (MRI) activity, and distribution of CSF monomers and dimers in HLA-G positive NIND, OIND and RRMS.

	CSF sHLA-G (ng/ml) median, IQR, mean ± SD (Total)	39kDa HLA-G monomer % (sHLA-G+)	78kDa HLA-G dimer % (sHLA-G+)	HLA-G like 53kDa % (sHLA-G+)
NIND (Total n=70; sHLA-G+ n=12)	0, 0-0, 1.2±7.2	12/12 (100%)	3/12 (25%)	0/12 (0%)
OIND (Total n=81; sHLA-G+ n=23)	0, 0-1.2, 5.1±12.1	23/23 (100%)	6/23 (26.1%)	2/23 (8.7%)
RRMS (Total n=80; sHLA-G+ n=58)	16, 0-32.3, 19.4±19.5^	58/58 (100%)	40/58 (69%)*≠†	6/58 (10.3%)
CA RRMS (Total n=49; sHLA-G+ n=36)	15, 0-26, 16.4±15.2	40/40 (100%)	25/40 (62.5%)	6/40 (15%)
CS RRMS (Total n=31; sHLA-G+ n=13)	18.6, 0-39.7, 24.1±24.3	22/22 (100%)	15/22 (68.2%)	0/22 (0%)
Gd+ RRMS (Total n=34; sHLA-G+ n=19)	2.7, 0-18.4, 10.8±14.1	19/19 (100%)	6/19 (31.6%)	6/19 (31.6%)
Gd- RRMS (Total n=46; sHLA-G+ n=39)	21, 11.0-41.7, 25.7±20.6°	39/39 (100%)	34/39 (87.2%)♦	0/39 (0%)

Total = total patients; sHLA-G+ = HLA-G positive patients; IQR = Interquartile range; SD = Standard deviation; Gd+ = MRI appearance of gadolium enhancing lesions; Gd- = no MRI evidence of gadolium enhancing lesions; CA = RRMS clinical active; CS = RRMS clinical stable; CSF sHLA-G levels (Mann-Whitney): ^RRMS vs. OIND and NIND ($p<0.00001$), °Gd- vs. Gd+ RRMS; 78kDa HLA-G dimer (Chi-square): *RRMS vs. OIND and NIND ($p<0.00001$), ≠RRMS vs. OIND ($p<0.001$), †RRMS vs. NIND ($p<0.01$), ♦Gd- vs. Gd+ RRMS.

Figure. Western Blot profiles of ELISA sHLA-G positive CSF samples from 8 patients with relapsing-remitting MS (MS), 4 patients with Other Inflammatory Neurological Disorders (OIND) and 3 patients with Non-Inflammatory Neurological Disorders (NIND) immunoprecipitated with both G233 and MEM-G9 antibodies and analysed under reducing (denaturating) and non-reducing (non-denaturating) conditions. The molecular weights were determined with the BenchMark (Invitrogen, CA, US) (Mk) pre-stained protein ladder (range 10-200 kD). 721.221G cell culture supernatants are the positive control (CTR). sHLA-G momers and dimers migrate at 39 kDa and 78 kDa, respectively, whereas additional HLA-G like molecules are recognized at 53 kDa. Upper blots show results obtained under reducing conditions in MS (a) and OIND e NIND (b) patients after G233 and MEM-G9 immunoprecipitation. Lower blots indicate the staining under non-reducing conditions in MS (c) and OIND e NIND (d) patients immunoprecipitated with G233.



235x161mm (300 x 300 DPI)

SUPPLEMENTARY MATERIALS

sHLA-G immunoprecipitation

721.221G cell culture supernatants (used as positive control) and CSF samples (100ul) were biotynilated with 0.2 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) in pH 8.0 PBS 1x for 30 min at 4°C. Samples were then immunoprecipitated for 2 hrs at RT with anti-HLA-G MoAb (G233, or MEMG9, specific for beta2-microglobulin conjugated HLA-G, Exbio, Praha, Czech Republic), washed twice in PBS 1x and incubated over night with protein G-Sepharose beads (Santa Cruz, CA,USA) at 4°C. The samples were washed twice and resuspended in 20ul of Laemli Buffer (BioRad, Segrate, MI, Italy).

Western Blot analysis

Immunoprecipitates were denatured at 100 °C for 5 min. Proteins were loaded with native or reducing (in presence of SDS) running buffers in 10% TGX-Pre-cast gel (Biorad, Segrate, MI, Italy), with subsequent electroblotting transfer onto a PVDF membrane (Millipore, MA, USA). The membrane was incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (1:5000; Amersham Biosciences, NJ, USA) and developed with the ECL kit (Amersham Biosciences, NJ, USA). The images were acquired by Geliance 600 (Perkin Elmer, Massachussets, USA).

Supplementary Table. Demographic and clinical features of Other Inflammatory Neurological Diseases (OIND) and Non-Inflammatory Neurological Diseases (NIND) patients.

Patients	n	Female:Male	Type of disease
OIND	81	55/26	
	27		Chronic inflammatory demyelinating polyneuropathy
	20		Acute inflammatory demyelinating polyneuropathy
	6		Herpes simplex virus-1 encephalitis
	5		Varicella-zoster virus encephalitis
	5		Viral meningitis
	4		Bacterial meningitis
	4		Inflammatory myelitis
	3		Neurolupus
	2		NeuroSjogren
	1		NeuroBechet
	1		HIV-related leukoencephalopathy
	1		Acute disseminated encephalomyelitis
	1		Post-infectious myelitis
	1		Post-infectious posterior reversible encephalopathy syndrome
NIND	70	49/21	
	18		Transient ischemic attack
	11		Headache
	9		Amyotrophic lateral sclerosis
	8		Vascular dementia
	6		Migraine
	3		Epilepsy
	3		Mild cognitive impairment
	3		Low grade glioma
	3		Parkinson disease
	2		Hereditary neuropathy
	2		Alzheimer disease
	1		compression neuropathy
	1		cervical spondylosis

Interestingly, HLA-G and its inhibitory receptors (ILT-2 and ILT-4) are found strongly up-regulated within and around MS lesions where microglia, macrophages and endothelial cells are recognized as the cellular sources [158]. This data agrees with our finding about HLA-G dimers in CFS since it is known that dimers can bind with major strength ILT-2 receptor [74]. Furthermore, cultured human MS microglial cells activated with Th1 proinflammatory cytokines have a higher HLA-G expression and a novel subpopulation of naturally occurring $CD4^+$ and $CD8^+$ Treg cells expressing HLA-G (HLA-G^+ Treg) [159] induced by IL-10 [160, 161] has been recently described in peripheral blood of MS patients with relapse. Taken together, these observations strenghten the evidence that HLA-G antigens are likely to be involved in the resolution of MS autoimmunity acting as anti-inflammatory molecules and suggest that HLA-G^+ Treg could play a key role in the development of a CNS immunosuppressive microenvironment at the sites of inflammation in MS.

1.2.3 HLA-G in other inflammatory and autoimmune diseases

HLA-G proves also to be an important biological marker in other pathologies, for example, gastrointestinal, allergic, and cutaneous diseases.

Ulcerativecolitis (UC) and Crohn's disease are characterized by a different sHLA-G expression pattern [162-165] by peripheral blood mononuclear cells. In particular, non-activated peripheral blood mononuclear cells from Crohn's disease patients produce spontaneously sHLA-G while those from UC patients and healthy donors do not. Furthermore, after stimulation with LPS, both cells from Crohn's disease and healthy subjects show sHLA-G production, while this does not happen in UC patients. The different HLA-G expression profiles observed in UC and Crohn's disease patients sustain the different aethiopathogenesis at the origin of these two diseases. In particular, the responses to therapies in UC and Crohn's disease correspond to different sHLA-G secretion levels [166]. When immunosuppressant therapy is administered, a normalization in the production of HLA-G molecules in Crohn's disease is obsverved, while it starts the release of HLA-G in UC patients. These data confirm the diversity in the behavior of these two pathologies and propose the analysis of sHLA-G levels with the final goal of distinguishing between UC and Crohn's disease patients and to monitor therapy. In particular, I investigated the association

between the HLA-G 14-bp deletion/insertion (DEL/INS) polymorphism and soluble (s)HLA-G production in Tunisian patients affected by Crohn's disease (CD). Furthermore, the presence of HLA-G dimer was analyzed by western Blot [167 paper attached]. We found a significant association concerning the genotype Ins/Ins for young-onset CD patients, but not with adult-onset CD patients. We observed also a significant increase in sHLA-G dosed by ELISA in CD patients compared to controls. Among sHLA-G positive patients, HLA-G dimers were found in the 43% of subjects present with a correlation to the advanced stages of the disease. These findings indicate that the 14-bp Del/Ins polymorphism of HLA-G gene and the presence of dimers are associated with the risk of CD and suggest a role for sHLA-G as prognostic marker for progressive disease.

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Association between sHLA-G and HLA-G 14-bp deletion/insertion polymorphism in Crohn's disease

Zidi Inès[1]*, Hamza Ben Yahia[1], Daria Bortolotti[2], Leila Mouelhi[1,3], Ahmed Baligh Laaribi[1,4], Shema Ayadi[3], Nour Zidi[5], Fatma Houissa[3], Radhouane Debbech[3], Abdellatif Boudabous[1], Taoufik Najjar[3], Dario Di Luca[2] and Roberta Rizzo[2]

[1]Laboratory Microorganismes et Biomolécules Actives, Sciences Faculty of Tunis, University of Tunis El Manar, Tunis, Tunisia
[2]Department of Experimental and Diagnostic Medicine, Section of Microbiology, University of Ferrara, Ferrara, Italy
[3]Gastroenterology Department, Charles Nicolle Hospital, Medicine Faculty of Tunis, University of Tunis El Manar, Tunis, Tunisia
[4]Laboratory of Microbiology-Immunology, UR02SP13, CHU Farhat Hached, Sousse, Tunisia
[5]Faculty of Medicine Ibn Al Jazzar, Sousse, Tunisia

*Correspondence to: Z. Inès, Laboratoire Microorganismes et Biomolécules Actives, Département de Biologie, Faculté des Sciences de Tunis, University of Tunis El Manar, 2092 Tunis, Tunisia. E-mail: ines.zidi@techemail.com

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Abstract

The aim of this study was to evaluate the association between the HLA-G 14-bp deletion/insertion (Del/Ins) polymorphism and soluble (s) HLA-G production in patients with Crohn's disease (CD). We analyzed also the sHLA-G molecules by ELISA and western blot in plasma samples. Among unselected patients, the 14-bp Del/Ins polymorphism was not significantly associated with increased CD risk neither for alleles ($P = 0.371$) nor for genotypes ($P = 0.625$). However, a significant association was reported between the 14-bp Del/Ins polymorphism and CD, in particular in young-onset CD patients for alleles [$P = 0.020$, odds ratio (OR) = 2.438, 95% confidence interval (CI): 1.13–5.25] but not with adult-onset CD patients. A significant association was reported concerning the genotype Ins/Ins for young-onset CD patients ($P = 0.029$, OR = 3.257, 95% CI: 1.08–9.77). We observed also a significant increase in sHLA-G dosed by ELISA in CD patients compared to controls ($P = 0.002$). The 14-bp Del/Del and 14-bp Del/Ins genotypes are the high HLA-G producers. Among sHLA-G^{positive} patients, a 43% of subjects present dimers of HLA-G. The presence of dimers seems to be related to the advanced stages of the disease. The 14-bp Del/Ins polymorphism is associated with an increased risk of CD particularly in young-onset CD patients and controls sHLA-G plasma levels. Dimers of sHLA-G are frequent in advanced disease stages. The above findings indicate that the genetic 14-bp Del/Ins polymorphism in the exon 8 of HLA-G gene is associated with the risk of CD and suggest a role for sHLA-G as prognostic marker for progressive disease.

Keywords: 14 bp, conformation, Crohn's disease, HLA-G, polymorphism

Introduction

Crohn's disease (CD), an inflammatory bowel disease, is characterized by a chronic inflammation commonly localized in ileocecal area (1). This auto-immune disease is associated in part by genetic background and also by immunologic factors (2). In fact, CD is linked to increased levels of T_h1 cytokines (including IFN-γ, tumor necrosis factor-alpha and IL-12), as well as higher concentrations of T_h17 cytokines (including IL-17A, IL-17F, IL-22, IL-21 and IFN-γ) (2, 3).

HLA-G is an immune-modulatory molecule located in the short arm of the chromosome 6. It is a non-classical HLA-I molecule characterized by low allelic polymorphism and a restrictive tissue expression in comparison with classical HLA-I antigens (4). After alternative splicing of the primary transcript, seven HLA-G isoforms could be obtained: four membranous isoforms (HLA-G1, G2, G3 and G4) and three soluble isoforms (HLA-G5, G6 and G7). HLA-G possesses an unpaired cysteine residue at position 42 on an external loop of the peptide binding groove that enables the dimerization (5). Leukocyte immunoglobulin-like receptors receptors have a greater affinity for the dimeric form that increases the signaling transduction (6).

HLA-G molecules mediate immunosuppressive functions through the inhibition of immune cells. Indeed, HLA-G inhibits the lysis of NK cells (7–9), the alloproliferation of $CD4^+$ T cells (10, 11) and the antigen presentation of dendritic cells (12). It enhances, in the other side, the production of regulatory T

cells (13) and the apoptosis of $CD8^+$ cells (14). Importantly, the 14-bp Del/Ins polymorphism in the 3′-untranslated region of HLA-G (rs66554220) controls mRNA stability (15). In particular, the insertion (Ins) has been associated with lower levels of HLA-G expression (16–18). Taking into account that the 14-bp Del/Ins polymorphism could influence HLA-G protein levels, and the reported implication of HLA-G molecules in patients with CD (19), first, we studied the contribution of this polymorphism on the susceptibility to CD in Tunisian samples, stratifying by disease onset, behavior, location and surgical resection.

Secondly, we analyzed the plasma levels of soluble (s) HLA-G and tried to correlate it to HLA-G 14-bp Del/Ins genotype either for CD patients or for healthy controls.

Methods

Patients

Blood samples were obtained from subjects with CD recruited from the Department of Gastroenterology in Charles Nicole Hospital of Tunis. Patients have not been treated by immunosuppressive therapeutics. Altogether, 44 patients (20 males and 24 females) were recruited with a mean age 36.89 ± 12.29 (SD; age range: 20–69 years) (Table 1). The sex ratio was evaluated to 0.83. CD diagnosis was based on clinical, radiological, endoscopic and histopathologic findings. Patients with uncertain diagnosis or with other auto-immune disease were excluded from the study. Several parameters were collected including age, family history, disease localization, smoking habits, surgical therapy, chemical therapy and extra-intestinal manifestations.

The control population consisted of 71 healthy subjects including 30 males and 41 females with a mean age 34.04 ± 11.58 (age range: 19–64 years).

All patients and control subjects were Tunisian. The study was approved by the local ethics committee. Subjects were stratified based on their age-at-CD onset in two groups: Young-onset CD (age ≤25 years) and Adult-onset CD (age >25 years). Patients were also stratified based on the disease behavior in inflammatory, stenotic phenotypes according to Montreal classification (20).

14-bp Del/Ins gene polymorphism

Genomic DNA was extracted from blood using the salting method. 14-bp Del/Ins genotyping was performed by PCR as previously described (21). Briefly, DNA was amplified with a set of primers: 5′-GTG ATG GGC TGT TTA AAG TGT CAC C-3′, 5′-GGA AGG AAT GCA GTT CAG CAT GA-3′. The 35 cycles of PCR was perform at 94°C for 30 s, 64°C for 60 s and 72°C for 60 s, and final cycle of 72°C for 10 min.

DNA fragments were electrophoresed on 3% agarose gels containing ethidium bromide. The insertion allele was visualized as 224-bp band, while the deletion allele was seen as 210-bp bands. Genotyping call rate exceeded 98%—no significant differences between cases and healthy controls.

Soluble HLA-G dosage by ELISA

sHLA-G levels [shedding HLA-G1 (sHLA-G1) molecules generated by metalloproteinases proteolytic cleavage (22) and HLA-G5 molecules] were measured in plasma of CD patients (n = 30) and controls (n = 25) in duplicate as previously reported in the Essen Workshop (23). We used the monoclonal antibody (mAb) MEM-G9 (Exbio, Praha, Czech Republic) as a capture antibody and the β2-microglobulin as the second specific antibody.

We measured also HLA-G5 levels according to the Essen Workshop (23), with the 5A6G7 (Exbio) as capture antibody and W6/32 (Exbio) as secondary antibody. The limit of sensitivity was 1.0 ng ml^{-1}.

sHLA-G immunoprecipitation and western blot analysis

221-G1 cell culture supernatants and samples were biotinylated with 0.2 mg ml^{-1} EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) in pH 8.0 PBS for 30 min at 4°C (17). Samples were then immunoprecipitated for 2 h at room temperature with anti-HLA-G MoAb (MEM-G9; Exbio), washed twice in PBS and incubated overnight with protein G-Sepharose beads (Santa Cruz, CA, USA) at 4°C. The samples were washed twice and suspended in 20 μl of Laemli Buffer (Bio-Rad, Segrate, Milan, Italy). The protein concentration in immunoprecipitates was quantified by the Bradford assay (Bio-Rad Laboratories) using plasma bovine albumin (Sigma-Aldrich) as the standard. The purified sHLA-G1 molecules obtained from untreated 221-G1 culture supernatants were used as positive control. Total protein was denatured at 100°C for 5 min. Proteins were loaded with or without reducing buffers in 10% TGX-Pre-cast gel (Bio-Rad), with subsequent electroblotting transfer onto a PVDF membrane (Millipore). The membrane was incubated with a HRP-conjugated streptavidin (Thermo Scientific, Rockford, IL, USA) and developed with the ECL kit (Amersham Biosciences, NJ, USA). The images were acquired by the Bio-Rad Gel Doc

Table 1. The characteristics of CD patients and controls

Characteristics	Patients	Controls	*P* value
Female *n* (%)/male *n* (%)	24 (54.5)/20 (57.7)	41 (45.5)/30 (42.3)	0.740[a]
Age [M ± SEM (25% P–75% P)]	36.89 ± 1.85 (27.00–47.00)	34.04 ± 1.37 (24.00–43.00)	0.118[b]
Disease onset: young (%)/adult (%)	18 (40.9)/26 (50.1)	—	
Disease duration [M ± SEM (25% P–75% P)]	6.57 ± 0.81 (2.00–8.00)	—	

M, mean; P, percentile; SEM, standard error of the mean.
[a]2 × 2 contingency table: χ^2 = 011.
[b]Mann–Whitney test: U = 1301.

(Bio-Rad, Milano, Italy). Monomers were detected at 39 kDa, while dimers at 78 kDa.

Statistical analysis

Statistical analysis was performed with SPSS (16.0) and by Graphpad prism 5. Comparison between baseline characteristics and levels of sHLA-G among patients and controls were performed estimated by Mann–Whitney *U*-test.

The differences in genotypic/allelic frequencies between patients and controls were evaluated by chi-square (χ^2) test (2×2 contingency table for alleles and 2×3 contingency table for genotypes). Pearson chi-square or Fisher's exact test were used to assess inter-group significance. Spearman test was used to test for correlations between quantitative variables.

14-bp polymorphism was tested for Hardy–Weinberg equilibrium using http://oege.org/software/hwe-mr-calc.shtml. Two-tailed P values <0.05 were considered statistically significant.

Results

Study population

A total of 44 patients with CD were included in this study. Table 1 summarizes their main demographic and clinical characteristics. No significant differences were noticed either for subgroups stratified by genders ($P = 0.113$) or for the participants' age ($P = 0.118$). Among patients, the mean of CD duration was 6.57 ± 0.81 years.

HLA-G genetic polymorphism typing

Allelic and genotypic frequencies of 14-bp HLA-G polymorphism among CD cases and control subjects are presented in Tables 2 and 3. No Hardy–Weinberg equilibrium deviation was observed in the control and CD samples for the 14-bp HLA-G polymorphism ($\chi^2 = 0.041$; $P = 0.839$).

No different allele and genotype frequencies were observed between CD patients and control subjects ($\chi^2 = 0.801$, $P = 0.371$) (Table 2). When we stratified according to CD young-onset ($n = 18$; mean age of CD onset: 25.56 ± 1.20 years, range: 20–38 years old) and adult-onset ($n = 26$; mean age of CD onset: 43.36 ± 1.97 years, range: 29–69 years old), we found an increased frequency of Ins allele in young-onset CD patients ($\chi^2 = 5.358$, $P = 0.020$) (Table 3) in comparison with controls. Similarly, there was an over-representation of Ins/Ins genotype ($\chi^2 = 4.718$, $P = 0.029$) (Table 3). In young-onset CD patients, the Ins allele was associated with a 2.438-fold [95% confidence interval (CI): 1.131–5.254] higher risk of CD susceptibility compared with Del allele. Moreover, when we considered the three genotypes separately, we evidenced a tendency to a statistical significance for the association between the three Del/Ins 14-bp genotypes and CD susceptibility in young-onset patients ($\chi^2 = 5.560$, $P = 0.062$). In particular, the Ins/Ins genotype was associated with 3.257-fold (95% CI: 1.086–9.770) increased risk for CD susceptibility compared with the Del/Del and Del/Ins genotypes (Table 3). No differences were observed in old-onset patients (data not shown).

Allelic and genotypic frequencies of 14-bp polymorphism in CD patients after stratification for clinical phenotypes

Patients with CD were stratified for disease behavior (inflammatory, stenotic or inflammatory and stenotic phenotype), disease location (ileum and colon/ileum only or colon only) and surgical resection (resection or not) (Table 4). The Ins allele was frequent in the stenotic phenotype (62.5%) and in patients with CD located in both colon and ileum (56.2%) in comparison with total CD patients (51.1%). However, no statistical significant association was found after the three cited stratifications. No correlation was found between 14-bp polymorphism and extra-intestinal manifestations (data not shown).

Increase of sHLA-G in CD patients

CD patients and controls analyzed for sHLA-G plasma levels presented similar age (mean ± SEM, patients: 38.6 ± 2.38 versus controls: 34.22 ± 1.58, Mann–Whitney: $P = 0.156$) and similar sex ratio [males/females, patients: 14/16 (ratio = 0.88) versus 26/32 (ratio = 0.81)].

The mean of sHLA-G was 5.20 (SEM = 1.06) ng ml^{-1} and 2.14 (SEM = 0.45) ng ml^{-1} in CD patients and controls, respectively (Fig. 1A) and were statistically different ($P = 0.002$). In addition, the number of sHLA-G^{positive} CD patients are enhanced ($n = 25/30$, 76.7%) compared with controls ($n = 25/58$, 43.1%) ($P = 3\times10^{-4}$).

Table 2. Distribution of allelic and genotypic frequencies of Del/Ins 14-bp polymorphism in CD patients (without stratification) and controls

14-bp Del/Ins (rs66554220)	Patients ($n = 44$), n (%)	Controls ($n = 71$), n (%)	χ^2	P value	OR (95% CI)
Alleles					
Del	43 (48.9)	78 (54.9)	0.80	0.37	1.27 (0.75–2.17)
Ins	45 (51.1)	64 (45.1)			
Genotypes[a,b]					
Del/Del	11 (25)	21 (29.6)	0.28	0.59	0.79 (0.34–1.86)
Ins/Ins	12 (27.3)	14 (19.7)	0.89	0.35	1.53 (0.63–3.70)
Del/Ins	21 (47.7)	36 (50.7)	0.09	0.76	0.89 (0.42–1.89)

14 bp, 14 base pairs; Del, deletion; Ins, insertion; OR, odds ratio.
[a]2×3 contingency table: $\chi^2 = 0.94$, $P = 0.63$.
[b]Hardy–Weinberg equilibrium: $\chi^2 = 0.04$, $P = 0.98$.

Table 3. Distribution of allelic and genotypic frequencies of Del/Ins 14-bp polymorphism in young-onset CD Patients (stratification by disease onset) and Controls

14-bp Del/Ins (rs66554220)	Patients (n = 18), n (%)	Controls (n = 71), n (%)	χ^2	P value	OR (95% CI)
Alleles					
Del	12 (33.3)	78 (54.9)	5.36	**0.02**	2.44 (1.13–5.25)
Ins	24 (66.7)	64 (45.1)			
Genotypes[a]					
Del/Del	2 (11.1)	21 (29.6)	—	0.14[b]	0.30 (0.06–1.44)
Ins/Ins	8 (44.4)	14 (19.7)	4.72	**0.03**	3.26 (1.08–9.77)
Del/Ins	8 (44.4)	36 (50.7)	0.23	0.64	0.78 (0.28–2.20)

14 bp, 14 base pairs; Del, deletion; Ins, insertion; OR, odds ratio.
[a]2 × 3 contingency table: $\chi^2 = 5.56$, $P = 0.06$.
[b]Fisher's exact test.

Table 4. Distribution of allelic and genotypic frequencies of Del/Ins 14-bp polymorphism in CD patients after stratification for clinical phenotypes

Genotype/allele	Disease behavior			Disease location		Resection or not	
	Inflammatory phenotype, n = 20/43 (%)	Stenotic phenotype, n = 12/43 (%)	Inflammatory and stenotic phenotype, n = 11/43 (%)	Location in both ileum and colon, n = 19/43 (%)	Location in ileum only or colon only, n = 24/43 (%)	No resection	Resection
Del	23 (57.5)	9 (37.5)	10 (45.5)	21 (43.8)	21 (55.3)	28 (50)	14 (50)
Ins	17 (42.5)	15 (62.5)	12 (54.4)	27 (56.2)	17 (44.7)	28 (50)	14 (50)
Del/Del	8 (40)	2 (16.7)	1 (9.1)	4 (16.7)	7 (36.8)	8 (28.6)	3 (21.4)
Ins/Ins	5 (25)	5 (41.7)	2 (18.2)	7 (29.2)	5 (26.3)	8 (28.6)	3 (21.4)
Del/Ins	7 (35)	5 (41.7)	8 (72.7)	13 (54.2)	7 (36.8)	12 (42.9)	8 (57.1)

Similarly, the mean of sHLA-G1 was increased in CD patients compared with controls (3.87 ± 0.92 versus 1.10 ± 0.32 ng ml^{-1}) ($P < 0.0001$, Fig. 1B). The number of positive plasma samples for sHLA-G1 was enhanced for CD patients (n = 21/30, 70% versus 12/58, 20.7%) ($P = 1.6 \times 10^{-5}$).

On the contrary, the levels of HLA-G5 were lower in CD patients compared with controls without reaching statistical significance (1.33 ± 0.67 versus 1.05 ± 0.31 ng ml^{-1}) ($P = 0.37$, Fig. 1C). The number of positive plasma samples for HLA-G5 was enhanced for controls (n = 16/58, 27.6% versus 5/30, 16.7%) ($P = 0.3$).

There was no significant association between age, sex, duration of illness with sHLA-G, sHLA-G1 or HLA-G5 levels (data not shown).

Association of sHLA-G to 14-bp Del/Ins polymorphism

According to previous studies (17, 24), the presence of one or two 14-bp Del alleles (14-bp Del/Del and 14-bp Del/Ins genotypes) is associated with higher HLA-G production. Similarly, we observed that CD patients with high-producer genotypes (14-bp Del/Del, Del/Ins) expressed increased sHLA-G mean plasma levels [7.36 ± 1.58 (SEM) ng ml^{-1}] in comparison with low producer genotype (14-bp Ins/Ins) (5.14 ± 1.00 ng ml^{-1}) even without reaching a statistical significance (P = 0.869) (Fig. 2A). Similar profile was found for sHLA-G1 concentrations, where high producer genotypes presented a mean of 6.17 ± 1.42 versus 4.32 ± 1.30 ng ml^{-1} for low producers, even without reaching a statistical significance (P = 0.282) (Fig. 2B). On the contrary, the 14-bp Del/Del and 14-bp Del/Ins genotypes presented lower concentrations of HLA-G5 (1.20 ± 0.81 ng ml^{-1}) compared with 14-bp Ins/Ins genotype (1.69 ± 1.12 ng ml^{-1}), even without reaching a statistical significance (P = 0.54) (Fig. 2C). The majority of high sHLA-G producers belong to adult-onset CD patients subgroup (71.9%) compared with low sHLA-G producers that mainly belong to young-onset CD patients subgroup (58.3%).

Association of sHLA-G dimers with advanced CD stages

Since HLA-G molecules have both monomeric and dimeric conformations, we performed western blot analysis on plasma samples positive for sHLA-G in ELISA. We easily distinguished the monomeric and dimeric conformations of sHLA-G (Fig. 3). The western blot analysis reported 43% of CD patients with sHLA-G dimers versus 78% for controls.

We evaluated the presence of sHLA-G dimers in association with clinical characteristics (Table 5). We observed a significant difference between genders in CD subgroups based on presence/absence of sHLA-G dimers (P = 0.04). Indeed, males present increased sHLA-G dimers than females (70 versus 30%) (P = 0.04). The absence of HLA-G dimers in patients with CD seems to be related to progressed stages

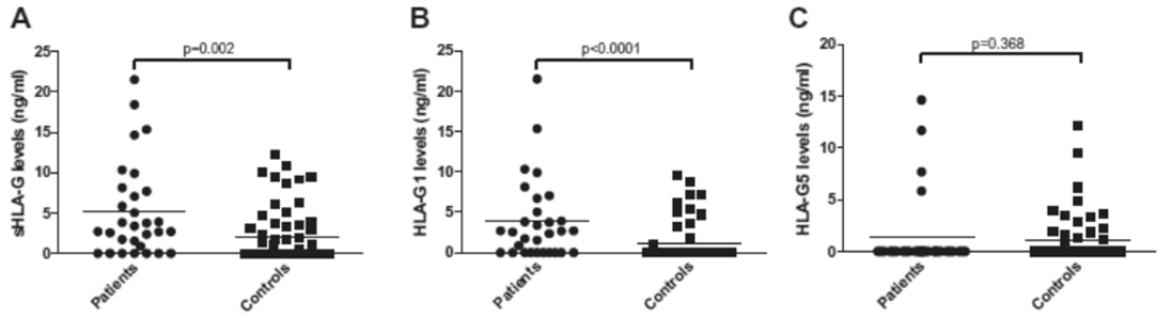

Fig. 1. Box-plot of serum sHLA-G (sHLA-G1 and HLA-G5) (A), sHLA-G1 (B) and HLA-G5 (C) concentrations in patients with CD and in healthy controls. Mean levels are indicated by horizontal lines. *P* values were obtained by Mann–Whitney test.

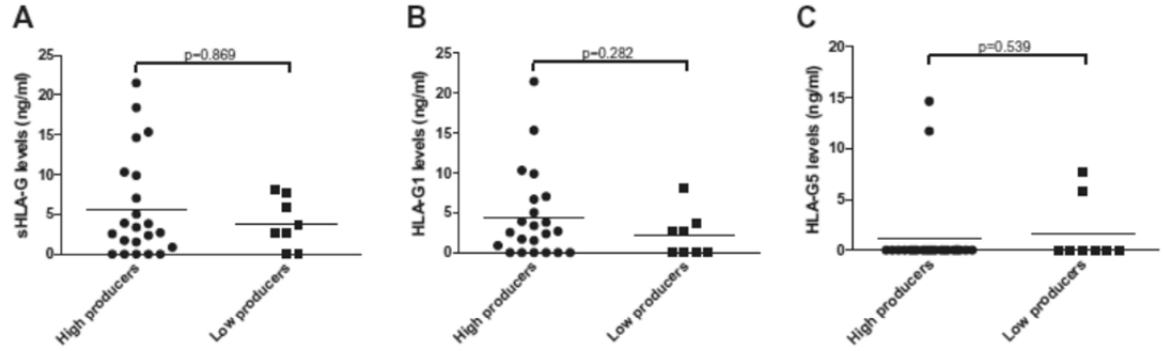

Fig. 2. Box-plot of serum sHLA-G (sHLA-G1 and HLA-G5) (A), sHLA-G1 (B) and HLA-G5 (C) concentrations in patients with CD subdivided according with HLA-G 14-bp Ins/Del genotypes. Mean levels are indicated by horizontal lines. *P* values were obtained by Mann–Whitney test. High producers: 14-bp Del/Del, Del/Ins; low producers: 14-bp Ins/Ins.

of CD characterized by an inflammatory phenotype (69.2% of CD patients with dimers) and an extended location covering the ileum or the colon (46.2% of CD patients with dimers) even without reaching a statistical significance (Table 5).

Discussion

Previous works have evaluated the role of HLA-G molecules in CD. In particular, Torres *et al.* (25) studied intestinal samples of CD patients and, by immunohistochemistry technique, demonstrated that CD intestinal biopsies did not present HLA-G expression. The distribution of the 14-bp Del/Ins polymorphism in CD patients was investigated by Glas *et al.* (26). They observed a significant increase of the Ins allele and the Ins/Ins genotype in those CD cases positive for ileocecal resection. Also Rizzo *et al.* (19) evaluated HLA-G expression in CD patients. Non-activated peripheral blood mononuclear cells from CD patients secrete spontaneously sHLA-G.

The present study was aimed at gaining further insight into the role of the genetic polymorphism HLA-G 14-bp Del/Ins in CD and the possible effect on HLA-G expression. Our results provide suggestive evidence for an association of this HLA-G polymorphism with the susceptibility to CD in young-onset CD patients. In fact, we observed an increased Ins allele and Ins/Ins genotype frequency in young-onset CD patients, where the Ins allele was associated with a 2.438-fold higher risk of susceptibility to CD compared with Del allele. Moreover, the Ins/Ins genotype was associated with 3.257-fold increased risk of CD susceptibility compared with the Del/Del and Del/Ins genotypes. These data sustain the role of HLA-G 14-bp Del/Ins polymorphism in CD. Similarly to Glas *et al.* (26), we found a tendency for an increase in Ins allele frequency in those CD patients with stenotic phenotype and location in both ileum and colon. These results could explain the maintenance of local inflammatory condition in CD patients, where an increase in Ins allele frequency could subtend to a lower HLA-G expression.

To confirm this point, we considered sHLA-G plasma levels and we observed significant higher levels of sHLA-G and in particular of sHLA-G1 isoform and an increased number of positive sHLA-G samples in CD patients in comparison with controls. As membranous HLA-G has not been described in intestinal samples from CD patients (25), we hypothesized that sHLA-G1 could not be issued from the local site of inflammation but from circulating immune cells including regulatory T cells (27, 28), and dendritic cells (29), that subtend a dysregulated immune response.

sHLA-G1 could derive from membrane-bound HLA-G1 cleavage due to metalloproteinases type 2, that are highly expressed in CD patients (30–32). This hypothesis is reinforced by the recent finding by Rizzo *et al.* that showed three possible highly specific cleavage sites for matrix metalloproteinase-2 (33). The systemic production of sHLA-G molecules

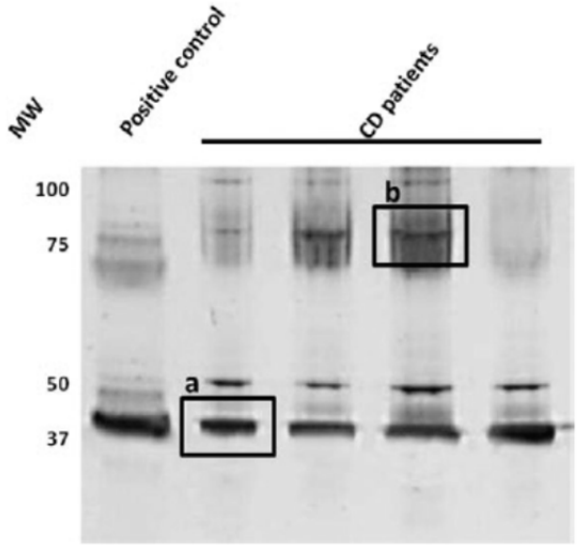

Fig. 3. sHLA-G expression and dimerization in patients with CD. Positive control corresponds to 221-G1 culture supernatant; (a) monomers of sHLA-G (sHLA-G1 and HLA-G5) (39 kDa); (b) dimers of sHLA-G (78 kDa). Samples were immunoprecipitated with MEM-G9 monoclonal antibody.

Table 5. The characteristics of CD patients sHLA-G^{positive} based on presence/absence of HLA-G dimers

	CD patients		*P* value[a]
	HLA-G dimers	No HLA-G dimers	
Female *n* (%)/male *n* (%)	3 (30)/7 (70)	10 (76.9)/3 (23.1)	**0.04**
Age [M ± SEM (25% P–75% P)]	40.80 ± 3.83 (32.75–49.25)	39.38 ± 4.13 (24.50–50.50)	0.80[b]
Disease duration [M ± SEM (25% P–75% P)]	9.20 ± 2.35 (1.00–16.75)	4.15 ± 0.66 (1.50–6.00)	0.15[b]
Disease behavior			
Inflammatory phenotype, *n* (%)	3 (30)	9 (69.2)	0.09
Stenotic phenotype, *n* (%)	2 (20)	1 (7.7)	0.56
Inflammatory and stenotic phenotype, *n* (%)	5 (50)	3 (23.1)	0.22
Disease location			
Location in both ileum and colon, *n* (%)	8 (80)	7 (53.8)	0.37
Location in ileum only or colon only, *n* (%)	2 (20)	6 (46.2)	0.38
Anal-perianal fistulas			
No, *n* (%)/yes, *n* (%)	6 (60)/4 (40)	12 (92.3)/1 (7.7)	0.12
Abscess			
No, *n* (%)/yes, *n* (%)	9 (90)/1 (10)	12 (92.3)/1 (7.7)	1
Resection or not			
No resection, *n* (%)	5 (50)	10 (76.9)	0.22
Resection, *n* (%)	5 (50)	3 (23.1)	

M, mean; P, percentile; SEM, standard error of the mean.
[a]2 × 2 contingency table: Fisher's exact text.
[b]Mann–Whitney test.

in CD patients represents a tentative to counteract the inflammatory condition.

As a confirm, of the previous data on the effect of HLA-G 14-bp Del/Ins polymorphism on HLA-G expression (16–18), we observed that patients with high-producer genotypes (Del/Del, Del/Ins) expressed increased sHLA-G mean level in comparison with sHLA-G low producer genotype (14-bp Ins/Ins) in CD patients. Similar profile was found for sHLA-G1 concentrations. We reported here a clear evidence of the correlation of Del allele to enhanced production of sHLA-G, while that low sHLA-G producers (Ins/Ins genotype) were included essentially in young-onset CD patients and were mainly characterized by HLA-G5 secretion. We could hypothesize a different cytokine environment, in young-onset CD patients, that could sustain HLA-G5 production also in low producer genotype. These results confirm the association of Ins allele

with low sHLA-G concentrations and sustain the role of this HLA-G polymorphism in controlling HLA-G expression also in a pathological condition as CD. However, the presence of higher levels of systemic sHLA-G in CD patients in comparison with controls could represent a tentative to counteract an inflammatory condition.

Since HLA-G could present monomeric and dimeric conformation, we evaluated the plasma composition in both controls and CD patients. We observed 43% of CD patients with sHLA-G dimers versus 78% for controls. These data are of extreme interest, since they could explain the contrasting results obtained with the levels of sHLA-G in plasma samples. In fact, even if we found higher levels of sHLA-G in CD plasma samples, they are mainly characterized by a monomeric conformation, that is known to have a lower affinity for inhibitory receptors (6). These results sustain the importance of evaluating not only HLA-G expression but also the monomer/dimer conformation. Additionally, the immunoprecipitation and western blot revealed some other extra-bands at 53 KDa that could be HLA-G-like molecules (34). When we correlated sHLA-G conformations with clinical characteristics, we observed the presence of sHLA-G dimers in CD patients with advanced stages of CD characterized by an inflammatory and stenotic phenotype and an extended location covering the ileum and the colon. We could hypothesize that sHLA-G dimers are increased in advanced stages of CD as an attempt to counteract inflammation.

To our knowledge, hitherto, this is the first study to describe the genotype of 14-bp Del/Ins polymorphism, the association with HLA-G expression and the monomer/dimer conformation in CD patients. Our data strengthen the functional role of HLA-G molecules in CD and suggest a potential use for clinical purposes as prognostic marker for progressive disease.

Acknowledgement

The authors declare that there are no conflicts of interests.

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The skin is characterized by a “skinimmunesystem (SIS),” where immune cells and humoral components support cutaneous inflammation. The deregulation of this skin defense mechanisms is evident in different inflammatory disorders of the skin, such as psoriasis, atopic dermatitis, pemfigo, vitiligo, and systemic sclerosis [168]. HLA-G protein normally is not expressed in the skin from healthy controls [169, 170], while ectopic HLA-G expression has been described in skin pathologies [–171-174].

Psoriasis is a chronic inflammatory skin disease characterized by an autoimmune component. Both membrane-bound and soluble HLA-G proteins have been detected in psoriatic skin lesions with the main compound characterized by macrophage lining at the dermoepidermal junctions [170]. It could be speculated that the up-regulation of HLA-G molecules by macrophages could represent an attempt to control auto-reactive T cells, induced by activated keratinocytes-derived cytokines/chemokines.

In fact, in this condition, HLA-G may modulate the activity of cytotoxic lymphocytes and promoting the development of Treg cells in order to prevent keratinocyte destruction [175]. Interestingly, psoriatic patients present lower plasma sHLA-G levels compared with controls [176], suggesting a difference in systemic HLA-G expression that could be associated with the IL-10 deficiency typical of psoriasis.

It is possible to identify three main therapies for Psoriasis: topical drugs, light therapy,and systemic medications. Evaluation of therapeutic effects on sHLA-G expression has shown an increase in plasmatic levels of systemic treated patients (efalizumab, cyclosporinA and acitretin) [176] and a significant association between HLA-G14bpDEL allele and 14bpDEL/DEL genotype with acitretin clinical out come [177 paper attached]. Thus, we can suppose a possible direct effect of HLA-G in antagonizing systemic Thelper1 activation that gives to HLA-G a potential role as a marker of response to acitretinin psoriatic patients.

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THERAPEUTIC HOTLINE

HLA-G 14-bp polymorphism: a possible marker of systemic treatment response in psoriasis vulgaris? Preliminary results of a retrospective study

Alessandro Borghi,* Roberta Rizzo,† Monica Corazza,* Alberto Maria Bertoldi,* Daria Bortolotti,† Giulia Sturabotti,* Annarosa Virgili* & Dario Di Luca†
**Department of Medical Sciences, Section of Dermatology and †Department of Medical Sciences, Section of Microbiology, University of Ferrara, Ferrara, Italy*

ABSTRACT: Human leukocyte antigen-G (HLA-G) is a nonclassical HLA class I molecule that exerts an immunosuppressive function. A 14-base pair (bp) sequence insertion/deletion (INS/DEL) polymorphism in the exon 8 at the 3′ untranslated region (UTR) modifies mRNA stability and protein production and has been shown to concur with efficacy of pharmacological treatments in immune-mediated conditions. The aim of this study was to assess for the first time the correlation between HLA-G 14-bp INS/DEL polymorphism with the response to systemic therapy in psoriatic patients. We retrospectively analyzed the HLA-G 14-bp INS/DEL polymorphism of *HLA-G* gene in patients with moderate to severe plaque psoriasis: 21 treated with acitretin, 16 with cyclosporine, 11 with anti-TNF-α. Patients who reached PASI 75 at weeks 10–16 were considered responders. Among patients treated with acitretin, we observed a significantly increased frequency of the HLA-G DEL allele and of the DEL/DEL genotype in responder patients when compared with nonresponders. An association between HLA-G genotype and response to cyclosporine and biologics was not found. The significant association between HLA-G 14-bp DEL allele and 14-bp DEL/DEL genotype and acitretin clinical outcome may suggest an advantage of this allele and propose this HLA-G polymorphism as a potential marker of response to acitretin in psoriatic patients.

KEYWORDS: 14-bp polymorphism, HLA-G, immune-modulation, pharmacogenetics, psoriasis, systemic therapy

Address correspondence and reprint requests to: Alessandro Borghi, MD, Department of Medical Sciences, Section of Dermatology, University of Ferrara, Via Savonarola 9, 44100 Ferrara, Italy, or email: alessandro.borghi@unife.it.

Introduction

Human leukocyte antigen-G (HLA-G) is a nonclassical major histocompatibility complex class I molecule, expressed as membrane and soluble

isoforms, that plays an immune regulatory role (1). HLA-G expression can be considered a negative feedback signal that down-regulates lymphoid reactions and inflammatory processes (2–4). Decreased levels of HLA-G molecules seem to contribute in increasing the susceptibility to autoimmunity and disease activity (5). We had previously shown significant lower soluble (s)HLA-G levels in the plasma of psoriatic patients in comparison with healthy controls (6). HLA-G down-modulation suggests the maintenance of a defective immune suppressive status in psoriatic patients, which may promote the development of inflammatory processes leading to psoriasis.

The HLA-G production is controlled by several polymorphisms both in the promoter and in the 3′ untranslated region (3′ UTR) modifying the affinity of gene targeted sequences for transcriptional or post-transcriptional factors, respectively (7). In particular, an insertion/deletion (INS/DEL) polymorphism of 14 base pairs (14bp) (rs1704) in the exon 8 at the 3′ UTR is confirmed to modify mRNA stability. The presence of the HLA-G INS allele has been associated with a lower mRNA transcription and a consequent decreased protein production (low HLA-G producer) (8). The importance of HLA-G 14-bp polymorphism was repeatedly confirmed by the association with autoimmune conditions (9,10). Consistent with this, previous studies showed that HLA-G 14bp genotypes may be useful to identify individuals at risk for host-transplantation complications (11). Moreover, HLA-G 14-bp polymorphism seems to be correlated with clinical efficacy of pharmacological treatments for autoimmune and inflammatory diseases (12).

The aim of the present study was to analyze the HLA-G 14-bp INS/DEL polymorphism in correlation with clinical response to systemic therapy in psoriatic patients.

Materials and methods

Patients

We retrospectively screened patients affected with moderate-to-severe plaque psoriasis, defined by Psoriasis Area and Severity Index (PASI) >10 at screening, treated with systemic therapy at our psoriasis outpatient unit between January 2009 and January 2013. Systemic treatments consisted of acitretin (at the daily dosage of 25–50 mg), cyclosporine (2.5–5 mg/kg/day) and anti-TNF-α agents (infliximab, etanercept, adalimumab) administered at the approved regimens.

The following data were collected from hospital clinical records: patients' demography, weight and height, waist circumference, comorbidities, lifelong history of the psoriasis, and disease severity before starting and throughout treatment evaluated by the PASI.

In conformity with guidelines (13), we considered the PASI 75 response between 10 and 16 weeks after the initiation of treatment, which corresponds to an improvement from baseline in the PASI of ≥ 75%, as primary measure for clinical efficacy. Patients reaching a reduction of less than 75% from the baseline PASI or a PASI 75 response after 16 weeks of treatment were considered as *nonresponders*. Exclusion criteria from this retrospective study were as follows: treatment discontinuation before completing a 10-week treatment course due to either poor tolerability or psoriasis worsening, administration of treatments at daily dosages different from those recommended by international guidelines (13), combination therapy, including two or more systemic treatments or a systemic agent and phototherapy. Clinical assessment and blood sampling were performed during routine clinics, with written informed consent and local ethical board approval.

Detection of HLA-G 14bp polymorphism

After completion of screening, ethylenediaminetetraacetic acid (EDTA) blood (7–10 mL) was obtained from the recruited patients. Genomic DNA was extracted from the EDTA blood using a Nucleon Bacc 3 Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. The HLA-G 14-bp polymorphism (14bp INS/DEL) was detected by real-time polymerase chain reaction (PCR) technique using the 7300 real-time PCR machine (Applied Biosystems, Monza, MB, Italy) performed as previously described (14).

Statistical analysis

The adherence to the Hardy–Weinberg equilibrium expectations was estimated by GENEPOP software (http://kimura.univ-montp2.fr/~rousset/Genepop.htm). To compare the frequencies of alleles and genotypes between the patients, we used the Fisher's exact test and the *p*-value was considered to be statistically significant when <0.05. A logistic regression analysis was performed to evaluate the effect of confounding variables.

Results

On the basis of inclusion and exclusion criteria, a total of 48 patients were evaluated: (i) 21 psoriatic patients treated with acitretin (18 males and 3 females), with a mean age of 57.7 years (range 33–81 years), (ii) 16 patients treated with cyclosporine (13 males and 3 females, mean age 48.3 years, range 37–75 years), (iii) 11 patients treated with anti-TNF-α (9 males and 2 females, mean age 64 years, range 28–81 years, 7 patients treated with etanercept, 3 with adalimumab, and 1 with infliximab). At the moment of starting acitretin therapy, the mean PASI score was 21.5 (range 11–57.3, S.D. 10.1); for patients treated with cyclosporine, the mean initial PASI score was 20.4 (10.2–35, S.D. 8.3) and for those treated with biologics was 22.2 (11–52.2, S.D. 12.9). Demographics and clinical features of the patients are reported in Table 1.

At weeks 10–16, the PASI 75 response (*responder patients*) was achieved in 11 patients treated with acitretin (52.4%), 15 patients treated with cyclosporine (93.7%), and 6 patients treated with biologics (54.5%).

The psoriatic population was in Hardy–Weinberg equilibrium for HLA-G 14-bp polymorphism. When patients were subdivided in relation to the clinical response to treatments, among those treated with acitretin, we observed a significantly increased frequency of the HLA-G 14-bp DEL allele (p = 0.008, Fisher's exact test; odds ratio (OR): 7.74, 95% confidence interval (CI): 1.72–34.79) and of the 14-bp DEL/DEL genotype (p = 0.05, Fisher's exact test) in *responder* patients when compared with *nonresponders* (Table 2a). Interestingly, the comparison between low/intermediate HLA-G producer genotypes (INS/INS, INS/DEL) and high HLA-G producer genotype (DEL/DEL) (8) confirmed a significant increase in the presence of a double dose of 14-bp DEL allele in *responder* patients when compared with *nonresponders* (p = 0.007, Fisher's exact test; OR = 24, 95% CI: 2.05–279.64). On the contrary, no association was found between HLA-G genotype and response to cyclosporine (Table 2b) and biologics (Table 2c).

The logistic regression model showed no significant correlation between clinical response to each of the analyzed treatments and patients' age, body mass index, mean disease duration, and PASI at baseline (p = NS, logistic regression test).

Discussion

In psoriatic patients, the reasons at the basis of a lack of clinical response to the available treatments are still unknown.

Based on these preliminary findings, the strong association between HLA-G 14-bp DEL allele and 14-bp DEL/DEL genotype and acitretin clinical outcome may propose both a pharmacogenetic role and a clinical advantage of the 14-bp DEL allele genotype in response to acitretin. On the contrary, HLA-G 14-bp INS/DEL polymorphism was not found to be associated with clinical response to cyclosporine and biologics. It may be speculated that these drugs, because of their specificity for the suppression of Th1 immune response, could be less influenced by HLA-G 14-bp INS/DEL polymorphism and consequent HLA-G expression than acitretin.

Undoubtedly, the small number of patients represents the major limitation of this investigation. Moreover, in the present study, sHLA-G levels were not measured; thus, an association between HLA-G polymorphism and HLA-G expression can only be supposed in agreement with previous reports (10,15). However, the preliminary genetic data collected with the present retrospective study present strong statistical associations and provide the first indication for larger prospective investigations.

Advances in our understanding of the clinical relevance of HLA-G 14-bp INS/DEL polymorphism and HLA-G expression in conditioning the clinical outcome of psoriasis treatments are necessary. If a role of this genetic polymorphism in acitretin response is confirmed, this could be helpful in order to preliminary select patients for an appropriate, individualized therapeutic management.

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Conflict of interest

The authors declare that they have no conflict of interest.

Table 1. Baseline demographic and clinical features of patients treated with acitretin, cyclosporine, and biologics

Patient	Gender	Age (years)	BMI (kg/m²)	Metabolic syndrome (yes/no)	Duration of disease (years)	Psoriatic arthritis (yes/no)	PASI at baseline	PASI 75 at 10–16 weeks (yes/no)	HLA-G polymorphism
1 aci	F	72	30.5	Yes	13	No	11.8	No	INS/DEL
2 aci	M	80	25.0	Yes	5	No	57.3	Yes	DEL/DEL
3 aci	M	49	24.7	No	20	Yes	25.2	No	INS/DEL
4 aci	M	75	25.0	No	22	No	18.0	No	INS/DEL
5 aci	F	79	24.0	No	20	No	24.0	Yes	DEL/DEL
6 aci	M	71	28.3	No	17	No	12.3	No	INS/DEL
7 aci	M	47	22.3	No	11	No	30.0	Yes	DEL/DEL
8 aci	M	37	27.7	No	20	No	20.0	Yes	DEL/DEL
9 aci	M	28	24.0	No	17	No	14.0	No	DEL/DEL
10 aci	M	65	28.0	No	20	Yes	15.0	No	INS/INS
11 aci	M	40	26.5	No	20	Yes	21.4	No	INS/DEL
12 aci	M	33	28.0	No	10	Yes	23.6	Yes	DEL/DEL
13 aci	M	75	30.0	No	15	No	29.8	Yes	INS/DEL
14 aci	M	56	25.0	No	22	No	18.6	Yes	INS/DEL
15 aci	M	70	26.5	No	25	No	18.0	Yes	DEL/DEL
16 aci	M	42	28.0	No	4	No	20.0	Yes	INS/DEL
17 aci	F	81	26.0	No	30	Yes	15.0	Yes	DEL/DEL
18 aci	M	44	28.0	No	14	No	28.2	No	INS/DEL
19 aci	M	79	28.0	No	38	Yes	12.7	No	INS/DEL
20 aci	M	49	26.5	No	11	Yes	26.6	No	INS/INS
21 aci	M	40	26.0	No	3	No	11.0	Yes	DEL/DEL
1 cyc	M	75	28.0	No	22	No	16.0	Yes	INS/DEL
2 cyc	M	71	28.3	No	17	No	10.5	Yes	INS/DEL
3 cyc	M	47	22.3	No	11	No	18.0	No	DEL/DEL
4 cyc	F	72	30.5	Yes	13	No	14.0	Yes	INS/DEL
5 cyc	M	37	27.7	No	20	No	30.8	Yes	DEL/DEL
6 cyc	M	46	30.8	Yes	7	No	14.5	Yes	INS/INS
7 cyc	M	41	27.6	No	11	No	35.0	Yes	INS/INS
8 cyc	M	65	28.0	No	20	Yes	26.0	Yes	INS/INS
9 cyc	M	49	27.0	Yes	17	No	18.0	Yes	INS/INS
10 cyc	M	67	31.2	Yes	12	No	25.5	Yes	INS/DEL
11 cyc	M	42	26.0	No	4	No	15.2	Yes	INS/DEL
12 cyc	F	48	26.2	No	14	No	33.0	Yes	INS/INS
13 cyc	M	54	26.0	No	21	No	14.0	Yes	DEL/DEL
14 cyc	F	48	29.3	No	14	No	10.2	Yes	INS/DEL
15 cyc	M	49	26.5	No	11	No	26.0	Yes	INS/INS
16 cyc	M	48	23.9	No	10	No	30.3	Yes	DEL/DEL
1 eta	F	72	30.5	Yes	13	No	13.0	No	INS/DEL
2 ada	M	52	25.9	No	20	No	18.8	Yes	INS/INS
3 inf	M	47	22.3	No	11	No	52.2	Yes	DEL/DEL
4 eta	M	59	30.0	No	15	No	20.7	No	INS/DEL
5 eta	M	28	25.0	No	17	No	11.0	Yes	DEL/DEL
6 ada	M	41	27.6	No	11	No	20.0	No	INS/INS
7 ada	M	67	31.2	Yes	12	No	12.0	No	INS/DEL
8 eta	F	81	25.0	No	30	Yes	16.5	Yes	DEL/DEL
9 eta	M	49	29.0	No	18	No	39.0	No	INS/DEL
10 eta	M	79	28.0	No	38	Yes	13.0	Yes	INS/DEL
11 eta	M	49	26.5	No	11	Yes	27.7	Yes	INS/INS

act, acitretin; ada, adalimumab; BMI, body mass index; cyc, cyclosporine; DEL, deletion; eta, etanercept; inf, infliximab; INS, insertion.

Table 2. Frequencies of HLA-G 14-bp alleles and genotypes in psoriatic patients treated with (a) acitretin, (b) cyclosporine and (c) biologics

(a) Acitretin	Acitretin responder number (%)	Acitretin non responder number (%)	Fisher's exact test *p*-value*
HLA-G INS/DEL allele			
DEL	19 (45)	9 (22)	0.008
INS	3 (7)	11 (26)	
HLA-G INS/DEL genotype			
DEL/DEL	8 (38)	1 (5)	0.05
INS/DEL	3 (14)	7 (33)	NS
INS/INS	0 (0)	2 (10)	

(b) Cyclosporine	Cyclosporine responder number (%)	Cyclosporine non responder number (%)	Fisher's exact test *p*-value*
HLA-G INS/DEL allele			
DEL	12 (40)	2 (100)	0.18
INS	18 (60)	0 (0)	
HLA-G INS/DEL genotype			
DEL/DEL	3 (20)	1 (100)	0.4
INS/DEL	6 (40)	0 (0)	NS
INS/INS	6 (40)	0 (0)	

(c) Biologics	Biologics responder number (%)	Biologics nonresponder number (%)	Fisher's exact test *p*-value*
HLA-G INS/DEL allele			
DEL	7 (58.33)	4 (40)	0.7
INS	5 (41.67)	6 (60)	
HLA-G INS/DEL genotype			
DEL/DEL	3 (50)	0 (0)	NS
INS/DEL	1 (16.67)	4 (80)	0.5
INS/INS	2 (33.33)	1 (20)	

The HLA-G 14-bp INS/DEL polymorphism was genotyped by real-time polymerase chain reaction performed as previously described (14).

*p-values were calculated using the INS allele and the INS/INS genotype as reference.

NS, not significant.

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Conclusions and perspectives

1. The key role of HLA-G in pathologies and infections and expected impact in treatment and prognosis

The data herein summarized suggest that HLA-G may play a key role in the onset of infectious and autoimmune diseases. In fact, the impication of HLA-G proteins in creating an impaired immune system response during autoimmunity and in the immune-escape mechanisms during viral infections has been here confirmed. Basing on that, it appears even more evident that understanding the functions of HLA-G in these disorders could help in the identification of new approaches to control HLA-G production [178]. HLA-G is characterized by a "double-faced" behaviour that explains how down/over-expression of HLA-G may not only act as an immunosuppressive and beneficial condition but may also sustain an unbalanced immune stimulation and autoimmunity. For example, during inflammatory cutaneous diseases there is a disproportional expression of HLA-G molecules that could lead to autoimmunity [176, 177]. Furthermore, several studies reported that intratecal sHLA-G could act as tolerogenic molecules in MS as confirmed by the disease remission associated to the presence of the molecule [150-158] and also a strong associatiation with RA severity and therapy response [45, 142] The comprehension of the specific mechanisms of action of HLA-G in the development and progression of inflammatory and autoimmune disorders could justify the use of HLA-G molecules as a marker of inflammation and drug treatment, providing new therapeutic perspectives.

Concerning viral and bacterial infections, the improvement in the comprehension of the role of HLA-G in immune-escape could help the development of new anti-viral treatments aimed to target HLA-G. This could represent an important advantage in therapy but also in prevention, above all when the infection could induce a neoplastic transformation [93] or, as in the case of CF patients infected by P. aeruginosa, where the possibility to modulate HLA-G may drastically influence the disease outcome and therapy response [123, 131]. In fact, HLA-G alteration is observed during both viral and bacterial infections and participates in the manteinance of the infective status.

In fact, as reported in this thesis, the literature and also the studies followed during my PhD, suggest that HLA-G could be implicated in both risk and disease exacerbation and chronicization since during these pathologic contiditions this antigen is characterized by an abberant expression connected to the different disease environment. In particular, HLA-G proteins could directly interact with immune cells or control the balance between Th1 and Th2 cytokines creating in this way a disequilibrium that would maintain inflammatory and immune-deregulated conditions and infections.

Diagnosis and prevention of diseases is mainly based on the identification of specific biological markers and drug targets. In view of this, the possibility of an easy and fast identification of molecules, for example in biological fluids, seems to be even more necessary. In recent years, different studies have demonstrated that HLA-G could fulfil this necessity [79-83]. In fact, HLA-G expression and levels in biological fluids, cells and tissues in different pathological conditions have been shown. Several authors reported that the level of soluble HLA-G and gene polymorphisms correlate with disease outcome and the therapeutic success of treatment [142, 143, 179, 180].
So in conclusion, HLA-G evaluation could represent a concrete help in outcome prediction and in supporting treatment decisions.

Other works

During my PhD course, I also worked on other topics connected to immune dysregulation in different pathological situations.
I investigated the role of KIR (Killer Immunoglobulin-like receptor) receptors in HPV infection and the association with cervical lesions (Paper 1, paper attached) and in herpesvirus infection in MS patients (Paper 2, paper attached).
I also evaluated the role of HLA-G molecules during CLL immune-escape (Paper 3, paper attached) and in the tolerogenic behaviour of multipotent stromal cells (Paper 4, paper attached). I also optimazed a Real Time PCR assay to analyse the +3142 C>G HLA-G polymorphism (Paper 5, paper attached).

Paper 1.

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Implication of *HLA-C* and *KIR* Alleles in Human Papillomavirus Infection and Associated Cervical Lesions

Roberta Rizzo, Valentina Gentili, Antonella Rotola, Daria Bortolotti, Enzo Cassai, and Dario Di Luca

Abstract

Human papillomavirus (HPV) regulation of host immune response leads to cervical lesions. In particular, natural killer (NK) cells are crucial for HPV control. Since specific HLA-I/KIR interactions modify NK cell activation, we analyzed *HLA-C* and *KIR* alleles in HPV infection and lesion development in 150 controls, 33 condyloma acuminatum, and 111 invasive cervical cancer (ICC) patients. We showed an increase in *HLA-C1/KIR2DL2* and *HLA-C1/KIR2DL3* pairs in HPV high-risk infected patients (OR 3.05, 3.24) with ICC (OR 1.33, 3.68). These data suggest *HLA-C* and *KIR* typing as risk marker for HPV infection and lesion evolution.

HIGH-RISK HUMAN PAPILLOMAVIRUS (HPV) infection is the most significant risk factor for the development of premalignant and malignant epithelial lesions of the cervix (12). The HPV types that infect the genital mucosa can be divided into two groups: high-risk HPV types 16, 18, 31, and 45, frequently found in cervical cancers; and low-risk HPV types 6 and 11 that infect the genital epithelia but are rarely detected in malignancies. The development of cervical cancer starts with viral infection but requires other factors for cell transformation, as an impaired host immune system. However, the exact causes that lead to tumor have not been fully understood. Recent but few studies suggest a relationship between *HLA-C* alleles and HPV-related cervical diseases (2,6,5,10,11). Since we previously documented an important implication of natural killer (NK) cell inhibitory receptors (KIRs) and their interaction with HLA ligands in viral infection control in a cohort of multiple sclerosis patients (7), we focused our attention on *HLA-C* and *KIR* as possible reliable markers for HPV infection and lesion development in benign (condyloma acuminatum) and invasive cervical carcinoma (ICC).

We detected the presence of HPV infection in biopsies from 33 women with condyloma acuminatum (M_{age} = 36.5 ± 10.5 years) and 111 ICC patients (M_{age} = 38.9 ± 16.4 years). We performed HPV high-risk (HPV-16, 18, 31, 33, 35, 39, 45, 52, 53, 56, 58, 59, 66, 70) screen with the Sacace Biotechnologies kit (Sacace Biotechnologies, Como, Italy) and nested polymerase chain reaction (PCR) for HPV low-risk (HPV-6, 11) detection (8). A total of 105 control women (M_{age} = 37.5 ± 11.6 years) were selected for the absence of HPV cervical infection and matched with the patient groups for age and risk of infection. All the subjects were recruited at the University Hospital of Ferrara, selected for a documented Caucasian and consistent geographical origin. They were nonsmokers, reported one sexual partner, and were not going through menopause. Clinical assessment and blood sampling were performed during routine clinics, with written informed consent and local ethical board approval. The extraction of DNA was performed on biopsies for HPV identification and on peripheral blood mononuclear cells for *HLA-C* and *KIR* typing using protease/SDS digestion, purification by fenol-chloroform followed by ethyl-ether extraction (8).

Due to limited DNA resources, we restricted to the identification of *HLA-C* allele groups into *HLA-C* group 1, characterized by Asn at position 80, and group 2, characterized by Lys at position 80 alleles, typed by PCR with specific primers (3). *KIR* alleles were genotyped by PCR by specific primers (4).

We estimated the frequencies of the HLA/KIR pairs by Arlequin 3.5.1.2 software. We analyzed the distribution of HLA-C alleles, genotypes, and pairs by StatView software package (SAS Institute, Inc., Cary, NC) and GraphPad Prism v6.0 software (Graphpad Software, San Diego, CA). Significance was assumed for $p < 0.05$. Bonferroni correction for multiple comparisons was applied when the p-value was significant.

The HPV analysis reported high-risk HPV types DNA in 100% ICC (111/111) and 9% condyloma (3/33) patients, while low-risk HPV types DNA in 100% condyloma patients (33/33). These data sustain a significant association between high-risk HPV types and ICC development and a strict correlation between condyloma and low-risk HPV types (condyloma vs. ICC = OR 203.5 [95% CI 48.2–858.4]). Three condyloma patients (9%) presented both high- and low-risk

Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Ferrara, Italy.

TABLE 1. ALLELIC, GENOTYPIC, AND PAIR FREQUENCIES OF *HLA-C* AND *KIR* IN CONTROL AND TEST GROUPS

	Control group HPV − (150)	*Test group HPV + (144)*	p_c[a]	*Test group HPV + low risk (30)*	*Test group HPV + high risk (111)*	p_c[b]
Allele n (%)						
HLA-C1	180 (60)	123 (42)	0.000048[c]	14 (23)	08 (49)[c]	0.0019[c]
HLA-C2	120 (40)	165 (58)		46 (77)	114 (51)	
Total	300	288		60	222	
Genotype n (%)						
HLA-C1:C1	53 (35)	40 (28)		3 (18)	37 (33)	
HLA-C1:C2	74 (49)	43 (30)	0.000003[c]	8 (24)	34 (31)	0.033[c]
HLA-C2:C2	23 (16)	61 (42)		19 (42)	40 (36)	
Total	150	144		30	111	
Pair n (%)						
HLA-C1/KIR2DL2	87 (29)	52 (18)		6 (10)	48 (22)	
HLA-C1/KIR2DL3	93 (31)	71 (25)	<0.0001[d]	8 (13)	60 (27)	
HLA-C2/KIR2DL2	102 (34)	70 (24)		10 (17)	56 (25)	0.000051[d]
HLA-C2/KIR2DL3	18 (6)	95 (33)		36 (60)	58 (26)	

[a]Control group HPV − vs. test group HPV +.
[b]Test group HPV + low risk vs. test group HPV + high risk.
[c]Fisher's exact test.
[d]Chi square test.
p_c, corrected *p*-value; HPV, human papillomavirus.

HPV infections. This condition was previously demonstrated in other studies, where most condyloma lesions contained multiple HPV types, including types associated with dysplastic epithelial abnormalities (1). The presence of multiple HPV types in a large percentage of condyloma lesions suggests that many individuals acquire additional HPV types at the time of infection with HPV type 6 or 11. Healthy controls presented no positivity for HPV DNA.

First, we considered the possible implication of *HLA-C* in modifying the risk of HPV infection. We compared HPV negative controls with all HPV positive patients (Test Group) (Table 1). We observed an increased risk of HPV infection in the presence of *HLA-C2* allele (OR 2.01 [95% CI 1.4–2.8]) with a higher frequency of both *HLA-C2* allele and *HLA-C2:-C2* genotypes in HPV positive patients in comparison with HPV negative controls ($p_c = 0.000048$ and 0.0000003 respectively). We then evaluated the effect of *HLA-C* alleles in high- and low-risk HPV infection (Table 1). We considered only the patients with one type (high or low risk) of HPV infection. We observed an increase in *HLA-C1* allele and *HLA-C1:-C1* genotype in high-risk HPV-infected patients in comparison with low-risk HPV-infected patients ($p_c = 0.0019$ and 0.033 respectively). These data suggest that the presence of *HLA-C1* alleles could facilitate high-risk HPV in comparison with low-risk HPV infection (OR 3.1 [95% CI 1.6–5.98]). We confirmed the results, comparing ICC patients with condyloma patients (Table 2). We observed an increase of *HLA-C1* alleles in ICC patients (49%), characterized by high-risk HPV infection, in comparison with condyloma (30%) patients ($p_c = 0.032$).

Taken together, these results support an implication of *HLA-C* alleles in HPV infection and lesion development.

Since HLA-C molecules are ligands for KIR2DL2, KIR2DL3, and KIR2DL1 NK receptors, we evaluated the frequencies of these inhibitory receptors. KIR2DL2 and/or KIR2DL3 receptors react with HLA-C1 epitope of HLA-C molecules; KIR2DL2 recognizes C2 in addition to C1, while KIR2DL1 strongly reacts with C2.

Since KIR2DL1 gene was present in the majority of the subject tested (98.4%; www.allelefrequencies.net/kir6006a.asp?kir_positives=on&kir_allele=2DL1), the calculations for this gene were not possible. We subdivided the subjects according to the presence or absence of *KIR2DL2* or *KIR2DL3* alleles, and analyzed the distribution of the corresponding *HLA-C/KIR* pairs. We reported a significant difference in the distribution of *HLA-C/KIR* pairs between control subjects and HPV positive individuals ($p_c < 0.0001$; Table 1). In particular, a decrease in *HLA-C2/KIR2DL2* pair was observed in HPV-positive subjects (24%) in comparison with controls (34%; OR 0.54 [95% CI 0.36–0.82]). When we subdivided HPV positive patients on the basis of low- and high-risk HPV types, we observed a significant difference in pair distribution ($p_c = 0.000051$; Table 1). In particular, an increase in *HLA-C1/KIR2DL2* and *HLA-C1/KIR2DL3* pairs was observed in high-risk HPV in comparison

TABLE 2. ALLELIC, GENOTYPIC, AND PAIR FREQUENCIES OF *HLA-C* AND *KIR* IN INVASIVE CERVICAL CANCER AND CONDYLOMA PATIENTS

	ICC (111)	*Condyloma (33)*	p_c
Allele n (%)			
HLA-C1	108 (49)	20 (30)	
HLA-C2	114 (51)	46 (70)	0.032[a]
Total	222	66	
Genotype n (%)			
HLA-C1:C1	37 (33)	6 (18)	
HLA-C1:C2	34 (31)	8 (24)	0.24[a]
HLA-C2:C2	40 (36)	19 (42)	
Total	111	33	
Pair n (%)			
HLA-C1/KIR2DL2	48 (22)	12 (18)	
HLA-C1/KIR2DL3	60 (27)	8 (13)	0.00018[a]
HLA-C2/KIR2DL2	56 (25)	10 (14)	
HLA-C2/KIR2DL3	58 (26)	36 (55)	

[a]Fisher's exact test.
ICC, invasive cervical cancer.

with low-risk HPV infection (OR 3.05 [95% CI: 1.2–8.04]; OR 3.24 [95% CI 1.33–7.89], respectively).

We then evaluated the role of *HLA-C/KIR* pairs in the development of neoplastic (ICC) and benign (condyloma) lesions. We observed a significant difference between ICC and condyloma subjects ($p_c = 0.00054$; Table 2). In particular, an increase in *HLA-C1/KIR2DL2* and *HLA-C1/KIR2DL3* pairs was observed in ICC in comparison with condyloma patients (OR 1.33 [95% CI 0.6–2.97]; OR 3.68 [95% CI 1.53–8.86], respectively).

These results suggest *HLA-C1/KIR2DL2* and *HLA-C1/KIR2DL3* pairs as risk factors for HPV high-risk infection, while the increased frequency of HLA-C2 alleles in HPV-positive patients in comparison with controls did not correspond to a similar increase in *HLA-C2/KIR2DL2* pair.

In summary, our results support the involvement of *HLA-C* alleles in HPV infection and lesion development. Although the presence of *HLA-C* group 2 alleles leads to an increased risk of HPV infection, the absence of an increased frequency of *HLA-C2/KIR2DL2* pair suggests no direct effect on NK cell activation control. On the other hand, HLA-C group 1 antigens are associated with high-risk HPV infection and neoplastic lesion development. Interestingly, the presence of the high inhibitory *HLA-C1/KIR2DL2* and *HLA-C1/KIR2DL3* interactions has a risk effect toward high-risk HPV infection and neoplastic lesion development. The increase in *HLA-C1/KIR2DL2* and *HLA-C1/KIR2DL3* pair frequencies in high-risk HPV-infected patients suggests a direct effect on NK cells. In particular, we hypothesize that *HLA-C1/KIR2DL2* and *HLA-C1/KIR2DL3* presence could maintain NK cell inhibition toward HPV infection sustaining the high-risk HPV-associated transformation and the development of pre-neoplastic lesions (9). The absence of such a strong interaction between *HLA-C/KIR* pairs and low-risk HPV infection could be due to the lower selective effect of the low-risk HPV infection that leads only to benign lesions.

Recent but few studies suggested a relationship between HLA-C alleles and HPV-related cervical diseases. Four case-control studies have previously examined the effects of variation at the HLA and KIR locus in women with CIN (2,5,6,10,11). In particular, Wang *et al.* (11) and Martin *et al.* (6) found that specific HLA ligands for inhibitory KIR were associated with modified risk of developing cervical neoplasia. Song *et al.* (10) found HLA-C*0303 to confer susceptibility to HPV-related cervical disease, whereas HLA-C*01 was protective against HPV-related cervical disease. Our data are in agreement with the previous results on HLA-C group 1 allele involvement in HPV-associated lesion development (6), and are unique in that they were from an Italian population.

In conclusion, our results strongly implicate *HLA-C* group 1 alleles in combination with *KIR2DL2* and *KIR2DL3* receptors as major determinants in high-risk HPV infection and neoplastic lesion development. Of course, these data need to be confirmed in a larger and prospective study and with functional experiments, but they appear to suggest *HLA-C* and *KIR* allele analysis as risk markers to be considered in the evaluation of HPV infection risk.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. Roberta Rizzo
Department of Medical Sciences
Section of Microbiology and Medical Genetics
University of Ferrara
Via Luigi Borsari, 46
Ferrara 44121
Italy
E-mail: rbr@unife.it

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Evaluation of the implication of KIR2DL2 receptor in multiple sclerosis and herpesvirus susceptibility

Nadia Ben Fredj [a,*], Roberta Rizzo [b], Daria Bortolotti [b], Faten Nefzi [a], Saber Chebel [c], Antonella Rotola [b], Mahbouba Frih-Ayed [c], Dario Di Luca [b], Mahjoub Aouni [a]

[a] Laboratory of Transmissible Diseases and Biological Active Substances, LR99-ES27, Faculty of Pharmacy, University of Monastir, Avicenne Street, 5000 Monastir, Tunisia
[b] Department of Medical Sciences, Section of Microbiology, University of Ferrara, Via L. Borsari, 46, 44121 Ferrara, Italy
[c] Department of Neurology, Fattouma Bourguiba University Hospital, Farhat Hached Avenue, 5000 Monastir, Tunisia

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ABSTRACT

To evaluate the possible effect of cell immunoglobulin-like receptors (KIRs) on viral infection in multiple sclerosis (MS) patients, we performed genotyping of KIR2DL2 and his HLA-C1 ligand and we analyzed the presence of all eight human herpesviruses (HHVs) in 60 MS patients and 112 healthy controls. Significantly higher frequencies were found for KIR2DL2 enhanced in the presence of its ligand HLA-C1 in MS patients. Moreover, a significant association was observed between an increase in HHV risk of infection in KIR2DL2 and HLA-C1 positive patient. Our results confirm a possible effect of KIR2DL2 on viral infection susceptibility in MS patients.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory immune-mediated disease causing demyelination and axonal damage in the central nervous system (CNS) (Hohlfled and Wekerle, 2004). Many efforts have been devoted to understand its etiology, focusing on the role of the adaptive immune system. However, recent evidences have shown that innate immunity is also important for MS initiation and progression (Gandhi et al., 2010).

Currently it is recognized that various types of regulatory cells can actively suppress autoimmune diseases. In experimental autoimmune encephalomyelitis (EAE) (Das et al., 1997; Olivares-Villagomez et al., 1998; Wolf et al, 1996; Zhang et al., 1997), the prototype autoimmune disease mediated by Th1 cells, cell depletion or adoptive transfer studies has established the role of regulatory αβT cells (Das et al., 1997; Olivares-Villagomez et al., 1998), B cells (Wolf et al, 1996), and NK cells (Zhang et al., 1997) in the regulation of the disease. However, it remains obscure how deeply involved are such regulatory cells in human Th1-mediated autoimmune diseases like MS.

Epidemiological studies indicate that exposure to an infectious agent, in combination with genetic predisposition, could be implicated in MS pathogenesis (Casetta and Granieri, 2000; Marrie, 2004; Sospedra and Martin, 2005; Ascherio and Munger, 2007).

Some circumstantial evidences suggest an association between MS and human herpesviruses (HHVs), which establish lifelong latent infections with occasional reactivation episodes, thus providing a persistent challenge for the immune system. We have previously reported a high frequency of HHV DNA presence in Tunisian patients affected with MS, as detected by nested PCR (Ben Fredj et al., 2012). Moreover, it has been shown an increased frequency of herpes simplex virus (HSV) (Ferrante et al., 2000; Hawkes et al., 2006) and Varicella Zoster virus (VZV) (Mancuso et al., 2007) in MS patients comparing with healthy controls. Epstein Barr virus (EBV) reactivation appears to be linked to MS activity (Wandinger et al., 2000; Serafini et al., 2007), and also human herpesvirus 6 (HHV-6) has been associated to tissue damage associated with MS lesions (Rotola et al., 2004; Fotheringham et al., 2005).

However, although several efforts have been made to identify a possible link between herpesvirus infection and disease, direct evidence for an infectious etiology in MS is still lacking (Sospedra and Martin, 2005). In particular, some researchers failed to identify EBV, HHV-6 and VZV in cerebrospinal fluids (CSF) from MS patients (Mirandola et al., 1999; Rodríguez Carnero et al., 2002; Burgoon et al., 2009; Sargsyan et al., 2010).

The most important effector of innate immunity towards viral infections is the natural killer (NK) cell. NK cells are cytotoxic lymphocytes involved in the innate immune defense against viral infections and tumor cells and in the regulation of adaptive immune responses (Carrol and Prodeus, 1998). NK cell deficiency is involved in viral infection sensibility. In particular, the vital role of NK cells in controlling virus

* Corresponding author. Tel.: +216 97 467 658; fax: +216 73 465 754.
E-mail address: benfredj_nadia@yahoo.fr (N. Ben Fredj).

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Table 1
Demographic and clinical data of MS patients.

Clinical variables	Values
Number	60
Sex (male/female)	22/38
Median age of recruitment	37.68 ± 11.27 (19–64)
Median age at onset	32.3 ± 10.46 (16–58)
Median disease duration	5.38 ± 3.91 (1–15)
Median EDSS	2.83 ± 11.27 (1–8)
Interferon beta therapy	32/60

infections was demonstrated in a patient completely lacking NK cells and sustaining severe herpesvirus infections (Biron et al., 1999).

Many studies suggest that NK cells might play a role in the regulation of MS (Takahashi et al., 2004; Hao et al., 2010) and other autoimmune diseases (Negishi, 1986; Yen, 2001), but despite all of the evidences, the mechanism by which NK cells could mediate immune regulation remains unclear (Shi and Van, 2006).

NK cell activity is the result of a delicate balance between activating and inhibitory signals, delivered by cell surface receptors belonging to several families, with the killer cell immunoglobulin-like receptors (KIRs) as one of the most important.

The KIR molecules are located on the NK cell membrane; however, it is the cytoplasmatic tail that defines their activating (short [S]) or inhibiting (long [L]) properties. The KIR gene cluster is located on chromosome 19q13 and consists of several genes and pseudogenes (Boyton et al., 2007), exhibiting considerable structural variation, resulting in all genes rarely being concomitantly present in one given individual. KIRs regulate the inhibition and activation of cell responses through recognition of polymorphic motifs on HLA class I molecules (HLA-A*03 and HLA-A*11 alleles, and alleles with the HLA-Bw4, HLA-C1 or HLA-C2 motifs) on target cells.

The association between KIR genes and autoimmune diseases has been widely studied and many of them have evaluated the correlation between incidence and progression of infectious and autoimmune diseases with the expression of particular KIRs in combination with MHC class I molecules. In particular, the high-affinity receptor/ligand pair KIR2DL2/HLA-C1 increases the risk of developing clinical HSV-1 (Moraru et al., 2012) and HHV-8 (Caselli et al., 2013) infection. Moreover, a recent study conducted by Rizzo et al. confirmed the implication of KIR2DL2 receptor in the control of NK cell activation towards herpesvirus infection in MS patients (Rizzo et al., 2012).

On the basis of this background, we performed a study with two objectives. First, we investigated the influence of KIR2DL2 gene on MS susceptibility and disease severity by determining the genetic interaction between KIR2DL2 and its HLA-C1 ligand in the Tunisian MS patients versus healthy controls. Second, we evaluated the possible effect of KIR2DL2 on viral infection.

2. Materials and methods

2.1. Patients and controls

Our sample set consisted of 60 unrealated patients affected by definite MS followed at the section of Neurology, Fattouma Bourguiba Hospital, Monastir, Tunisia and 112 healthy controls (HC). All MS patients were diagnosed with defined MS according to the criteria proposed by McDonald et al. (2001). The demographic and clinical characteristics of MS patients are shown in Table 1.

The control samples were randomly selected from the Regional Center for Blood Transfusion, CHU Farhat Hached, Sousse, Tunisia, and were sex and age matched with MS patients.

This study was approved by the local ethics committee and all of the participants gave informed consent before the experimental procedures.

2.2. KIR2DL2 and HLA-C1 genotyping

Genomic DNA was extracted from peripheral blood samples collected on EDTA anticoagulant using spin column technique of QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, eluted in 100 μl of water and subsequently quantified using Nanodrop spectrophotometer (UV–Visible NanoDrop 1000; Thermo Fisher Scientific Inc.) and standardized to 100 ng/ml.

DNA was amplified by specific oligonucleotide primers for KIR2DL2 (KIR2DL2 for: CCA TGA TGG GGT CTC CAA A; rev: GCC CTG CAG AGA ACC TAC A). PCR reaction was performed as previously described (Uhrberg et al., 1997). Briefly, 100 ng of DNA was added to a 25 μl reaction mixture containing: reaction Buffer (Roche, Basel, Switzerland) 1×; each dNTP (Roche, Basel, Switzerland) 0.2 mM; $MgCl_2$ (Roche, Basel, Switzerland) 1.5 mM; Taq polymerase (Roche, Basel, Switzerland) 0.75 units; and 0.5 μM of each forward and reverse primer. Cycling parameters were as follows: initial denaturation for 5 min at 95 °C; then 20 s at 97 °C, 45 s at 62 °C, and 90 s at 72 °C for the first five cycles; and then 20 s at 95 °C, 45 s at 60 °C, and 90 s at 72 °C for 25 cycles. Amplifications were performed in a MyCycler thermal cycler (Bio-Rad). Amplification products were 296 bp. The amplification products were analyzed by ethidium bromide-stained agarose gel electrophoresis.

The typing of HLA-C1 (HLA-CAsn80), the KIR2DL2 ligand, was performed by PCR. Briefly, 100 ng of genomic DNA was amplified by specific oligonucleotide primers for HLA-C1 (HLA-CAsn80 for: CGA GTG AGC CTG CGG AAC; rev: GC CCA CTT CTG GAA GGT TCC). PCR

Table 2
KIR2DL2 and HLA-C1 genes carrier frequencies in Tunisian MS patients and controls.

	MS patients, observed/n (%)	Controls, observed/n (%)	p value	OR (CI 95%)	Statistical power
KIR2DL2	45/60 (75%)	50/112 (44.6%)	0.0002	3.72 (1.77–7.91)	0.98
HLA-C1	54/60 (90%)	62/112 (55.3%)	$<10^{-5}$	7.26 (2.71–20.48)	

Table 3
Interaction between KIR2DL2 and his ligand HLA-C1 in Tunisian MS patients and controls.

Haplotypes	MS patients, observed/n (%)	Controls, observed/n (%)	p value	OR (CI 95%)	Statistical power
2DL2 +/C1 +	42/60 (70%)	34/112 (30.4%)	0.004	6.59 (1.61–31.21)	0.98
2DL2 +/C1 −	3/60 (5%)	16/112 (14.23%)	0.06	–	
2DL2 −/C1 +	12/60 (20%)	28/112 (25%)	0.5	–	
2DL2 −/C1 −	3/60 (5%)	34/112 (30.4%)	0.03	4.86 (1.11–24.28)	

Table 4a
KIR2DL2/HLA-C1 haplotypes and age of onset.

Haplotypes	Age of onset groups (per year)						
	[17–30]	[31–40]	≥41	p value	OR (CI 95%)	Statistical power	Sample size estimation
2DL2+/C1+	18/29 (62.06%)	14/18 (77.77%)	7/13 (53.84%)	NS	–		
2DL2+/C1−	2/29 (6.89%)	0/18 (0%)	1/13 (7.69%)	NS	–		
2DL2−/C1+	7/29 (24.13%)	3/18 (16.66%)	5/13 (38.46%)	NS	–	0.2	288
2DL2−/C1−	2/29 (6.89%)	1/18 (5.55%)	0/13 (0%)	NS	–		

reaction was performed as previously described (Du et al., 2007). 25 μl of the PCR reaction mixture was prepared at a final concentration of: reaction Buffer (Roche, Basel, Switzerland) 1×; each dNTP (Roche, Basel, Switzerland) 0.2 mM; $MgCl_2$ (Roche, Basel, Switzerland) 1.5 mM; Taq polymerase (Roche, Basel, Switzerland) 0.75 Units; 0.5 μM of each forward and reverse primer, following the PCR conditions: initial denaturation for 3 min at 95 °C; then 20 s at 94 °C, 20 s at 66 °C, and 90 s at 72 °C for the first eight cycles; then 35 cycles of 20 s at 94 °C, 20 s at 63 °C, and 90 s at 72 °C and a final extension at 72 °C for 10 min. PCR product size is 1.344 bp. The amplification products were analyzed by ethidium bromide-stained agarose gel electrophoresis.

Amplifications were performed in a MyCycler thermal cycler (Bio-Rad).

2.3. Human herpesviruses PCR assays

We analyzed MS patients for the presence of all eight human herpesviruses. The presence of HSV-1, HSV-2, VZV, EBV, HCMV, HHV-6, HHV-7 and HHV-8 was evaluated on peripheral blood DNA as previously described (Ben Fredj et al., 2012).

2.4. Statistical analysis

Statistical analyses were performed using statistical software (Epi Info software version 3.2.2). Carrier frequencies of KIR2DL2 genes and HLA-C1 alleles were compared between MS patients and healthy controls by Pearson χ^2 test or by Fisher's exact test, estimating odds ratios (OR) with 95% confidence intervals (CI). Comparisons between KIR2DL2 genes and clinical characteristics of MS patients were performed either by Pearson χ^2 or by Fisher's exact test for the categorical variables and by Mann–Whitney or by Kruskal–Wallis tests for the quantitative variables. Power analysis and sample size estimation were performed with ClinCalc software (clincalc.com). Results were considered significant at a p value <0.05.

3. Results

3.1. KIR2DL2 and HLA-C1 frequencies

The possible association between MS and KIR-HLA genotype has been investigated by molecular typing of HLA-C1 and KIR2DL2 genes in a homogeneous group of 60 Tunisian patients then, results were compared to those obtained in 112 unrelated HC with the same genetic background.

KIR2DL2 and HLA-C1 frequencies of MS patients and healthy controls are reported in Table 2. Results revealed a significant increase in the frequency of KIR2DL2 gene in MS patients (75%) when compared with control subjects (44.6%) ($p = 0.0002$) (Power: 0.98). The increased frequency of KIR2DL2 allele in MS patients resulted in a significant OR of 3.72 (CI: 1.77–7.91). Regarding HLA-C1 frequencies, a statistically significant difference likewise was observed between MS patients and HC (90% versus 55.3%, $p < 10^{-5}$), (OR = 7.26, CI: 2.71–20.48).

Since KIR2DL2 and HLA-C1 molecules are a receptor-ligand pair, we investigated whether there was evidence for statistical interaction. Interestingly, our analysis yielded a significant support for an interaction effect of HLA-C1 with KIR2DL2 receptor (Table 3). We observed an increased frequency of KIR2DL2+/HLA-C1+ haplotype in MS population (70%) in comparison with control subjects (30.4%) ($p = 0.004$) (OR = 6.59 CI: 1.61–31.21) (Power: 0.98). On the opposite, we showed a significant decrease in KIR2DL2-/HLA-C1-haplotype in MS patients (5%) in comparison with controls (30.4%) (OR: 4.86; CI: 1.11–24.28).

3.2. KIR2DL2 and clinical variables

Since KIR2DL2+/HLA-C1+ haplotype was associated with MS condition, we evaluated the possible correlation with clinical variables. No significant associations were discovered for age at onset, disease duration and EDDS score (Table 4a, 4b, 4c), considering both KIR2DL2 and KIR2DL2/HLA-C1 haplotype positivities. The results obtained presented a power of 0.2, 0.14 and 0.3, respectively. In order to exclude

Table 4b
KIR2DL2/HLA-C1 haplotypes and disease duration.

Haplotypes	Duration disease groups (per year)						
	[1–4]	[5–9]	≥10	P value	OR (CI 95%)	Statistical power	Sample size estimation
2DL2+/C1+	19/31 (61.29%)	11/15 (73.33%)	9/14 (64.28%)	NS	–		
2DL2+/C1−	2/31 (6.45%)	0/15 (0%)	1/14 (7.14%)	NS	–		
2DL2−/C1+	8/31 (25.8%)	4/15 (26.66%)	3/14 (21.42%)	NS	–	0.14	381
2DL2−/C1−	2/31 (6.45%)	0/15 (0%)	1/14 (7.14%)	NS	–		

Table 4c
KIR2DL2/HLA-C1 haplotypes and EDSS score.

Haplotypes	EDSS score groups						
	[1–2]	[3–4]	≥5	p value	OR (CI 95%)	Statistical power	Sample size estimation
2DL2+/C1+	20/34 (58.88%)	15/19 (78.94%)	4/7 (57.14%)	NS	–		
2DL2+/C1−	3/34 (8.82%)	0/19 (0%)	0/7 (0%)	NS	–		
2DL2−/C1+	9/34 (26.47%)	4/19 (21.05%)	2/7 (28.57%)	NS	–	0.3	180
2DL2−/C1−	2/34 (5.88%)	0/19 (0%)	1/7 (14.28%)	NS	–		

Table 5a
KIR2DL2 and total HHVs (HHV-1 to HHV-8) in MS patients.

	HHVs+, observed/n (%)	HHVs−, observed/n (%)	p value	OR (CI 95%)	Statistical power	Sample size estimation
KIR2DL2+	26/45 (57.8%)	19/45 (42.2%)	0.65	1.56 (0.42–5.92)	0.33	304
KIR2DL2−	7/15 (46.7%)	8/15 (55.3%)				

Table 5b
HLA-C1 and total HHVs (HHV-1 to HHV-8) in MS patients.

	HHVs+, Observed/n (%)	HHVs−, Observed/n (%)	p value	OR (CI 95%)	Statistical power	Sample size estimation
HLAC-1+	31/54 (57.4%)	23/54 (42.6%)	0.24	2.7 (0.37–23.51)	0.3	398
HLAC-1-	2/6 (33.33%)	4/6 (66.66%)				

definitely the associations, we would need a total of 288, 381 and 180 subjects, respectively.

3.3. KIR2DL2 and herpesviruses

Since Rizzo et al. (2012) observed the influence of KIR2DL2 + /HLA-C1 + haplotype in HSV-1 susceptibility in MS patients and Caselli et al. (2013) reported a significant association between KIR2DL2/HLA-C1 haplotype and HHV8 infection in type 2 diabetes patients, we evaluated the possible influence of this haplotype on HHV infection in our MS population. Firstly, we subdivided the patients according to the positivity for almost one HHV. We observed 55% of MS patients positive for at least one HHV. A slight increase in HHV risk of infection in KIR2DL2 positive patients was observed with an OR = 1.56 (CI: 0.42–5.92) (Table 5a) but without reaching a significant p value (p = 0.65). Similarly, a slight increase in HHV risk of infection in HLA-C1 positive patients was observed with an OR = 2.7 (CI: 0.37–23.51) (Table 5b) but without reaching a significant p value (p = 0.24). When we considered the haplotypes, we observed an increase in KIR2DL2 + /HLA-C1 + haplotype in HHV infected patients (73%) in comparison with uninfected subjects (66%) (OR: 1.33; CI 95%:0.44–4.04) (Table 5c). The results obtained presented a power of 0.33, 0.3 and 0.3, respectively. In order to exclude definitely the associations, we would need a total of 304, 398 and 1830 subjects, respectively.

Then, we considered the single HHV infections. We observed a 6.66% of MS patients with VZV infection, a 28.33% with EBV, a 5% with HHV-6 and a 28.33% with HHV-7 infection. No positive samples to HSV-1, HSV-2, CMV or HHV-8 were detected in MS patients. We obtained a doubled risk for EBV infection for KIR2DL2 positive patients (OR = 1.81 CI: 0.38–9.6) (Table 6a). These results presented a power of 0.96, supporting the statistical significance. When we considered the haplotypes, we observed an increase in KIR2DL2 + /HLA-C1 + haplotype in EBV infected patients (84%) in comparison with uninfected subjects (65%) (OR: 2.5; CI 95%:0.62–10.1) (Table 6c). The results obtained presented a power of 0.23, and needs an increase to 111 subjects to confirm the data. (See Table 6b.)

No correlations were observed with other HHVs, possibly because of the low percentages of positive MS patients for the other HHVs, that did not allow to reach a statistical significance.

4. Discussion

Natural killer (NK) cells are the fast-acting effector lymphocytes of innate immunity that respond to infection (Biron et al., 1999; French and Yokoyama, 2003), tumor (Diefenbach and Raulet, 2002), and allogeneic hematopoietic cell transplantation (Parham and McQueen, 2003; Dupont and Hsu, 2004) while remaining tolerant to healthy cells.

NK cells discriminate between self and non-self through a battery of inhibitory and activating receptors (Kumar and McNerney, 2005; Lanier, 2005).

This potentially self-destructive effector function is mainly controlled by the interactions between KIR receptors expressed by NK cells and HLA class I ligands expressed by healthy cells (Gumperz et al., 1995; Colonna et al., 1997; Fan et al., 2001; Moretta et al., 2004).

Several reports have studied the role of NK cells in MS. NK cells regulate the function of antigen-specific T cells in MS (Takahashi et al., 2004) and correlate with MS disease activity (Lorentzen et al., 2009), NK cells mediate the immunomodulatory effects of MS therapy (Bielekova et al., 2006; Saraste et al., 2007) and reductions of NK functional activity often precede MS relapse (Kastrukoff et al., 1999; Takahashi et al., 2004).

In this study, we investigate the link between NK cells and herpesvirus infection in a cohort of MS patients and healthy controls. KIR2DL2 and his ligand HLA-C1 polymorphisms as well as HHV prevalence

Table 5c
Interaction between KIR2DL2/HLA-C1 haplotypes and HHVs in Tunisian MS patients.

Haplotypes	HHVs+, observed/n (%)	HHVs−, observed/n (%)	p value	OR (CI 95%)	Statistical power	Sample size estimation
2DL2 +/C1 +	24/33 (72.72%)	18/27 (66.66%)	0.61	1.33 (0.44–4.04)		
2DL2-/C1 +	7/33 (21.21%)	5/27 (18.51%)	0.79	1.18 (0.33–4.26)		
2DL2 +/C1-	2/33 (6.06%)	1/27 (4.7%)	0.6	1.7 (0.14–19.56)	0.3	1830
2DL2-/C1-	0/33 (0%)	3/27 (11.11%)	0.085	NA		

Table 6a
KIR2DL2 and EBV in MS patients.

	EBV+, observed/n (%)	EBV−, observed/n (%)	p value	OR (CI 95%)	Statistical power
KIR2DL2+	14/45 (31.1%)	31/45 (68.9%)	0.31	1.81 (0.38–9.6)	0.96
KIR2DL2-	3/15 (20%)	12/15 (80%)			

Table 6b
HLA-C1 genes carrier frequencies and EBV in Tunisian MS patients.

	EBV+, observed/n (%)	EBV−, observed/n (%)	p value	OR (CI 95%)
HLAC-1+	17/54 (31.48%)	37/54 (68.51%)	0.12	NA
HLAC-1-	0/6 (0%)	6/6 (100%)		

were analyzed, to assess whether interactions between KIR2DL2 gene and its HLA-C1 ligand may contribute to the pathogenesis of MS. Our results revealed a statistically high frequency of KIR2DL2 in MS patients with respect to controls ($p = 0.0002$, OR = 3.72 (CI: 1.77–7.91)), suggesting the implication of this inhibitory receptor in MS disease. This finding was enhanced in the presence of its ligand HLA-C1 ($p < 10^{-5}$, OR = 7.26 (CI: 2.71–20.48)), since a significant p values and high OR was found in KIR2DL2 +/HLA-C1 + patients ($p = 0.004$, OR = 6.59 (CI: 1.61–31.21)).

Three previous studies analyzed the contribution of KIR genes and their ligand in MS patients, reaching divergent conclusions. The Garcia-Leon study revealed that the KIR2DL5 and KIR3DS1 genes confer a slight susceptibility to MS (García-León et al., 2011). Whereas Lonrentzen et al. found a lower KIR2DL1 and a higher KIR2DS4 and KIR3DL1 carrier frequency among MS patients compared to controls (Lorentzen et al., 2009).

Meanwhile, Fusco et al. found a lower frequency of KIR2DS1 and KIR2DL5 as well as a higher frequency of KIR2DS4 in MS patients (Fusco et al., 2010), attributing the relationship with KIR2DL5 to the linkage with KIR2DS1.

The clinical manifestations in our patients don't seem to be associated with KIR2DL2 gene although Lorentzen et al. found more severe disease among MS patients carrying the inhibitory KIR2DL2 and the activating KIR2DS2 genes compared to non-carriers (Lorentzen et al., 2009). Furthermore, García-León et al. found patients carrying the KIR2DS5 gene had a lower age at onset, indicating that even though this gene does not predispose to the disease, it may contribute to an earlier appearance of clinical symptoms. Besides, in the same study, patients carrying the KIR2DL1 gene presented a higher progression index (current EDSS/disease duration) (García-León et al., 2011).

These discrepancies between studies suggest an implication of KIR/HLA genetic background not in MS disease but in related conditions. The absence of correlation between KIR2DL2 and disease status (EDSS, disease duration, age of onset) in our study, could be explained by the fact that KIR2DL2 is not a disease gene.

On the basis of previous results, by supporting a genetic association between KIR2DL2 and susceptibility to viral infections (Estefanía et al., 2007; Moraru et al., 2012; Rizzo et al., 2012), we focused on the possible effect of KIR2DL2 on viral infection. A significant OR was observed, signifying that there is a slight increase in HHV risk of infection in KIR2DL2 positive patients. The same results were found with EBV with a doubled risk for EBV infection for KIR2DL2 positive patients.

None of these associations yielded a significative p, indicating that the size of our cohort was not large enough to obtain strong p values. These results therefore need to be confirmed in a larger cohort, as indicated by power analysis and sample size estimation.

A recent study carried out by Rizzo et al. using peripheral blood mononuclear cells from MS patients and controls treated with CpG sequences and infected in vitro with HSV-1, showed that MS patients positive for the inhibitory KIR2DL2 receptor and its ligand HLA-C1 had increased sensitivity to in vitro HSV infection and decreased NK cell degranulation. Moreover, KIR2DL2 positive MS patients without HLA-C1 ligand behaved as KIR2DL2 negative individuals, therefore, the lack of NK cell activation takes place only in the context of a functional complex between KIR2DL2 receptor and its HLA-C1 ligand.

In fact, the presence of HLA-C1 molecules induces inhibitory signals in KIR2DL2 positive NK cells from MS patients, creating an anergic environment with significant lack of IFN-gamma production and consequent absence of NK cell activation and for the lack of innate protection to HSV infection (Rizzo et al., 2012).

It is noteworthy that genotyping other KIR genes as well as their ligand may be of interest and will provide supplementary information about the implication of KIR receptor in the control of NK cell activation towards herpesvirus infection in multiple sclerosis patients. Moreover, these results need confirmation by studies on additional samples from the same population and from other different ethnicity.

In summary, we have confirmed the possible effect of KIR2DL2 on viral infection in MS patients, supporting the possibility to exploit this receptor for modulating NK cell activation as an important strategy in the management of viral infections in MS patients.

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Table 6c
Interaction between KIR2DL2/HLA-C1 haplotypes and EBV in Tunisian MS patients.

Haplotypes	EBV +, Observed/n (%)	EBV -, Observed/n (%)	p value	OR (CI 95%)	Statistical power	Sample size estimation
2DL2+/C1+	14/17 (82.35%)	28/43 (65.11%)	0.22	2.5 (0.62–10.1)	0.23	111
2DL2-/C1+	3/17 (17.64%)	9/43 (20.93%)	1			
2DL2+/C1-	0/17 (0%)	3/43 (6.97%)	0.55	NA		
2DL2-/C1-	0/17 (0%)	3/43 (6.97%)	0.55	NA		

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Paper 3

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Early Release Paper

HLA-G is a component of the CLL escape repertoire to generate immune suppression: impact of HLA-G 14 bp (rs66554220) polymorphism

by Roberta Rizzo, Valentina Audrito, Paola Vacca, Davide Rossi, Davide Brusa, Marina Stignani, Daria Bortolotti, Giovanni D' Arena, Marta Coscia, Luca Laurenti, Francesco Forconi, Gianluca Gaidano, Maria Cristina Mingari, Lorenzo Moretta, Fabio Malavasi, and Silvia Deaglio

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HLA-G is a component of the chronic lymphocytic leukemia escape repertoire to generate immune suppression: impact of HLA-G 14 bp (rs66554220) polymorphism

Running title: HLA-G polymorphism in CLL

Roberta Rizzo[1], Valentina Audrito[2,5], Paola Vacca[3], Davide Rossi[4], Davide Brusa[5], Marina Stignani[1], Daria Bortolotti[1], Giovanni D'Arena[6], Marta Coscia[7], Luca Laurenti[8], Francesco Forconi[9], Gianluca Gaidano[4], Maria Cristina Mingari[3,10], Lorenzo Moretta[11], Fabio Malavasi[2,12] and Silvia Deaglio[2,5]

[1]Department of Medical Sciences, Sections of Microbiology and Medical Genetics, University of Ferrara, Ferrara, Italy; [2]Department of Medical Sciences, University of Turin, Turin, Italy; [3]Department of Experimental Medicine, University of Genoa, Genoa Italy; [4]Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy; [5]Human Genetics Foundation (HuGeF), Turin, Italy; [6]Department of Onco-Hematology, IRCCS ''Centro di Riferimento Oncologico della Basilicata,'' Rionero in Vulture, Italy; [7]Division of Hematology, University of Torino, Azienda Ospedaliera Città della Salute e della Scienza di Torino, Torino, Italy; [8]Institute of Hematology, Catholic University of the Sacred Heart, Rome; [9]Cancer Sciences Unit, CRUK Centre, University of Southampton & Haematology Department, SUHT, Southampton, United Kingdom; [10]AOU San Martino–Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy; [11]Giannina Gaslini Institute, Genoa, Italy; and [12]Research Center for Experimental Medicine, Città della Salute e della Scienza Hospital, Turin, Italy

Correspondence

Silvia Deaglio, MD, PhD, Department of Medical Sciences, University of Turin School of Medicine & Human Genetics Foundation (HuGeF), via Nizza, 52, 10126 Torino, Italy.
E-mail: silvia.deaglio@unito.it

Roberta Rizzo, PhD, Department of Medical Sciences, Section of Microbiology, University of Ferrara, via Borsari 46, 44121 Ferrara, Italy. Email: roberta.rizzo@unife.it.

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Abstract

This work investigates the possibility that HLA-G, a molecule modulating innate and adaptive immunity, is part of an immune escape strategy of chronic lymphocytic leukemia cells. A 14 base pair insertion/deletion polymorphism (rs66554220) in the 3'-untranslated region of *HLA-G* influences mRNA stability and protein expression. The analysis of a cohort of chronic lymphocytic leukemia patients confirms that del/del individuals are characterized by higher levels of surface and soluble HLA-G than those of the other two genotypes. In line with its role in immunomodulation, the percentage of regulatory T lymphocytes is higher in del/del patients than in patients with the other genotypes and correlates with the amounts of surface or soluble HLA-G. Furthermore, addition of sHLA-G-rich plasma from chronic lymphocytic leukemia patients induces natural killer cell apoptosis and impairs natural killer cell lysis, with effects proportional to the amount of soluble HLA-G added. Lastly, the presence of *HLA-G* 14 base pair polymorphism is of prognostic value, with del/del patients showing reduced overall survival, as compared to the other genotypes.

These results suggest that i) the *HLA-G* 14 bp polymorphism influences the levels of surface and soluble HLA-G expression, and that ii) the over-expression of HLA-G molecules contributes to create tolerogenic conditions.

Introduction

Chronic lymphocytic leukemia (CLL), the most common type of adult leukemia in Caucasian populations, is characterized by the progressive accumulation of mature $CD5^+/CD23^+$ B cells in the peripheral blood and lymphoid organs (1). Several observations point to immune escape as a relevant mechanism of tumor promotion. First, CLL cells express high levels of immunomodulatory factors [*e.g.*, TGF (2) and IL-10 (3)], which suppress response to antigens and influence activation, expansion, and effector functions of T lymphocytes (4). Second, an increase in the numbers of circulating regulatory T cells (Treg) parallels disease progression (5, 6). Lastly, adenosine production in the extracellular milieu by selected leukemic subpopulations shields the CLL clone from the actions of the immune system (7).

The aim of this work is to investigate the role of human leukocyte antigen G (HLA-G) as a further strategy adopted by CLL cells to evade immune defenses and to create protected niches where to grow and expand. HLA-G is a non-classical major histocompatibility complex (MHC) Class I product with limited sequence variability. It is exclusively expressed in tissues where the immune system needs to be constantly suppressed, including cytotrophoblast from early gestation placentas, amniotic cells, endothelial cells of chorionic blood vessels, thymic epithelial cells and corneas (8). HLA-G is a tolerogenic molecule, which inhibits cytolysis mediated by natural killer (NK) cells or T lymphocytes, induces T cell apoptosis and blocks transendothelial migration of NK cells (9). These functions are exerted upon binding the killer cell immunoglobulin-like receptor (KIR)2DL4 and the immunoglobulin-like transcript (ILT)2 and (ILT)4 ligands (10, 11). Hence, the immunosuppressive features of HLA-G are functional in pregnancy, organ transplantation, autoimmune diseases, and cancer immune escape (12).

The *HLA-G* gene encodes seven isoforms generated through alternative splicing: four are membrane-bound (namely, HLA-G1, -G2, -G3, and -G4), while three (HLA-G5, -G6, and -G7) are soluble and represent the counterparts of HLA-G1, -G2 and -G3, respectively. An alternative

mechanism to generate soluble HLA-G (sHLA-G) forms is represented by proteolytic cleavage of the membrane molecules (13).

HLA-G is characterized by different polymorphisms at the 5' upstream regulatory and the 3' untranslated regions (UTR) (14). One of these, characterized by the deletion/insertion (del/ins) of 14 base pairs (14 bp) (rs66554220), is responsible for mRNA stability and consequently protein production (15). The presence of the 14bp sequence is associated with unstable mRNA and reduced sHLA-G protein production (16). This polymorphism is implicated in autoimmune and chronic inflammatory conditions (8), while its role in cancer growth and progression is still controversial.

The role of HLA-G products in CLL patients has been evaluated in previous studies, which however focused exclusively on the expression either of the membrane or of the secreted isoform of the molecule. The results obtained indicate that i) HLA-G expression at transcription and protein levels is increased in CLL cells as compared to normal B lymphocytes (17-19); and ii) HLA-G expression correlates with worse clinical outcome in CLL (20, 21).

This work was undertaken with the aim of assessing the impact of the *HLA-G* 14 bp polymorphism on expression of the membrane and soluble forms of the HLA-G protein, and its role in promoting immune escape in a wide and well-characterized cohort of CLL patients.

Methods

Patients and controls

Five hundred and six individuals with a confirmed diagnosis of CLL were enrolled at diagnosis into a retrospective study and typed for *HLA-G* rs66554220 polymorphism. Patient characteristics are reported in Supplementary Table 1. Blood samples from patients or non-leukemic individuals were obtained after written informed consent in accordance with local institutional guidelines and the Declaration of Helsinki. The study has been approved by the Human Genetics Foundation Ethical Committee.

Peripheral blood mononuclear cells (PBMCs) and purified B lymphocytes were obtained as described (22).

Flow cytometric analyses

Antibodies used for flow cytometry are detailed in Supplementary Materials and Methods. Data were acquired using a FACSCanto II (BD Biosciences, Buccinasco, Italy) or Gallios (Beckman Coulter) flow cytofluorimeters, processed with DIVA v6.1.3 (BD Biosciences), and analyzed with FlowJo Version 9.01 softwares (TreeStar, Ashland, OR). At least 10,000 events were analyzed for each sample.

HLA-G 14 bp polymorphism typing

Genomic DNA was extracted from PBMCs using a DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy). The *HLA-G* 14 bp polymorphism was genotyped by PCR (23, 24).

Soluble HLA-G detection by ELISA

Measurements of sHLA-G1 and HLA-G5 levels were performed as reported (25). After both ELISA measurements, the amount of sHLA-G1 was expressed as the difference between sHLA-G1/HLA-G5 and HLA-G5 concentrations (26).

Isolation and culture of NK cells

NK cells were isolated from PBMCs or from decidua were obtained as described (27) (28). Purified NK cells were cultured on allogeneic irradiated feeder cells in the presence of IL-2 (100 U/ml) and PHA (1.5 ng/ml, Gibco, Life Technologies) (27).

NK cell apoptosis

Freshly isolated or IL-2 activated NK cells were incubated with different serum samples from CLL patients representative of the different 14 bp HLA-G polymorphism. After overnight incubation, NK cell apoptosis was measured using annexin V and propidium iodide (Invitrogen, Eugene, OR, USA).

NK cytolytic activity

IL-2-activated NK cells were tested for cytolytic activity in a 4 h ^{51}Cr-release assay against the K562 cell line (27).

Statistical analyses

Overall survival (OS) was measured from date of sampling to date of death (event) or last follow-up (censoring). Survival analysis was performed by the Kaplan-Meier method. The crude association between time-fixed exposure variables at diagnosis and survival was estimated by log-rank. Statistical significance was defined as p value $<.05$. Statistical tests were performed using the GraphPad Prism 6.0 software (Graphpad Software, San Diego, CA, USA) and the SPSS software v20.0 (Chicago, IL).

Results

Effects of *HLA-G* 14 bp polymorphism on membrane and soluble protein expression in CLL patients

The hypothesis behind this work is that patients with a del/del genotype of the 14 bp polymorphism are characterized by a more stable *HLA-G* mRNA, resulting in higher levels of the molecule on the cell surface and in biological fluids (16).

The hypothesis was tested by determining the effects of the 14 bp polymorphism on the expression of HLA-G protein at the surface of CLL B cells obtained from 126 patients. The observed levels of HLA-G expression on $CD19^{+}/CD5^{+}$ CLL B lymphocytes were highly variable (mean ± SEM 7.35 ± 1.13%, Figure 1A, B). Patients with a del/del genotype had a trend towards increased levels (n=51, mean 8.97 ± 1,85) of surface HLA-G, even if a comparison with ins/del (n=48, mean 6.38 ± 2) or ins/ins (n=27, mean 6.03 ± 1.77) patients was not statistically significant (Figure 1C). However, when divided into quartiles, 25.4% of the 126 patients presented a surface HLA-G expression by leukemic cells above the third quartile (third quartile value: 9.5%). Of these, 58% had a del/del, 23% an ins/del and 19% an ins/ins genotype ($p<0.0001$, Chi-Square test, Figure 1D).

Attention was next focused on HLA-G plasma levels assayed by ELISA assay in a cohort of 60 patients and 60 sex and age matched controls. Results indicate a marked variability in concentration in both CLL patients (mean ± SEM, 19.71 ± 2.83 ng/ml) and controls (mean ± SEM, 17.28 ± 23.64 ng/ml), without statistical differences between the two groups (Figure 2A). When CLL patients and controls were divided according to the genotype, del/del patients (n=27) displayed significantly higher levels of sHLA-G (mean 30.82 ± 4.8 ng/ml), than ins/del (n=18, mean 13.68 ± 3.67 ng/ml, p=0.015, Mann Whitney test) or ins/ins patients (n=15, mean 6.95 ± 2.74 ng/ml, p=0.003, Mann Whitney test, Figure 2B). Del/del controls showed a tendency towards a higher production of sHLA-G (n=22, mean 22.06 ± 27.32ng/ml) when compared to ins/del (n=25, mean 16.93 ± 24.87 ng/ml) or ins/ins (n=13, mean 9.88 ± 11.90 ng/ml) individuals, without reaching a statistically significant difference (Supplementary Figure 1A). After dividing sHLA-G levels into quartiles,

25% of CLL patients and of controls presented sHLA-G expression above the third quartile (third quartile value CLL patients: 31.75 ng/ml; third quartile value controls: 24.40 ng/ml). Of these, in the CLL cohort 80% presented a del/del genotype, while ins/del and ins/ins genotypes were 13% and 7%, respectively ($p<0.0001$, Fisher exact test, Figure 2C). When considering controls, 46% presented a del/del, 46% an ins/del and 8% an ins/ins genotype ($p<0.0001$, Fisher exact test, Supplementary Figure 1B).

We then asked whether sHLA-G levels correlate with HLA-G expressed on the membrane of CLL cells. As expected, the levels of expression of sHLA-G and membrane HLA-G presented a positive correlation (n=60, rho=0.4, p=0.003, Spearman Correlation test), in line with a relationship between surface HLA-G expression and release (Figure 2D). The analysis of covariance reported an independent effect of 14 bp polymorphism (p=0.003) and surface HLA-G expression (p=0.02) on sHLA-G plasma levels. Using an isoform-sensitive ELISA, sHLA-G1 was predominant in the plasma of CLL patients, indicating a derivation from shedding of the membrane form rather than from alternative splicing (sHLA-G1 *vs* HLA-G5 isoforms, p=0.001; Mann Whitney test, Figure 2E).

The conclusion is that patients with a del/del genotype present significantly higher sHLA-G levels and tend to express more membrane HLA-G on leukemic cells than the other genotypes.

Effects of *HLA-G* 14 bp polymorphism on the number of circulating T lymphocytes in CLL patients

CLL development and progression is paralleled by a progressive impairment of the host immune defenses, with clinically manifest immune defects of the T cell compartment. The next step of the work was to determine whether high levels of surface and sHLA-G would reflect the immune defects characterizing CLL. To this aim, the composition of T cell subsets was assessed in 52 CLL patients divided according to *HLA-G* 14 bp polymorphism.

$CD4^+$ and $CD8^+$ and T cell percentages were not significantly different in the three *HLA-G* 14 bp genotypes (Figure 3A, B). On the contrary, the number of Tregs, defined as

$CD4^+/CD25^{high}/CD127^{low}$), was significantly higher in the del/del genotype (mean 6.97% ± 0.88), as compared to the ins/ins group (mean 3.23% ± 0.69, p=0.006, Mann Whitney test, Figure 3C). Heterozygous patients displayed intermediate values, not significantly different from both ins/ins and del/del homozygous patients (mean 5.77 % ± 0.78). The percentage of Treg positively correlate with the levels of expression of surface HLA-G on CLL cells (n=33, rho=0.4, p=0.04, Spearman Correlation test), suggesting that the amount of HLA-G expressed by leukemic cells could impact on the frequency of Tregs (Figure 3D). In line with this observation was the finding that the percentage of circulating Tregs is higher in CLL with surface HLA-G expression >9.5% (third quartile) compared to CLL in which surface HLA-G expression was <9.5% (mean 6.95% ± 0.98 *vs* mean 4.31% ± 0.52, p=0.03, Mann Whitney U test; Figure 3E).

No statistically significant correlation could be detected between sHLA-G levels and the frequency of Tregs, likely due to the limited sample analysed (n=13, not shown). However, CLL patients in which sHLA-G levels were >31.75 ng/ml (third quartile), presented a trend towards a higher percentage of Tregs than patients in which sHLA-G were <31.75 ng/ml (mean 5.1% ± 1.44 *vs* mean 3.37% ± 0.52, p=0.30, Mann Whitney test, Figure 3F).Together, these data suggest that HLA-G expression is linked to an expansion of Tregs, as partly observed in other models(29).

Effects of HLA-G polymorphism on NK cell function

An alternative mechanism through which HLA-G molecules suppress the immune response is their inhibitory effect on NK cell activation and cytotoxic functions, mediated through the KIR2DL4 ligand (11). Therefore, we analyzed the possible implication of sHLA-G molecules in plasma samples on composition, activation and functional activities of NK cells in CLL samples. The reference control was represented by healthy individuals matched for gender and age.

The number of circulating NK cells (gated as $CD56^+/CD3^-$) was sharply decreased in CLL patients (n=9, mean 2.53% ± 0.88) as compared to controls (n=11, mean 11.52% ± 1.22, p<0.0001, Mann Whitney test, Figure 4A).

The cytolytic activity of NK cells isolated from CLL patients (n=9) was tested after culture for 2-4 weeks in the presence of IL-2. Cells were then assessed for their ability to lyse the K562 target cell line in a ^{51}Cr-release cytolytic assay at different Effector:Target (E:T) ratios (from 40:1 to 0,25:1). Controls were IL-2-activated NK cells from healthy donors. As shown in Figure 4B, the NK-mediated cytolytic activity in CLL patients was lower than that in healthy donors. Thus, for example, 40% target cell lysis was obtained at 2.5:1 E:T ratio from healthy donors and at 20:1 for NK cells from CLL patients (Figure 4B).

We then asked whether KIR2DL4, the main HLA-G ligand (30), was expressed by NK cells. While resting NK cells from normal donors and from CLL patients lacked KIR2DL4, the ligand was induced upon IL-2-mediated NK cell activation, in agreement with published data (30) (Figure 4C). Representative expression plots are reported in Supplementary Figure 2, where decidual NK cells were used as positive control. No differential expression in KIR2DL4 was noted in HLA-G typed patients (not shown). The next step was to test whether sHLA-G present in plasma could induce apoptosis or inhibit the cytolytic function of NK cells obtained from normal donors. To answer this question, we used plasma from CLL patients containing different levels of sHLA-G to interfere with the viability and cytotoxic activity of NK cells. Plasma from CLL patients significantly compromised viability of NK cells from normal donors, by inducing apoptosis. The effect was directly correlated to sHLA-G levels in plasma, with significantly lower survival in the presence of plasma from patients with sHLA-G>31,75 ng/ml as compared to patients with undetectable sHLA-G (Figure 4D).

A significant inhibition of NK cell function was detected in the presence of CLL plasma samples with detectable sHLA-G ($p<0.0001$, Mann Whitney test, Figure 4E). At an E:T ratio of 10:1 donor NK cells in the absence of CLL plasma efficiently killed target cells (mean 85% ± 1.63), while in the presence of CLL plasma samples with detectable sHLA-G, the cytolytic activity was sharply reduced (mean 27.38% ± 4.72). CLL plasma samples with no sHLA-G were used as control. The presence of NK cell inhibition also in the presence of CLL plasma samples with undetectable

sHLA-G suggests the presence of other factors implicated in NK cell activation control. However, the degree of inhibition correlates with the concentrations of sHLA-G (Figure 4F). An inverse correlation between sHLA-G levels in CLL plasma samples (n=22) and the cytolytic activity of NK cells expressed as % cell lysis could be measured (rho=-0.49, p=0.02, Spearman Correlation test, Figure 4G). Consistent with the notion that CLL patients with del/del polymorphism display higher levels of sHLA-G than the other genotypes, plasma from del/del patients presented a higher inhibition than from the other genotypes. Thus, at 10:1 E:T ratio, del/del plasma inhibited NK cell lysis by 57.9%, while the effects exerted by ins/del and ins/ins plasma were 35.8% and 5.3%, respectively ($p<0.0001$, Fisher exact test, Figure 4H).

Proof of principle: the *HLA-G* 14 bp polymorphism influences survival of CLL patients

The results obtained so far indicate that the *HLA-G* 14 bp polymorphism dictates the amount of HLA-G protein present on the cell surface and in the plasma of CLL patients and that the molecule modulates quantitatively and qualitatively T and NK immunocompetence. In consideration of the immunosuppressive features of HLA-G, patients characterized by a del/del genotype would have higher levels of HLA-G levels, would be more immunosuppressed and ultimately present a worse clinical outcome. This issue was approached by testing the frequency of the HLA-G 14 bp polymorphism in 506 CLL patients (Supplementary Table 1). The genotype frequencies were in Hardy-Weinberg equilibrium: 176/506 patients (34.8%) were del/del homozygous, 81/506 (16%) were ins/ins homozygous and the remaining 249/506 (49.2%) were ins/del heterozygous. None of the demographic (age and sex), clinical (disease stage, splenomegaly, lymph node size), laboratory (LDH or β_2m levels), or molecular variables (CD38, ZAP-70, IgHV mutational status, chromosomal aberrations) showed a preferential association with the HLA-G polymorphism (Supplementary Table 1). By survival analysis, patients harboring the del/del genotype were characterized by shorter survival than patients harboring the ins/del and/or ins/ins genotype ($p=0.027$, Log-rank test, Figure 5A). Consistently, CLL patients with sHLA-G levels above the third quartile presented lower survival (median, 63.9 months) compared to patients with sHLA-G

below the third quartile (median, 71.5 months, p=0.0215, Log-rank test; Figure 5B), and CLL patients with plasma samples showing inhibitory effects on NK cells showed a trend towards a lower survival than patients whose plasma lacked inhibitory effects (p=0.147, Log-rank test, not shown).

Discussion

HLA-G is a non-classical HLA protein that works by modulating main functions of NK cells and of Tregs. HLA-G-mediated signals are critical in mediating tolerance during specific ontogenetic moments (i.e., pregnancy). HLA-G may be expressed by tumor cells as part of a strategy to evade the action of the innate and adaptive immune system.

To test whether this may happen also in CLL cells, HLA-G expression was tested in a large cohort of CLL patients with well-defined molecular and clinical characteristics and with survival data available. The originality of the work is that the analyses of surface or sHLA-G expression were flanked by the characterization of the 14 bp polymorphism in the 3' UTR untrascribed region. This point was dictated by evidence derived from different models, which allows to conclude that this polymorphism accurately predicts the amount of transcribed protein, either bound to the membrane or released in biological fluids.

The results indicate an association between the del/del genotype and increased levels of plasma HLA-G molecules. This association is apparent only in CLL patients, as plasma from age- and sex-matched controls failed to reach a statistically significant association between the del/del genotype and the amount of plasma HLA-G.

The situation on the cell membrane is less defined: del/del patients are more frequent in the group expressing higher levels of the molecule, even if a statistical significance is not reached. A potential explanation for this finding lies in the relative instability of HLA-G molecules on the CLL cell membrane. Results showing a correlation between membrane and sHLA-G levels favor the view that the main mechanism for the generation of sHLA-G is shedding rather than alternative splicing. This was confirmed by the analysis of HLA-G isoforms, which highlight a dominance of sHLA-G1, generated by proteolytic cleavage of membrane HLA-G1. No preference of isoform according to the 14 bp polymorphism was noted, in agreement with published data (31, 32).

A reasonable hypothesis to explain this result is that the membrane form represents a transitory step. A consequence is that quantification of soluble rather than surface HLA-G may be more accurate.

This is also in line with previous studies, which found that sHLA-G levels in plasma were higher in CLL patients than in healthy controls (17, 18). A second issue favoring the view that quantification of soluble rather than membrane HLA-G is an informative and dependable assay derives from old and new facts linked to the unique lipid structure of the CLL surface (33, 34), potentially increasing the instability of the membrane form. In agreement with this in myeloma cells mHLA-G is released from the cell membrane also in microparticles (35). These findings suggest that a quantification of sHLA-G may be clinically useful and more informative than surface analysis on tumor cells.

The second set of results obtained in this work may be considered as tiles composing a tolerogenic mosaic, where HLA-G molecules represent a link between innate and adaptive immunity. Accordingly, the presence of a del/del genotype (i.e., with high sHLA-G levels) is paralleled by an expansion of Tregs in the circulation. Supportive data come from other models, where HLA-G is reported to induce Tregs. As an example, PBMC exposed to sHLA-G5 acquire regulatory features, inhibiting allo-proliferative responses exerted by other T lymphocytes. It is also known that patients receiving combined liver/kidney transplants show high levels of sHLA-G5, which correlate with increased percentages of suppressor T cells (36). Similarly, stem-cell transplanted patients present high sHLA-G5 in the peripheral blood, with a simultaneous expansion of $CD4^+/CD25^+/CD152^+$ T lymphocytes with suppressive activity. Indirect confirmation in the CLL model may be deduced from data showing that an increase in Tregs positively correlates with the presence of clinical and biological features of aggressive disease (37).

NK cells obtained from CLL patients present a lower cytotoxic activity when compared to NK cell populations obtained from healthy donors of a comparable age. This issue suggests that leukemic cells directly affect NK cell viability and/or activity. This would be achieved through binding of HLA-G to the KIR2DL4 ligand, which becomes expressed once NK cells are activated in the presence of IL-2. Our working hypothesis is that ectopic expression of HLA-G contributes to block NK cell functions. With the aim of reproducing physiological conditions the experiments were performed using whole plasma instead of purified HLA-G. The assumption was confirmed by

incubating NK cells obtained from normal donors with plasma from CLL patients containing variable amounts of sHLA-G. This was followed by a marked induction of NK cell apoptosis, which was proportional to the amount of sHLA-G present in plasma. Furthermore, also lysis inhibition was proportional to the amount of sHLA-G. Noteworthy, CLL plasma samples with undetectable sHLA-G were able to moderately induce NK cell apoptosis and to reduce NK cell cytotoxicity. These results suggest the presence of other factors than sHLA-G that are able to control NK cell activation in CLL condition. As expected, NK cell function of del/del patients was more impaired than that of ins/del or ins/ins patients.

In conclusion, data obtained in this work provide two different sets of information. The first one indicates that the 14 bp polymorphism influences quantitative analyses of sHLA-G. The amount of sHLA-G in plasma has a prognostic value, suggesting that this specific assay may be relevant in the management of CLL patients, rather than the mere measurement of membrane HLA-G. The second set of results indicates that the presence of HLA-G molecules in the neoplastic environment, either soluble or bound to the membrane, creates a favorable setting for CLL expansion. As a proof, the evaluation of the impact of 14 bp polymorphism on the clinical outcome of the disease shows that del/del patients display a lower overall survival than ins/del or ins/ins patients.

Taken together, our results sustain the view that HLA-G molecules are part of the escape strategies designed by CLL cells and indicates that a quantitative analysis of sHLA-G levels may be of clinical relevance in the management of CLL patients.

Authorship and Disclosures: RR, VA, PV, DR, DBr, MS, DBo: performed research; GDA, MC, LL, FF: contributed patient samples; GG, MCM, LM: designed research and interpreted data; FM, SD: designed research, interpreted data and together with RR wrote the paper. The authors declare no competing financial interests.

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Figure Legends

Figure 1. Distribution of membrane HLA-G in a cohort of 126 CLL patients typed for the 14 bp polymorphism. (**A**) Density plots represent the gating strategy. Left panel indicates the morphological gate, right panel shows the staining for CD19 and CD5. (**B**) Histograms represent surface HLA-G expression in $CD19^{+}/CD5^{+}$ CLL B lymphocytes of three representative patients. (**C**) Patients were then divided according to the 14 bp polymorphism into del/del, ins/del and ins/ins categories. Box plots represent the distribution of mHLA-G in the different categories. (**D**) Graph representing the percentage of patients expressing mHLA-G above (black bars) or below (open bars) the third quartile (9.5%).

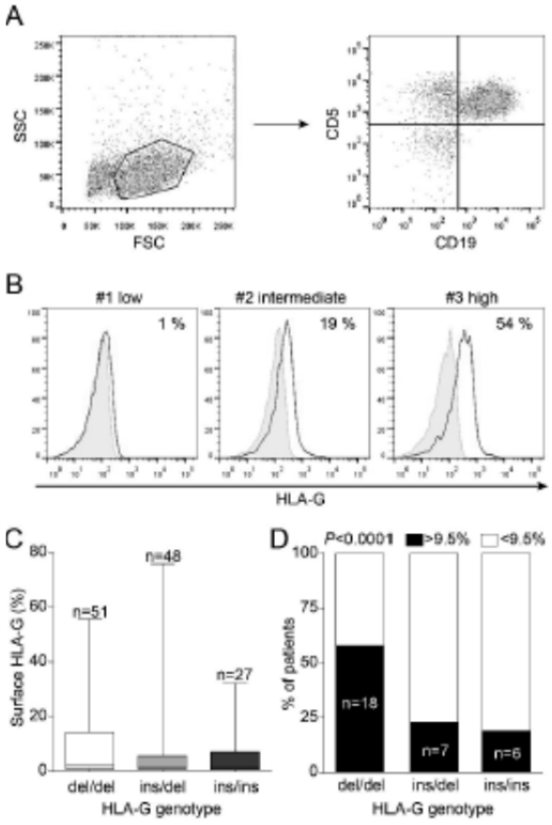
Figure 2. Quantification of soluble HLA-G levels in a cohort of 60 CLL patients typed for the 14 bp polymorphism. (**A**) Box plot analysis summarizing data obtained with a quantitative ELISA assay performed on 60 plasma samples from CLL patients and 60 plasma samples from control subjects (controls). The lower and upper limit of the box define the first and third quartiles, respectively, while the line inside the box represents the median. Whiskers identify minimum and maximum values. (**B**) Graph showing soluble HLA-G levels in CLL patients divided according to the 14 bp polymorphism into del/del, ins/del and ins/ins categories. (**C**) Graph representing the percentage of CLL patients expressing soluble HLA-G above (black bars) or below (open bars) the third quartile (31.75 ng/ml) in the three genotypes (**D**) Regression lines showing a positive correlation between the percentage of CLL cells expressing HLA-G on the cell surface and the amount of soluble HLA-G in the plasma. Spearman's coefficient (rho) and the corresponding p value is listed. (**E**) Box plot showing the results of an ELISA assay using isoform specific antibodies to discriminate between soluble HLA-G1 (derived from shedding of the membrane form) and soluble HLA-G5 (derived from alternative splicing).

Figure 3. Evaluation of the T cell compartment in CLL patients typed for the 14 bp polymorphism. Percentage of total $CD4^{+}$ (**A**) and $CD8^{+}$ (**B**) circulating T lymphocytes and Tregs

(**C**) in CLL patients divided according to the 14 bp polymorphism. Tregs were defined as $CD4^{+}/CD25^{high}/CD127^{low}$. (**D**) Regression line showing a positive correlation between membrane HLA-G and the percentage of circulating Tregs. Spearman's coefficient (rho) and the corresponding p values are listed. (**E**) Percentage of Tregs in CLL patients expressing membrane HLA-G above or below the third (9.5%) quartile. (**F**) Percentage of Tregs in CLL patients expressing soluble HLA-G above or below the third (31.75 mg/ml) quartile.

Figure 4. Evaluation of the NK cell compartment in CLL patients typed for the 14 bp polymorphism. (**A**) Percentage of circulating $CD56^{+}/CD3^{-}$ NK cells in 7 CLL patients and in 11 healthy donors (controls) with a comparable age and male:female ratio. (**B**) ^{51}Cr cytotoxicity assay comparing the lytic potential of *in vitro* IL-2-activated NK cells from controls (circles) or CLL patients (squares) against the K562 target cell line. (**C**) Expression of KIR2DL4 in resting and IL-2-activated NK cells from CLL patients. Data are expressed as MFI ratio. (**D**) Percentage of NK cell viability in the presence of sHLA-G-high and sHLA-G-low plasma from CLL patients. (**E**) Inhibition of cytolytic activity of IL-2-activated HD-NK cells against K562 target cells in the presence or absence of plasma obtained from CLL patients. (**F**) The inhibitory effect of CLL plasma on NK cell lysis was studied in patients with high levels of soluble HLA-G and compared to patients with undetectable soluble HLA-G. (**G**) Regression line showing a negative correlation between the amount of soluble HLA-G and the percentage of cell lysis. Spearman's coefficient (rho) and the corresponding p value is listed. (**H**) Graph representing the percentage of NK cell lysis inhibition obtained using plasma derived from patients categorized on the basis of HLA-G genotype.

Figure 5. Kaplan-Meyer curves showing overall survival in 506 CLL patients. (**A**) Kaplan-Meier estimates of overall survival according to according to *HLA-G* 14 bp genotype. (**B**) Kaplan-Meier estimates of overall survival according to according to soluble HLA-G levels.


A
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#2 intermediate
#3 high
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19 %
54 %
HLA-G
C
Surface HLA-G (%)
n=51
n=48
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del/del
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D
P<0.0001
>9.5%
<9.5%
% of patients
n=18
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n=6
del/del
ins/del
ins/ins
HLA-G genotype

FIGURE 1

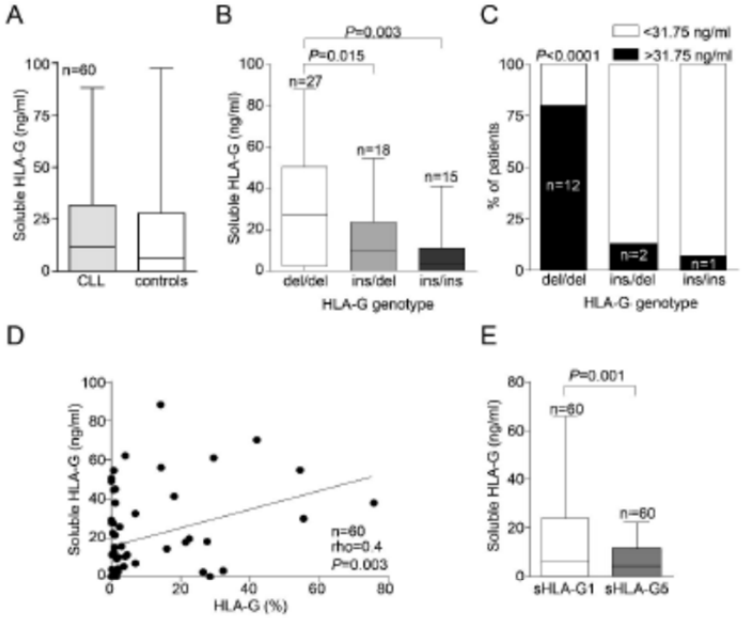

A
B
C
n=60
Soluble HLA-G (ng/ml)
CLL
controls
P=0.003
P=0.015
n=27
n=18
n=15
del/del
ins/del
ins/ins
HLA-G genotype
<31.75 ng/ml
>31.75 ng/ml
P<0.0001
% of patients
n=12
n=2
n=1
D
E
n=60
rho=0.4
P=0.003
HLA-G (%)
P=0.001
sHLA-G1
sHLA-G5

FIGURE 2

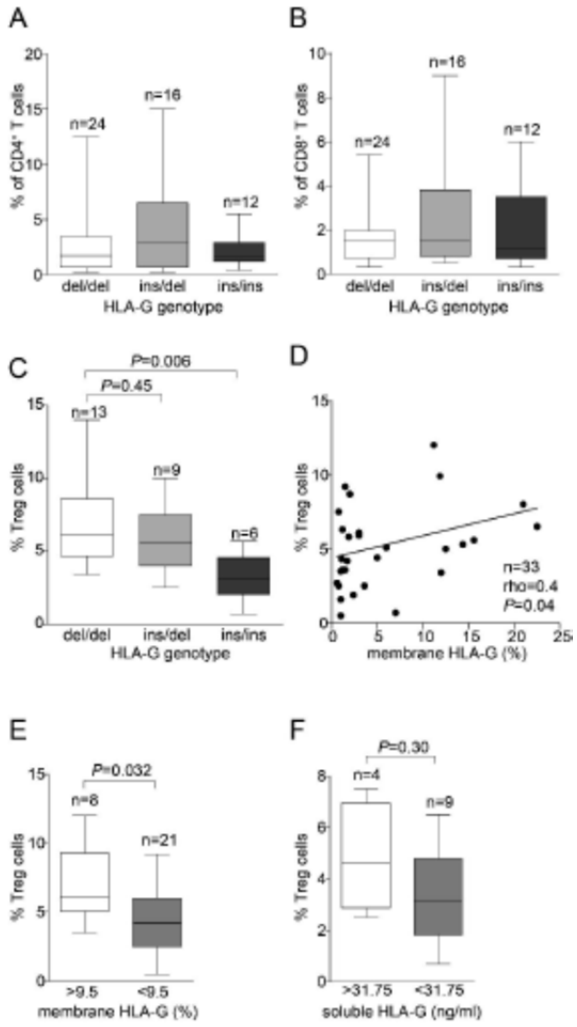

A
% of CD4+ T cells
n=24
n=16
n=12
del/del
ins/del
ins/ins
HLA-G genotype
B
% of CD8+ T cells
n=24
n=16
n=12
del/del
ins/del
ins/ins
HLA-G genotype
C
P=0.006
P=0.45
% Treg cells
n=13
n=9
n=6
del/del
ins/del
ins/ins
HLA-G genotype
D
% Treg cells
n=33
rho=0.4
P=0.04
membrane HLA-G (%)
E
P=0.032
% Treg cells
n=8
n=21
>9.5
<9.5
membrane HLA-G (%)
F
P=0.30
% Treg cells
n=4
n=9
>31.75
<31.75
soluble HLA-G (ng/ml)

FIGURE 3

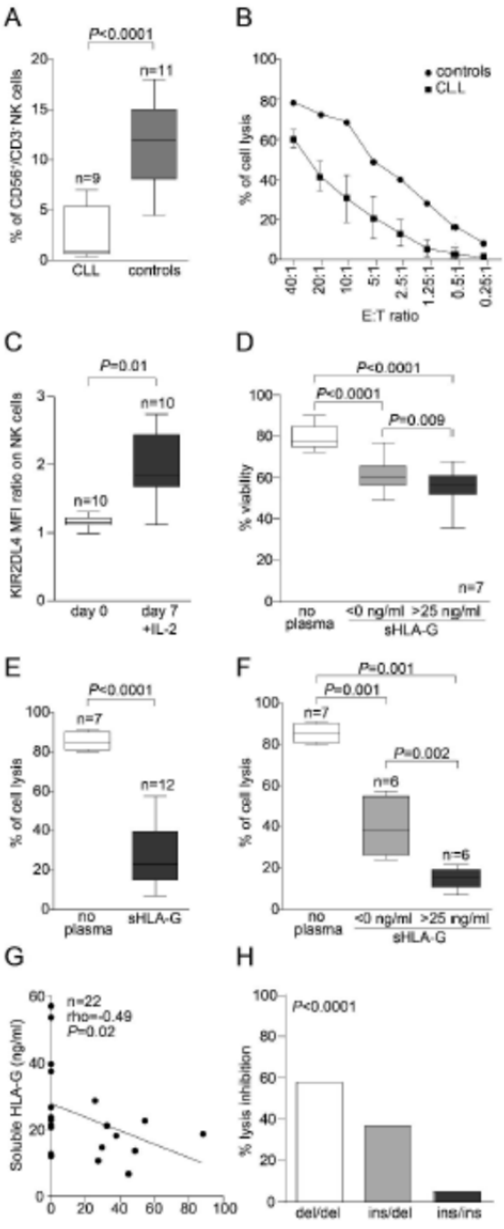

A
P<0.0001
n=11
n=9
% of CD56+/CD3- NK cells
CLL
controls
B
controls
CLL
% of cell lysis
40:1
20:1
10:1
5:1
2.5:1
1.25:1
0.5:1
0.25:1
E:T ratio
C
P=0.01
n=10
n=10
KIR2DL4 MFI ratio on NK cells
day 0
day 7 +IL-2
D
P<0.0001
P<0.0001
P=0.009
% viability
n=7
no plasma
<0 ng/ml
>25 ng/ml
sHLA-G
E
P<0.0001
n=7
n=12
% of cell lysis
no plasma
sHLA-G
F
P=0.001
P=0.001
n=7
P=0.002
n=6
n=6
% of cell lysis
no plasma
<0 ng/ml
>25 ng/ml
sHLA-G
G
n=22
rho=-0.49
P=0.02
Soluble HLA-G (ng/ml)
H
P<0.0001
% lysis inhibition
del/del
ins/del
ins/ins

FIGURE 4

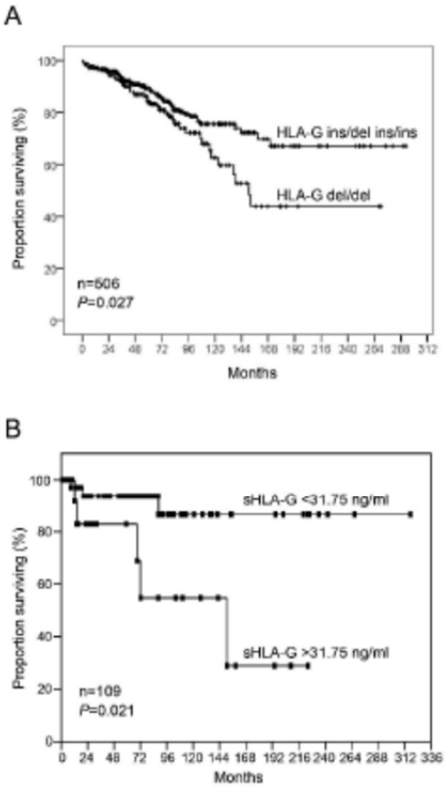

A
Proportion surviving (%)
HLA-G ins/del ins/ins
HLA-G del/del
n=506
P=0.027
Months
B
Proportion surviving (%)
sHLA-G <31.75 ng/ml
sHLA-G >31.75 ng/ml
n=109
P=0.021
Months

FIGURE 5

Supplementary Table 1. Demographic, clinical, laboratory and molecular variables of the CLL population subdivided according to rs 66554220 polymorphism.

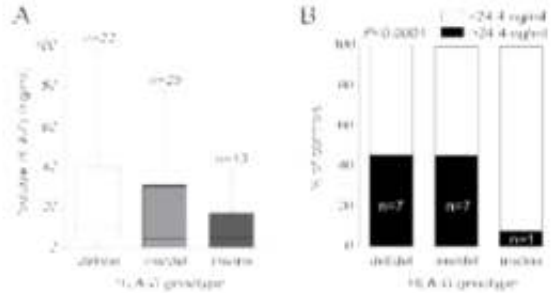
		HLA-G ins/ins n(%)	HLA-G ins/del n(%)	HLA-G del/del n(%)	Total	p value
CD38	≤30%	40 (49)	158 (63)	108 (61)	306	
	>30%	41 (51)	91 (37)	68 (39)	200	0.076
	Total	81	249	176	506	
ZAP70	≤20%	42(52)	132 (53)	96 (55)	270	
	>20%	39 (48)	117 (47)	80 (45)	236	0.19
	Total	81	249	176	506	
FISH	13q-/normal/+12	54 (67)	157 (63)	118 (67)	368	
	17p-/11q-	27 (33)	92 (37)	58 (33)	138	0.66
	Total	81	249	176	506	
***IGHV* homology**	≤98%	40 (49)	157 (63)	104 (59)	301	
	>98%	41 (51)	92 (37)	72 (41)	205	0.09
	Total	81	249	176	506	
Age (years)	<65	31 (38)	100 (40)	64 (36)	195	
	≥65	50 (62)	149 (60)	112 (64)	311	0.73
	Total	81	249	176	506	
Binet Stage	A	51 (63)	190 (76)	125 (71)	366	
	B - C	30 (37)	59 (24)	51 (29)	140	0.06
	Total	81	249	176	506	
Sex	F	41 (51)	100 (40)	73 (41)	214	
	M	40 (49)	149 (60)	103 (59)	292	0.25
	Total	81	249	176	506	
Lymphocytes	$<15x10^9/l$	50 (62)	132 (53)	97 (55)	279	
	$>15 x10^9/l$	31 (38)	117 (47)	79 (45)	192	0.39
	Total	81	249	176	506	
Lymph Node Size	≤3cm	71 (88)	198 (79)	143 (81)	412	
	>3cm	10 (12)	51 (21)	33 (19)	94	0.26
	Total	81	249	176	506	
Splenomegaly	No	52 (64)	190 (76)	134 (76)	386	
	Yes	29 (36)	59 (24)	42 (24)	120	0.075
	Total	81	249	176	506	
B2M	<2.5 mg/l	44 (54)	100 (40)	82 (47)	226	
	>2.5 mg/l	37 (46)	149 (60)	94 (53)	280	0.07
	Total	81	249	176	506	
LDH	<500 U/l	50 (62)	210 (84)	145 (82)	405	
	>500 U/l	31 (38)	39 (16)	31 (18)	101	0.11
	Total	81	249	176	506	

The genotypes ins/ins and ins/del were considered together as low and medium HLA-G producers and compared with the del/del high HLA-G producer genotype.

FISH, fluorescence in situ hybridization; *IGHV*, immunoglobulin heavy variable gene; B2M, beta-2-microglobulin.

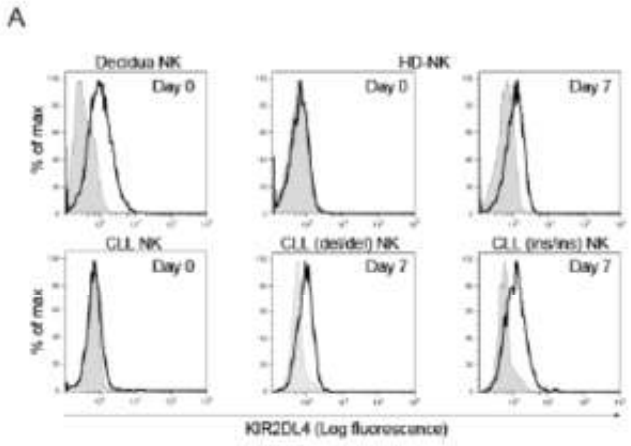
p values were obtained by chi square test or Fisher's exact test, when appropriate.

Supplementary Figure 1. Quantification of soluble HLA-G levels in a cohort of 60 controls typed for the 14 bp polymorphism. **(A)** Graph showing soluble HLA-G levels in control subjects divided according to the 14 bp polymorphism into del/del, ins/del and ins/ins categories. **(B)** Graph representing the percentage of control subjects expressing soluble HLA-G above (black bars) or below (open bars) the third quartile (24.4 ng/ml) in the three genotypes.



SUPPLEMENTARY FIGURE 1

Supplementary Figure 2. Phenotypic analysis of KIR2DL4 on NK cells. (A) NK cells from decidual tissue (dNK), healthy donor (HD-NK) and 2 representative CLL patients were analyzed for surface expression of KIR2DL4 at day 0 and after 7 days of culture in IL-2. Staining with secondary antibody alone is shown (gray profile). One representative experiment out of 10 performed.



SUPPLEMENTARY FIGURE 2

Supplementary Materials and Methods

Antibodies for flow cytometric analyses

Antibodies used were anti-HLA-G-PE (clone 87G), -CD19-FITC, -CD5-FITC, -CD4-PE-Cy5 (all from eBioscience, Milan, Italy), -CD38-PE (EXBIO Praha, Vestec, Czech Republic), -ZAP-70-AlexaFluor488 (Life Technologies, Invitrogen, Monza, Italy), -CD8-FITC (Biolegend, Milan, Italy), -CD3-PerCP, -CD56-APC, -CD19-APC (Miltenyi Biotech, Calderara di Reno, Italy). KIR2DL4 (R&D Systems, Abingdon, UK) was highlighted using a PE-conjugated rabbit anti-mouse secondary antibody (Southern Biotechnologies, Birmingham, AL). Tregs were detected using anti-CD4-PerCP/-CD25-PE/-CD127-AlexaFluor647 mix (Biolegend).

Multipotent stromal cells skew monocytes towards an anti-inflammatory function: a role for HLA-G molecules

We have read with interest the paper by Melief *et al.*[1] reporting the ability of multipotent stromal cells (MSCs) to skew monocytes towards an anti-inflammatory IL-10 producing phenotype by production of IL-6 and preventing the differentiation of monocytes towards antigen-presenting immunogenic cells. The authors concluded their paper proposing "the hypothesis that MSC, by inducing IL-10 production in monocyte-derived cells, play a powerful regulatory role in multiple anti-inflammatory mechanisms, which could explain their clinical benefits in immunotherapy". The authors suggest "that IL-6 is important but not the only factor". In fact, previous studies demonstrated that prostaglandin E and not IL-6 seems to represent the key inhibitory mediator.[2] Moreover, several studies have reported the ability of MSCs, when co-cultured with activated peripheral blood mononuclear cells (PBMC) or directly activated by exogenous IL-10, to modulate membrane bound and soluble HLA-G antigens.[3-6] HLA-G antigens are non-classical HLA-class I molecules characterized by tolerogenic and anti-inflammatory functions. In particular, both membrane and soluble HLA-G molecules have been shown to inhibit natural killer cell (NK) and $CD8^+$ T-cell mediated cytolysis, $CD4^+$ T-lymphocyte proliferation and dendritic cell maturation. Moreover, the expression of HLA-G antigens has been associated to the induction of regulatory T cells.[6]

The production of sHLA-G molecules by MSCs[3-6] has also been suggested, in addition to other mechanisms, as a rationale for the immunomodulatory properties of MSCs in preventing graft-*versus*-host disease (GVHD). In particular, through *in situ* immune-histochemical studies and by a multiparametric cytofluorimetric approach, useful to distinguish MSC and monocytes in co-culture conditions, we have observed a significant correlation between the presence of increased levels of HLA-G and IL-10 in the MSC co-cultures with PBNCs and a significant correlation with lymphoproliferative inhibition.[4]

Several studies have demonstrated that HLA-G modulation is of benefit in organ transplantations, autoimmune diseases and pregnancy where the downregulation of the immune response is essential for a positive outcome. On the other hand, the presence of HLA-G antigens has been associated to clinical negative consequences in tumor and in viral infections where the tolerogenic function of these molecules permits immune-escape.[7] The documented production of IL-10 by monocytes in the presence of MSC could trigger the production of HLA-G molecules by both monocytes and MSC.[3,4] Our data and the results of Melief *et al.*[1] support these factors as key mechanisms for MSC-induced immune-regulation.

Diana Campioni,[1] Daria Bortolotti,[2] Olavio R. Baricordi,[3] and Roberta Rizzo[2]

[1]Department of Medical Sciences, Hematology Section, S. Anna Hospital, Ferrara; [2]Department of Medical Sciences, Section of Microbiology, University of Ferrara; and [3]Department of Medical Sciences, Section of Medical Genetics, University of Ferrara, Ferrara, Italy

Correspondence: rbr@unife.it

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Key words: multipotent stromal cells, HLA-G molecules, skew, monocytes, anti-inflammatory.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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Paper 5

TISSUE ANTIGENS
IMMUNE RESPONSE GENETICS

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BRIEF COMMUNICATION

An accurate and reliable real time SNP genotyping assay for the HLA-G +3142 bp C>G polymorphism

D. Bortolotti[1], V. Gentili[1], L. Melchiorri[2], A. Rotola[1] & R. Rizzo[1]

1 Department of Experimental and Diagnostic Medicine, Section of Microbiology, University of Ferrara, Ferrara 44121, Italy
2 Department of Experimental and Diagnostic Medicine, Section of Medical Genetics, University of Ferrara, Ferrara 44121, Italy

Key words
3′ untranslated region; human leukocyte antigen-G; miRNA; single-nucleotide polymorphism genotyping

Correspondence
Roberta Rizzo
Department of Experimental and Diagnostic Medicine
Section of Microbiology
University of Ferrara
Via Luigi Borsari, 46
Ferrara 44121
Italy
Tel: +39 053 245 5382
Fax: +39 053 297 4470
e-mail: rbr@unife.it

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Abstract

Human leukocyte antigen (HLA)-G is a non classical HLA class I antigen with immuno-modulatory functions. The HLA-*G* gene is characterized by a +3142C>G variant in the 3′ untranslated region which is suggested to control protein production and to be associated with pathological conditions. DNAs form 221 randomly selected healthy subjects were genotyped for HLA-*G* +3142C>G polymorphism by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (*Bae*GI), real-time PCR and sequencing. The 19% of the PCR-RFLP heterozygous samples were genotyped as 3142GG by real-time PCR and sequencing. This disagreement is caused by digestion efficiency in PCR-RFLP. This real-time PCR method will guarantee an accurate genotyping for future research and clinical purposes, where large cohorts should be tested.

Human leukocyte antigen (HLA)-G is a non classical HLA class I molecule with a physiological tissue-restricted distribution in cytotrophoblast (1), amniotic cells (2), thymus (3) and endothelial cells of chorionic blood vessels (4). HLA-G molecules are generated by an alternative splicing of the primary transcript of the gene: HLA-G exists as four membrane-bound (HLA-G1, -G2, -G3 and -G4) and three secreted soluble isoforms (HLA-G5, -G6, -G7) (5, 6). HLA-*G* exhibits low allelic polymorphisms in comparison with classical HLA class I genes, with only 47 alleles (IMGT HLA database, April 2012) and 14 proteins. HLA-G is characterized by tolerogenic functions, inducing apoptosis of activated CD8+ T cells (7), acting on T regulatory cells (8), modulating the activity of natural killer cells (9) and of dendritic cells (10) and blocking allo-cytotoxic T lymphocyte response (11). These immuno-regulatory functions are mediated by the interaction of HLA-G molecules with specific inhibitory receptors: ILT-2 (LILRB1/CD85j), ILT-4 (LILRB2/CD85d), CD8 and KIR2DL4 (CD158d) expressed by immune cells (12). HLA-G expression is differently modulated during inflammatory diseases, viral infections, cancer and organ transplantation (13).

The HLA-G production is controlled by several polymorphisms both in the promoter and in the 3′ untranslated region modifying the affinity of gene-targeted sequences for transcriptional or post-transcriptional factors, respectively (14). One single-nucleotide polymorphism (SNP) C>G at the +3142 bp position (rs1063320) has recently been explored by Tan et al. (15), who showed that this marker may be related to susceptibility to asthma as well as influencing HLA-G expression. The presence of a guanine at the +3142 position may influence the expression of the *HLA-G* locus by increasing the affinity of this region for the microRNAs, miR-148a, miR-148b and miR-152, therefore decreasing the mRNA availability by mRNA degradation and translation suppression. The influence of the +3142G allele was demonstrated by a functional study in which HLA-G high-expressing JEG-3 cells were transfected with miR-148a, decreasing soluble HLA-G levels (15). The contrasting results obtained by Manaster et al. (16), who reported the absence of +3142C>G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression.

Currently, the HLA-G +3142 polymorphism typing can be performed by PCR-RFLP with specific primers (Figure 1) and *Bae*GI as restriction enzyme (17, 18). This technique has been used in association studies between HLA-G +3142C>G

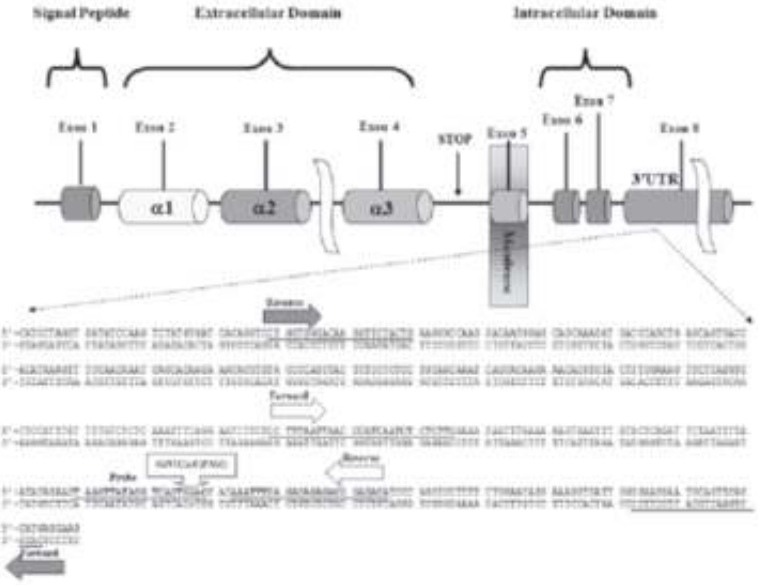

Figure 1 Position of the PCR-RFLP and real time primers in the HLA-G 3′ untranslated region lexon 8l. The forward and reverse primers are marked in grey and underlined (PCR-RFLP) and in white and bold underlined (real-time PCR). The VIC and FAM MGB probes for real time PCR are shown.

polymorphism and two diseases: systemic lupus erythematosus (SLE) and sickle cell disease (SCD). The authors showed an increase of the +3142G allele frequency in SLE patients (17) and the underrepresentation of the +3142C allele in SCD patients hepatitis C positive and with history of respiratory tract infections (18).

We performed the PCR-RFLP on randomly selected DNAs from 221 healthy subjects extracted using a Nucleon Bacc 3 Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to manufacturer's instructions. HLA-G +3142 polymorphism was analyzed on 100 ng of genomic DNA added to a final volume of 25 μl, with final concentrations as follows: PCR buffer 1×, 2.0 mM $MgCl_2$; 0.2 mM of each dNTP; 1.0 U of Taq-polymerase and 10 pmol of each primer (Figure 1; +3142F-5′CTGGTGGGACAAGGTTCTACTG-3′, +3142R-5′-CATGCTGAACTGCATTCCTTCC-3′) (18). Thermocycling conditions were: 94°C for 5 min; 32 cycles of 94°C for 30 s, 65.5°C for 30 s and 72°C for 60 s followed by a final extension step at 72°C for 5 min. The amplified PCR products were cleaved with 3 U of *Bae*GI (New England Biolabs Inc., Ipswich, MA), according to manufacturer's instructions and visualized on 1.5% agarose gel, with amplicon sizes of 406 bp for the C allele and 316 and 90 bp for the G allele.

The same samples were analyzed by real-time PCR assay for genotyping of the HLA-G +3142C>G polymorphism in exon 8 (Figure 1). We performed the analysis using the 7300 real-time PCR system (Applied Biosystems, Bedford, MA). The forward primer, 3142for, was 5′-CCTTTAATTAACCCATCAATCTCTCTTG-3′ and the reverse primer, 3142rev, was 5′-TGTCTCCGTCTCTGTCTCAAATTT-3′. The MGB probe used for detection of the 3142C allele was 5′-VIC-TAAGTTATAGCTCAGTGGAC-3′ (3142CFVIC) and the MGB probe for the 3142G allele was 5′-FAM-TAAGTTATAGCTCAGTGCAC-3′ (3142GFAM) (Figure 1). Each probe has a non fluorescent quencher at the 3′ end. Amplification was performed with 0.625 μl Assay mix 40× (Applied Biosystems), 12.5 μl PCR master mix 2× and 5 μl genomic DNA in a total volume of 25 μl. The PCR microtiter plates were centrifuged at 3800 *g* for 2 min

Table 1 Comparison between 221 DNAs from randomly selected healthy control subjects genotyped for *HLA-G* 3142C>G polymorphism with the use of the traditional PCR-RFLP method and the new real-time polymerase chain reaction (PCR) method. The three genotypes are reported as CC, CG and GG

	PCR-RFLP		Real-time PCR	
	N	Frequency (%)	*N*	Frequency (%)
+3142C>G Genotype	221		221	
CC	41	18.6	41	18.6
CG	130	58.8	105	47.5
GG	50	22.6	75	33.9

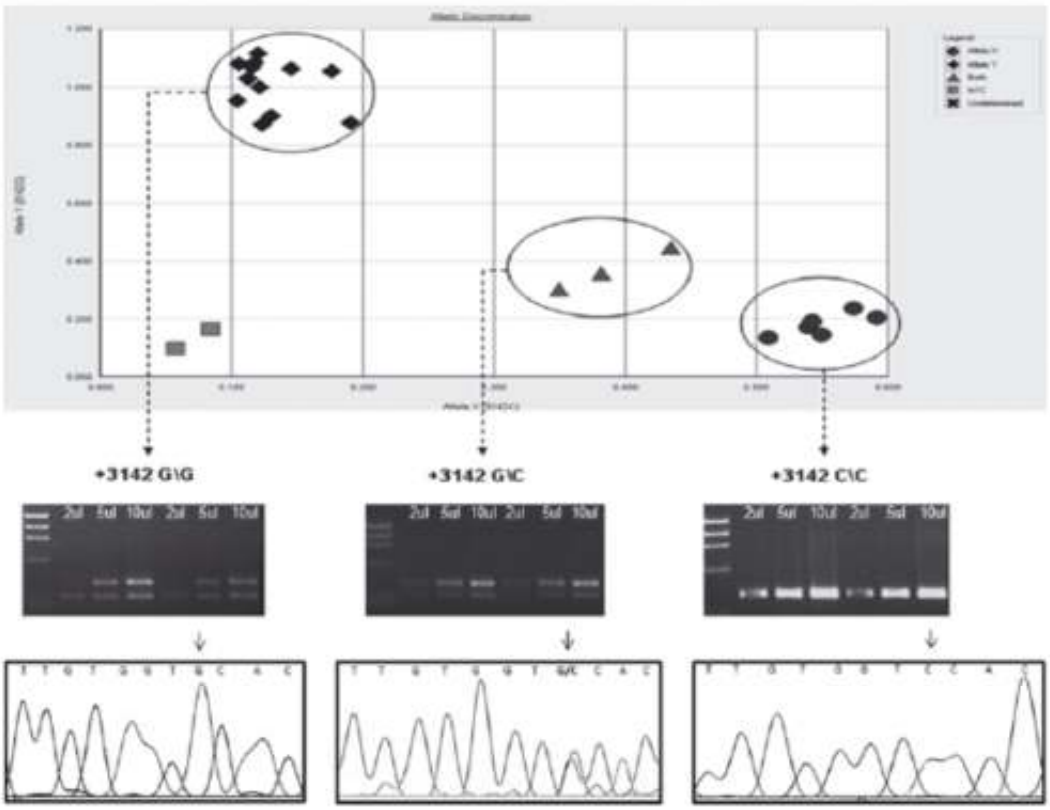

Figure 2 Representative results from the real-time PCR of HLA-G +3142C>G SNP genotyping illustrated by a scatter plot. The probes were labeled with VIC (+3142C allele) and FAM (+3142G allele) fluorocromes. Each probe has a non fluorescent quencher at the 3' end. Grey squares represent negative controls, which consist of sterile water without genomic DNA. Representative results from PCR-RFLP and sequencing are reported below. PCR-RFLP: amplification products from each +3142 genotype were diluted (1:2, 1:4, 1:10) in a total volume of 20 µl, restricted with *Bae*GI enzyme (New England Biolabs) and visualized on 1.5% agarose gel, with amplicon sizes of 406 bp for the C allele and 316 and 90 bp for the G allele. Sequencing: the amplified PCR products obtained with the primers of PCR-RFLP protocol were sequenced by an ABI 3130 automated sequencer (Applied Biosystems). The sequencing confirmed the real time results, with a 3142GG genotype for the discordant samples.

is a swing rotor. Before the amplification, a pre-read run was performed at 60°C for 1 min. The amplification protocol was: an initial step at 95°C for 10 min followed by the amplification step at 92°C for 15 s and 60°C for 60 s for 40 cycles. A post-run reading at 60°C for 1 min was performed.

The frequencies of the three genotypes are reported in Table 1. The genotypes followed the Hardy–Weinberg (HD) distribution for the real-time PCR ($\chi^2 = 0.158$, $P =$ non significant), while the PCR-RFLP results were not in HD equilibrium ($\chi^2 = 7.036$, $P = 0.0079$). This disagreement was confirmed by a kappa (κ) value, measure of the agreement between two tests, of 0.81 (confidence interval 95%: 0.746–0.883). Since only a κ value of 1.00 is acceptable for a complete agreement, we evaluated the causes of these different results. In particular, we observed the 19% of the PCR-RFLP heterozygous samples that were genotyped as 3142GG homozygous by real time technique. The cause of this discrepancy was evaluated performing the enzymatic digestion on different dilutions (1:2, 1:4, 1:10) of the PCR amplification products. The 3142CC and 3142CG DNAs presented the same results at the different dilutions (Figure 2), while the 3142GG DNAs produced contrasting results after the enzymatic digestion on the basis of the sample dilutions. The 3142GG typing was obtained only with the digestion of 1:10 dilution of the amplification product, while 1:2 and 1:4 dilutions were misleading and suggest a 3142CG typing. These results are strictly connected with PCR amplification and enzymatic efficiency. Since different amplification product concentrations

could modify the final typing and we could not know *a priori* the protocol efficiency, it is of extreme importance to perform PCR-RFLP assay on different dilution of the amplification products.

The accuracy of the real time test was confirmed by the 4.5% intra- and inter-assay coefficient of variations, the sensitivity of 95.5% and the specificity of 100%. The DNAs were also genotyped by sequencing (Figure 2). The amplified PCR products obtained with the primers of PCR-RFLP protocol were sequenced by an ABI 3130 automated sequencer (Applied Biosystems). The sequencing confirmed all the real-time PCR results, with a 3142GG genotype for the discordant samples.

Since alterations in the frequency of HLA-G +3142C>G polymorphism are suggested to have a significant impact in different pathological conditions, such as SLE (17), SCD (18) and asthma (15), it is necessary to univocally evaluate the different genotypes. Moreover, the interaction between this polymorphism and miRNAs suggests this gene position as a possible therapeutic target for HLA-G expression modulation (19, 20). The real-time PCR method presented in this study will guarantee an accurate genotyping for future research and clinical purposes, where large cohorts should be tested.

Conflict of interest

The authors have declared no conflicting interests.

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New Insights into HLA-G and Inflammatory Diseases

Roberta Rizzo[*,1], Daria Bortolotti[1], Olavio R Baricordi[2] and Enrico Fainardi[3]

[1]*Department of Experimental and Diagnostic Medicine, Section of Microbiology, University of Ferrara, Ferrara 44121, Italy*

[2]*Department of Experimental and Diagnostic Medicine, Section of Medical Genetics, University of Ferrara, Ferrara 44121, Italy*

[3]*Neuroradiology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliera-Universitaria, Arcispedale S. Anna, Ferrara 44121, Italy*

Abstract: Human leucocyte antigen-G (HLA-G) is a non-classical HLA class I molecule with an important role at the fetus-maternal interface, preventing fetus recognition and abortion. The role of HLA-G as an immune-modulatory and anti-inflammatory molecule has led to investigate its role in pathological conditions. In these years, HLA-G has been shown to have an important implication in inflammatory pathologies. The focus of this review is to up-date the scientific knowledge on the expression of HLA-G molecules in inflammatory conditions.

Keywords: HLA-G, immunity, immune cells, inflammation, pathology, regulation.

INTRODUCTION

Inflammation is a localized protective reaction to allergic or chemical irritation, injury and/or infections. Inflammation is characterized by pain, heat, redness, swelling and loss of function that result from dilation of the blood vessels leading to an increased blood supply and intercellular spaces, movement of leukocytes, protein and fluids into the inflamed regions. Inflammatory response could be subdivided into : i) acute inflammation, with a rapid onset and a short duration. It is considered the main defense mechanism aimed at killing of bacteria, virus and parasites and facilitating wound repairs. It is characterized by the exudation of fluids and plasma proteins and the migration of leukocytes, most notably neutrophils into the injured area; ii) chronic inflammation, with a more prolonged duration and the presence of fibrosis and tissue necrosis. The persistence of a chronic inflammation increases the development of degenerative diseases such as rheumatological diseases, atherosclerosis, heart diseases, asthma, multiple sclerosis (MS), diabetes, inflammatory bowel diseases (IBD) [1, 2], because of the persistent immune cell reactivity and cytokine secretion. The regulation of a chronic inflammation is strictly connected to the presence of anti-inflammatory factors, that interact with immune cells and target transcription elements to modulate immune cell activation.

One of this anti-inflammatory components is suggested to be the Human leukocyte antigen-G (HLA-G). HLA-G is a major histocompatibility complex class I antigen encoded by a gene on chromosome 6p21. It differs from classical HLA class I molecules due to limited polymorphism in the coding region with 47 allele (IMGT HLA database, April 2012) and 14 proteins and a restricted tissue distribution. Seven HLA-G isoforms exist due to alternative splicing and differential association with β2-microglobulin; two of these are found on cell surface and in biological fluids: membrane-bound G1 and soluble G5, that lacks the trans-membrane and intracellular domains of G1 (Fig. 1) [3]. HLA-G possesses an unpaired cysteine residue at position 42 on an external loop of the peptide binding groove that enables the dimerisation [4, 5]. HLA-G monomers are recognized by the inhibitory receptors LILRB1 and LILRB2 and by KIR2DL4 [6]. LILR receptors have a greater affinity for the dimeric form with an increase in signalling potential *via* LILRB1 [7] in NK cells [8]. The interaction of HLA-G molecules with inhibitory receptors induces apoptosis of activated CD8+ T cells [9] modulates the activity of natural killer (NK) cells [10, 11] and of dendritic cells [12, 13], blocks allo-cytotoxic T lymphocyte response [14, 15] and induces expansion of suppressor T cell populations such as CD4+CD25+FoxP3+ regulatory T (Treg) cells [16, 17] (Fig. 1). The HLA-G production is controlled by several polymorphisms both in the promoter and in the 3' untranslated region (3' UTR) modifying the affinity of gene targeted sequences for transcriptional or post-transcriptional factors, respectively [18]. A 14 base pair (14bp) insertion/deletion (INS/DEL) polymorphism in exon 8 involves mRNA stability and expression (rs1704) [19, 20] (Fig. 2). In particular the DEL allele stabilizes the mRNA with a consequent higher HLA-G expression [20]. One single nucleotide polymorphism (SNP) C>G at the +3142bp position (rs1063320) has recently been explored by Tan and coauthors [21] (Fig. 2). The presence of a guanine at the +3142 position may influence the expression of the HLA-G locus by increasing the affinity of this region for the microRNAs miR-148a, miR-148b and miR-152, therefore decreasing the mRNA availability by mRNA degradation and translation suppression. The influence of the +3142G allele has been demonstrated by a functional study in which HLA-G high-expressing JEG-3 choriocarcinoma-derived cells have been transfected with miR-148a, decreasing soluble HLA-G levels [21]. The contrasting results obtained by Manaster and coauthors [22], who have

*Address correspondence to this author at the Department of Experimental and Diagnostic Medicine, Section of Microbiology, University of Ferrara, Via Luigi Borsari, 46, 44121 Ferrara, Italy; Tel. +390532455382; Fax: +390532974470; E-mail: rbr@unife.it

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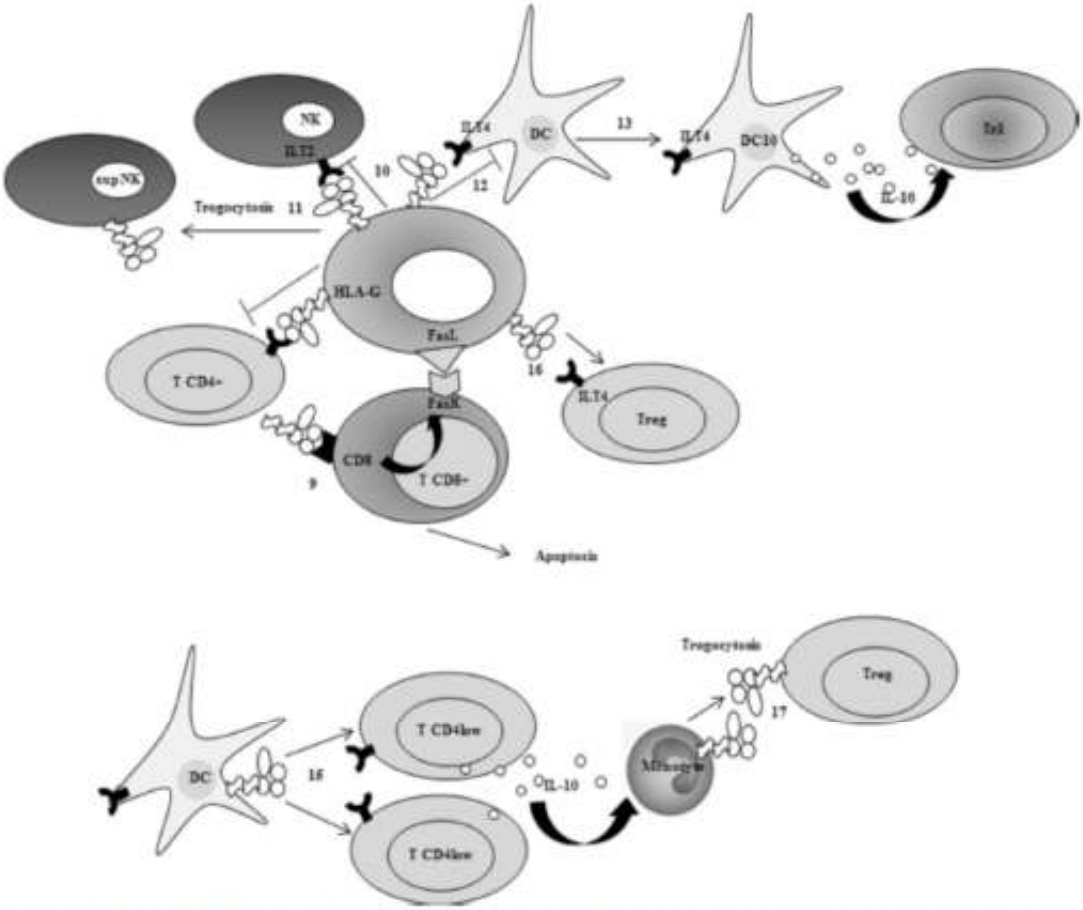

Fig. (1). HLA-G immune cell targets. HLA-G is an anti-inflammatory molecule inhibiting and controlling immune cell activation. supNK: suppressive NK cells; Tr1: type 1 regulatory T cells; DC: dendritic cell; Treg: regulatory T cell; FasR: Fas receptor; DC10: IL-10-differentiated dendritic cells. The corresponding references are reported.

reported the absence of +3142C>G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression. HLA-G is a stress-inducible gene. Heat shock, hypoxia and arsenite induce an increase of the different HLA-G alternative transcripts [23-25]. The indolamine 2,3-dioxygenase (IDO), an enzyme which metabolizes tryptophan [26], induces HLA-G expression during monocyte differentiation into dendritic cells [27]. The anti-inflammatory and immunosuppressive interleukin (IL)-10 has been correlated with concomitant HLA-G expression [28, 29]. Transactivation of HLA-G transcription has also been demonstrated by leukemia inhibitory factor (LIF) [30] progesterone [31] and Methotrexate [32] cell exposure. Furthermore, interferon (IFN)-a, -ß and –γ enhance HLA-G cell-surface expression by tumors or monocytes [33, 34]. HLA-G role in immune-tolerance has been discovered studying its expression in trophoblast cells at fetus-maternal interface [35]. The importance of HLA-G production by placental trophoblasts is evident in pre-eclampsia, a disorder that evolves in the second half of pregnancy and affects 2-7% of all pregnancies with varying severity and unexplained recurrent spontaneous abortion (RSA). Several studies have found an aberrant or reduced expression of both HLA-G mRNA and protein in pathological in comparison with control placentas [36-38] with a possible implication in fetal protection and vascular events.

HLA-G expression has been documented in few tissues during physiological conditions, such as cornea, thymus, erythroid and endothelial precursors [39-41] and in a variable percentage of serum/plasma samples from healthy subjects [42], where the main producers are activated CD14positive monocytes [43]. A modified expression of HLA-G molecules have been observed during "no-physiological" conditions, such as viral infection [44-47], cancer [48-53], transplantation [54-57], inflammatory and

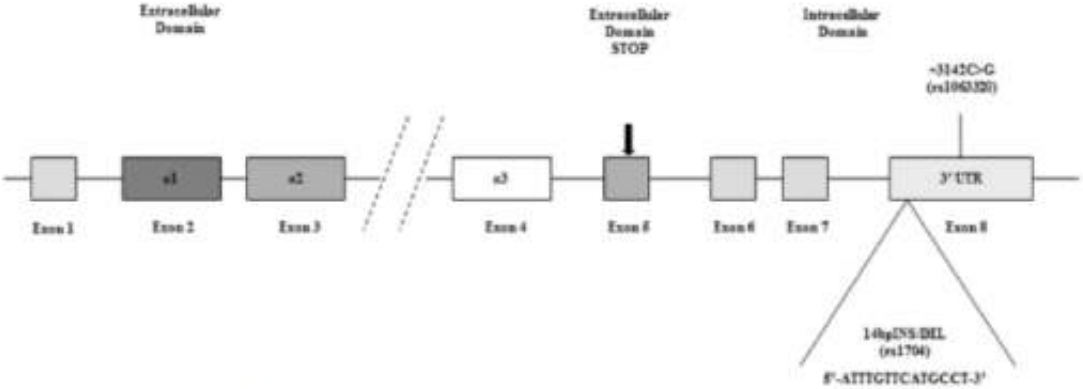

Fig. (2). Organization of the HLA-G gene. Exons and the corresponding protein domains are shown in detail. The HLA-G insertion/deletion 14 base pair (14bp INS/DEL) and the single nucleotide +3142C>G polymorphisms are located in the 3' untranslated region of the exon 8.

autoimmune diseases [58, 59]. Recently, the role of HLA-G molecules in inflammatory diseases has gained a scientific and clinical interest, for the possibility to be proposed as molecular biomarker and a possible therapeutical target.

This review will present recent findings on HLA-G molecules in different inflammatory conditions (Table 1).

CUTANEUS DISEASES AND HLA-G

The skin is not a simply passive barrier but it is characterized by an intricate and complex system, called "skin immune system" (SIS), where several cell types (keratinocytes, dendritic cells, monocytes/macrophages, granulocytes, mast cells and T lymphocytes) together with humoral components (antimicrobial peptides, complement, immunoglobulins, cytokines and other bioactive small molecules) translate the epidermal insult into cutaneous inflammation through the coordinated action of innate and acquired immunity components. The disregulation of skin defense mechanisms appears to be implicated in the pathology of a large variety of inflammatory disorders of the skin, such as psoriasis, atopic dermatitis, pemfigo, vitiligo and systemic sclerosis [60]. HLA-G protein is not normally expressed in the skin or on the cells residing in the skin [61-65]. Proximal nail matrix seems to be an exception, in fact HLA-G is up-regulated and could maintain this immune privileged niche [66]. Ectopic HLA-G expression has been described in inflammatory skin pathologies.

Psoriasis (Online Mendelian Inheritance in Man, OMIM, #177900) is a chronic inflammatory skin disease with an important autoimmune implication that usually appears as oval-shaped, erythematous plaques covered with silvery scales. Microscopically, psoriasis is characterized by hyperproliferation of epidermis, premature maturation of keratinocytes, incomplete cornification of the stratum corneum with retention of nuclei, increased blood flow to the surface of the skin, and increased infiltration of inflammatory cells, including dendritic cells, macrophages, and T cells [67]. Both membrane-bound and soluble HLA-G proteins have been detected in psoriatic skin lesions with the main compound characterized by macrophage lining at the dermo-epidermal junctions [68]. The up-regulation of HLA-G molecules by macrophages could represent a tentative control of auto-reactive T cells, induced by activated keratinocytes-derived cytokines/chemokines. HLA-G may prevent keratinocyte destruction modulating the activity of cytotoxic lymphocytes and promoting the development of Treg cells [69]. Interestingly, significantly lower plasma sHLA-G levels have been found in psoriatic patients compared to controls [70], suggesting a difference in systemic HLA-G expression that could be associated with the IL-10 deficiency, that has been hypothesized to be a central phenomenon in the pathogenesis of psoriasis [71]. Psoriasis management can be divided into three main types: topical drugs, light therapy and systemic medications. The evaluation of the treatment effect on sHLA-G expression reported increased plasmatic levels in systemic treated patients (efalizumab, cyclosporin A, acitretin), suggesting a direct effect in antagonizing systemic T helper 1 activation.

Atopic dermatitis (AD) (OMIM, #603165) is a chronic relapsing inflammatory skin disorder associated with environmental, mainly food allergy, and genetic factors [72]. The identification of the responsible molecules would lead to identification of children who require early preventive intervention.

Early phase of spontaneous AD lesions are mainly characterized by Th2 cell infiltrate and IL-10 is produced in large amounts by peripheral blood monocytes and infiltrating lesional cells [73]. HLA-G has been found in AD dermal infiltrate, mainly by T cells and to a lesser extent by macrophages and dendritic cells, suggesting a role in the inhibitory pathways implicated in the spontaneously favorable evolutions that may occur in the course of AD as well as in the local observed immunodeficiency [74].

Pemphigus vulgaris (PV) (OMIM, #169610) is a potentially fatal blistering disease caused by autoantibodies to desmoglein skin adhesion proteins. The exact molecular pathogenesis of the disease remains unclear, even though it

Table 1. Present Literature on HLA-G in Inflammatory Diseases

Citation Number*	Reference	Samples	Techniques	Results
CUTANEOUS DISEASES				
68	Cardili RN *et al.*, *Br J Dermatol*, 2010	Tissues from 30 untreated patients with psoriasis and 32 controls	Immunohistochemistry	HLA-G molecule are expressed in affected areas, irrespective of the severity of the clinical variants.
70	Borghi A *et al.*, *Arch Dermatol Res*, 2008	Plasma from 65 patients with moderate-to-severe plaque psoriasis	ELISA	Lower plasma levels of both sHLA-G and IL-10 in psoriatic group compared to controls and in local treated patients in comparison with systemic treated patients
76	Yan F *et al.*, *Iran J Allergy Asthma Immunol*, 2008	Skin tissue sections from normal and autoimmune pemphigus vulgaris (PV) individuals	Immunohistochemistry, RT-PCR	PV cells express detectable HLA-G molecules at both transcriptional and translational levels. Reduction in HLA-G2 and an increase in HLA-G1 transcripts in epidermal cells of PV patients as compared to normal cells
77	Gazit E *et al.*, *Hum Immunol*, 2004	DNA samples from pemphigus patients and matched controls in Jewish population	26 HLA-G SNPs panel	Four markers are found informative. Significant differences in 14bpINS/DEL polymorphism
78	Jalali A *et al.*, *Indian J Dermatol*, 2010	22 vitiligo patients and 24 healthy controls	Immunohistochemistry	Negative correlation between HLA-G expression and vitiligo
79	Kim JJ *et al.*, *Arch Dermatol Res*, 2011	Blood samples from 241 patients with non-segmental vitiligo (NSV) and 395 healthy controls	PCR-RFLP, Direct sequencing	HLA-G SNP (rs1736936) has a significant association with NSV
MUSCULAR DISEASES				
83	Wiendl H *et al.*, *Hum Immunol*, 2003	Human myoblasts from patients and controls	Flow cytometry, cytotoxicity test, SDS-PAGE	Muscle-related HLA-G expression in pathologic conditions
HEART DISEASES				
92	Lin A *et al.*, *Tissue Antigens*, 2007	Blood samples from 117 idiopathic dilated cardiomyopathy (IDC) patients and age and sex matched 401 unrelated healthy controls	PCR	IDC patients have increased frequencies of 14bp DEL/DEL and 14bp DEL when compared with healthy controls
93	Boiocchi C *et al.*, *Mol Med Report*, 2012	Blood samples from 664 patients with coronary artery disease (CAD) and 345 matched controls	PCR-RFLP, REAL TIME-PCR	The frequency of 14bp INS/INS genotype is higher in CAD patients compared to the controls
REUMATHOLOGICAL DISEASES				
94	Lee HM *et al.*, *Arthritis Res Ther*, 2011	BM-derived mononuclear cells from 9 rheumatoid arthritis (RA) and 10 osteoarthritis (OA) patients	GEPs by DNA microarray	HLA-G gene is up-regulated in RA patients
95	Vet TD *et al.*, *Tissue Antigens*, 2008	Blood samples from 106 juvenile idiopathic arthritis (JIA) patients, 265 RA patients, 356 healthy adults and 85 healthy children	PCR	Increased frequency of the 14bp DEL allele in JIA patients
97	Verbruggen L *et al.*, *Hum Immunol*, 2006	Plasma samples from 106, 80 women and 26 men, RA patients and control plasma samples were obtained from 56 healthy men and 48 women	ELISA	sHLA-G levels are lower in RA patients in comparison with controls. sHLA-G levels increase in correlation with disease activity and are affected by the presence of disease-predisposing HLA
98	Prigione I *et al.*, *Rheumatology*, 2011	Sera from 58 JIA patients and 54 healthy donors; T, B cells and monocytes from peripheral blood and synovial fluids from 12 JIA patients	ELISA, Flow cytometry	Serum sHLA-G concentration is lower JIA patients than in controls
101	Rizzo R *et al.*, *Pharmacogenet Genomics*, 2006	Peripheral blood mononuclear cells from healthy individuals and non-MTX-treated RA patients	ELISA, PCR	MTX induces the sHLA-G molecules. A significant association is observed between sHLA-G highest levels and 14bp DEL/DEL genotype
106	Rosado S *et al.*, *Hum Immunol*, 2008	Peripheral blood and cutaneous biopsies from 50 systemic lupus erythematosus female (SLE) patients and 59 healthy female donors	Flow cytometry, immunohistochemistry	SLE patients present higher levels of HLA-G than healthy controls
112	Vet TD *et al.*, *Lupus*, 2009	Blood samples from 293 patients with SLE and 460 healthy controls	PCR	SLE patients with 14bp INS/DEL genotype exhibit lower disease activity indexes

(Table 1) contd.....

Citation Number*	Reference	Samples	Techniques	Results
CNS INFLAMMATORY DISEASES				
121	Fainardi E *et al.*, *J Neuroimmunol*, 2003	CSF from 50 relapsing-remitting multiple sclerosis (MS) patients, 36 patients with other inflammatory neurological disorders (OIND) and 41 with non-inflammatory neurological disorders (NIND)	ELISA, MRI	CSF mean levels are significantly higher in MS and OIND than in NIND for sHLA-I and in MS than in controls for sHLA-G. sHLA-G is more represented in MS patients without lesional activity on MRI scans.
122	Fainardi E *et al.*, *Mult Scler*, 2006	CSF from 109 MS patients, 91 OIND, 92 NIND and 82 healthy controls	ELISA, MRI	sHLA-G production is significantly more frequent in MS patients than in controls
124	Fainardi E *et al.*, *Mult Scler*, 2008	CSF from 65 relapsing-remitting MS patients, 64 OIND and 64 NIND	ELISA, MRI	In MS patients with no evidence of MRI disease activity, a trend towards an inverse correlation is found between CSF concentrations of sHLA-G and sFas
125	Wiendl H *et al.*, *Brain*, 2005	Brain specimens from 11 MS patients, 2 meningitis and 2 Alzheimer's disease and 6 non-pathological CNS controls. Cultured human microglial cells and CSF of MS patients and controls	Immunohistochemistry	Expression of HLA-G and its receptor ILT2 on CNS cells and in areas of microglia activation
128	Huang YH *et al.*, *Ann Neurol*, 2009	CSF from 64 MS patients, 9 patients with other neurological diseases, and 20 healthy donors	Flow cytometry, Immunohistochemistry	HLA-G(pos) T(reg) are found enriched in the inflamed CSF of MS patients and in inflammatory demyelinating lesions of MS brain specimens. HLA-G(pos) T(reg) derived from peripheral blood are functionally unimpaired in MS.
GASTROINTESTINAL INFLAMMATORY DISEASES				
133	Torres MI *et al.*, *Int Immunol*, 2004	Intestinal biopsies from 24 ulcerative colitis (UC) and 19 Crohn's disease (CD) patients	Immunohistochemistry	Presence of HLA-G on the surface of intestinal epithelial cell in patients with UC
134	Glas J *et al.*, *Int Immunol*, 2007	Peripheral blood anti-coagulated with EDTA from 371 CD patients, 257 UC patients and 739 controls	PCR	The 14bp INS/DEL genotype is significantly increased, whereas the 14bp DEL/DEL genotype is significantly decreased in UC patients
135	Rizzo R *et al.*, *Inflamm Bowel Dis*, 2008	Supernatants of unstimulated and bacterial lipopolysaccharide (LPS)-stimulated cultures of peripheral blood mononuclear cells from 30 healthy subjects, 10 CD, and 18 UC patients	ELISA	Spontaneous secretion of sHLA-G in CD patients but not in UC and healthy subjects
ALLERGIC DISEASES				
139	Nicolae D *et al.*, *Am J Hum Genet*, 2005	129 asthmatic families	Linkage analysis, Immunohistochemistry	HLA-G as a novel asthma and bronchial hyperresponsiveness susceptibility gene
140	White SR *et al.*, *Eur Respir J*, 2010	Bronchoalveolar lavage (BAL) fluids obtained from 12 non-asthmatic control subjects and 15 subjects with mild persistent asthma	ELISA	sHLA-G levels are increased in the BAL fluid of asthmatic subjects compared with controls
141	Tahan F *et al.*, *Int Arch Allergy Immunol*, 2006	Plasma from 53 asthmatic and 18 healthy children	ELISA	Significantly higher levels of sHLA-G in atopic asthmatics
142	Zheng XQ *et al.*, *Hum Immunol*, 2010	Plasma from 72 atopic asthma patients and 76 normal controls	PCR, ELISA	HLA-G 14-bp INS/DEL polymorphism is not significant difference between asthmatic patients and controls. Plasma HLA-G in atopic asthma patients is higher compared with that of controls
143	Rizzo R *et al.*, *J Allergy Clin Immunol*, 2005	Culture supernatants of LPS-activated peripheral blood mononuclear cells from 24 healthy subjects and 20 patients with moderate to severe persistent asthma	ELISA	LPS stimulation induces the secretion of sHLA-G in all healthy subjects, whereas in patients with asthma, the levels of sHLA-G are significantly lower
144	Mapp CE *et al.*, *Clin Exp Allergy*, 2009	(LPS)-stimulated peripheral blood mononuclear cells from 20 subjects with isocyanate asthma, 16 asymptomatic subjects exposed to isocyanates, 18 subjects with non-occupational allergic asthma, and 26 control subjects	ELISA	Spontaneous production of sHLA-G by PBMCs is higher in subjects with isocyanate-asthma compared with asymptomatic-exposed controls
148	Ciprandi G *et al.*, *Int Arch Allergy Immunol*, 2009	Sera from 25 perennial allergic rhinitis patients and 50 healthy non-allergic subjects	ELISA	Allergic patients have higher levels of sHLA-G than controls
151	Ciprandi G *et al.*, *J Biol Regul Homeost Agents*, 2010	Sera from 47 symptomatic allergic patients and 50 healthy subjects	ELISA	Children with allergic rhinitis have higher levels of sHLA-G than controls or children with allergic asthma

(Table 1) contd.....

Citation Number*	Reference	Samples	Techniques	Results
DIABETES				
153	Eike MC *et al., Genes Immun*, 2009	2321 type 1 diabetes families of multiple (mostly Caucasian) ethnicities	Linkage analysis	A SNP in the vicinity of HLA-G is associated with diabetes
154	Abediankenari S *et al., Iran J Allergy Asthma Immunol*, 2007	Dendritic cell from 20 diabetic patients and 20 control subjects	Flow cytometry	Lower levels of HLA-G molecules in dendritic cells from diabetic patients

*Reported in the Reference section.
Abbreviations: ELISA: enzyme immunosorbent assay; PCR: polymerase chain reaction; RT-PCR: reverse transcription PCR; SNP: single nucleotide polymorphism; PCR-RFLP: PCR-restriction fragment length polymorphism; SDS-PAGE: Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis GEPs: Gene expression profiles; MRI: magnetic resonance imaging.

seems that dysregulation of cytokine signalling may be implicated in the disease development [75]. Skin tissue sections from PV patients express detectable HLA-G molecules at both transcriptional and translational levels, while control sections present only HLA-G transcription [76]. Moreover, the deletion variant of the HLA-G 14bp INS/DEL polymorphism, which leads to increased concentration of HLA-G, has been observed with higher frequency in PV patients in comparison with controls [77].

Vitiligo (OMIM, #606579) is an acquired cutaneous disorder with gradual skin depigmentation produced by the deterioration of melanocyte functions. Several hypotheses have been proposed to explain this dysfunction: autoimmunity, auto-cytotoxicity, impairment in the melanocytes but the pathogenesis remains partially understood. Vitiligo is associated with lower HLA-G expression, which seems to maximize active disease [78]. Moreover, a polymorphism in the HLA-G promoter region (rs1736936) has been related to non-segmental vitiligo and to the age of onset [79].

It is interesting to note that inflammatory cutaneous diseases present a disproportional expression of HLA-G molecules with respect to controls and this could be implicated in the defective development of tolerance to self-antigens, which may predispose to autoimmunity. So it appears that down/over-expression of HLA-G may not only act as an immunosuppressive and beneficial molecule but it may also sustain an unbalanced immune stimulation and autoimmunity.

MUSCULAR DISEASES AND HLA-G

Muscle injury and regeneration is characterized by an acute damage, producing an innate inflammatory response that boosts proliferative stage of myogenesis at the onset of regeneration. Release of cytokines during the Th1 inflammatory response, especially Tumor-necrosis factor-alpha (TNF-α) and IL-6, has a strong influence on the normal progression [80, 81]. However, in the absence of transition from the Th1 response to the Th2 response, the regenerative process is arrested sustaining a chronic inflammation where presentation of the muscle antigens and activation of a cellular immune response would be more likely. This condition allows the development of autoimmune reactions which lead to myopathies. The inflammatory myopathies (OMIM, #160500) may be caused by an allergic reaction, exposure to a toxic substance, the presence of a concomitant disease or virus infection. The chronic inflammatory myopathies are thought to be autoimmune disorders, against blood vessels, normal muscle fibers, and connective tissue in organs, bones and joints. Under physiologic conditions muscle fibers are negative for HLA class I antigens, which are upregulated under pathologic conditions, thus rendering muscle a possible target for the recognition by cytotoxic CD8 positive T cells. Cultured muscle cells are able to present antigens to CD4 positive and CD8 positive T cells, supporting the role of muscle fibers in initiating and perpetuating inflammatory responses [82]. HLA-G is expressed in muscle fibers in inflammatory myopathies and IFN-gamma induces HLA-G mRNA transcription and transduction in myoblasts cultured from control subjects and patients [83]. These data, together with the improved heart graft acceptance in the presence of HLA-G expression at heart muscle [84, 85] and the ability to induce HLA-G expression in vascular smooth muscle cells by exposure to progesterone [86], support the immune-regulatory relevance of HLA-G that could protect muscle fibers from cell-mediated injury in autoimmune muscle disorders.

HEART DISEASES AND HLA-G

Inflammation is an important component in the pathogenesis of many common cardiovascular diseases. In most cases, the role of inflammation is a natural response to injury and an important mechanism for healing and tissue repair. However, the inflammatory response can be either inadequate or overwhelming, leading to direct injury or severe host disease. Patients with chronic heart failure (HF) are characterized by systemic inflammation, with raised circulating levels of several inflammatory cytokines [87, 88] that is not accompanied by a corresponding increase in anti-inflammatory cytokines such as IL-10 [89]. Recent researches suggest that this systemic inflammation may play a role in the development and progression of the disorder, not only by promoting myocardial dysfunctions, but also by inducing pathogenic consequences in other organs and tissues [90]. Circulating HLA-G is up-regulated in patients diagnosed with HF, suggesting a tentative regulation of the inflammatory condition [91].

Accumulating data have revealed an important inflammatory component in the pathogenesis of idiopathic dilated cardiomyopathy (IDC) (OMIM, #115200). 117 IDC patients have been HLA-G genotyped for the 14 bp INS/DEL polymorphism [92]. IDC patients have shown an increased frequency of 14 bp DEL/DEL genotype, high

HLA-G producer, and DEL allele when compared with healthy controls.

On the contrary, the evaluation of 664 patients with coronary artery disease (CAD) (OMIM, #608320) has presented an increased frequency of 14bp INS/INS genotype, low HLA-G producer, in the presence of a persistent angiographic CAD. The impaired HLA-G production could be a risk factor in the control of the disease [93]. These results suggest a different role of HLA-G molecules in cardiovascular diseases, depending on the pathological feature. HLA-G could be used as risk factor and as immune-modulating agent for disorder control.

RHEUMATOLOGICAL DISEASES AND HLA-G

Rheumatic disease is a general term used to describe more than 100 conditions that affect the joints (rheumatoid arthritis), connective tissues (scleroderma, systemic lupus erythematosus) and vessels (vasculitis). Rheumatic diseases are inflammatory and autoimmune diseases, that are the second most common cause of disability after musculoskeletal injuries. Rheumatoid arthritis (RA) (OMIM, #180300) is an autoimmune disease caused by the immune system attacking synovial cells. RA causes inflammation, pain and stiffness in the joints that can lead to a loss of function. A combination of genetic and environmental factors may increase the risk. Bone marrow (BM) mononuclear cells from RA patients present an abnormal regulatory networks in the immune response [94]. Gene expression profiles (GEPs) in BM-derived RA mononuclear cells showed 1,910 down-regulated and 764 up-regulated gene, which include HLA-G gene. Several studies have evaluated the role of HLA-G polymorphisms in RA susceptibility without a final common result. The evaluation on 256 RA patients and 356 healthy controls genotyped for the *HLA-G* 14bp INS/DEL polymorphism has reported no differences in allelic and genotypic frequencies and no correlation with disease characteristics [95]. Two-promoter single-nucleotide polymorphisms (SNPs) (rs1736936, -1202T/C and rs2735022, -586C/T) in HLA-G gene have presented no association with the development of RA in Korean population [96]. On the contrary, the analysis of 106 patients with juvenile idiopathic arthritis (JIA) (OMIM, #604302), the most common form of persistent arthritis in children, have shown a significant correlation between the 14bp DEL allele and JIA susceptibility in girls when compared with controls of the same gender. The different results obtained support the presence of different physiopathogenic elements between RA and JIA [95]. Serum sHLA-G concentration is significantly lower in RA [97] and JIA patients than in controls [98] while higher sHLA-G is present in JIA synovial fluids (SF) [98]. Decreased sHLA-G serum concentration may lead to a chronic activation of inflammatory cells and contribute to the development of autoimmune disease. The high release of HLA-G in the inflamed synovium may be related to the recruitment of activated HLA-G positive immune cells that could interact with immune inhibitory ILT-2 receptor, that is upregulated in SF, and maintain a chronic inflammatory response. In fact, it has been reported that the production of HLA-G molecules is enhanced in synovial fibroblasts from inflamed joints [99]. Interestingly, in a group of RA patients with HLA complex shared epitopes associated with the disease, especially HLA-DRB1*01, *04, and *10, the sHLA-G levels are higher and positively correlated with disease activity parameters, such as C-reactive protein and the number of swollen joints [97]. The highest sHLA-G concentrations may represent the inability to control inflammation and confirm the upregulation of HLA-G gene transcription observed by GEPs analysis [94].

The HLA-G 14bp INS/DEL polymorphism has also been evaluated as pharmacogentic marker of RA therapy. Methotrexate (MTX), the major disease-modifying anti-rheumatic drug (DMARD), is implicated in the increased production of IL-10 in patients with RA, which correlates with better therapeutic response [100]. IL-10 is able to induce HLA-G secretion by peripheral blood monocytes (PBMCs) activated by lipopolysaccharides (LPS), with the highest levels in the 14bp DEL/DEL genotype [29]. The analysis of the HLA-G 14bp INS/DEL polymorphism in 156 MTX-treated RA patients has shown an increase of the 14bp DEL/DEL genotype in the responder group, characterized by a reduction in disease activity score (DAS)28 measured before and after six months of treatment with MTX [101]. In contrast to this study, there are two researches with negative results: i) 130 RA patients have presented no significant difference in 14bp DEL/INS allelic and genotypic distribution in patients responsive to MTX (DAS28 < 3.2) [102]; ii) 186 RA patients, who have never been treated with MTX, have prospectively been followed and have been considered as responders with a DAS28 of up to 2.4 after six months of treatment [103]. No significant association between HLA-G 14bp INS/DEL and MTX efficacy has been observed. Comparing these studies, the opposite results may reflect population differences in gene expression, that could influence the power of association studies and lead to different levels of association. In addition, the different doses of MTX and the different cut-off used for RA therapy response definition could affect the results obtained.

Scleroderma (OMIM, #18175) is an autoimmune rheumatic disease of the connective tissue. Patients with scleroderma produce too much collagen causing the connective tissue to harden. Scleroderma can be mild, affecting only the outer skin, known as localized scleroderma, or more severe causing blood vessels and organs to harden, known as systemic sclerosis, SSc. SSc is a clinically heterogeneous, systemic disorder which affects the connective tissue of the skin, internal organs and the walls of blood vessels. It is characterized by alterations of the microvasculature, disturbances of the immune system and by massive deposition of collagen and other matrix substances in the connective tissue. The course and even the initial events in the pathogenesis of SSc are still poorly understood. The presence of inflammatory infiltrates, mainly CD4 positive T cells, around blood vessels and at sites of active connective tissue formation suggests their pathogenetic role together with an increased secretion of Th1 cytokines [104]. The skin biopsies from patients with SSc with a longer survival, lower frequency of vascular cutaneous ulcers, telangiectasias and inflammatory polyarthralgia present HLA-G molecule expression [105], suggesting a role in immune control.

Systemic lupus erythematosus (SLE) (OMIM, #601744) is a systemic autoimmune disease of the connective tissue that can affect any part of the body. SLE symptoms are usually chronic and relapsing characterized by periodic episodes of inflammation. The immune response is mainly characterized by Th2-cell predominance. Rosaso and coauthors [106] and Chen and coauthors [107] have shown higher sHLA-G and IL-10 levels in SLE patients in comparison with healthy controls, while Rizzo and coauthors [108] have observed lower sHLA-G concentrations in SLE patients. The differences in sHLA-G levels in these two papers could be due to the difference in the analyzed samples (serum or plasma), since it is known that the highest sHLA-G levels are recovered from plasma sample in comparison with serum collected from the same subjects because of a trapping phenomenon, during clot formation, that could subtract sHLA-G from the serum [109]. As a proof, Monsiváis-Urenda and coauthors [110] have evidenced a diminished expression of HLA-G in monocytes and in mature CD83positive dendritic cells from SLE patients compared with healthy controls. In addition, monocytes from SLE patients have shown a diminished induction of HLA-G expression in response to IL-10. Finally, lymphocytes from SLE patients have displayed a lower acquisition of HLA-G (by trogocytosis) from autologous monocytes compared to controls. Interestingly, ILT-2 receptor expression is increased on lymphocytes from SLE patients, in particular in CD3positive cells, CD19positive cells, CD56positive cells and related to IL-10 and anti-DNA antibodies [107]. These results confirm the presence of a HLA-G impaired expression in patients with SLE and a possible role in the pathogenesis. Using SNP mapping approach, HLA-G gene is reported as a novel independent locus with SLE interaction [111]. In particular, HLA-G 14bp INS/DEL polymorphism and HLA-G +3142 C>G SNP have been analysed in SLE population. SLE patients have shown a higher frequency of 14bp INS allele and 14bp INS/INS genotype [108]. Moreover, 14bp INS/INS patients have presented the highest disease activity [112]. On the contrary, the evaluation of HLA-G 14bp INS/DEL polymorphism in a SLE Brazilian population has failed to present an association [113]. The +3142G allele and the +3142 GG genotype frequencies are increased among SLE patients as compared with controls [114]. These data sustain the role of HLA-G molecules in the control of SLE condition, in particular several results sustain the lower HLA-G expression as a risk factor for SLE development.

Behçet's syndrome (OMIM, # 109650) is a rare immune-mediated systemic vasculitis with mucous membrane ulceration and ocular involvements. The HLA-G*010101 allele is associated with a reduced risk of BD while HLA-G*010102, G*0105N alleles and 14bp INS/DEL polymorphism are associated with an increased risk of BD [115, 116].

Kawasaki disease (OMIM, # 611775) is an acute, self-limited vasculitis of infants and children characterized by prolonged fever, polymorphous skin rash, erythema of the oral mucosa, lips, and tongue, erythema of the palms and soles, bilateral conjunctival injection and cervical lymphadenopathy. Without treatment, approximately 15–25% of patients with Kawasaki disease will develop coronary artery aneurysms, making this disease the leading cause of acquired heart disease among children in developed countries. Although an infectious agent is highly suspected, the aetiology of the disease is largely unknown. However, it has been established that inflammation is a central feature of Kawasaki disease. Several lines of evidence suggest that genetic and immunological factors play important roles in disease susceptibility and outcomes. Interestingly, non-synonymous SNP (C>A) of the HLA-G gene (rs12722477, Leu134Ile) is significantly associated with Kawasaki disease [117].

Sarcoidosis (OMIM, # 181000) is a systemic inflammatory granulomatous disease associated with an accumulation of CD4 positive T cells and a Th1 immune response. The aetiology is unknown but at the molecular level several studies have shown HLA associations (HLA-DRB1*1101) [118]. 47 patients with sarcoidosis have been analysed for different HLA-G alleles/polymorphisms [119]. 14bp INS allele has been observed more often in sarcoidosis patients than in controls. Only rare and weak expression of HLA-G has been observed in granulomas from sarcoidosis patients, supporting the genetic results.

CENTRAL NERVOUS SYSTEM (CNS) INFLAMMATORY DISEASES AND HLA-G

The central nervous system (CNS) is considered an immune-privileged compartment that maintains an adaptable immune surveillance system. Dysregulated immune function within the CNS contributes to the development of brain tumor growth, and robust immune activation results in excessive inflammation.

Multiple sclerosis (MS) (OMIM, #126200) is a chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) with an unknown aetiology that is widely considered to be autoimmune in nature [120]. Epidemiological studies indicate that exposure to an environmental factor, such as an infectious agent, in combination with genetic predisposition, could be implicated in MS pathogenesis [120]. MS is currently believed to be mediated by autoreactive CD4positive T helper 1 (Th1) cells which traffic across the blood–brain barrier (BBB) and migrate into the CNS after activation [120]. These cells orchestrate a combined attack of both innate and acquired immune responses directed against myelin proteins consisting of a cooperation among monocytes, macrophages (innate immune system), B cells, CD4positive T cells and CD8positive T cells (acquired immune system) and resulting in CNS inflammation leading to myelin damage and axonal loss. The presence of CSF detectable levels of sHLA-G in RRMS (relapsing-remitting MS) patients and, occasionally, in other inflammatory neurological disorders (OIND) and non-inflammatory neurological disorders (NIND) has been reported, for the first time by Fainardi and coauthors [121]. In addition, CSF levels of sHLA-G are higher in RRMS than in controls and increased, in association with IL-10 values, in RRMS patients without than in those with magnetic resonance imaging (MRI) evidence of disease activity [121]. Of note, in RRMS patients, CSF concentrations of sHLA-G and IL-10 are positively correlated with inactive MRI disease and CSF IL-10 titers are more elevated in patients with than in those without CSF measurable levels of sHLA-G. These data suggest that CSF sHLA-G levels may

modulate MS disease activity acting as anti-inflammatory molecules under the control of IL-10 CSF levels which may enhance sHLA-G production. The existence of high CSF concentrations of sHLA-G in MS patients and their association with clinical and MRI stable disease have been repeatedly confirmed in subsequent investigations in which: i) an intrathecal production of sHLA-G is more frequent in MS than in inflammatory and non-inflammatory controls and predominated in clinically and MRI inactive compared to clinically and MRI active MS [122]; ii) sHLA-G concentrations reciprocally fluctuate in CSF and serum of MS patients because they are decreased in serum of clinically stable MS and increased in CSF of MRI inactive MS [123]; iii) CSF levels of HLA-G5 and not those of sHLA-G1 isoforms are more increased in MS than controls and in MS patients without MRI appearance of disease activity than in those with MRI Gd-enhancing lesions [124]; and iv) CSF values of sHLA-G and antiapoptotic sFas molecules are inversely correlated in MS patients with no evidence of MRI disease activity, since CSF concentrations of sFas are lower in MS than in controls and in MRI inactive than in MRI active MS [124]. RNA and protein HLA-G expression on peripheral blood monocytes is lower in MS patients than in healthy donors and increases after the first month of treatment with IFN-b [43]. Interestingly, HLA-G and its inhibitory receptors (ILT-2 and ILT-4) are strongly up-regulated within and around MS lesions where microglia, macrophages and endothelial cells are recognized as the cellular sources [125]. Furthermore, protein HLA-G expression is higher on cultured human MS microglial cells after activation with Th1 proinflammatory cytokines and a novel subpopulation of naturally occurring CD4positive and CD8positive regulatory T cells expressing HLA-G (HLA-Gpos Treg) has been recently described in peripheral blood of MS patients with relapse [126].

Further studies have demonstrated that IL-10 contributes to mediate the suppressive activity of CD4positive HLA-G^{pos} T_{reg} [127] which are highly represented in CSF and inflammatory brain lesions of MS patients as activated central memory T cells capable of migrating from the periphery to intratecal compartment due to the expression of CCR5 [128]. These results strengthen the assumption that an association between HLA-G antigens and MS.

Collectively, these observations provide evidence that HLA-G antigens are likely involved in the resolution of MS autoimmunity acting as anti-inflammatory molecules, and suggest that HLA-G positive Treg could play a role in the development of a CNS immunosuppressive microenvironment at the sites of inflammation in MS.

GASTROINTESTINAL INFLAMMATORY DISEASES AND HLA-G

Inflammatory bowel disease (IBD) (OMIM, #266600) is the general term for Crohn's disease (CD) and ulcerative colitis (UC), two chronic inflammatory disorders of the intestine which have different clinical, morphological, and immunological characteristics. The cause of IBD is unknown, but recent results strongly suggest that both CD and UC are due to an inappropriate or exaggerated immune response to normal constituents of the gut bacterial flora. In IBD, the tissue damage occurs in areas that are heavily infiltrated with activated CD4 positive T lymphocytes. Th1 signals as IL-6 and IL-12 are markedly elevated in CD patients [129, 130]. On the contrary, CD4 positive T lymphocytes from UC patients produce more IL-5 [131, 132], suggesting that Th2-type cytokines may play a role in UC.

Torres and coauthors [133] have studied intestinal samples of UC and CD patients and, by using immunohistochemistry technique, demonstrated that while UC intestinal cells presented HLA-G on their surface, CD intestinal biopsies did not. This result combined with high levels of IL-10 found in the lamina propria of the colon of UC patients, suggested that HLA-G can regulate the mucosal immune responses in UC. The distribution of the 14bp INS/DEL polymorphism in UC and CD has been investigated by Glas and coauthors [134]. They have observed an increase of both 14bp DEL/INS and 14bp INS/INS genotypes and a consequent decrease of the high producer genotype (14bp DEL/DEL) in UC subjects in comparison with CD patients. Also Rizzo and coauthors [135] have found a different HLA-G expression in UC and CD patients. Non activated peripheral blood mononuclear cells from CD patients secrete spontaneously sHLA-G while those from UC patients and healthy donors do not. Furthermore, after stimulation with LPS, both cells from CD and healthy subjects show sHLA-G production, while this does not happen in UC patients. This defective production in UC patients seems to be due to an altered secretion of IL-10 in response to inflammation. The different HLA-G expression profiles in UC and CD patients sustain the different aethiopathogenesis at the origin of these two diseases and propose sHLA-G and IL-10 levels as diagnostic parameters to facilitate the diagnosis of UC and CD patients.

Celiac disease (OMIM, #212750) (CD) is a gluten sensitivity that induces an autoimmune inflammatory disease that damages the villi in the small intestine of genetically predisposed subjects. Both genetic and environmental factors contribute to the development of CD. The primary HLA association for CD is conferred by class II HLA-DQ genes, that preferentially bind gluten. Approximately 90 % of the CD patients express the HLA-DQ2 molecule, encoded by DQA1*05/DQB1*02 genes, and the majority of the remaining patients express HLA-DQ8, encoded by DQA1*03/DQB1*03:02 genes [136, 137]. Patients with active CD are characterized by significantly high levels of pro-inflammatory cytokines, such as IFN-gamma, IL -1beta, tumor necrosis factor-alpha, IL-6 and IL-8, and also of Th-2 cytokines such as IL-4 and IL-10, compared with normal controls [138]. Torres and coauthors [139] have shown the presence of HLA-G in biopsies from celiac patients and have observed higher sHLA-G amount in comparison with control subjects. The evaluation of the 14bp INS/DEL polymorphism in a group of 522 celiac patients [140], stratified for the presence of HLA-DQ2, has demonstrated an increase in the 14bp DEL/DEL genotype in comparison with controls. These data suggest that the 14bp DEL allele could increase the risk of gut inflammation and probably sustain a chronicization. During the disease, HLA-G expression seems to be strictly correlated with the disease specific cytokine environment, that could induce, UC and celiac disease, or down-regulate, CD condition, HLA-G production.

ALLERGIC DISEASES AND HLA-G

Allergic disorders, such as asthma, now afflict roughly 25% of people in the developed world. In allergic subjects, persistent or repetitive exposure to allergens, which typically are intrinsically innocuous substances common in the environment, results in chronic allergic inflammation. This in turn produces long-term changes in the structure of the affected organs and substantial abnormalities in their function.

Asthma (OMIM, #600807) is a chronic disease affecting approximately 300 million people worldwide, with 180.000 deaths resulting annually from severe asthma attacks. Asthma is characterized by chronic inflammation in the airway, which consequently narrows more easily in response to a variety of triggers than the airway of a healthy individual. Nicolae and coauthors [141] have suggested the role of HLA-G as a potential asthma and bronchial hyperresponsiveness susceptibility gene. In particular, susceptibility varies depending on whether the mother has asthma or bronchial hyper-responsiveness (BHR). A G/G genotype at SNP -964G/A in the promoter region has been associated with asthma in the offspring of mothers with either asthma or BHR, whereas the A/A genotype has been associated with asthma in the offspring of asthma- and BHR-free mothers. Tanz and coauthors [17] have discovered an association between +3142 C>G (rs1063320) and asthma. HLA-G5 is expressed by airway epithelium and is present in the bronchoalveolar lavage (BAL) fluid from asthmatic patients [141, 142]. In addition to the local presence in airways, sHLA-G may also be found in asthmatic subjects outside the lung. The plasma sHLA-G levels are higher in atopic asthmatic children than in both non-atopic, asthmatic and non-atopic, non-asthmatic children [143]. A more recent study has examined plasma sHLA-G levels in 72 children with atopic asthma and 76 non-atopic, normal controls [144]. Circulating plasma sHLA-G is higher in the atopic, asthmatic children. The 14bp INS/DEL polymorphism has no impact on plasma sHLA-G levels in the atopic, asthmatic children. Thus, circulating HLA-G may be important as a biomarker and could potentially modulate immune function more broadly, while the local abundance in airways may have a more direct relationship with immune modulation in the mucosa. There are also *in vitro* evidences that the presence of HLA-G may differ in asthma. sHLA-G expression by peripheral blood mononuclear cells is reduced in asthmatic patients [145] while it is increased in asthma induced by isocyanates [146]. This different behavior may represent differences in biological roles in different disease contexts. A loss of HLA-G could reduce immunosuppression and perpetuate inflammation, whereas increased HLA-G in asthma could be an attempt to reassert immunosuppression. Interestingly, HLA-G is differentially expressed during the lung development [147], suggesting a potential role in lung inflammation induction and chronicization.

Allergic rhinitis (AR) (OMIM, #607154) is characterized by a Th2 polarized immune response. sHLA-G molecules are increased in sera of patients with pollen-induced allergic rhinitis studied outside the pollen season [148], during the pollen season [149] and in perennial allergic rhinitis patients [150]. Interestingly, sublingual immunotherapy (SLIT) for allergic rhinitis is able to reduce sHLA-G serum levels in pollen allergic patients [149, 150], suggesting a clinical implication as biomarker of response to SLIT. Interestingly, children with AR have significantly higher levels of sHLA-G molecules than normal controls or children with allergic asthma [151]

DIABETES AND HLA-G

Immunologic abnormalities are associated with type 1 (OMIM, #2221000) and type 2 (OMIM, #125853) diabetes. T cells are believed to be the major cause of autoimmune disease in type 1 diabetes, leading to the destruction of pancreatic islets. Many factors can enhance insulin resistance, including genetics, a sedentary lifestyle, obesity, and other conditions, such as chronic inflammation or infection. Increases in inflammation, such as activation of monocytes and inflammatory molecules (C-reactive protein, plasminogen activator inhibitor-1 and cytokines) have been reported in insulin-resistant states without diabetes. One possible mechanism is that abnormal levels of metabolites, such as lipids, fatty acids, and various cytokines from the adipose tissue, activate monocytes and increase the secretion of inflammatory cytokines, enhancing insulin resistance. It has been shown that higher levels of sHLA-G, linked to a typical biomarker of insulin resistance like IL-6, seems to characterize subjects with an impaired glucose metabolism [152]. These data suggest a possible implication of HLA-G antigens in diabetic condition. In fact, a SNP in the vicinity of HLA-G has been associated with type 1 diabetes [153]; dendritic cells from type 1 diabetic patients produce lower HLA-G molecules in response to IFN-beta [154] in comparison with control subjects. Interestingly, HLA-G has been detected in some secretory granules and found to be upregulated at the cell surface of primary islet cells which are stimulated to secrete insulin [155]. Since many autoantigens in islet immunity are components of secretory granules and activation of autoreactive T cells depends upon the surface density of antigen/HLA complexes, it could be suggested that unwanted activation of low-affinity cytotoxic T cells at the sites of insulin exocytosis may be prevented by the locally clustered HLA-G molecules. An impaired HLA-G expression at pancreatic islets could sustain T cell activation and diabetes induction.

CONCLUSIONS

This review has focused on the role of HLA-G molecules in inflammatory diseases. Taken together, these studies suggest that HLA-G could be implicated in both risk and disease chronicization, where this antigen is characterized by an impaired expression depending on the different disease environment.

In fact, HLA-G proteins seem to be involved in the regulation of the immune system during autoimmune and allergic conditions, such as gastrointestinal, skin, neurological and rheumatic diseases. In particular, in these disorders, HLA-G proteins could directly interact with immune cells or control the balance between Th1 and Th2 cytokines. What remains to be understood is whether the production of HLA-G is an attempt to restore a proper balance in inflammatory cells and cascades that have been activated, or is a part of the on-going pathogenesis by its

repression of immunologically active cells, or *via* a shift towards the Th2 phenotype.

The comprehension of the specific role and mechanisms of action of HLA-G molecules in the development and progression of inflammatory disorders could justify the use of HLA-G molecules as a marker of inflammation and drug treatment and open up new therapeutic perspectives. The identification of pharmacological strategies with the aim of control the HLA-G production could be a concrete possibility to improve inflammatory diseases prevention and therapy. Moreover, the definition of the role of HLA-G genetic polymorphisms as risk and pharmacogenetic markers could sustain the clinical relevance of HLA-G typing in the laboratory routine.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

DISCLOSURE

This manuscript is an extended /updated version of your previously published manuscript.

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REVIEW

Impact of HLA-G analysis in prevention, diagnosis and treatment of pathological conditions

Daria Bortolotti, Valentina Gentili, Antonella Rotola, Enzo Cassai, Roberta Rizzo, Dario Di Luca

Daria Bortolotti, Valentina Gentili, Antonella Rotola, Enzo Cassai, Roberta Rizzo, Dario Di Luca, Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, 44121 Ferrara, Italy

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Correspondence to: Roberta Rizzo, PhD, Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Via Luigi Borsari, 46, 44121 Ferrara, Italy. rbr@unife.it

Telephone: +39-532-455382 Fax: +39-532-974470
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Abstract

Human leukocyte antigen-G (HLA-G) is a non-classical HLA class I molecule that differs from classical HLA class I molecules by low polymorphism and tissue distribution. HLA-G is a tolerogenic molecule with an immune-modulatory and anti-inflammatory function on both innate and adaptative immunity. This peculiar characteristic of HLA-G has led to investigations of its role in pathological conditions in order to define possible uses in diagnosis, prevention and treatment. In recent years, HLA-G has been shown to have an important implication in different inflammatory and autoimmune diseases, pregnancy complications, tumor development and aggressiveness, and susceptibility to viral infections. In fact, HLA-G molecules have been reported to alternate at both genetic and protein level in different disease situations, supporting its crucial role in pathological conditions. Specific pathologies show altered levels of soluble (s)HLA-G and different *HLA-G* gene polymorphisms seem to correlate with disease. This review aims to update scientific knowledge on the contribution of HLA-G in managing pathological conditions.

Key words: Human leukocyte antigen-G; Pathology; Prognosis; Diagnosis; Treatment; Marker

Core tip: Human leukocyte antigen-G (HLA-G) is a tolerogenic molecule. HLA-G has been shown to have important implications in different pathological conditions where it is reported to alternate at both protein and genetic level. The peculiar immunoregulatory function of HLA-G and its dysregulation in different diseases have led to investigation of its role in pathological conditions in order to define possible uses in diagnosis, prevention and treatment. This review aims to update scientific knowledge on the contribution of HLA-G in managing pathological conditions.

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INTRODUCTION

Diagnosis and prevention of diseases is mainly based on the identification of specific biological markers and drug targets. In view of this, the possibility of easy and fast identification of molecules, for example in biological fluids, seems to be even more necessary.

In recent years, different studies have demonstrated that human leukocyte antigen-G (HLA-G), a non-classical class I molecule, could fulfil this necessity[1-3]. In fact, HLA-G expression and levels in biological fluids, cells and tissues in different pathological conditions have been shown. Several authors reported that the level of soluble

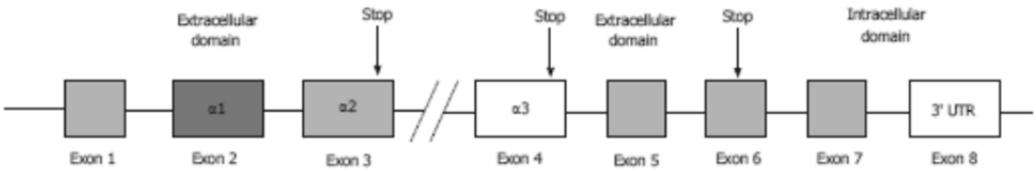

Figure 1 Human leukocyte antigen-G gene. UTR: Untranslated region.

HLA-G and gene polymorphisms correlate with disease outcome and the therapeutic success of treatment[4-6].

HLA-G MOLECULE

HLA-G is a major histocompatibility complex class I antigen encoded by a gene on chromosome 6p21. It differs from classical HLA class I molecules by its restricted tissue distribution and limited polymorphism in the coding region. To date, 50 alleles (IMGT HLA database, August 2013) and 16 proteins are known. The gene structure of HLA-G is homologous to other HLA class I (Ia) genes consisting of 7 introns and 8 exons coding the heavy chain of the molecule. Exon 1 encodes the peptide signal, while exons 2, 3 and 4 encode the extracellular α1, α2 and α3 domains, respectively. Exons 5 and 6 encode the transmembrane and cytoplasmic domains of the heavy chain. Exon 7 is always absent from mature mRNA due to the stop codon in exon 6; exon 8 is not translated (Figure 1). Seven HLA-G isoforms exist due to mRNA alternative splicing and differential association with β2-microglobulin; two of these are found on the cell surface and in biological fluids: Membrane-bound G1 and soluble G5, which lacks the trans-membrane and intracellular domains of membrane-bound G1 (Figure 1)[7]. HLA-G possesses an unpaired cysteine residue at position 42 on an external loop of the peptide binding groove that enables the dimerisation[8,9]. HLA-G monomers are recognized by the inhibitory receptors LILRB1 and LILRB2 and by KIR2DL4[10]. LILR receptors have a greater affinity for the dimeric form that increases the signaling transduction, especially in natural killer (NK) cells[11,12]. The interaction of HLA-G molecules with inhibitory receptors induces apoptosis of activated Crohn's disease ($CD8^+$) T cells[13], modulates the activity of NK cells[13,14] and of dendritic cells (DC)[15,16], blocks allo-cytotoxic T lymphocyte response[17] and induces expansion of suppressor T cell populations, such as $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells[18,19]. Moreover, HLA-G is expressed at high levels on DC-10 cells, human DCs with tolerogenic activity and an outstanding ability to produce interleukin (IL)-10[16]. Interestingly, the expression of membrane-bound HLA-G1 and that of its receptors is up-regulated by IL-10 on DC-10 and the expression of high levels of membrane-bound HLA-G1, ILT4 and IL-10 by DC-10 is critical to the generation of allergen-specific Tr1 cells by DC-10[16].

The HLA-G production is controlled by several polymorphisms, both in the promoter and in the 3' untranslated region (3' UTR), modifying the affinity of gene targeted sequences for transcriptional or post-transcriptional factors, respectively[20].

Twenty-nine single nucleotide polymorphisms (SNPs) have been identified in the HLA-G promoter region which may be involved in the regulation of HLA-G expression, considering that many of these polymorphisms are within or close to known or putative regulatory elements. The HLA-G 5' upstream regulatory region (URR) is unique among the *HLA* genes[21] and is unresponsive to NF-κB[22] and interferon (IFN)-γ[23] due to the presence of a modified enhancer A and a deleted interferon-stimulated response element (ISRE). A locus control region located -1.2 kb from exon 1 exhibits a binding site for CREB1 factor, which also binds to two additional cAMP response elements at -934 and -770 positions from the ATG. In addition, a binding site ISRE for interferon response factor-1 is located at the -744 base pair (bp) position[24] and is involved in HLA-G transactivation following IFN-β treatment[24]. The HLA-G promoter also contains a heat shock element at the -459/-454 position that binds heat shock factor-1[25] and a progesterone receptor binding site at -37 bp from ATG[26]. Several promoter region polymorphisms coincide with or are close to known or putative regulatory elements and thus may affect the binding of HLA-G regulatory factors[27]. The -725 C > G/T SNP is very close to ISRE, in which the -725 G allele is associated with a significantly higher expression level compared with the others[28]. The polymorphic sites at the 5' URR are frequently in linkage disequilibrium with the polymorphic sites identified at the 3' UTR, some of them influencing alternative splicing and mRNA stability.

A 14 bp insertion/deletion (INS/DEL) polymorphism (rs66554220) in exon 8 involves mRNA stability and expression[29,30]. In particular, the DEL allele stabilizes the mRNA with a consequent higher HLA-G expression[30,31]. The presence of an adenine at position +3187 modifies an AU-rich motif in the HLA-G mRNA and decreases its stability[32]. One SNP C > G at the +3142 bp position (rs1063320) affects the expression of the HLA-G locus by increasing the affinity of this region for the microRNAs (miR)-148a, miR-148b and miR-152, therefore decreasing the mRNA availability by mRNA degradation and translation suppression[33]. The influence of the +3142G allele has been demonstrated by a functional study in which HLA-G high-expressing JEG-3 choriocarcinoma-derived cells have been transfected with miR-148a, decreasing soluble HLA-G levels. The

discordant results obtained by Manaster *et al*[34], who have reported the lack of +3142 C > G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression. Other SNPs are identified as implicated in miRNA interaction. In particular, +3003, +3010, +3027 and +3035 SNPs are targets for miR-513a-5p, miR-518c*, miR-1262 and miR-92a-1*, miR-92a-2*, miR-661, miR-1224-5p and miR-433 miRNAs[35]. The miR-2110, miR-93, miR-508-5p, miR-331-5p, miR-616, miR-513b, and miR-589* miRNAs target the 14bp INS/DEL fragment region and miR-148a, miR-19a*, miR-152, mir-148b, and miR-218-2 target the +3142 C/G polymorphism.

HLA-G is a stress-inducible gene; heat shock, hypoxia and arsenite increase different HLA-G alternative transcripts[25,36,37]. The indoleamine 2,3-dioxygenase, an enzyme which metabolizes tryptophan, induces HLA-G expression during monocyte differentiation into DCs[38]. The anti-inflammatory and immunosuppressive IL-10 has been correlated with concomitant HLA-G expression[30,39]. Transactivation of HLA-G transcription has also been demonstrated by leukemia inhibitory factor[40], progesterone[26] and methotrexate[41] cell exposure. Furthermore, IFN-α, -β and -γ enhance HLA-G cell-surface expression by tumors or monocytes[42,43]. HLA-G expression could be acquired by trogocytosis, where a "donor" cell that expresses membrane HLA-G exchanges membrane parts containing HLA-G with a "recipient" cell that is not expressing HLA-G molecules. In this particular situation, "recipient" cells will acquire and make use of membrane HLA-G molecules from a "donor" HLA-G positive cell without the activation of HLA-G gene. Trogocytosis of antigen presenting cell HLA-G1 by T cells in humans makes T cells unresponsive[44]. It has been shown that HLA-G1 can be acquired by NK cells from tumor cells. NK cells that acquire HLA-G1 stop proliferating, are no longer cytotoxic and behave like suppressor cells capable of inhibiting other NK-cell functions[14].

HLA-G's role in immune-tolerance was discovered studying its expression in trophoblast cells at the fetus-maternal interface[45]. The importance of HLA-G production by placental trophoblasts is evident in pre-eclampsia and unexplained recurrent spontaneous abortion (RSA). Several studies have found an aberrant or reduced expression of both HLA-G mRNA and protein in pathological compared with control placentas[46-48], with a possible implication in fetal protection and vascular events.

HLA-G expression has been documented in a few tissues during physiological conditions, such as cornea, thymus, erythroid and endothelial precursors[49-51], and in a variable percentage of serum/plasma samples from healthy subjects[52] where the main producers are activated CD14 positive monocytes[53]. A modified expression of HLA-G molecules has been observed during "no-physiological" conditions, such as viral infection[54-57], cancer[58,59], transplantation[60-64], inflammatory and autoimmune diseases[65,66].

Thus, a growing body of evidence has indicated HLA-G as a suitable key factor in different pathologies. In fact, the immune-modulation by HLA-G may exhibit two distinct effects in pathological conditions: It could be protective in inflammatory and autoimmune diseases[2,25-67], or on the other hand it could be dangerous, for example in tumors or infectious diseases[54-56,58,59]. Based on this evidence, the role of HLA-G in inflammatory and autoimmune diseases has gained considerable clinical interest for the possibility of exploiting it as a molecular biomarker and a therapeutic target.

HLA-G AND PATHOLOGICAL CONDITIONS: PERSPECTIVES IN PREVENTION, DIAGNOSIS AND TREATMENT

Given the immunomodulatory nature of HLA-G molecule, it could be considered a good reference parameter for prevention, diagnosis and treatment in autoimmune and inflammatory diseases.

HLA-G has been analyzed in different pathologies. In this review, we focus on the importance of HLA-G analysis in common and debilitating pathologies characterized by a dysregulation in host immune system in which HLA-G plays a central role.

HLA-G impact in rheumatic disease

Rheumatic disease is a general term used to describe numerous conditions that affect the joints [rheumatoid arthritis (RA)], connective tissues [scleroderma, systemic lupus erythematosus (SLE)] and vessels (vasculitis). Rheumatic diseases are inflammatory and autoimmune diseases, the second most common cause of disability after musculoskeletal injuries. RA (OMIM, #180300) is caused by the immune system attacking synovial cells and treatments include disease modifying anti-rheumatic drugs (DMARDs) and, more recently, biological agents. An important goal of RA therapy has shifted to initiate treatment early and aggressively to achieve remission or low disease activity as quickly as possible. This "treat-to-target" concept has been shown to maximize long-term healthy life[68,69].

Interestingly, RA patients present with an abnormal regulatory network in the immune response, which includes *HLA-G* gene[70]. Serum sHLA-G protein concentration is significantly lower in RA[71] patients than in controls. The decreased sHLA-G concentrations may lead to a chronic activation of inflammatory cells and contribute to the development of the disease. The evaluation of sHLA-G molecules at the specific inflammation site of the synovia reported higher levels of sHLA-G in RA[72] patients. The release of HLA-G in the inflamed synovium may be related to the recruitment of activated HLA-G positive immune cells and the local production by activated synovial fibroblasts[73] that could interact with immune inhibitory receptors and maintain a chronic inflammatory response. These data suggest that there is

a different production of HLA-G molecules on the basis of the local and systemic environments, characterized by different molecular factors and cell types. Interestingly, a recent work confirmed the role of HLA-G molecules in RA. The authors used an intracutaneous treatment of HLA-G monomer or dimer molecules in collagen-induced arthritis model mice. These molecules produced excellent anti-inflammatory effects with a single, local administration[74]. Notably, the dimer exhibited higher immunosuppressive effects than the monomer due to the higher dimer affinity for PIR-B, the mouse homolog of the LILRBs. The HLA-G 14 bp INS/DEL polymorphism has been evaluated as a pharmacogenetic marker of MTX therapy[41]. The authors showed an increase of the 14 bp DEL/DEL genotype in the responder group, characterized by a reduction in disease activity score (DAS28) measured before and after six months of treatment with MTX. In contrast to this study, there are two researches with negative results: (1) 130 RA patients responsive to MTX did not show a significant difference in 14 bp DEL/INS allelic and genotypic distribution (DAS28 < 3.2)[75]; and (2) 186 RA patients, previously untreated with MTX, were prospectively followed up and considered as responders with a DAS28 of up to 2.4 after six months of treatment[76]. No significant association between HLA-G 14 bp INS/DEL and MTX efficacy was observed. Comparing these studies, the discordant results may reflect population differences in gene expression that could influence the power of association studies and lead to different levels of association. In addition, the different doses of MTX and the different cut-off used for RA therapy response definition could affect the results obtained.

Rizzo *et al*[2] evaluated the possible role of HLA-G molecules as biomarkers for RA treatment in a follow-up study. Twenty-three early RA (ERA) patients were analyzed during a 12 mo follow-up disease treatment for sHLA-G levels in plasma samples, mHLA-G and ILT2 expression on peripheral blood CD14 positive cells, and typed HLA-G 14 bp DEL/INS polymorphism. Interestingly, the authors observed that ERA patients with low sHLA-G and membrane HLA-G expression suffered a more severe disease. In fact, sHLA-G levels inversely correlated with DAS28 and ultrasonographic power Doppler scores, used to define the severity and progression of the disease. Interestingly, sHLA-G up-modulation is evident after 3 mo of DMARDs therapy, while a significant reduction in tumor necrosis factor-α levels is evident after 9 mo therapy when a clear amelioration of the disease is evident, with a high specificity for HLA-G detection in EA condition. Moreover, the implication of the HLA-G 14 bp INS/DEL polymorphism is confirmed as the presence of the DEL allele characterizes the patients with a significant improvement in disease status.

SLE (OMIM, #601744) is a systemic autoimmune disease of the connective tissue that can affect any part of the body. Rosado *et al*[77] and Chen *et al*[78] showed higher sHLA-G and IL-10 levels in SLE patients in comparison with healthy controls, while Rizzo *et al*[44] observed lower sHLA-G concentrations in SLE patients. The differences in sHLA-G levels in these two papers could be due to the difference in the analyzed samples (serum or plasma) since it is known that the highest sHLA-G levels are recovered from plasma samples compared with serum collected from the same subjects because of a trapping phenomenon during clot formation that could subtract sHLA-G from the serum[79]. As a proof, Monsiváis-Urenda *et al*[80] evidenced a diminished expression of HLA-G in monocytes and in mature CD83 positive DCs from SLE patients compared with healthy controls. In addition, monocytes from SLE patients showed a decreased induction of HLA-G expression in response to IL-10. Finally, lymphocytes from SLE patients displayed a lower acquisition of HLA-G (by trogocytosis) from autologous monocytes compared to controls. Interestingly, ILT-2 receptor expression is increased on lymphocytes from SLE patients, in particular, in CD3 positive cells, CD19 positive cells, CD56 positive cells and related to IL-10 and anti-DNA antibodies[78]. These results confirm the presence of a HLA-G impaired expression in patients with SLE and a possible role in the pathogenesis. Using a SNP mapping approach, HLA-G gene is reported to be a novel independent locus with SLE interaction[81]. In particular, HLA-G 14 bp INS/DEL polymorphism and HLA-G +3142 C > G SNP were analyzed in a SLE population. SLE patients showed a higher frequency of 14 bp INS allele and 14 bp INS/INS genotype[66]. Moreover, 14 bp INS/INS patients presented the highest disease activity[82]. On the contrary, the evaluation of HLA-G 14 bp INS/DEL polymorphism in a SLE Brazilian population failed to present an association[83], while the +3142 G allele was found to be associated with SLE susceptibility[84]. The +3142 G allele and the +3142 GG genotype frequencies are increased among SLE patients compared with controls[85]. These data support the role of HLA-G molecules in the control of the SLE condition and in particular several results sustain the lower HLA-G expression as a risk factor for SLE development.

HLA-G impact in central nervous system inflammatory diseases

Multiple sclerosis (MS) (OMIM, #126200) is a chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) with unknown etiology that is widely considered to be autoimmune in nature[86]. The presence in CSF of detectable sHLA-G levels in relapsing-remitting MS (RRMS) patients and, occasionally, in other inflammatory neurological disorders and non-inflammatory neurological disorders was reported for the first time by Fainardi and coauthors[87]. In addition, sHLA-G levels in CSF are higher in RRMS than in controls and increased, in association with IL-10 values, in RRMS patients without than in those with magnetic resonance imaging (MRI) evidence of disease activity[88]. The importance of sHLA-G level evaluation as a biomarker for MS is confirmed[89]. Of note, in RRMS

patients, CSF concentrations of sHLA-G and IL-10 are positively correlated with inactive MRI disease and CSF IL-10 titers are more elevated in patients with than in those without CSF measurable levels of sHLA-G. These data suggest that CSF sHLA-G levels may modulate MS disease activity acting as anti-inflammatory molecules under the control of IL-10 CSF levels which may enhance sHLA-G production together with the influence due to HLA-G polymorphisms[67]. The existence of high CSF concentrations of sHLA-G in MS patients and their association with clinical and MRI stable disease have been repeatedly confirmed in subsequent investigations in which: (1) An intrathecal production of sHLA-G is more frequent in MS than in inflammatory and non-inflammatory controls and predominated in clinically and MRI inactive compared to clinically and MRI active MS[88]; (2) sHLA-G concentrations reciprocally fluctuate in CSF and serum of MS patients because they are decreased in the serum of clinically stable MS and increased in CSF of MRI inactive MS[65]; (3) CSF levels of HLA-G5 and not those of sHLA-G1 isoforms are increased in MS compared to controls and in MS patients without MRI appearance of disease activity than in those with MRI Gd-enhancing lesions[90]; and (4) CSF values of sHLA-G and antiapoptotic sFas molecules are inversely correlated in MS patients with no evidence of MRI disease activity since CSF concentrations of sFas are lower in MS than in controls and in MRI inactive than in MRI active MS[91]. Interestingly, HLA-G and its inhibitory receptors (ILT-2 and ILT-4) are strongly up-regulated within and around MS lesions where microglia, macrophages and endothelial cells are recognized as the cellular sources[91]. Furthermore, protein HLA-G expression is higher on cultured human MS microglial cells after activation with Th1 proinflammatory cytokines and a novel subpopulation of naturally occurring CD4 positive and CD8 positive Treg cells expressing HLA-G (HLA-Gpos Treg) has been recently described in peripheral blood of MS patients with relapse[92].

Further studies demonstrated that IL-10 contributes to mediating the suppressive activity of CD4 positive HLA-G^{pos} T_{reg}[93] which are highly represented in CSF and inflammatory brain lesions of MS patients as activated central memory T cells capable of migrating from the periphery to intrathecal compartment due to the expression of CCR5[94]. These results strengthen the assumption of an association between HLA-G antigens and MS.

Collectively, these observations provide evidence that HLA-G antigens are likely to be involved in the resolution of MS autoimmunity acting as anti-inflammatory molecules and suggest that HLA-G positive Treg could play a role in the development of a CNS immunosuppressive microenvironment at the sites of inflammation in MS.

HLA-G impact in other inflammatory and autoimmune diseases

HLA-G proves to also be an important biological marker in other pathologies, for example, gastrointestinal and allergic diseases and diabetes.

Inflammatory bowel disease (OMIM, #266600) is the general term for CD and ulcerative colitis (UC), two chronic inflammatory disorders of the intestine which have different clinical, morphological and immunological characteristics.

Torres *et al*[95] studied intestinal samples of UC and CD patients and, by using an immunohistochemistry technique, demonstrated that while UC intestinal cells presented with HLA-G on their surface, CD intestinal biopsies did not. This result combined with high levels of IL-10 found in the lamina propria of the colon of UC patients suggested that HLA-G can regulate the mucosal immune responses in UC. The distribution of the 14 bp INS/DEL polymorphism in UC and CD was investigated by Glas *et al*[96]. They observed an increase of both 14 bp DEL/INS and 14 bp INS/INS genotypes and a consequent decrease of the high producer genotype (14 bp DEL/DEL) in UC subjects in comparison with CD patients. Also, Rizzo *et al*[97] found a different HLA-G expression in UC and CD patients. Non activated peripheral blood mononuclear cells from CD patients spontaneously secrete sHLA-G, while those from UC patients and healthy donors do not. Furthermore, after stimulation with LPS, both cells from CD and healthy subjects show sHLA-G production, while this does not happen in UC patients. This defective production in UC patients seems to be due to an altered secretion of IL-10 in response to inflammation. The different HLA-G expression profiles in UC and CD patients sustain the different etiopathogenesis at the origin of these two diseases. This hypothesis is sustained by the different modulation of HLA-G observed in the two pathologies after therapy[98]. On the basis of this evidence, it is possible to propose sHLA-G and IL-10 levels as diagnostic parameters to facilitate the diagnosis of UC and CD patients.

Asthma (OMIM, #600807) is a chronic disease affecting approximately 300 million people worldwide, with 180000 deaths resulting annually from severe asthma attacks. Asthma is characterized by chronic inflammation in the airway, which consequently narrows more easily in response to a variety of triggers than the airway of a healthy individual. Nicolae *et al*[99] suggested the role of HLA-G as a potential asthma and bronchial hyperresponsiveness (BHR) susceptibility gene. In particular, susceptibility varies depending on whether the mother has asthma or BHR. A G/G genotype at SNP -964G/A in the promoter region was associated with asthma in the offspring of mothers with either asthma or BHR, whereas the A/A genotype was associated with asthma in the offspring of asthma- and BHR-free mothers. Tan *et al*[33] discovered an association between +3142 C > G (rs1063320) and asthma. HLA-G5 is expressed by airway epithelium and is present in the bronchoalveolar lavage fluid from asthmatic patients[100,101]. In addition to the local presence in airways, sHLA-G may also be found in asthmatic subjects outside the lung. The plasma sHLA-G

levels are higher in atopic asthmatic children than in both non-atopic, asthmatic and non-atopic, non-asthmatic children[101]. The 14 bp INS/DEL polymorphism has no impact on plasma sHLA-G levels in the atopic, asthmatic children. Thus, circulating HLA-G may be important as a biomarker and could potentially modulate immune function more broadly, while the local abundance in airways may have a more direct relationship with immune modulation in the mucosa. There is also *in vitro* evidence that the presence of HLA-G may be different in an asthma condition in comparison with physiological status. sHLA-G expression by peripheral blood mononuclear cells is reduced in asthmatic patients[102] while it is increased in asthma induced by isocyanates[103]. This different behavior may represent differences in biological roles in different disease contexts. A loss of HLA-G could reduce immunosuppression and perpetuate inflammation, whereas increased HLA-G in asthma could be an attempt to reassert immunosuppression. Interestingly, HLA-G is differentially expressed during the lung development[104], suggesting a potential role in lung inflammation induction and chronicization.

Allergic rhinitis (AR) (OMIM, #607154) is characterized by a Th2 polarized immune response. sHLA-G molecules are increased in sera of patients with pollen-induced AR studied outside the pollen season[105], during the pollen season[106] and in perennial AR patients[107]. Interestingly, sublingual immunotherapy (SLIT) for AR is able to reduce sHLA-G serum levels in pollen allergic patients[108,109], suggesting a clinical implication as a biomarker of response to SLIT. Interestingly, children with AR have significantly higher levels of sHLA-G molecules than normal controls or children with allergic asthma[110].

HLA-G impact in pathological pregnancies

During human pregnancy, the maternal immune system recognizes and eliminates alloantigens derived from bacteria or virus, but it tolerates genetically different fetal cells, especially extravillous trophoblast cells invading the maternal decidua or entering the spiral arteries. The expression of HLA-G antigens by trophoblasts is of major importance in protecting the fetus from the semiallogeneic response of the mother[111].

The lack of an established immunological tolerance in pregnancy results in an immune response against paternal antigens expressed by the fetus at the placenta, causing severe health problems for both the fetus and the mother. Complications during pregnancy may affect the woman, the fetus, or both. Miscarriage, RSA and pre-eclampsia account for the most frequent pregnancy complications[112] and the dysregulation of the immunological control at the fetal-maternal interface seems to play a role in these pregnancy complications.

Interestingly, there is a reduced expression of both HLA-G mRNA and protein in pathological compared with control placentas[46-48,113]. In pregnant women, there is a peak of sHLA-G levels in plasma samples in the first trimester that is not evidenced in complicated pregnancies[114,115]. In particular, pregnant women with low sHLA-G plasma levels are characterized by a relative risk of 7.12 of developing placental abruption[116].

The lower secretion of HLA-G by maternal immune cells seems to be in part influenced by HLA-G gene polymorphisms, affecting mRNA stability. In particular, the HLA-G 14 bp ins allele decreases mRNA stability[29,117] and protein production[30,39,118-120]. The HLA-G 14 bp INS/DEL polymorphism seems to affect the fetal HLA-G expression as independent studies have reported fetuses carrying the homozygous genotype for the 14 bp INS allele with a significantly increased risk of pre-eclampsia[121-124]. In addition, the 5' URR seems to be implicated in pathological pregnancies[125]. The confirmed role of HLA-G molecules during pregnancy suggests a potential use in clinical practice. Most pregnancy complications are controversial in terms of diagnosis and treatment. As an example, pre-eclampsia can mimic and be confused with many other diseases and none of the signs are specific. The lower levels of sHLA-G detected in maternal plasma and the HLA-G polymorphism association could assist clinicians in an accurate and reliable diagnosis. Moreover, the HLA-G genetic background of the mother could be an a priori sign of an increased risk of complication during pregnancy. These women could be identified and proposed for a stricter follow-up. It is noteworthy that with an appropriate and timely treatment, the success rate is approximately 80%. Therefore, the use of HLA-G as a biological and genetic marker could improve the management of pregnant women. Moreover, the ability to control HLA-G expression in pathological pregnancies and in women with a high risk of pregnancy complications and infertility could be a tool to cure and prevent these conditions with a deep impact, not only for the individual but also for society.

Until now, more than 15000 embryo culture supernatants have been evaluated for sHLA-G expression, with a positive correlation with embryo implantation rate and pregnancy outcome[126]. However further research is needed to investigate HLA-G in assisted reproductive technologies, but recent studies suggest that sHLA-G is a good candidate as a valuable non-invasive embryo marker to improve pregnancy outcome[127]. Three aspects should be taken into consideration: (1) The recognition of a common sHLA-G detection protocol; (2) The necessity to identify a standardized range for positivity; and (3) The comprehension of the factors involved in the differential expression of sHLA-G between equal stage embryos originating from the same woman.

HLA-G impact in tumors

A high frequency of HLA-G surface expression and increased sHLA-G serum levels has been detected in both hematological and solid tumors. HLA-G and sHLA-G expression correlates with a poor clinical outcome in tumor patients, suggesting a role in the immune escape mechanism of tumors. The frequency of HLA-G expression varies between different types of cancer and even between

different studies in the same type of tumor, probably due to the criteria of patient selection and the methodology used. In hematological malignancies, HLA-G expression was documented with a higher frequency in acute myeloid leukemia cases[128], B and T acute lymphoid leukemia and chronic B lymphocyte leukemia[1,129].

HLA-G expression is frequent in choriocarcinoma[45,130,131], breast[132-135], endometrial[136], and ovarian cancers[137]. In digestive tumors, HLA-G expression was described in esophageal squamous cell carcinoma[138], colorectal cancer[139,140], gastric cancer[139], and liver cancer[141]. In relation to increased membrane HLA-G expression in cancer, higher circulating sHLA-G concentrations were described in patients suffering from different types of cancer[142,143].

These data suggest that HLA-G levels might be used as a diagnostic tool to distinguish between malignant and benign tumors and during disease follow-up. Moreover, HLA-G might serve as a possible marker for tumor sensitivity to chemotherapy and as a prognostic marker for advanced disease stage and clinical outcome. HLA-G assay, either in biological fluids or in biopsies, may have a clinical value in diagnosis, staging, or prognosis of cancer, but prospective validation studies should be conducted in order to use it as a biomarker.

Indeed, it would be important to suppress its immune-suppressive expression in cancer. HLA-G blockade in those tumors that express it remains an attractive therapeutic strategy against cancer. Targeting HLA-G-expressing cancer cells would be also important for maximize the efficacy of anticancer therapies. An experimental approach to target HLA-G-expressing cells in a renal cell carcinoma model was the use of HLA-G- derived peptides based on the binding motif to the HLA-A24[144]. HLA-G peptides induced a cytotoxic attack against HLA-G-expressing HLA-A24 tumor cells, suggesting that HLA-G-mediated suppression can be overcome using peptide-derived immunotherapy.

HLA-G impact in viral infections

Host immune defence mechanisms are efficient at eliminating most viral infections. However, some viruses have developed multiple strategies for subverting host immune defences, thus facilitating their spread in the host[145]. Virus-infected cells are protected against attack by NK cells by HLA-G, providing a long-term immunosuppression function. It may be, therefore, that the diminished immune function induced by HLA-G in the host sometimes leads to an advantage for virus progression by helping viruses subvert the host's antiviral defences[146].

Human immunodeficiency virus type 1 (HIV-1) infection is associated with severe and progressive loss of the immune function in infected persons. It is known that HIV-1 protects infected cells from T lymphocytes and NK cell recognition and lyses by classical HLA-A and B down-regulation and non-classical HLA-G molecule up-regulation, respectively. Since the immunoregulatory ability of HLA-G has become known, the involvement of this molecule in the progression of HIV-1 infection has been widely examined. Studies have focused on the expression of HLA-G in monocytes, which are relevant as reservoirs of HIV-1, and in lymphocytes, which are more susceptible to be infected by HIV-1. Monocytes obtained from HIV-1 seropositive patients expressed HLA-G, although only a small proportion of healthy individuals express this molecule[147]. This might be a consequence of highly active antiretroviral therapy (HAART) since a greater proportion of monocytes expressing HLA-G was observed in patients undergoing HAART compared to untreated[148]. T cells obtained from HIV-1 seropositive individuals were found to express HLA-G at a higher proportion[149] and behave as HLA-G$^+$ Treg.

Human cytomegalovirus (HCMV) is a herpes virus causing widespread, persistent human infection in a delicate balance between the progression of the virus and the defences of the host[150]. HCMV has evolved a number of independent strategies to evade the immune system. HLA-G is produced during viral reactivation in macrophages and astrocytoma cells[56] and the percentage of HLA-G-positive monocytes and sHLA-G levels in patients with active HCMV infection were both dramatically higher than in healthy individuals[151]. The up-regulation observed in HLA-G is probably related to a virus-encoded homologue of human IL-10 (cmvIL-10)[151], which prevents NK cell recognition of infected cells.

Evidence also supports a role of HLA-G in human papilloma virus (HPV) infections. In fact, HLA-G may play a role in mediating HPV infection risk[152] and facilitate cervical cancer development[153].

The ability of specific neurotropic viruses to induce the formation of HLA-G in infected neurons, thus conferring protection against NK cells, was demonstrated. For example, herpes simplex virus-1 and Rhabdovirus[154], trigger the expression and up-regulation of membrane and soluble HLA-G molecules in actively infected neurons.

There is also some evidence that HCV and HBV viruses use HLA-G as a strategy to evade the immune response[155-158].

In summary, one of the main mechanisms of virus evasion is the induction of changes in levels of the classical HLA-G proteins. This enables the virus to prevent infected cells from being recognized and attacked by CTL and NK cells. The main challenge would be to block HLA-G up-modulation by viral infection in order to allow the recognition by immune cells.

CONCLUSION

This review has underlined the importance of HLA-G molecules in pathological conditions.

The literature data suggest that HLA-G could be implicated in both risk and disease chronicization where this antigen is characterized by an impaired expression depending on the different disease environment.

In fact, HLA-G proteins seem to be involved in the

Table 1 Summary of the main studies on human leukocyte antigen G and pathological conditions

Topics	HLA-G genetics and polymorphism	Protein	Ref.
Autoimmune and inflammatory pathologies			
Rheumatoid arthritis		Lower plasma sHLA-G levels than in controls	71
		Higher sHLA-G levels in the synovia	73
		Plasma level of sHLA-G correlates with disease activity parameters	2
	Increase in 14 bp DEL/DEL genotype frequency in responsive patients to MTX treatment		41
	Increase in 14 bp DEL allele frequency in patients with improved disease status		2
Systemic lupus erythematosus		Higher level of sHLA-G and IL-10 in plasma that in controls	77,78
	Higher frequency of 14 bp INS allele and 14 bp INS/INS genotype than in controls	Lower concentration of sHLA-G in serum than in controls	66
		Decrease in HLA-G expression in monocytes and DCs	80
	14 bp INS/INS genotype is associated to the highest disease activity		82
	+3142 G allele and +3142 GG genotype are more frequent in SLE and associated to SLE susceptibility		84,85
Multiple sclerosis		sHLA-G levels in MS CSF are higher than in controls	87,88
	sHLA-G levels in MS could be influenced by HLA-G 14 bp and +3142 C < G polymorphisms		67
		sHLA-G level are increased in serum of CPS of MRI inactive MS	65,90
		HLA-G expression in monocytes is lower than in controls	91
		Presence of HLA-Gpos Treg cells in peripherial blood	92
Inflammatory bowel disease Crohn's disease and ulcerative colitis		HLA-G is present on UC intestinal cells but not in CD biopsies	95
	14 bp INS/DEL and 14 bp INS/INS are increased in UC in comparison with CD patients		96
		PBMCs from CD patients secrete spontaneously sHLA-G	97
		Different modulation of HLA-G by therapy in UC and CD	98
Asthma	-964 G < A and +3142 C < G SNPs are associated with asthma	Expression of HLA-G in airway epithelium and airway system	99,100
		sHLA-G plasma levels are higher in atopic asthmatic children	101
		sHLA-G secretion is increased in asthma induced by isocyanates	103
Allergic rhinitis		Higher sHLA-G serum levels than controls	106-110
Pathological pregnancy		Decreased HLA-G expression in placenta than uncomplicated pregnancies	46-48,112,113
Pre-eclampsia	Increased 14 bp INS/INS genotype frequency than uncomplicated pregnancies		120-125
Tumors		Increased HLA-G expression in tumor cells	1,45,128-143
		Higher sHLA-G serum levels than controls	142
Viral infection		Increased HLA-G expression in viral infected cells	145
HIV-1		Increased HLA-G expression in infected monocytes and T cells	147
HCMV		Increased HLA-G expression in infected monocytes	56
		Increased sHLA-G serum levels than controls	151

sHLA-G: Soluble human leukocyte antigen G; IL-10: Interleukine-10; SNPs: Single nucleotide polymorphisms; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; MS: Multiple sclerosis; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; AR: Allergic rhinitis; HCMV: Human cytomegalovirus; HIV-1: Human immunodeficiency virus-1; MRI: Magnetic resonance imaging; HLA-G: Human leukocyte antigen G; INS/DEL: Insertion/deletion; CPS: Chronic fatigue syndrome.

regulation of the immune system during autoimmune and allergic conditions, such as gastrointestinal, skin, neurological, rheumatic diseases, in pathological pregnancies and in the immune escape mechanisms during viral infections and tumor transformation. In particular, in these disorders, HLA-G proteins could directly interact with immune cells or control the balance between Th1 and Th2 cytokines. In fact, a disequilibrium in this setting would maintain an inflammatory and immune-deregulated condition.

The comprehension of the specific role and mechanisms of action of HLA-G antigens in the development and progression of inflammatory and autoimmune disorders could justify the use of HLA-G molecules as a marker of inflammation and drug treatment and open up new therapeutic perspectives. Moreover, the definition of the role of HLA-G genetic polymorphisms as risk and pharmacogenetic markers could sustain the clinical relevance of HLA-G typing in the laboratory routine. In particular, the possibility to use simple, non-invasive and standardized tools for HLA-G analysis makes it quickly transferable to the health care system practice. These could help in pathology outcome prediction and support treatment decisions.

As reported in Table 1, there are still contrasting results that need to be taken into consideration. The present challenge is to confirm whether HLA-G molecules have a potential role in prevention and diagnosis of pathological conditions. The perspective to identify pharmacological strategies to control the HLA-G production would represent a concrete possibility to improve the control of inflammation and to guide the therapeutic approach. In fact, the possible use of HLA-G as a therapeutic target is of extreme interest.

The ability to modulate HLA-G molecules on the cell surface and to administer HLA-G molecules[74] seems to be at the basis of these cell therapies, suggesting the importance of further studies on HLA-G role in pathological conditions and the possibility of having a controlled modification of the HLA-G level according to disease status and pregnancy complications.

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HLA-G molecules in autoimmune diseases and infections

Roberta Rizzo[1], Daria Bortolotti[1], Silvia Bolzani[1] and Enrico Fainardi[2]*

[1] Section of Microbiology and Medical Genetics, Department of Medical Sciences, University of Ferrara, Ferrara, Italy
[2] Neuroradiology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliero-Universitaria Arcispedale S. Anna, Ferrara, Italy

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***Correspondence:**
Roberta Rizzo, Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Via Luigi Borsari, 46, Ferrara 44121, Italy
e-mail: rbr@unife.it

Human leukocyte antigen (HLA)-G molecule, a non-classical HLA-Ib molecule, is less polymorphic when compared to classical HLA class I molecules. Human leukocyte antigen-G (HLA-G) was first detected on cytotrophoblast cells at the feto-maternal interface but its expression is prevalent during viral infections and several autoimmune diseases. *HLA-G* gene is characterized by polymorphisms at the 3′ un-translated region and 5′ upstream regulatory region that regulate its expression and are associated with autoimmune diseases and viral infection susceptibility, creating an unbalanced and pathologic environment. This review focuses on the role of HLA-G genetic polymorphisms, mRNA, and protein expression in autoimmune conditions and viral infections.

Keywords: HLA-G, inflammation, autoimmunity, infection, regulation

INTRODUCTION

Human Leukocyte Antigen-G (HLA-G) is a functional molecule belonging to class Ib human leukocyte antigens (HLA) characterized by a non-covalent link between β_2-microglobulin (β2m) and glycoprotein heavy chain. The gene is located within Major Histocompatibility Complex (MHC) locus on chromosome 6 (1, 2). HLA-G products show some peculiar features for which they are considered as non-classical HLA-I antigens: (1) the limitation of their allelic polymorphism (3); (2) the expression of seven isoforms represented by four membrane-bound (G1, G2, G3, and G4) and three soluble (G5, G6, and G7) proteins (4); and (3) the restriction of their tissue distribution (5). Polymorphisms at the 5′ upstream regulatory region and at the 3′ UTR of the *HLA-G* gene play an important role in the regulation of HLA-G production (6). Mainly, two polymorphisms at the 3′ UTR: a deletion/insertion (*DEL/INS*) of 14 base pairs (*14bp*) polymorphism (rs371194629) and a *C* > *G* single-nucleotide polymorphism (SNP) at the *+3142bp* position (rs1063320) (7) (**Figure 1**) are able to affect mRNA stability *in vivo* and protein production and implicated in pathological conditions: *14bpINS* allele is associated with mRNA instability (8, 9); *+3142G* allele creates a binding site for three microRNAs (miRNAs) (*miR-148a*, *miR-148b*, and *miR-152*) reducing soluble protein production (10). These observations suggest that *14bpINS/INS* and *+3142G/G* genotypes are associated with a lower HLA-G production than *14bpDEL/INS* and *DEL/DEL*, *+3142C/G*, and *C/C* genotypes (8, 10).

Membrane-bound HLA-G1 and soluble HLA-G5 (HLA-G5) represent the mainly expressed and investigated HLA-G isoforms (1) and are currently supposed to be the most important and functional isoforms (11). However, while HLA-G5 molecules are actively secreted as soluble isoforms, HLA-G1 proteins could be released by proteolytic shedding from cell surface (sHLA-G1) via matrix metalloproteinase-2 (MMP-2) (12–16). HLA-G can exist as β2m-associated and -free monomers (17, 18) and as disulfide-linked dimers or multimers (17, 19, 20). HLA-G disulfide-linked dimers are linked by disulfide bonds between two cysteine residues at position 42 of the HLA-G alpha-1 domain (19–21) and present higher affinity for ILT-2 and ILT-4 receptors compared to monomers (22, 23). Placental trophoblast cells (24), thymus (25), cornea (26), nail matrix (27), pancreas (28), erythroid, and endothelial precursors (29) present a physiological expression of HLA-G molecules. However, HLA-G can be ectopically expressed also on monocytes (30), in transplantation, tumors, viral infections, and autoimmune diseases (1, 2). HLA-G antigens are currently considered as immune-modulatory molecules due to their role in preserving immune tolerance at the feto-maternal interface (31), promoting graft tolerance (32), reducing inflammatory and immune responses (33), favoring tumors (34), and virus infection via immune escape (35). Both membrane-bound and soluble HLA-G antigens exert their immune-suppressive properties: (a) inhibiting the activity and inducing apoptosis of cytotoxic $CD8^+$ T cells and NK cells (36–38); (b) inhibiting the proliferation of $CD4^+$ T cells that are shifted to an immune-suppressive profile (39, 40); (c) inhibiting antigen-presenting cells and B cell differentiation (41, 42); (d) inducing a Th2 polarization (43); and (e) inducing regulatory T cells (44) and Interleukin (IL)-10 secreting dendritic cells (DC10) (45) (**Figure 2**). The interactions between HLA-G proteins and their specific inhibitory receptors ILT-2 (LILRB1/CD85j), ILT-4 (LILRB2/CD85d), and KIR2DL4 (CD158d) expressed by immune cells (46) account for the effects of these molecules on immune cells.

Moreover, HLA-G expression is up-regulated by the secretion of anti-inflammatory cytokines such as IL-10 which, in its turn, is enhanced by HLA-G (30). For these reasons, the implication of HLA-G molecules in inflammatory, immune-mediated, and infective conditions has been investigated (47, 48). The knowledge of

FIGURE 1 | Human leukocyte antigen-G gene. UTR, un-translated region.

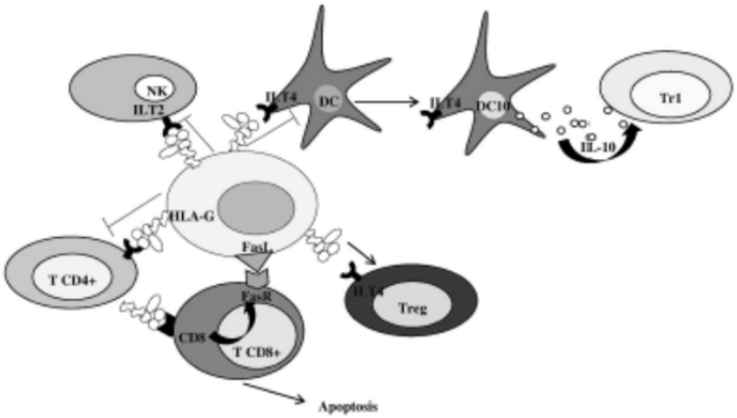

FIGURE 2 | Human leukocyte antigen-G is an anti-inflammatory molecule inhibiting and controlling immune cell activation. NK, natural killer cells; Tr1, type 1 regulatory T cells; DC, dendritic cell; Treg, regulatory T cell; FasR, Fas receptor; DC10, IL-10-differentiated dendritic cells.

the interactions between HLA-G molecules and immune mechanisms and their implication in pathological conditions may assist in improving our knowledge on the mechanisms at the basis of several autoimmune diseases and viral infections.

HLA-G AND GASTROINTESTINAL DISEASES

Celiac disease is a gluten sensitivity, which induces an inflammation that damages the villi in the small intestine of genetically predisposed subjects. Both genetic and environmental factors contribute to the development of celiac disease (CD). Torres and coauthors (49) have shown the presence of HLA-G in biopsies from celiac patients and have observed higher sHLA-G amounts in comparison with control subjects. The evaluation of the *14bp INS/DEL* polymorphism in a group of 522 celiac patients (50), subdivided accordingly with the presence of HLA-DQ2 molecule, encoded by *DQA1*05/DQB1*02* genes, has demonstrated an increased frequency of the *14bp INS/INS* genotype in comparison with controls. These data suggest that the *14bp INS* allele may increase the risk of gut inflammation, most likely leading to chronicity. Ulcerative colitis (UC) and Crohn's disease are characterized by a different sHLA-G expression pattern (51) by peripheral blood mononuclear cells. Non-activated peripheral blood mononuclear cells from Crohn's disease patients secrete spontaneously sHLA-G while those from UC patients and healthy donors do not. Furthermore, after stimulation with LPS, both cells from Crohn's disease and healthy subjects show sHLA-G production, while this does not happen in UC patients. The different HLA-G expression profiles in UC and Crohn's disease patients sustain the different aethiopathogenesis at the origin of these two diseases. In particular, the responses to therapies in UC and Crohn's disease correspond to different sHLA-G secretion levels (52). The immunosuppressant therapy normalizes the production of HLA-G molecules in Crohn's disease while it starts the release of HLA-G in UC patients. These data confirm the diversity in the behavior of these two pathologies and propose the analysis of sHLA-G levels with the final goal of distinguishing between UC and Crohn's disease patients and to monitor therapy.

HLA-G AND RHEUMATOLOGIC DISEASES

Rheumatic diseases are inflammatory and autoimmune diseases, which are the second most common cause of disability after musculoskeletal injuries. Rheumatoid arthritis (RA) is an autoimmune disease caused by the immune system attacking synovial cells. A combination of genetic and environmental factors may increase the risk of RA. Gene expression profiles (GEPs) in bone marrow-derived RA mononuclear cells (53) have shown 1,910 down-regulated and 764 up-regulated gene, which include the *HLA-G* gene. Several studies have evaluated the role of *HLA-G* polymorphisms in RA susceptibility without reaching a final common result. The evaluation on 256 RA patients and 356 healthy controls genotyped for the *HLA-G 14bp INS/DEL* polymorphism has reported no differences in allelic and genotypic

frequencies and no correlation with disease characteristics (54). The analysis of two SNPs (rs1736936, −1305G/A and rs2735022, −689A/G) in *HLA-G* promoter in the Korean population has not presented any connection to the development of RA (55). The evaluation in a Brazilian cohort documented the implication of 3′ UTR polymorphisms in RA follow-up (56). The authors have observed a significant association of the *−762C > T*, *−716T > G*, *−689A > G*, *−666G > T*, *−633G > A*, *−486A > C*, and *−201G > A* (*rs1632946*; *rs2249863*; *rs2735022*; *rs35674592*; *rs1632944*; *rs1736933*; and *rs1233333*) SNPs with the disease. The analysis of 106 patients with juvenile idiopathic arthritis (JIA) has shown an association between JIA female susceptibility and the 14 bp DEL allele. These different associations support the presence of different pathogenic elements between RA and JIA (54). RA (57) and JIA patients present lower serum sHLA-G concentration than in controls (58), with a possible contribution to the chronicity of the inflammation. On the contrary, JIA synovial fluids showed higher sHLA-G levels than controls (SF) (56). Since we have observed that HLA-G molecules are enhanced in synovial fibroblasts from inflamed joints (59) and that high sHLA-G levels correlate with disease activity (57), we may suggest an impaired control of immune reaction at joint, which characterizes JIA disease. The *HLA-G 14bp INS/DEL* polymorphism has also been evaluated as a marker for RA therapy. Methotrexate (MTX), a disease-modifying anti-rheumatic drug (DMARD), induces an increased production of IL-10 in RA patients with a better therapeutic response (60) and is able to enhance HLA-G secretion by peripheral blood mononuclear cells (61). Interestingly, the *14bp DEL/DEL* genotype is increased in RA patients with a good response to MTX therapy (62), with a possible implication in the control of immune activation. It must be underlined, however, that contrasting results have been obtained (63, 64), possibly due to a different dosage of MTX, a different cut-off value for RA therapy response assessment. Scleroderma (SSc) is an autoimmune rheumatic disease of the connective tissue (65). Only SSc patients with a longer survival, lower frequency of vascular cutaneous ulcers, telangiectasias, and inflammatory polyarthralgia present HLA-G molecule expression in skin biopsies (66) suggesting an implication of this molecule on the control of immune response at the skin level.

Systemic lupus erythematosus is a systemic autoimmune disease of the connective tissue that can affect any part of the body. The immune response is mainly characterized by Th2-cell predominance. Rosado and coauthors (67) and Chen and coauthors (68) have shown higher sHLA-G and IL-10 levels in systemic lupus erythematosus (SLE) patients in comparison with healthy controls, while Rizzo and coauthors (69) have observed lower sHLA-G concentrations in SLE patients (70). Interesting, the analysis of monocytes and mature CD83 positive dendritic cells from SLE patients has evidenced a diminished expression of HLA-G in comparison with healthy controls (71), a lower HLA-G expression in response to IL-10 and a lower HLA-G trogocytosis from autologous monocytes compared with controls. Using the SNPs mapping approach, *HLA-G* gene is recognized as a novel independent locus for SLE (72). In particular, *HLA-G 14bp INS/DEL* polymorphism and *HLA-G +3142C > G* SNP have been analyzed in a SLE population. SLE patients showed a higher frequency of *14bp INS* allele and *14bp INS/INS* genotype (69) and the heterozygote group showed lower systemic lupus erythematosus disease activity index (SLEDAI) indexes than homozygous groups (73). On the contrary, the evaluation of *HLA-G 14bp INS/DEL* polymorphism in a SLE Brazilian population did not present an association (74), while the *+3142G* allele and the *+3142 GG* genotype frequencies were increased among SLE patients as compared with controls (75, 76). These data sustain a possible role of HLA-G expression in modifying SLE condition. Behçet (BD) and Kawasaki diseases are autoimmune vasculitis. The *HLA-G*01:01:01* allele is associated with a reduced risk of BD while *HLA-G*01:01:02* and *G*01:05N* alleles are associated with an increased risk of BD (77, 78). Non-synonymous SNP *(+755A/C)* of the *HLA-G* gene (rs12722477, *G*01:04*) is significantly associated with Kawasaki disease (79). These data suggest an influence of *HLA-G* polymorphisms in determining disease risk, possibly affecting HLA-G production and consequently inflammation status.

HLA-G AND CUTANEOUS DISEASES

The skin is characterized by a "skin immune system (SIS)," where immune cells and humoral components support cutaneous inflammation. The deregulation of skin defense mechanisms is evident in a large variety of inflammatory disorders of the skin, such as psoriasis, atopic dermatitis, pemfigo, vitiligo, and systemic sclerosis (80). HLA-G protein is not expressed in the skin from healthy controls (81, 82). Ectopic HLA-G expression has been described in skin pathologies (83–86).

Psoriasis is a chronic inflammatory skin disease with an autoimmune component. Both membrane-bound and soluble HLA-G proteins have been detected in psoriatic skin lesions with the main compound characterized by macrophage lining at the dermo-epidermal junctions (82). The up-regulation of HLA-G molecules by macrophages could represent an attempt to control auto-reactive T cells, induced by activated keratinocytes-derived cytokines/chemokines. HLA-G may prevent keratinocyte destruction by modulating the activity of cytotoxic lymphocytes and promoting the development of Treg cells (87). Interestingly, significantly lower plasma sHLA-G levels have been found in psoriatic patients compared with controls (88), suggesting a difference in systemic HLA-G expression that could be associated with the IL-10 deficiency typical of psoriasis. Psoriasis management can be divided into three main types: topical drugs, light therapy, and systemic medications. Evaluation of therapeutic effects on sHLA-G expression has shown an increase in plasmatic levels of systemic treated patients (efalizumab, cyclosporin A, and acitretin) (88) and a significant association between *HLA-G 14bp DEL* allele and *14bp DEL/DEL* genotype with acitretin clinical outcome (89). We can suppose a possible direct effect of HLA-G in antagonizing systemic T helper 1 activation and with a potential role as a marker of response to acitretin in psoriatic patients.

Pemphigus vulgaris is a blistering disease caused by autoantibodies to desmoglein skin adhesion proteins. Skin tissue sections from pemphigus vulgaris (PV) patients express detectable HLA-G molecules at both transcriptional and translational levels, while control sections present only HLA-G transcription (90). Moreover, the HLA-G *14bp DEL* allele has been observed with higher frequency in PV patients in comparison with controls in a Jewish

population (91). These data suggest that HLA-G expression could be a detrimental factor for the development of PV.

HLA-G AND DIABETES

Type 1 and type 2 diabetes present immunologic defects that enhance insulin resistance as a result of genetics sedentary lifestyle, obesity, and other conditions, such as chronic inflammation or infection. It has been shown that higher levels of sHLA-G are frequent in subjects with an impaired glucose metabolism (92). These data suggest a possible implication of HLA-G antigens in the diabetic condition. In fact, SNPs *rs4122198*, *rs2394186*, *rs1619379*, and *rs1611133* near the *HLA-G* gene have been associated with type 1 diabetes (93); dendritic cells from type 1 diabetic patients produce lower HLA-G molecules in response to IFN-beta (94) in comparison with control subjects and the *HLA-G 14bp INS-INS* genotype might contribute to the development of high blood pressure in type 2 diabetes (95).

Interestingly, HLA-G has been found in some secretory granules and on the cell surface of primary islet cells induced to secrete insulin (28). On the basis of these data, it could be hypothesized that an impaired HLA-G expression at pancreatic islets could sustain T cell activation and onset of diabetes.

HLA-G IN MULTIPLE SCLEROSIS

Multiple sclerosis is the prototypic autoimmune disease of the central nervous system (CNS) characterized by chronic inflammatory demyelination and neurodegeneration of unidentified origin (96). Multiple sclerosis (MS) typically occurs in young adults and manifests in women twice as frequently as in men with neurological symptoms and signs, called relapses, which are usually disseminated in space and time (97). About the 80% of MS patients present a disease onset with a relapsing–remitting (RR) form followed by a secondary progressive (SP) course that arises after years, whereas MS starts with a primary progressive (PP) form in approximately the 20% of subjects (98). However, the recent proposed criteria (99) suggest that the coexistence of multi-focal lesions in the periventricular white matter on T2-weighted Magnetic Resonance Imaging (MRI) scans with or without Gadolinium (Gd) enhancement on T1-weighted MRI scans are needed for the diagnosis of MS. Based on epidemiological studies, exposure to an environmental factor, e.g., an infectious agent, in genetically predisposed individuals is currently thought to be crucial for MS pathogenesis (100) in which the traffic into the CNS of activated auto-reactive $CD4^+$ T helper 1 (Th1) cells plays a central role (96, 101, 102). The initiation of brain inflammation is due to the activation of microglia by infiltrating $CD4^+$ T cells leading to the generation of Th1-mediated immune responses (IL-12/IFN-γ and IL-23/IL-17), while the resolution of neuroinflammation is triggered by astrocytes, which promote anti-inflammatory Th2-polarized responses (IL-10 and TGF-β) and the elimination of infiltrating immune cells through Fas/FasL-dependent apoptosis (96, 101) (**Figure 3**).

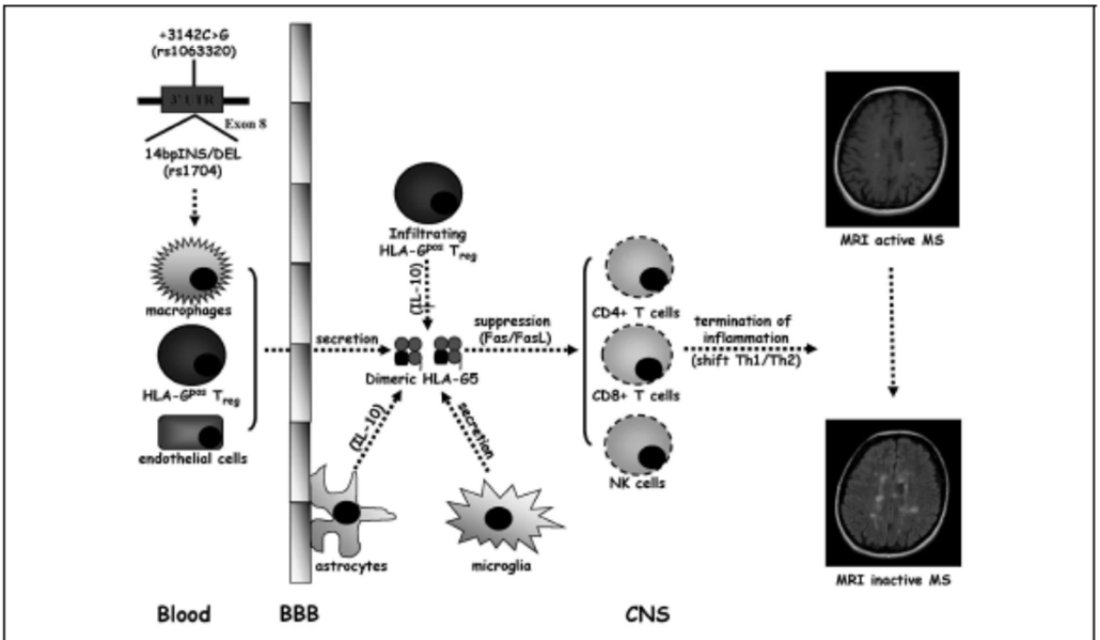

FIGURE 3 | Intrathecal immune milieu in MS. The secretion of HLA-G5 in dimeric form by macrophages and HLA-G^{pos} T_{reg} infiltrating the central nervous system (CNS) across the blood–brain barrier (BBB), endothelial cells, and microglia, sustained by a IL-10 release by astrocytes, may promote the suppression of $CD4^+$ Th1 cell activity and the apoptotic removal of $CD8^+$ T cells and NK cells that favor the formation of an anti-inflammatory intrathecal microenvironment leading to the termination of MS inflammation.

A growing body of evidence indicates that sHLA-G antigens may have a tolerogenic role in MS (102, 103). Cerebrospinal fluid (CSF) detectable sHLA-G has been detected in RRMS patients with higher levels in comparison with other inflammatory neurological disorders (OIND), non-inflammatory neurological disorders (NIND), and controls (104). Furthermore, higher CSF sHLA-G levels have been detected in RRMS without MRI evidence compared to those with MRI active disease. Notably, a positive correlation between CSF concentrations of sHLA-G and IL-10 has been found in MS patients without MRI evidence of active disease. Therefore, CSF levels of sHLA-G may act, together with IL-10, as anti-inflammatory molecules to regulate MS disease activity. The association between elevated CSF sHLA-G levels and clinical and MRI appearance of MS stable disease is supported by the intrathecal synthesis of sHLA-G in MS clinically and MRI inactive patients (105). We have found higher CSF levels of HLA-G5 and not of sHLA-G1 isoforms compared with controls and in presence rather than in absence of MRI Gd enhancing lesions (106) and an as well as inverse correlation between CSF levels of sHLA-G and anti-apoptotic sFas molecules in MS patients without MRI disease activity (107). Collectively, these results suggest a strong correlation between high CSF levels of sHLA-G antigens and the resolution of MS autoimmunity probably related to the anti-inflammatory properties of these molecules. The impact of HLA-G in MS pathogenesis was recently confirmed by other studies, which demonstrated that: (a) Th1 and Th2 cytokine production and $CD4^+$ T cell proliferation are suppressed by HLA-G from MS patient peripheral blood monocytes during the first month of treatment with IFN-β (108); (b) MS disease activity during pregnancy may be modulated by tolerogenic properties of sHLA-G since post-partum serum sHLA-G levels are higher in MS patients without clinical attacks (109); and (c) microglia, macrophages, and endothelial cells located within and around MS lesions present a strong immunohistochemical expression of HLA-G and its inhibitory receptors (ILT-2 and ILT-4), with an elevated protein HLA-G expression on cultured human microglial cells after activation with Th1 pro-inflammatory cytokines (110). Meanwhile, a novel subpopulation of naturally occurring $CD4^+$ and $CD8^+$ regulatory T cells of thymic origin expressing HLA-G ($HLA\text{-}G^{pos}$ T_{reg}), has been characterized in MS patients with a suppressive activity through the secretion of HLA-G5 and the shedding of sHLA-G1 (111–113). Overall, these data sustain anti-inflammatory properties of sHLA-G molecules, and in particular HLA-G-5 isoform, which could lead to the remission of MS autoimmunity. Although it has been demonstrated that SNP rs4959039, a SNP in the downstream un-translated region of HLA-G gene is independently associated with MS susceptibility (114), the possible link between *HLA-G* genetic polymorphisms and MS has not been intensively explored (102, 103). Conflicting results have been obtained. Although no association between *HLA-G* gene polymorphism and MS or severity of the disease has been initially found (115), *14bpINS* and *−725G* (rs1233334) alleles have been shown to be related to MS (116). However, a recent study, evaluating the influence of *14bpDEL/INS* and *+3142C > G* HLA-G polymorphisms on CSF and serum sHLA-G production, has documented a correlation between HLA-G genetic polymorphisms and sHLA-G concentrations in both CSF and serum (117). These findings indicate that CSF and serum sHLA-G levels in MS could be affected by two main HLA-G polymorphisms. Moreover, preliminary results from our laboratory have demonstrated that, MS patients present dimeric sHLA-G form more frequently than control, in particular in MRI inactive MS patients (unpublished data), suggesting that large amounts of biologically active dimeric sHLA-G form could be released in CSF of MS patients, possibly induced by pharmacological treatment (118). Nevertheless, in a recent study no association was found between serum sHLA-G levels, disability progression, disease MRI activity, and time to conversion from clinically isolated syndrome (CIS) to clinically definite MS (119). These findings suggest that the use of sHLA-G levels in CSF should be taken into consideration as a prognostic marker for monitoring disease conversion, activity, progression, and response to therapy.

HLA-G IMPACT IN VIRAL INFECTIONS

Even if host immune system present several mechanisms to control viral infections, the viruses have developed several strategies to counteract host immune defenses (120). HLA-G seems to be implicated in viral immune-escape from Natural Killer cells (121).

Human immunodeficiency virus type 1 (HIV-1) up-regulates HLA-G molecules and down-regulates classical HLA-A and -B. Studies have focused on the expression of HLA-G in monocytes, which are relevant as reservoirs of HIV-1, and in lymphocytes, which are more susceptible to infection by HIV-1. Monocytes from HIV-1 seropositive patients express HLA-G (122) with a possible association with antiretroviral therapy (HAART), since patients undergoing HAART present higher levels of HLA-G expression on monocytes in comparison with untreated and healthy subjects (122, 123). T cells obtained from HIV-1 seropositive individuals have been found to express HLA-G at a higher proportion (124) and behave like HLA-G+ Treg. Furthermore, on the basis of *HLA-G* genetics, it would seem that the *HLA-G 14bpINS* and *+3142G* polymorphisms affect the susceptibility to HIV (125) but not mother–child transmission (126) in African population.

Human cytomegalovirus is a herpes virus that persists in the host (127) by means of several strategies to evade the immune system. HLA-G expression is evidenced during viral reactivation in macrophages and astrocytoma cells (35) and the levels of expression on monocytes an in serum is higher during active human cytomegalovirus (HCMV) infection (128). This up-regulation is proposed to be associated with virus-encoded homologs of humanIL-10 (cmvIL-10) (129), which prevents NK cell recognition of infected cells.

There is also evidence to support also a role of HLA-G molecules in susceptibility and outcome of human papilloma virus (HPV) infections. The alleles HLA-G *14bp INS*, *+1537C* (rs12722477), *G*01:01*, *G*01:04*, and *G*01:06* have been associated with both high-grade squamous intraepithelial lesions and cervical cancer, while *HLA-G 14bp DEL* and *+3142C* alleles have been identified as protective (130–135). These results are in agreement with the low levels of HLA-G5 expression in cervical cancer (136). On the other hand, two researches recognized *HLA-G 14bp DEL* allele and *+3142C* as associated with increased risk of cervical cancer (137, 138), in agreement with increased expression

of HLA-G in cervical cancer tissues (139) and with the spontaneous de-methylation of *HLA-G* promoter that allows immune-evasion and the development of precancerous cervical lesions (140). HLA-G has been also implicated in nasal polyposis development in the presence of HPV infection (141). Nasal polyps with HPV11 infection have shown HLA-G expression on epithelial cells, while no HLA-G expression has been observed in HPV negative polyps.

Neurotropic viruses such as herpes simplex virus-1 (HSV-1) and Rabdovirus (RABV) (142) induce the expression and up-regulation of membrane and soluble HLA-G molecules in actively infected neurons with a consequent protection toward host NK cells.

Hepatitis C virus (HCV) and Hepatitis B virus (HBV) seems to induce HLA-G expression to control host immune response (125, 143–148).

On the basis of these results, HLA-G proteins are expressed by virally infected cells as a mechanism to evade host immune control, preventing T cell and NK cell activation. The main challenge would be to block HLA-G up-regulation by viral infection, in order to allow the recognition by immune cells.

INTERACTION OF HLA-G MOLECULES WITH OTHER HLA-Ib MOLECULES

Other HLA-Ib molecules have been identified: HLA-E and HLA-F (149, 150) characterized by a low genetic diversity as well as by a particular expression pattern, structural organization and functional profile.

Similar to HLA-G, HLA-E forms a complex with β2-microglobulin. HLA-E is known to play an important role as immune-modulator during pregnancy and transplantation (151), inhibiting immune responses by its interaction with $CD8^+$ T cell receptors (TCRs) (152) and with the CD94/NKG2A inhibitory receptors of NK cells (153). Meanwhile, this molecule may present non-self antigens activating immune response (154).

Similar to other HLA molecules, HLA-F can form a complex with beta2 microglobuli and three splicing variants have been described. While the presence of HLA-G and HLA-E has been recently correlated with physiological and pathological conditions, the clinic-pathological significance of HLA-F is limited. HLA-F is expressed by peripheral blood B cells upon activation (155) and is detected in embryonic tissues, including the extravillous trophoblasts invading maternal deciduas, and in spermatozoids (156, 157) and in the serum of patients affected by tumors (158).

Only few data are available on the interaction of HLA-G molecules with the other HLA-Ib antigens. In physiological conditions, HLA-G molecules interact with HLA-E and co-operate to inhibit NK cells, mainly at feto-maternal interface, via interaction with ILT-2 and CD94/NKG2A, respectively (159). In pathological condition, the interaction between these two molecules facilitates the escape of tumor cells from NK cell recognition (160). In MS, HLA-G and HLA-E molecules are expressed by resident CNS cells and interact with NK cell and cytotoxic lymphocytes (161). HLA-G, -E, and -F expression by trophoblasts correlates with the protection of the fetus from destruction by the maternal immune system, suggesting a co-operation for fetal tissue preservation.

CONCLUSION

This review aims to focus on the key role of HLA-G molecules in autoimmune diseases and viral infections. The data herein summarized suggest that HLA-G may have a crucial role in the creation of an impaired immune response that characterizes these pathological conditions.

In fact, it appears even more evident that HLA-G proteins are involved in the regulation of the immune system during autoimmunity, such as gastrointestinal, skin, rheumatic and neurological diseases and in the immune-escape mechanisms during viral infections.

Here, we have reviewed a series of experimental and epidemiological studies that support the direct influence of HLA-G proteins on the balance of immune settings. On this basis, understanding the function of HLA-G in these disorders could help in the identification of new approaches to control HLA-G production.

For example, it is interesting to note that inflammatory cutaneous diseases present a disproportional expression of HLA-G molecules with respect to controls and that this could generate autoimmunity. Thus it appears that down/over-expression of HLA-G may not only act as an immunosuppressive and beneficial molecule but may also sustain an unbalanced immune stimulation and autoimmunity. With reference to bowel diseases especially, it appears clear that the different HLA-G expression levels could help in the differential diagnosis and consequently in the choice of appropriate treatment.

Furthermore, several studies have evidenced the possible role of sHLA-G antigens as a tolerogenic molecules in MS since their intrathecal production is associated with disease remission. It is of extreme importance to evaluate the role of HLA-G antigens in MS pathogenesis, in particular if they are implicated in disease progression or if they represent an indirect manifestation of MS inflammation of CNS. Still to be clarified are the functional differences between HLA-G5 and sHLA-G1, and whether dimers and monomers exert a different function in MS inflammatory disease activity. As far as viral infections are concerned, HLA-G could be considered a target for anti-viral treatment, so increased knowledge in this field could contribute to identifying different therapeutic strategies.

Collectively, the results emerging from the literature confirm the importance of the HLA-G molecule in the pathogenesis and progression of immune-based diseases and infections, underlining the relevance of its investigation with the aim to developing new therapeutic strategies and clinical markers. Meanwhile, the analysis of the interactions between HLA-G and other HLA-Ib molecules may be useful to understand the mechanisms for the creation of immune-suppressive microenvironments.

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Review Article

Some Basic Aspects of HLA-G Biology

Estibaliz Alegre,[1] Roberta Rizzo,[2] Daria Bortolotti,[2] Sara Fernandez-Landázuri,[1] Enrico Fainardi,[3] and Alvaro González[1]

[1] *Department of Biochemistry, University Clinic of Navarra, Pamplona, Spain*
[2] *Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Ferrara, Italy*
[3] *Neuroradiology Unit, Sant'Anna Hospital, Ferrara, Italy*

Correspondence should be addressed to Alvaro González; agonzalah@unav.es

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Human leukocyte antigen-G (HLA-G) is a low polymorphic nonclassical HLA-I molecule restrictively expressed and with suppressive functions. HLA-G gene products are quite complex, with seven HLA-G isoforms, four membrane bound, and other three soluble isoforms that can suffer different posttranslational modifications or even complex formations. In addition, HLA-G has been described included in exosomes. In this review we will focus on HLA-G biochemistry with special emphasis to the mechanisms that regulate its expression and how the protein modifications affect the quantification in biological fluids.

1. Introduction

Human leukocyte antigen-G (HLA-G) is a major histocompatibility complex class I antigen encoded by a gene on chromosome 6p21. It differs from classical HLA class I molecules by its restricted tissue distribution and limited polymorphism in the coding region. HLA-G role in immune tolerance was uncovered studying its expression in trophoblast cells at fetus-maternal interface [1]. Several studies have found an aberrant or reduced expression of both HLA-G mRNA and protein in pathological conditions such as preeclampsia [2] or recurrent spontaneous abortion [3] in comparison with normal placentas. HLA-G expression has been documented in few tissues during physiological conditions, such as cornea, thymus, erythroid, and endothelial precursors [4–6], and in a variable percentage of serum/plasma samples from healthy subjects [7] where the main producers seem to be activated CD14+ monocytes [8]. An ectopic expression of HLA-G molecules has been observed during "no-physiological" conditions, such as viral infection [9–12], cancer [13], transplantation [14–18], and in inflammatory and autoimmune diseases [19–21]. Thus, a growing body of evidence has indicated HLA-G as a suitable key actor in different pathologies. In fact, HLA-G may exhibit two distinct effects in pathological conditions: it could be protective in inflammatory and autoimmune diseases [22] or it could be dangerous, for example, in tumors or infectious diseases.

2. HLA-G Expression and Regulation

The HLA-G production is controlled by several polymorphisms both in the promoter and in the 3′ untranslated region (3′ UTR) that modify the affinity of gene targeted sequences for transcriptional or posttranscriptional factors, respectively [24]. Twenty-nine single nucleotide polymorphisms (SNPs) have been identified in the HLA-G promoter region, which may be involved in the regulation of HLA-G expression, considering that many of these polymorphisms are within or close to known or putative regulatory elements (Figure 1). The *HLA-G* 5′ upstream regulatory region (URR) is unique among the *HLA* genes [25] and is unresponsive to NF-κB [25] and IFN-γ [26], due to the presence of a modified enhancer A (enhA) and a deleted interferon-stimulated response element (ISRE). A locus control region (LCR) located −1.2 kb from exon 1 exhibits a binding site for CREB1 factor, which also binds to two additional cAMP response elements at −934 and −770 positions from the ATG start codon. In addition, an ISRE for IFN response factor-1 (IRF-1) is located at

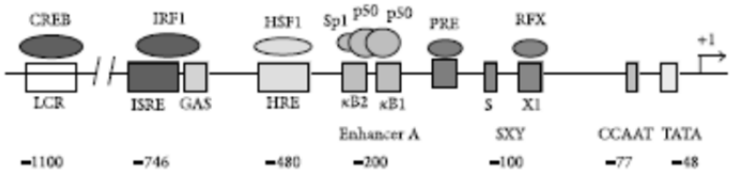

Figure 1: HLA-G unique promoter region. Enhancer A element (KB1, KB2, Sp1): NF-KB; interferon-stimulated regulatory element (ISRE); interferon regulatory factor (IRF); interferon-gamma activated site (GAS); SXY region; progesterone response element (PRE); hypoxia response element (HRE).

the −744 bp position [24] and is involved in *HLA-G* transactivation following IFN-β treatment [27]. The *HLA-G* promoter also contains a heat shock element at the −459/−454 position that binds heat shock factor-1 (HSF-1) [28] and a progesterone receptor binding site at −37 bp from ATG start codon [29]. Several promoter region polymorphisms coincide with or are close to known or putative regulatory elements and thus may affect the binding of *HLA*-G regulatory factors [30]. The −725 C>G/T SNP is very close to ISRE, and the −725 G allele is associated with a significantly higher expression level compared with the other alleles [31]. The polymorphic sites at the 5′ URR are frequently in linkage disequilibrium (LD) with the polymorphic sites identified at the 3′ UTR, some of them influencing alternative splicing and mRNA stability [25].

A 14 base pair (14 bp) insertion/deletion (INS/DEL) polymorphism (rs66554220) in exon 8 involves mRNA stability and expression [32]. In particular, the DEL allele stabilizes the mRNA with a consequent higher HLA-G expression [33, 34]. The presence of an adenine at position +3187, modifying an AU-rich motif in the *HLA-G* mRNA, decreases its stability [35]. One single nucleotide polymorphism (SNP) C>G at the +3142 bp position (rs1063320) has been explored by Tan and coauthors [36]. The presence of a guanine at the +3142 position may influence the expression of the HLA-G locus by increasing the affinity of this region for the microRNAs miR-148a, miR-148b, and miR-152, therefore decreasing the mRNA availability for translation by mRNA degradation and translation suppression. The influence of the +3142G allele has been demonstrated by a functional study in which HLA-G high-expressing JEG-3 choriocarcinoma-derived cells have been transfected with miR-148a, decreasing soluble HLA-G levels. The contrasting results obtained by Manaster and coauthors [37], who have reported the absence of +3142 C>G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression. Other SNPs have been identified as implicated in miRNA interaction. In particular, +3003, +3010, +3027, and +3035 SNPs are influenced by miR-513a-5p, miR-518c*, miR-1262 and miR-92a-1*, miR-92a-2*, miR-661, miR-1224-5p, and miR-433 miRNAs [35]. The miR-2110, miR-93, miR-508-5p, miR-331-5p, miR-616, miR-513b, and miR-589* miRNAs target the 14 bp INS/DEL fragment region, and miR-148a, miR-19a*, miR-152, mir-148b, and miR-218-2 also influence the +3142 C/G polymorphism.

HLA-G is a stress-inducible gene: heat shock, hypoxia, and arsenite increase different HLA-G alternative transcripts [28, 38]. The indolamine 2,3-dioxygenase (IDO), an enzyme which metabolizes tryptophan, induces HLA-G expression during monocyte differentiation into dendritic cells [39]. Interestingly, HLA-G exerts its immune tolerogenic function towards T cell alloproliferation following an independent pathway from IDO [40]. Nitric oxide-dependent nitration of both cellular and soluble HLA-G protein decreases total HLA-G cellular protein content and expression on the cell surface, while it increases HLA-G shedding into the culture medium. This effect was posttranscriptional and the result of metalloprotease activity [41–43]. Several evidences indicate that the soluble HLA-G1 (sHLA-G1) form is generated through the shedding of the membrane bound HLA-G1 by metalloproteinase (MP) [44–47]. In particular, matrix metalloproteinase-2 (MMP-2), a zinc-containing and calcium-requiring endopeptidase known for the ability to cleave several extracellular matrix constituents, as well as nonmatrix proteins, is responsible for HLA-G1 membrane-shedding via three possible highly specific cleavage sites [48].

The anti-inflammatory and immunosuppressive interleukin- (IL-) 10 has been correlated with concomitant HLA-G expression [33]. Transactivation of HLA-G transcription has also been demonstrated by leukemia inhibitory factor (LIF) [49] and methotrexate cell exposure [50]. Furthermore, interferon (IFN)-α, -β, and -γ enhance HLA-G cell-surface expression by tumors or monocytes [51, 52]. HLA-G expression could be acquired by trogocytosis, where a "donor" cell that expresses membrane HLA-G exchanges membrane parts containing HLA-G with a "recipient" cell that is not expressing HLA-G molecules. In this particular situation, "recipient" cells will acquire and make use of membrane HLA-G molecules from a "donor" HLA-G positive cell without the activation of HLA-G gene transduction into protein. Trogocytosis of HLA-G from antigen presenting cell (APC) by T cells in humans makes these T cells unresponsive [53]. It has been shown that NK cells can acquire HLA-G1 from tumor cells, which provokes an arrest of NK cells proliferation and cytotoxic activity,

behaving like suppressor cells capable of inhibiting other NK cell functions [54].

3. HLA-G Transcription Products

To date, 50 alleles (IMGT HLA database, December 2013) and 16 proteins are known. Seven HLA-G isoforms exist due to mRNA alternative splicing and differential association with β2-microglobulin (β2-m). Four of them are found on the cell surface (HLA-G1, -G2, -G3, and -G4), while the other three are soluble forms released from the cell (HLA-G5, -G6, and -G7), due to the lack of the transmembrane and intracellular domains of membrane-bound HLA-G (Figure 2). The HLA-G 14 bp INS/DEL polymorphism is involved in the expression of both HLA-G1 and HLA-G5 isoforms, with decreased HLA-G1 and HLA-G5 concentrations in 14 bp INS samples in comparison with 14 bp DEL samples [32, 34].

The overall structure of HLA-G resembles other class I MHC molecules, in which a heavy chain comprised of three extracellular domains is noncovalently associated with β2-m (Figure 2). A nine-residue self-peptide is bound within a cleft formed by two alpha-helices and a beta-sheet floor. An extensive network of contacts is formed between the peptide and the binding cleft, leading to a constrained mode of binding reminiscent of that observed in HLA-E [65].

4. HLA-G Receptors

HLA-G exerts its immunomodulatory functions through the interaction with multiple receptors such as LILRB1 (ILT2/CD85j), LILRB2 (ILT4/CD85d), and KIR2DL4 (CD158d), which are differentially expressed by immune cells. The interaction of HLA-G molecules with inhibitory receptors induces apoptosis of activated CD8+ T cells [66], modulates the activity of NK cells [67] and dendritic cells (DC) [68], blocks allocytotoxic T lymphocyte response, induces expansion of T cell populations such as $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells [69] and $CD3^+CD4^{low}Foxp3^-$ and $CD3^+CD8^{low}Foxp3^-$ [70], and inhibits Vγ9Vδ2 T-cell proliferation and cytotoxicity without inducing apoptosis [71]. Moreover, HLA-G is expressed at high levels on DC-10 cells, human DCs with tolerogenic activity and an outstanding ability to produce IL-10 [72]. Interestingly, the expression of membrane-bound HLA-G1 and its receptors is upregulated by IL-10 on DC-10 and the expression of high levels of membrane-bound HLA-G1, ILT4, and IL-10 by DC-10 is critical to the generation of allergen-specific Tr1 cells by DC-10.

Whereas LILRB1 is expressed by NK cells, T cells, DCs, and decidual macrophages, LILRB2 expression is restricted to monocytes, macrophages, and DCs. These receptors can bind both classical and nonclassical HLA-I molecules [73, 74]. However, they present more affinity for HLA-G than for classical HLA-I molecules [75]. Also, HLA-G interaction with LILRB1 on NK cells and the resultant inhibitory function do not require tumor cell lipid raft integrity [76]. This differs from classical HLA-I, which are recruited in lipid rafts upon receptor engagement [77].

LILRB1 and LILRB2 possess 4 extracellular domains (D1–D4) and four and three immunoreceptor tyrosine-based inhibitory motifs (ITIMs), respectively, in their long cytoplasmic tails. These ITIM motifs confer them inhibitory characteristics, contrary to other LILR family receptors with activating properties that lack these ITIM motifs and possess an Arg residue in the transmembrane domain [74]. Interaction of LILRB1 and LILRB2 with their ligands causes phosphorylation of these ITIMS and recruitment of SHP phosphatases that initiate the inhibitory cascade. The D1 and D2 domains mediate the interaction of these receptors with HLA-I molecules and in the case of LILRB1 that occurs with the α3-domain and β2-m [74]. In fact, β2-m free HLA-G molecules are not recognized by LILRB1 [78]. However, in the case of LILRB2, it seems that interactions of these receptors with HLA-I molecules implicate the conservative residues of α3-domain but not of β2-m [73, 74]. HLA-G can form dimers that bind to LILR receptors with even a higher affinity than HLA-G monomers [79], being able to bind two receptors simultaneously [80].

Another HLA-G receptor is KIR2DL4 or CD158d, the only receptor of the killer cell immunoglobulin-like receptors (KIR) family that is expressed in all NK cell types [67]. KIR family includes receptors with activating properties and receptors with inhibitory properties. KIR2DL4 has unique structural properties among the rest of KIR receptors: it possesses a long cytoplasmic tail characteristic of inhibitory receptors, a charged amino acid in the transmembrane domain similarly to activating KIR receptors (reviewed [81]), and a mixed structure in the extracellular part with D0 and D2 domains. Contrary to other KIR receptors, KIR2DL4 expression is transitory on NK cell surface, with a main expression in endosomes, reached by an endocytic process. KIR2DL4 seems to participate to HLA-G endocytosis when it is transient expressed on NK cell surface, as both HLA-G and KIR2DL4 can be simultaneously colocalized in endosomes [82]. This could explain why soluble HLA-G or anti-KIR2DL4 antibodies, but not solid-phase bound antibodies, can induce cytokine secretion by NK resting cells. However, KIR2DL4 expression can be induced by IL-2 and its activation upon antibodies engagement provokes a weak cytotoxic activity with a strong IFN-γ production [83].

In vitro studies have shown that KIR2DL4 is able to interact with β2-m free HLA-G molecules, inducing IFN-γ production [84] and increasing NK cell cytotoxicity [19]. Contrary to LILR receptors, KIR does not bind HLA-I molecules through its α3 domain but through α1 and α2 domains which are much more polymorphic than α3 domain [85, 86]. This could account for the broader specificity of LILR receptors in comparison with KIR2DL4 that binds specifically HLA-G and no other HLA-I molecules. Also, structural studies suggest that KIR2DL4 cannot bind HLA-G dimers due to steric reasons [22].

The expression of LILRB1, LILRB2, and KIR2DL4 can be induced by HLA-G without any costimulatory requirement, which indicates that it can occur independently from any immune response [87].

Besides these receptors, HLA-G can also bind to CD8 without TCR interaction, provoking NK cells and activated

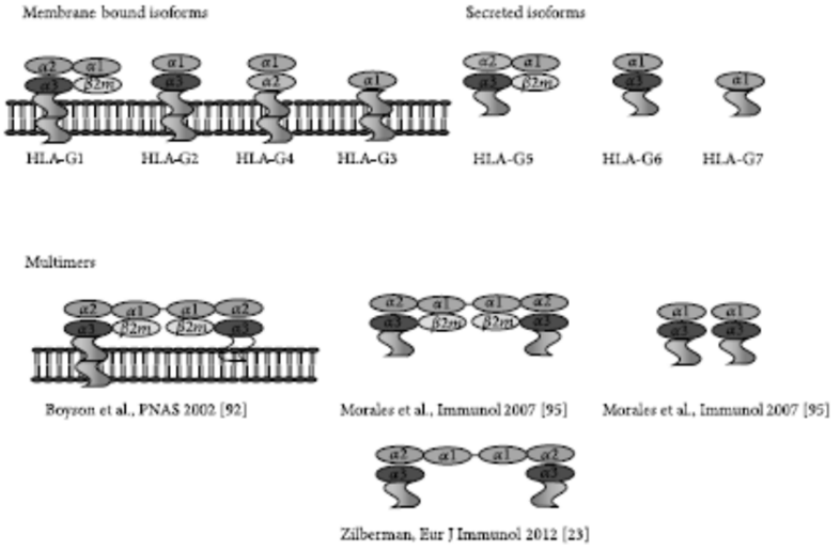

FIGURE 2: HLA-G isoforms and conformations. Membrane and soluble HLA-G isoforms are reported as monomeric and dimeric conformation Zilberman, Eur J Immunol 2012 [23].

CD8+ T cells apoptosis, and FasL upregulation and secretion [88]. Another putative HLA-G receptor is CD160. Interaction of HLA-G with CD160 expressed by endothelial cells induces the apoptosis of these cells [89] and inhibits cell proliferation, migration, and tubule formation [90], inhibiting the angiogenic process.

5. Posttranslational Modifications of HLA–G Molecule

Although most studies are related to β2-m bound HLA-G molecules that correspond to the originally described structure, several results have demonstrated the existence of modified variants of this structure. For example, expression of β2-m free HLA-G, which can be originated by dissociation of HLA-G complete isoforms [45], has been demonstrated in different tissues such as placenta [78] or pancreatic endocrine cells [91].

Besides Cys residues in α2 and α3 domains that allow intramolecular disulphide bonds, HLA-G molecule presents other important Cys residues. Cys42 in α1 domain and Cys147 in α2 domain can form intermolecular disulphide bonds giving rise to HLA-G dimers that can be observed by SDS/PAGE under nonreducing conditions [92]. These structures have been observed for all HLA-G isoforms except HLA-G3 [93]. It has been estimated that about 40% of HLA-G molecules at trophoblastic cells surface are in a dimeric form; meanwhile, only a small fraction of soluble HLA-G would be constituted by HLA-G dimers [94]. Even more, villous cytotrophoblast cells can produce dimers of β2-m free HLA-G5 molecules [95].

Immunoblot analysis with 4H84 antibody rends bands of diverse molecular weights (35–50 kDa) due to a glycosylation of HLA-G through an Asn residue (Asn86). This modification has been observed for both soluble and membrane bound HLA-G [96]. Another posttranslational modification observed in HLA-G is nitration in Tyr residues. Presence of 3-nitrotryrosine in HLA-G has been demonstrated in vivo in biological fluids both in monomeric and multimeric form [42] and in vitro after treatment with NO donors, which also increase HLA-G shedding by metalloproteases [43]. The detection of this modified HLA-G may characterize HLA-G synthesized at sites of inflammation where there is an important peroxide production.

Recently, HLA-G of molecular weights (70–76 kDa) higher than those expected were observed in biological fluids even when SDS/PAGE prior to western blot was performed under reducing conditions [64]. These molecules were associated with β2-m and could form dimers through disulphide bonds. The importance of these structures resides in the fact that they are not equivalently recognized by anti-HLA-G antibodies and can originate discrepancies in HLA-G quantification results. These molecules were later identified as ubiquitinated HLA-G molecules [97] with an intracellular origin demonstrated by their presence in exosomes, which are microvesicles of 50–100 nm originated from the endolysosomal pathway and secreted by many different cell types [98].

These particles carries mRNA, miRNA, and proteins, such as classical HLA-I molecules [98], and can exert distant immune functions [98]. Exosomes could act as a mechanism

to spread HLA-G tolerogenic functions because HLA-G presence has been demonstrated in exosomes produced by melanoma cells [99] and by early and term placenta [100]. Furthermore, in serum from pregnant women HLA-G can be detected incorporated into exosomes [101].

6. Analytical Challenging in Soluble HLA-G Analysis

Searching in PubMed with the words HLA-G and ELISA there are 175 papers published until November, 2013, measuring soluble HLA-G in different biological fluids, including serum, plasma, and exudates. From these papers, it is clear that the measurement of soluble HLA-G is a potential biomarker for diagnostic and/or prognostic in some physiopathological situations, such as obstetric complications or cancer [102]. In addition detectable levels of soluble HLA-G in medium from embryo culture are associated with success in vitro fertilization. For this reason, the disposal of a good and widely accepted method to measure the soluble HLA-G levels is of crucial importance to achieve a good translation of results between different laboratories. Most are in-house ELISA assays (Table 1) using as capture antibody the mAb MEM-G/9, which has been raised against recombinant human HLA-G refolded with β2-m and peptide [56]. Other ELISAs are designed to measure exclusively HLA-G5 and/or -G6 using anti-pan HLA-I antibody W6/32 as detection antibody and the antibody 5A6G7 as capture antibody [103], that reacts with the intron 4, which is exclusive of these two isoforms [104]. As detection antibody most assays use an anti-β2-m antibody or W6/32. These assays perform very well in vitro using cell cultures, but the procedure for HLA-G measurement is far from being resolved in vivo, and it has been a source of conflicting results and interesting discussions [105–108]. More than 15 years have passed since first reports of a method for measuring HLA-G [55] and meanwhile some important efforts have been carried out by several authors to validate a method and a standard to measure soluble HLA-G [58]. However, four main problems remain to be solved: the identification of the main circulating HLA-G molecules in vivo, the obtaining of a purified standard widely available, the selection of the antibodies used in the procedure, and the sensitivity of the methodology.

Probably the most important issue is related to the types of HLA-G molecules present in biological fluids, as we do not know yet the predominant isoform and whether it circulates free or included in microvesicles, that is, exosomes [64], or if they are mainly free molecules or associated with β2-m, or even the influence of modifications such as dimerization [92], nitration [42], or ubiquitination [97]. The presence of these altered structures could be more relevant in cancer where there is a deeply altered microenvironment. Probably, the predominant structures in biological fluids are the dimeric or multimeric forms, considering that the extracellular redox status is more oxidized than the redox status and that there is a low proportion of free SH groups from the Cys in circulation [41]. It is not known if these proteins react equally with different antibodies employed to measure HLA-G in ELISA. Assuming the statement that only shed HLA-G1 and HLA-G5 are released to circulation, we and others have calculated the amount of sHLA-G1 by the difference between the concentrations of sHLA-G1/HLA-G5 (using MEM-G/9 as capture mAb) and HLA-G5 (using 5A6G7 as capture mAb) [109]. However, under the new vision of circulating HLA-G molecules we cannot be sure now that this always occurs in vivo. Unexpected results probably due to anomalous structures were already documented in the Wet-Workshop for Quantification of Soluble HLA-G held in 2004 [58]. In this workshop it was observed in some samples that there were HLA-G 5A6G7-immunoreactive molecules that were not recognized by MEM-G/9. These different structures were later elucidated to be new high molecular weight HLA-G complexes [64].

A second important problem is the lack of a widely available purified HLA-G molecule that could serve as a standard. The only commercial soluble HLA-G available kit nowadays for quantitative measurement (EXBIO Praha, Czech Republic) uses a sHLA-G standard calibrator in terms of arbitrary units/mL, but its equivalence to a protein concentration or biological activity is unknown. A high useful method to produce a protein is by plasmid transfection in bacteria, and both HLA-G1 and a fusion protein have been produced by this methodology [110]. As synthetized in a prokaryotic model, there are not the posttranslational modifications produced in eukaryotes, mainly glycosylation [96], and probably their conformation is not equivalent to the native protein. For example, the fusion proteins produce inhibition in NK cells only at levels much higher than the native protein. HLA-G5 molecules purified from detergent lysates of SP9 cells transfected with HLA-G5 and human β2-m have been used as a standard [58], while others have purified the protein from HLA-G transfected cell culture supernatants by affinity chromatography [111]. Also, other studies use dilutions of tested cell supernatants as standard, but the concentrations obtained cannot be extrapolated to other studies [109]. A standard widely available that could serve for data comparison between different laboratories could be of interest, so data could be transferred between papers. Until this standard becomes available, HLA-G level comparisons between different laboratories should be taken with caution. Same precautions should be taken when transferring the reference values that depend on not only both the standard and methodology used, but also on the population studied.

The third issue is related to the capability of the antibodies to recognize all forms of HLA-G. Most of the anti-HLA-G antibodies used in the ELISA recognize the native protein, are very specific, and do not react with other HLA-I molecules (Table 1) as it has been discussed elsewhere [112]. We do not know yet if the reaction is equimolar with all HLA-G molecules, and probably some proteins could be underrecognized. For example, it was recently shown by flow cytometry that MEM-G/9 can also react with HLA-G3, but the intensity of the signal is weaker than with HLA-G1 [113]. Some HLA-G complexes are underrecognized by MEM-G/9 and react better with the anti-HLA-G antibody G-233 [64]. In addition, although HLA-G polymorphism is quite low with only 16

Table 1: Examples of methods developed for measuring soluble HLA-G.

Type	Standard	Capture antibody	Detection antibody	Detection	Detection limit	Reference
ELISA-sandwich	HLA-GI/LCL 722.221 transfected cells	W6.32 after depletion with TP25.99	anti-β2-m	Colorimetric	2.1 ng/mL	[55]
ELISA-sandwich	None	87G, BFL1 or MEM-G/9	W6/32	Colorimetric	O.D.	[56]
ELISA-sandwich	HLA-G transfected CHO cells	G233	56B	Colorimetric	1 ng/mL	[57]
ELISA-sandwich	HLA-G5 protein derived from insect SP9 cells	MEM-G/9	anti-β2-m	Colorimetric	5 ng/mL	[58]
ELISA-sandwich	HLA-G5 protein derived from insect SP9 cells	5A6G7	W6/32	Colorimetric	5 ng/mL	[58]
Luminex	HLA-G5 and beta2 m transfected SP9 cells	MEM-G9	anti-β2-m	Fluorescence	0.3 ng/mL	[59]
ELISA-sandwich	HLA-G transfected LCL 721.221 cells	MEM-G/9	W6/32	Fluorescence	1 ng/mL	[60]
Bio-Plex	HLA-G5 transfected HeLa cells	MEM-G/9	W6/32	Fluorescence	0.3 ng/mL	[61]
ELISA-sandwich	HLA-G transfected LCL 721.221 cells	MEM-G/9	W6/32	Chemiluminescence	2 ng/mL	[62]
ELISA-sandwich	Purified HLA-G	HGY (noncommercial)	Polyclonal anti-HLA-G	Colorimetric	1 U/mL	[63]
ELISA-sandwich	HLA-GI transfected LCL-721.221 cells	G233	anti-β2-m	Colorimetric	4 ng/mL	[64]

proteins described to date, we do not know yet how they affect the binding to the antibodies. Of particular interest is that although the capture antibody in ELISA is HLA-G specific, only a few authors have used a specific antibody for HLA-G as detection antibody [63]. Instead, as we mentioned before, the detection antibody used in most of the ELISAs is an anti-β2-m antibody. The fact that HLA-GI and -G5 from cell cultures are complexed with β2-m does not imply that the same occurs always in vivo in all clinical situations. Some of HLA-G released by the embryo is not bound to β2-m [95], so these molecules would not be detected in this type of assays. Although some authors have used the anti-HLA-G mAb 4I1B4 in ELISA [114], its use is not recommended as it can produce some nonspecific reactions with classical HLA-I molecules, under certain methodological conditions [113].

Finally, another problem to be solved is the sensitivity of the method. An important issue is that neither the functional sensitivity nor the analytical sensitivity is usually reported. Most methods are sandwich-ELISAs with colorimetric detection, whose reported detection limit is in the order of 1–10 ng/mL, and soluble HLA-G levels are below this detection limit in many occasions. Thus, it is not known if there is no circulating HLA-G or if the procedure is not sensitive enough for quantification of low HLA-G levels. Some authors have improved the methodology, using fluorescence detection or with procedures based on microspheres technology. The detection limit decreased one order of magnitude compared to the colorimetric based ELISA methodology [59, 61]. This last methodology seems more appropriate for measuring HLA-G in media from embryo culture during in vitro fertilization [59].

7. Conclusions

HLA-G is a molecule that has been deeply studied during the last two decades where the almost exclusive expression in placenta has been well documented. When the HLA-G gene is expressed, it can produce seven isoforms that exert immune-suppressive functions by binding to its receptors. However, there are some important basic concepts in its biochemistry that remain not well explained yet. Among them, the knowledge of the regulation of the protein expression is a corner stone to understand how it can be expressed ectopically in different pathological situations. This could help to induce HLA-G in a tissue when a suppressive action is convenient (e.g., organ transplantation) or to suppress it when its expression is harmful (e.g., tumor). Also, along recent years multiple HLA-G protein modifications have been described, such as HLA-G dimers that bind LILRB receptors with an affinity even higher than monomers, or nitration.

Moreover, high molecular weight molecules of HLA-G have been described as HLA-G complexed with ubiquitin. Furthermore, circulating HLA-G has also been observed as included in exosomes. The complete identification of these circulating HLA-G structures would improve not only the knowledge of this molecule but also the design of better methods for analysis. These are important questions that should be elucidated in order to understand the biology of HLA-G and to clarify some discrepant results.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Matrix metalloproteinase-2 (MMP-2) generates soluble HLA-G1 by cell surface proteolytic shedding

Roberta Rizzo · Alessandro Trentini · Daria Bortolotti · Maria C. Manfrinato · Antonella Rotola · Massimiliano Castellazzi · Loredana Melchiorri · Dario Di Luca · Franco Dallocchio · Enrico Fainardi · Tiziana Bellini

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Abstract Human leukocyte antigen-G (HLA-G) molecules are non-classical HLA class I antigens with an important role in pregnancy immune regulation and inflammation control. Soluble HLA-G proteins can be generated through two mechanisms: alternative splicing and proteolytic release, which is known to be metalloprotease mediated. Among this class of enzymes, matrix metalloproteinases (MMPs) might be involved in the HLA-G1 membrane cleavage. Of particular interest are MMP-2 and MMP-9, which regulate the inflammatory process by cytokine and chemokine modulation. We evaluated the effect of MMP-9 and MMP-2 on HLA-G1 membrane shedding. In particular, we analyzed the in vitro effect of these two gelatinases on the secretion of HLA-G1 via proteolytic cleavage in 221-G1-transfected cell line, in JEG3 cell line, and in peripheral blood mononuclear cells. The results obtained by both cell lines showed the role of MMP-2 in HLA-G1 shedding. On the contrary, MMP-9 was not involved in this process. In addition, we identified three possible highly specific cleavage sites for MMP-2, whereas none were detected for MMP-9. This study suggests an effective link between MMP-2 and HLA-G1 shedding, increasing our knowledge on the regulatory machinery beyond HLA-G regulation in physiological and pathological conditions.

Keywords HLA-G · Matrix metalloproteinase · Protein shedding · Inflammation

Abbreviations

HLA-G	Human leukocyte antigen-G
MPase	Metalloproteinase
MMP	Matrix metalloproteinase
ADAM	A disintegrin and metalloproteinase

Introduction

Human leukocyte antigen-G (HLA-G) molecules are non-classical HLA class I antigens with an important role in pregnancy immune regulation and inflammation control [1]. On one hand, the expression of HLA-G molecules is fundamental for creating a tolerogenic environment at the maternal–fetal interface [2–4] and in transplanted patients [5–9]. On the other hand, the presence of HLA-G molecules facilitates tumors [10–13], virus immuno-escape [14], and is implicated in the pathogenesis of several diseases such as multiple sclerosis [15–20], rheumatoid arthritis [21, 22], and psoriasis [23].

The functions of HLA-G molecules are carried out through the interaction with inhibitory receptors, ILT-2

Roberta Rizzo and Alessandro Trentini have contributed equally to this study.

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R. Rizzo (✉) · D. Bortolotti · A. Rotola · L. Melchiorri · D. Di Luca
Section of Microbiology and Medical Genetics, Department of Medical Sciences, University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy
e-mail: rbr@unife.it

A. Trentini · M. C. Manfrinato · M. Castellazzi · F. Dallocchio · T. Bellini
Department of Biomedical and Surgical Sciences, University of Ferrara, 44121 Ferrara, Italy

E. Fainardi
Neuroradiology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliera-Universitaria di Ferrara, 44121 Ferrara, Italy

(LILRB1/CD85j), ILT-4 (LILRB2/CD85d), CD8, and KIR2DL4 (CD158d), at the surface of immune cells [24–29]. The ligation of HLA-G with these receptors induces the apoptosis of activated CD8+ T cells [30], acts on T regulatory cells [31], modulates the activity of natural killer cells [32] and of dendritic cells [33, 34], and blocks allo-cytotoxic T lymphocyte response [35].

HLA-G exists as four membrane-bound (HLA-G1, -G2, -G3, and -G4) and three secreted soluble isoforms (HLA-G5, -G6, and -G7) generated by alternative splicing of the primary transcript [36]. In addition, the HLA-G1 transmembrane isoform can produce a soluble form (sHLA-G1), by proteolytic shedding, which retains all the functions of the membrane counterpart [37]. Several lines of evidence indicate that the sHLA-G1 form is generated through the shedding of the membrane-bound HLA-G1 by metalloproteinase (MPase) pathways [37–40]. However, the identity of this membrane-bound MPase is still unknown, although potential candidates seem to belong to a disintegrin and metalloproteinase family [41]. In addition to these proteases, soluble MPases might be also involved in the shedding of HLA-G1: some of the largest families of such proteases are matrix metalloproteinases (MMPs). MMPs are zinc-containing and calcium-requiring endopeptidases known for their ability to cleave several extracellular matrix constituents as well as non-matrix proteins [42, 43]. Increased expression of MMPs was observed in several human diseases such as cutaneous epithelial tumors [44], colon cancer [45], multiple sclerosis [46, 47], and Duchenne muscular dystrophy [48], suggesting an implication of these enzymes in the immune defense, inflammation, and repair mechanisms [49]. In particular, MMP-9 and MMP-2, two MPases that belong to the gelatinases family, are able to regulate the inflammatory process by cytokine and chemokine activation/inactivation [49–51].

Taking into account the ability of both HLA-G and gelatinases to modulate immune response during inflammatory conditions, in the present work, we planned a focused approach to evaluate the effect of MMP-9 and MMP-2 on HLA-G1 membrane shedding. In particular, we analyzed the effect of these two gelatinases on the secretion of HLA-G1 via proteolytic cleavage.

Materials and methods

Transfected cell lines

Parental 721.221B-LCL HLA null lympho-blastoid cell line and HLA-G1-transfected 221-G1 cell lines were used for the study. HLA-G transfectants were described elsewhere [52]. The parental 721.221 cell line and transfectant were maintained in RPMI 1640 medium supplemented with glutamine and antibiotics and with or without (FBS-free experiments) 10 % fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT, USA). 221-G1 transfectants were selected in the presence of antibiotic G418.

Jeg3 cell culture

Human choriocarcinoma trophoblastic cells (JEG-3) were used as positive control for the constitutive expression of HLA-G [53]. The cells were cultured in RPMI medium (Sigma-Aldrich, St. Louis, M, USA) with glutamine and antibiotics with 10 % FBS and maintained at 37 °C in a sterile humid atmosphere under 5 % CO_2 and 95 % O_2. No FBS was added for the FBS-free experiments.

PBMC culture

Peripheral blood mononuclear cells (PBMCs) from five healthy donors were isolated from whole blood by ficoll gradient (Cederlane, Hornby, ON, Canada) and resuspended in RPMI medium (EuroClone, Milano, Italy), 100 U/ml penicillin, and 100 U/ml streptomycin (Sigma-Aldrich) with 10 % FBS. No FBS was added for the FBS-free experiments. 10^6 PBMCs were supplemented with 20 ng/ml of exogenous recombinant IL-10 [54] (PeproTech Inc., Rocky Hill, NJ, USA) to induce HLA-G expression.

mRNA preparation

Total cellular RNA was prepared from each cell culture with TRIzol reagent (Life Technologies, NY, USA) as described [55]. The RNA samples were digested with DNase. The quality and quantity of RNA samples were assessed by a 1 % agarose gel electrophoresis, followed by ethidium bromide staining. These mRNA samples were immediately used for cDNA synthesis or stored frozen at −80 °C until use.

RT-PCR reactions and analyses

To analyze the presence of HLA-G mRNA, 2 μg mRNA was reverse transcribed for each sample using a SuperScript™ First-Strand Synthesis System (Invitrogen, San Giuliano Milanese, MI, Italy) according to manufacturer's instructions. The quantification of RNA was checked with b-actin amplification by RT-PCR (for-5′-GCTGCTATC ACTTAGACCTCA-3′; rev-5′-CTTGTCACAGTGCAGCT CAC-3′) [56]. HLA-G mRNA was evaluated by RT-PCR (for-5′-AACCCTCTTCCTGCTGCTCT-3′; rev-5′-CTCCT TTTCAATCTGAGCTCTTCT-3′) with the following cycling parameters: 40 cycles of 30 s at 94 °C, 30 s at 55 °C, and 2 min at 68 °C, final extension at 68 °C for 5 min obtaining a 291-bp amplified fragment [57]. Jeg3

cell was used as HLA-G-positive control. The mRNA relative quantification was evaluated by a Geliance 600 system (Perkin Elmer, MA, USA).

Cytometric analysis

For flow cytometric analysis, cells (10^6) were washed and incubated for 30 min on ice in 100 µl of phosphate buffered saline (PBS) containing 1 % FBS, 10 mM sodium azide, and appropriately diluted fluorescent mAb. After two washes with cold washing buffer, cells were then washed, fixed in 2 % formaldehyde, and analyzed by flow cytometry with a FACSCount flow cytometer (Becton–Dickinson, San Jose, CA, USA) using standard settings and CellQuest software (Becton–Dickinson, San Jose, CA, USA) for data analysis. The membrane-bound HLA-G antigens were detected by anti-HLA-G FITC monoclonal antibodies (MoAbs) (87G; MEM-G9, Exbio, Praha, Czech Republic); HLA class I molecules were stained by W632-FITC MoAb (Exbio, Praha, Czech Republic). Beta-tubulin was analyzed by anti-beta-tubulin-FITC MoAb (Milli-Mark, Temecula, CA, USA) after cell fixation and permeabilization with Cytofix/Cytoperm™ Kit (BD Biosciences, San Jose, CA, USA). CD14 staining was performed by anti-CD14-PE MoAb (Sigma-Aldrich). Anti-isotype controls (Exbio) were performed.

sHLA-G ELISA

Soluble HLA-G (sHLA-G) levels in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) as previously reported [17, 19, 20], using as capture antibody the MoAb MEM-G9 (Exbio), which recognizes the HLA-G molecule in β_2-microglobulin-associated form, or 5A6G7 MoAb (Exbio), which recognizes the HLA-G5 and -G6 isoforms. Transfected 221-G1 or HeLa-G5 cell (kindly provided by Prof. E.Weiss, Institut fur Anthropologie und Genetik, LMU, Munchen, Germany) culture supernatants were used as standards. The intra-assay coefficient of variation (CV) was 1.4 % and the inter-assay CV was 4.0 %. The limit of sensitivity was 1.0 ng/ml.

sHLA-G immunoprecipitation

221-G1 cell culture supernatants were biotinylated with 0.2 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) in pH 8.0 PBS for 30 min at 4 °C [58]. Samples were then immunoprecipitated for 2 h at RT with anti-HLA-G MoAb (MEMG9, Exbio), washed twice in PBS, and incubated overnight with protein G-Sepharose beads (Santa Cruz, CA, USA) at 4 °C. The samples were washed twice and suspended in 20 µl of Laemli Buffer (BioRad, Segrate, MI, Italy).

Western blot analysis

The protein concentration in immunoprecipitates was quantified by the Bradford assay (Bio-Rad Laboratories) using serum bovine albumin (Sigma-Aldrich) as the standard. The purified sHLA-G1 molecules obtained from untreated 221-G1 culture supernatants were used as positive control. Total protein was denatured at 100 °C for 5 min. Proteins were loaded with reducing buffers in 10 % TGX-Pre-cast gel (Biorad), with subsequent electroblotting transfer onto a PVDF membrane (Millipore). The membrane was incubated with a horseradish peroxidase (HRP)-conjugated streptavidin (Thermo Scientific, Rockford, IL, USA) and developed with the ECL kit (Amersham Biosciences, NJ, USA). The images were acquired by the Geliance 600 (Perkin Elmer).

Cell treatments

For the MMPs' effect, cells were placed in serum-free medium and treated with different amounts (1, 2, 4, 6 ng/ml) of recombinant human MMP-9 or MMP-2 (R&D System) and pre-activated with 1 mM APMA (GE Healthcare, Milan, Italy) for 2 h at 37 °C. The activation of both MMP-9 and MMP-2 was confirmed by zymography (Supplementary Fig. 1). The MMPs' function was determined by commercially available activity assay systems (GE Healthcare) as previously described [59]. Then, the APMA was removed by the Biogel-P6 (BioRad) gel filtration. For the MMPs' inhibition, cells were treated with 10 mM EDTA (Calbiochem, Merck Millipore, Darmstadt, Germany) [28]. Cycloheximide at 20 µg/ml (Sigma) was used for protein synthesis inhibition.

SDS-PAGE and Western blot

We performed an SDS-PAGE on the culture supernatants from untreated and MMP-2-treated 221-G1 cells. The gel was stained with the Colloidal Blue Staining Kit (Invitrogen). The gel was then electroblotted onto a PVDF membrane (Millipore), increasing the blot transfer time 1.5 times, to overcome the interference of the Coomassie staining [60]. The membrane was incubated with MEMG1 MoAb (Exbio), revealed with HRP-conjugated antimouse antibody (Amersham Biosciences, NJ, USA), and developed with the ECL kit (Amersham Biosciences, NJ, USA). The images were acquired by the Geliance 600 (Perkin Elmer).

Cell viability

The evaluation of the cell viability was conducted using Trypan blue dye (Lonza, Italy) by preparing a 1:1 dilution of the cell suspension using a 0.4 % Trypan Blue solution. Cells were observed by microscope: Non-viable cells were visualized in blue; viable cells were unstained.

HLA-G1 sequence analysis

The primary sequence of human HLA-G1 (Uniprot identifier Q8WLS1) was compared with known MMP-9 and MMP-2 cleavage recognition sites retrieved from the MEROPS database and the literature to identify specific recognition sites for both proteases [61], using the Site-Prediction comparison program [62]. Sites with homology >60 % (from the BLOSUM 62 matrix score), and giving an *N*-terminal fragment of at least 30 kDa, were considered in the analysis. Moreover, cleavage sites after the amino acid 281 (305) were excluded due to the presence of a transmembrane sequence not accessible to these proteases. Secondary structure and residue solvent accessibility were calculated by the program.

Statistical analysis

The differences in HLA-G1 levels were evaluated by the Mann–Whitney U test using Stat View software (SAS Institute Inc, Cary, NC, USA). p value was considered to be statistically significant when <0.05.

Results

Basal HLA-G1 shedding by 221G1 and 7721.221 cell lines

Firstly, we confirmed that 7221.221 B-LCL cell line did not express any HLA antigen (Fig. 1a, b), while 721.221-HLA-G1-transfected cell line (221-G1) constitutively expresses membrane HLA-G1 as confirmed by flow cytometry (Fig. 1a) and mRNA (Fig. 1b) analysis. 221-G1 cells are characterized by a basal HLA-G1 shedding in complete medium, that decreased in the absence of FBS (Fig. 1c), which is known to contain proteases. Since the HLA-G1 membrane expression (Fig. 1d) and cell viability (data not shown) were not affected by the absence of serum, we selected this culture condition to reduce the HLA-G1 constitutive shedding and avoid the presence of exogenous gelatinases. To be sure that no HLA-G5 was expressed, we performed the analysis of cell culture supernatants with a specific HLA-G5 ELISA assay, with 5A6G7 MoAb. No HLA-G5 molecules were detected (Supplementary Fig. 2a).

Induced HLA-G1 shedding via MMPs

We added exogenous MMP-2 and MMP-9 to 221-G1 FBS-free cultures and quantified the amount of membrane and shedded HLA-G1. This in vitro approach allowed the selective evaluation of HLA-G1 shedding without the presence of other exogenous confounding molecules. Thus, we performed time course/dose dependence experiments. As depicted in Fig. 2a, b there was increased HLA-G1 shedding after the addition of MMP-2, but not of MMP-9. In particular, MMP-2 had a time-dependent effect on HLA-G1 shedding from 2 to 6 h of incubation with 2 ng/ml as the lowest concentration that induced a significant sHLA-G1 increase, with a peak after 6 h (Fig. 2a; sHLA-G1 221-G1: 5.8 ng/ml; sHLA-G1 221-G1 + MMP-2 2 ng/ml: 20.9 ng/ml) ($p < 0.001$; Mann–Whitney U test). On the contrary, the addition of MMP-9 did not affect the constitutive HLA-G1 shedding, which showed comparable levels with the untreated cells (Fig. 2b; sHLA-G1 221-G1: 5.8 ng/ml; sHLA-G1 221-G1 + MMP-9 2 ng/ml: 5.3 ng/ml) (p = NS; Mann–Whitney U test). Of note, the levels of active MMP-2 and MMP-9 did not change over time (Fig. 2c).

Indeed, the increase of sHLA-G1 in the culture supernatants of 221-G1 MMP-2-treated cells, detected by the ELISA technique, correlated with a decreased percentage of membrane HLA-G1-expressing cells, visualized by flow cytometry (Fig. 3a, b). In particular, we evidenced 30 % of cells without HLA-G membrane expression after 6 h of MMP-2 exposure. On the contrary, HLA-G-positive cells presented the same level of HLA-G expression as in the untreated condition.

The Western blot analysis (Fig. 3c) of cell culture supernatants showed the presence of a band at 39 kDa confirming the ability of MMP-2 to shed a HLA-G molecule with a native dimension and folding, as the anti-HLA-G MEMG9 MoAb is able to recognize native HLA-G molecules [63]. We could not exclude the fact that MMP-2 could further process HLA-G molecules in lower MW proteins. For this, we performed an SDS-PAGE of the cell culture supernatants and stained the proteins with colloidal coomassie blue. We observed the presence of bands at 250, 100, and 10 kDa in both MMP-2-treated and untreated 221-G1 cell culture supernatants. The 39 kDa band is present in all the culture supernatants, with higher levels in 221-G1 cells treated with MMP-2 for 6 h. On the contrary, a band at 25 kDa is present in MMP-2-treated 221-G1 cells, while it is faint in untreated 221-G1 cell culture. This additional band could originate from HLA-G1 degradation. To answer to this question, we electroblotted the gel and

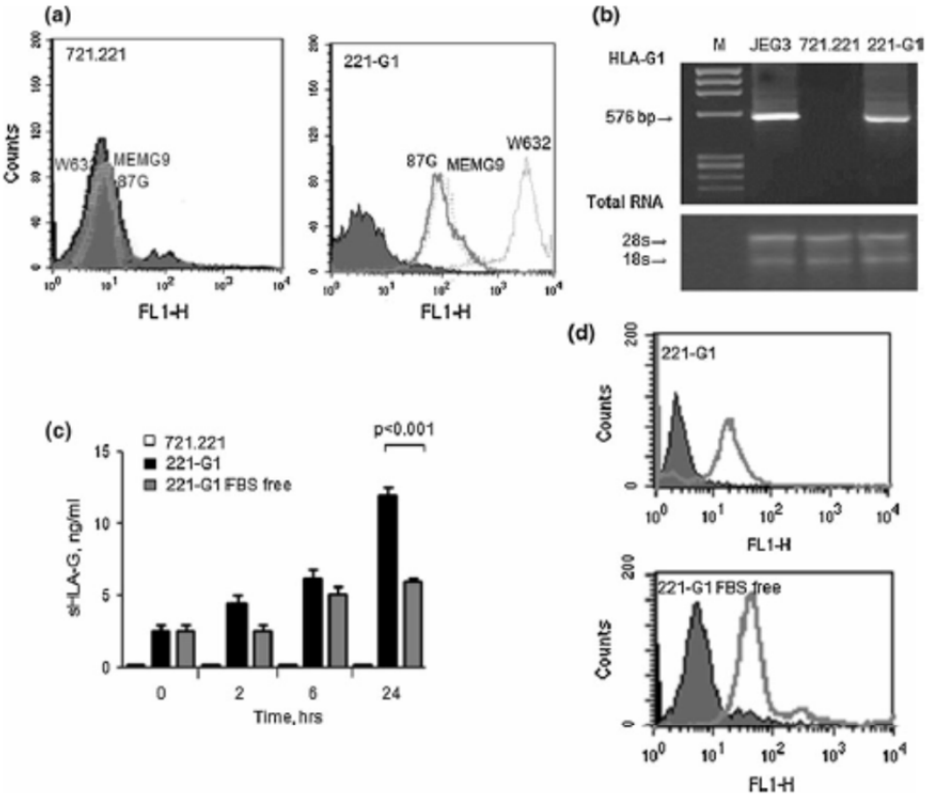

Fig. 1 HLA-G molecule expression in 721.221 and 221-G1 cell lines. **a** Representative flow cytometry of 221-G1 and 721.221 cells with anti-HLA-G FITC (87G, *gray line peak*, MEM-G9, *dotted line peak*, Exbio) and anti-HLA-I FITC (W632, *thin line peak*, Exbio) MoAbs. The *gray peak* corresponds to anti-isotype control; **b** HLA-G-specific mRNA quantification in 721.221 and 221G1 cell lines. JEG3 cell line was used as positive control. *M* DNA ladder marker (IX Marker, Roche); **c** sHLA-G levels in culture supernatants of 7221.221 (*white histogram*) and of 221-G1 in complete medium (*black histogram*) or in FBS-free medium (*gray histogram*) during a time point experiment (0, 2, 6, 24 h); **d** representative flow cytometry with anti-HLA-G FITC (87G, *white peak*, Exibio) MoAb of 221-G1 in complete medium or in FBS-free medium after 24-h culture. The *gray peak* corresponds to anti-isotype control. Mean ± SD is reported. Significant *p* values obtained by Mann–Whitney U test are reported

stained with MEMG1 MoAb, specific for HLA-G molecules. The results showed that the 39 kDa band corresponded to HLA-G1. The highest bands at 250 and 100 kDa are not stained with MEMG1 MoAb. The lowest band at 10 kDa could contain degraded HLA-G that lacks the antigenic epitope for MEMG1. A faint band at 25 kDa is evidenced in both Coomassie SDS-PAGE and Western blot analysis and it could correspond to a degraded HLA-G molecule, as previously suggested [64].

EDTA affected HLA-G1 shedding via MMP-2

To confirm the activity of MMP-2 in HLA-G1 shedding, we treated 221-G1 cells with EDTA, a non-specific MMP inhibitor [28]. The addition of EDTA in the presence or absence of MMP-2 did not affect cell viability (data not shown). Of note, EDTA suppressed the effect of MMP-2, reducing HLA-G1 shedding as confirmed by the unmodified sHLA-G1 (Fig. 4a) and HLA-G1 membrane (Fig. 4b, c) expression in comparison with untreated 221-G1 cells. Both MMP-2 and EDTA treatments did not induce HLA-G5 expression (Supplementary Fig. 2b).

Cycloheximide treatment

Since HLA-G1 mRNA transduction into protein could replace shedded HLA-G1 molecules at the surface of 221-G1 cells incubated with MMP-2, we treated 221-G1 cells with both MMP-2 and cycloheximide, a protein synthesis inhibitor [40]. The cycloheximide treatment did not

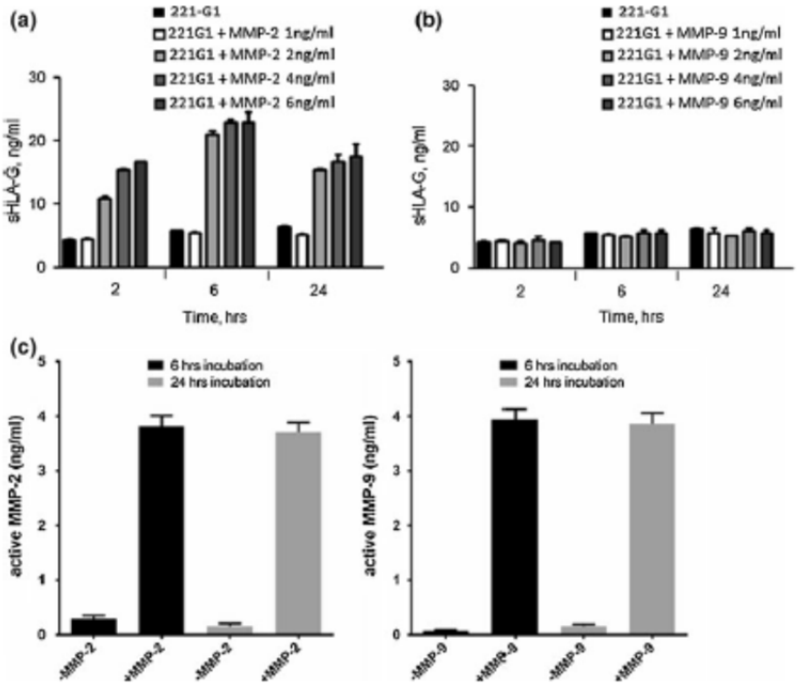

Fig. 2 HLA-G molecule shedding by MMPs. Evaluation of sHLA-G levels in 221-G1 FBS-free cultures during a time point experiment (2, 6, 24 h) without or with **a** MMP-2 or **b** MMP-9; **c** active MMP-2 (*left panel*) and MMP-9 (*right panel*) levels in supernatant culture of 221-G1 cells without (−MMP-2; −MMP-9) or with MMP2 or MMP-9 treatment (+MMP-2 or +MMP-9) after 6 h and 24 h of incubation. Mean ± SD is reported

affect cell viability (data not shown). On the contrary, protein transcription was reduced as documented by the decrease in house-keeping beta-tubulin protein content (Fig. 4d). HLA-G1 shedding was not modified by the addition of cycloheximide (Fig. 4e) to MMP-2 treatment. On the contrary, the expression of membrane-bound HLA-G1 decreased 36 or 10 % with MMP-2 or cycloheximide treatment, respectively (Fig. 4f) in comparison with untreated 221-G1 cells. The addition of both cycloheximide and MMP-2 in 221-G1 culture reduced by 60 % the membrane-bound HLA-G1 (Fig. 4f, g, $p < 0.0001$) in comparison with untreated 221-G1 cells. The reduction of HLA-G membrane expression with Cycloheximide and MMP-2 was 2-fold higher than the MMP-2 exposure alone. Both MMP-2 and Cycloheximide treatments did not induce HLA-G5 expression (Supplementary Fig. 2c).

Induced HLA-G1 shedding via MMP-2 in JEG3 cell lines

JEG3 cell line is characterized by a constitutive expression of HLA-G antigens. Thus, we treated JEG3 with MMP-2 and MMP-9 in time point experiments. As illustrated in Fig. 5a, MMP-2 had a time-dependent effect, with a significant increase in soluble HLA-G levels measured by ELISA, indicating a MMP-2-specific shedding. On the contrary, no modification occurred after MMP-9 treatment. Since we could not exclude the secretion of the HLA-G5 isoform [65] produced by mRNA alternative splicing, we evaluated the levels of HLA-G5 by ELISA with 5A6G7 MoAb (Fig. 5b). The HLA-G5 levels did not change after MMP-2 treatment, accounting for a specific HLA-G1 shedding induced by MMP-2 (Fig. 5c). The increased HLA-G1 shedding corresponded to a reduced HLA-G1 membrane expression (Fig. 5d, e).

Induced HLA-G1 shedding via MMP-2 in peripheral blood mononuclear cells

Peripheral blood mononuclear cells present low levels of basal membrane HLA-G expression. For this, we induced HLA-G expression by IL-10 treatment [54]. The addition of IL-10 induced HLA-G expression only on the surface of CD14+ cells and the treatment with MMP-2 reduced HLA-

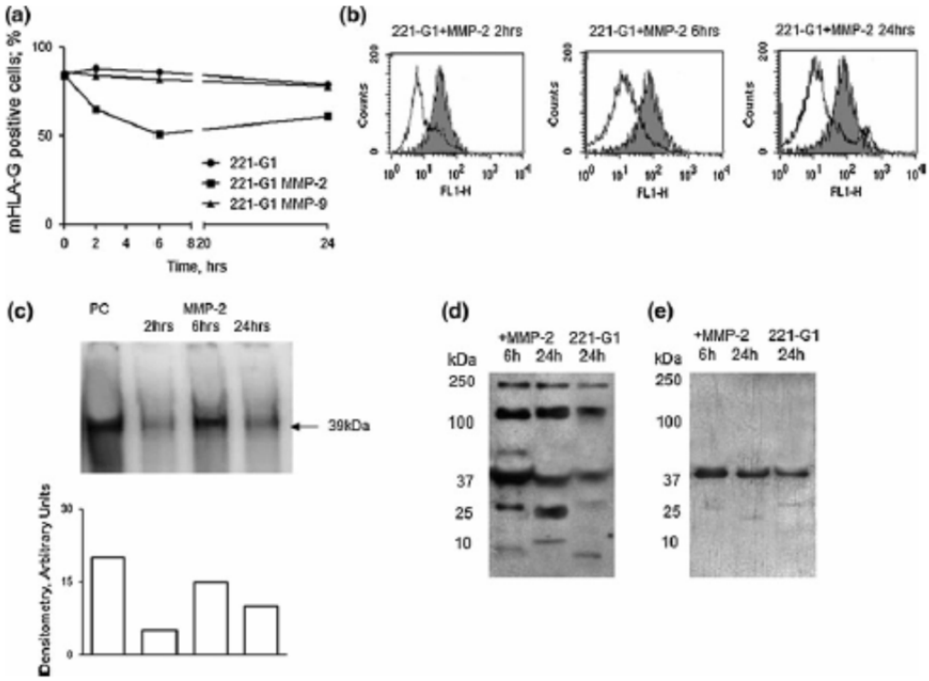

Fig. 3 HLA-G expression after MMPs' treatment. **a** Percentage of membrane HLA-G-positive 221-G1 cells, evaluated by flow cytometry with anti-HLA-G FITC (87G, Exibio) MoAb, without treatment or after 2 ng/ml MMP-2 or MMP-9 exposure for 2, 6, 24 h. **b** Flow cytometry with anti-HLA-G FITC (87G, *white peak*, Exibio) MoAb of 221-G1 in FBS-free medium after 2-, 6-, or 24-h culture with MMP-2 2 ng/ml (*white peak*). The *gray peak* corresponds to basal HLA-G membrane expression. **c** Western blot analysis of 221-G1 culture supernatants in FBS-free medium with 2 ng/ml MMP-2 in denaturing conditions, after immunoprecipitation with anti-beta2 microglobulin-associated HLA-G MoAb (MEMG9, Exbio). The positivity for HLA-G molecule was shown at 39 kDa. The densitometry results are reported as arbitrary units. *PC* positive control. **d** SDS-PAGE with Coomassie Blue staining of the culture supernatants of 221-G1 cells without (221-G1) or with MMP-2 (+MMP-2) exposure. **e** Western blot of the SDS-PAGE (**d**) stained with MEMG1 moAb (Exbio)

G1 membrane expression (Fig. 6a, b). As illustrated in Fig. 6c, after 6 h of incubation with MMP-2, there was a significant increase in soluble HLA-G levels measured by ELISA, indicating a MMP-2-specific shedding. Since we could not exclude the secretion of the HLA-G5 isoform [65] produced by mRNA alternative splicing, we evaluated the levels of HLA-G5 by ELISA with 5A6G7 MoAb (Fig. 6d). The HLA-G5 levels did not change after MMP-2 treatment, accounting for a specific HLA-G1 shedding induced by MMP-2 (Fig. 6e). On the contrary, no modification occurred after MMP-9 treatment (data not shown).

Analysis of HLA-G1 sequence for MMP-9 and MMP-2 cleavage recognition sequences

Comparison of the amino acid sequence of human HLA-G1 with MMP-9 and MMP-2 cleavage sequences retrieved from the MEROPS database (Fig. 7a, b, respectively) revealed the presence of three overlapping regions recognized by both proteases (Table 1, sequences from S1 to S3; Fig. 7c, in orange, Or). Since we did not find any increase in the shedding of membrane HLA-G1 in the presence of MMP-9, we can exclude these three regions as possible cleavage sites. Moreover, based on the data presented in the literature, we did not find any sequence preferentially recognized by MMP-9 (with Arg at both P2 and P1 sites, and Ser/Thr at P2′) [66]. On the contrary, we identified three possible cleavage sites with consensus motives (I/LXXXHy, XHySXL, and HXXXHy, from P3 to P1′), more selective for MMP-2 over MMP-9 [61]. In particular, the sequences S4 and S6 (Table 1; Fig. 7c, sequences in pink, Pi) seemed appropriate candidates for the specific cleavage from MMP-2, since the residues belonging to the sequence S5 were computed as not accessible to the solvent (buried residues, b). Interestingly, sequence S4 (HEGL) is similar to the cleavage site for MMP-2 within laminin-5 [67], characterized by good accessibility to the solvent.

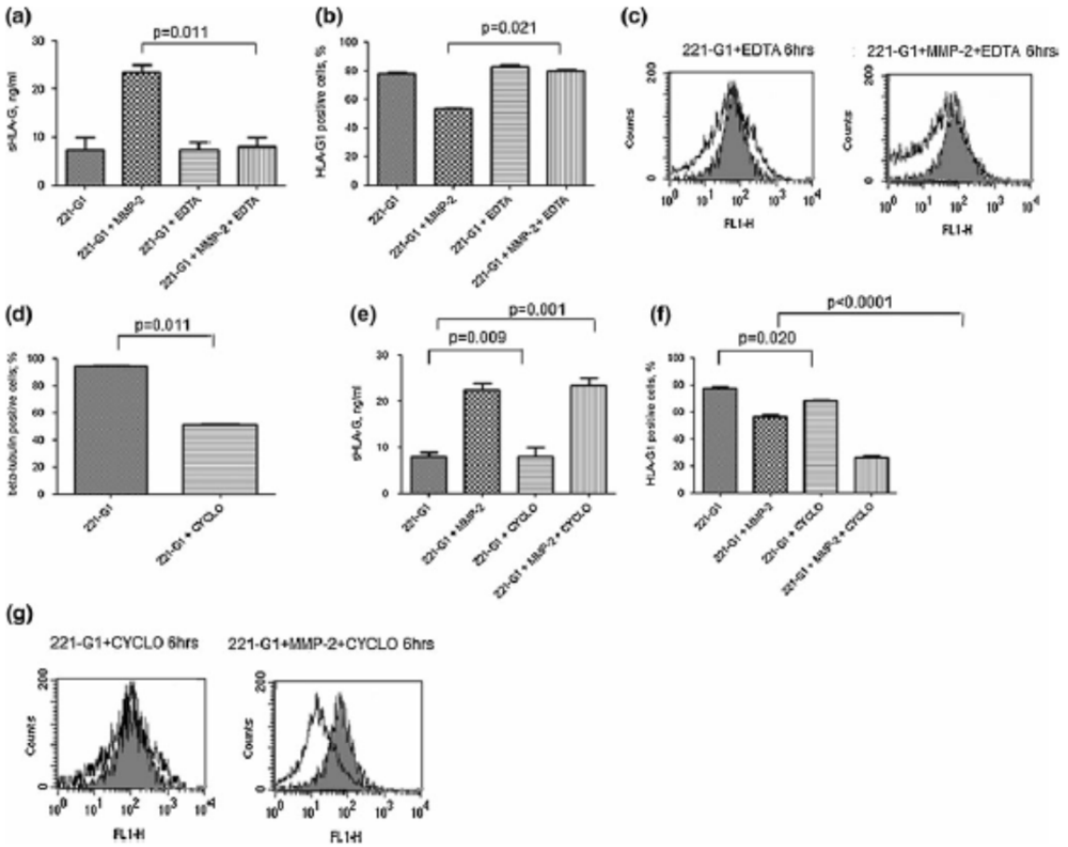

Fig. 4 MMP-2 blocking. 221-G1 alone, in the presence of MMP-2, EDTA (a non-specific MMP inhibitor), MMP-2 and EDTA for 6-h culture were evaluated for: **a** HLA-G1 shedding by ELISA test; **b**, **c** membrane HLA-G1 expression by flow cytometry with anti-HLA-G FITC (87G, Exibio) MoAb. Protein transduction inhibition. 221-G1 alone, in the presence of MMP-2, cycloheximide (CYCLO, protein synthesis inhibitor), MMP-2, and CYCLO for 6-h culture were evaluated for **d** house-keeping protein tubulin expression with anti-tubulin FITC (*Milli-Mark*) MoAb before (*gray histogram*) and after (*black and white histogram*) CYCLO treatment; **e** HLA-G1 shedding by ELISA test; **f**, **g** membrane HLA-G1 expression by flow cytometry with anti-HLA-G FITC (87G, Exibio). Mean ± SD is reported. Significant values obtained by Mann–Whitney U test are reported

Discussion

It is known that soluble HLA-G proteins can be generated through two mechanisms: alternative splicing and proteolytic release [36], which is known to be metalloprotease mediated [38]. Based on our results, we suggest a clear implication of MMP-2 in membrane HLA-G1 shedding.

The treatment of HLA-G1-expressing cells with active MMP-2 increased the basal shedding. This process seems to be MMP-2 dependent. In fact, the proteolytic shedding of HLA-G1 by MMP-2 was abolished by EDTA, a non-specific inhibitor of MMPs, and more pronounced in the presence of cycloheximide, a protein synthesis inhibitor. In contrast, the treatment with active MMP-9 did not affect the shedding of membrane-bound HLA-G1 protein, confirming that despite the high homology and overlapping substrate recognition profiles of these two proteases [61], this process was MMP-2 specific. Although the sequence of the protein shows several non-specific cleavage sites recognized by almost all MMPs (with the generic sequence PXX-X_{Hy}), we identified three possible highly specific cleavage sites for MMP-2, whereas none for the MMP-9 were detected. Of course, it will be necessary to determine the C-terminal sequence of the MMP-2-shed HLA-G1 protein to clearly identify the exact cleavage site.

It is noteworthy that the link we found between MMP-2 and HLA-G1 in transfected cells is strengthened by the results obtained in JEG3 cell lines, characterized by a

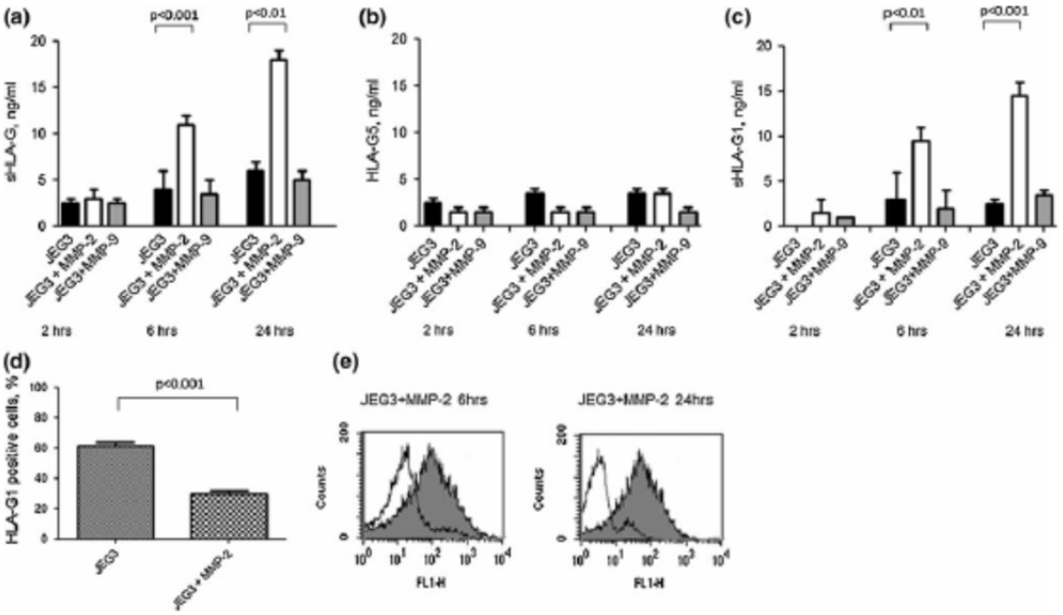

Fig. 5 HLA-G shedding in JEG3 cell lines. Evaluation of JEG3 during a time point experiment (2, 6, 24 h) without or with MMP-2 or MMP-9 exposure for **a** total sHLA-G, **b** HLA-G5 isoform levels, **c** sHLA-G1 isoform levels in culture supernatants; **d**, **e** membrane HLA-G1 expression by flow cytometry with anti-HLA-G FITC (87G, Exibio) MoAb. Mean ± SD is reported. Significant *p* values obtained by Mann–Whitney U test are reported

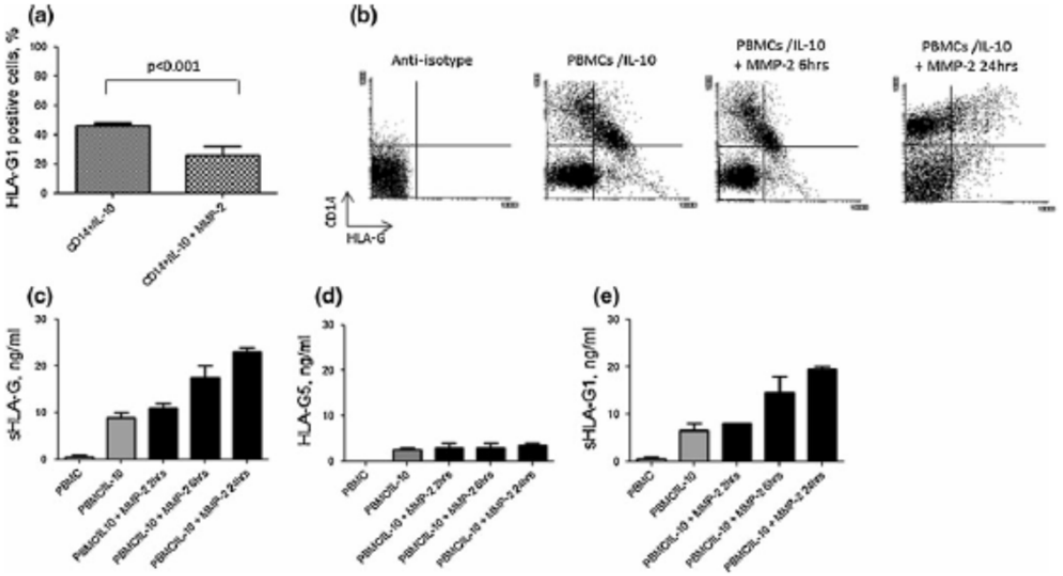

Fig. 6 HLA-G shedding in PBMCs. PBMCs were pre-treated with IL-10 to induce $CD14^+$ cell HLA-G expression. Evaluation of PBMCs during a time point experiment (2, 6, 24 h) without or with MMP-2 exposure for **a**, **b** membrane HLA-G1 expression by flow cytometry with anti-HLA-G FITC (87G, Exibio) MoAb; **c** total sHLA-G, **d** HLA-G5 isoform levels, and **e** sHLA-G1 isoform levels in culture supernatants. Mean ± SD is reported. Significant *p* values obtained by Mann–Whitney U test are reported

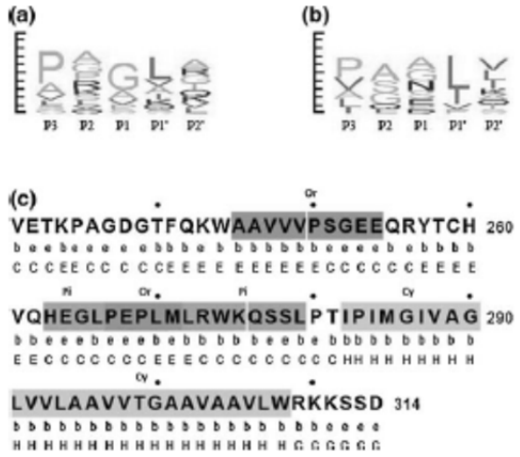

Fig. 7 Theoretical analysis for possible MMP-9 and MMP-2 cleavage sites within HLA-G1 sequence. Logo representation of the consensus motif retrieved from the MEROPS database and recognized by **a** MMP-9 and **b** MMP-2. **c** The sequences recognized by both MMP-9 and MMP-2 are highlighted in *orange* (or), whereas the sequences recognized only by MMP-2 are highlighted in *pink* (Pi). In *cyan* (Cy) is reported the transmembrane sequence of the HLA-G1. In this panel the solvent accessibility and the secondary structure predictions are also reported; *b* residue theoretically not accessible; *e* residue theoretically exposed; *C* chain loop; *E* extended β-strand; *H* helix. (Color figure online)

constitutive expression of HLA-G antigens. Indeed, the MMP-2-specific shedding was confirmed by the rise in the sHLA-G1 levels in the cell cultures and the decrease in membrane HLA-G expression. The same results were obtained in primary PBMCs, where MMP-2 was able to reduce $CD14^+$ cell HLA-G membrane expression and increase sHLA-G1 secretion, whereas MMP-9 did not show a considerable effect.

To note, all the cultures where tested for the presence of HLA-G5, and non-increase in the secretion of this isoform, obtained by mRNA alternative splicing, was documented.

In a previous work, Diaz-Lagares et al. [68] showed that MMP-3 and MMP-8, but not MMP-9 or MMP-2, were responsible for the increased shedding of HLA-G1 from transfected U-937 cells. This seems to be in contrast with our results, where MMP-2 is implicated in HLA-G shedding. However, MMP-2 is known to be expressed at very low levels in resting U-937 cells [59, 69–71] and increases only in differentiated macrophages [70]. It is possible that Diaz-Lagares et al. [68] failed to evidence MMP-2 implication in membrane HLA-G shedding as they worked in a low MMP-2 concentration environment. The results from Diaz-Lagares et al. [68] and our data suggest a complex implication of several MMPs (MMP-3, MMP-8, MMP-2) in HLA-G shedding, which could change the levels of sHLA-G on the basis of the specific MMP expression.

Interestingly, there was lower HLA-G shedding after 24 h of MMP-2 treatment compared with 6 h in 221-G1 cells. One should expect higher HLA-G shedding as long as the incubation time increased. SDS-PAGE of 221-G1 cell culture supernatants (Fig. 3d) showed an increase of the two bands at 10 and 25 kDa after the 24-h incubation, which is likely to be HLA-G specific [64], with a corresponding decrease of the native HLA-G 39 kDa band. We could hypothesize that MMP-2 continued the degradation of shedded HLA-G molecules, resulting in a lower detection of sHLA-G by the available MoAbs, which are specific to the native conformation. On the contrary, the increase in sHLA-G1 was time dependent in JEG3 and primary PBMCs, suggesting a higher resistance of native HLA-G to MMP-2 degradation in comparison with recombinant molecules from transfected 221-G1 cells. This observation is of importance, taking into consideration the necessity of the native HLA-G conformation to interact with the specific receptors (ILT-2, ILT-4).

Our results add novel aspects on the possible immunemodulatory function of MMP-2, supporting its role as an anti-inflammatory molecule. In fact, it has been previously suggested that MMP-2 can act as an anti-inflammatory molecule by dampening the pro-inflammatory cytokine signal [49, 51] : Our findings add a new point in the regulation of physiological and pathological conditions through the production of MMP-2-dependent soluble HLA-

Table 1 Possible cleavage sites for MMP-9 and MMP-2 in HLA-G1 primary sequence

Code	Position (amino acid)	Site	*N*-fragment (kDa)	Similarity maxscore (MMP-9/MMP-2)	Similarity maxsite (MMP-9/MMP-2)	Protease
S1	245–249	AAV.VV	28.5	65.000/60.000	AAA.VL/SAA.IV	MMP-9/2
S2	250–254	PSG.EE	28.9	88.889/84.615	PAG.EE/PSG.ES	MMP-9/-2
S3	267–271	PEP.LM	30.9	70.370/81.481	PRP.LV/PEP.LS	MMP-9/-2
S4	263–266	HEG.L	30.5	–/68.182	$-/HXXX_{Hy}$	MMP-2
S5	272–275	LRW.K	31.6	–/79.167	$-/L(I)XXX_{Hy}$	MMP-2
S6	276–279	QSS.L	32.1	–/70.588	$-/X_{Hy}SXL$	MMP-2

The amino acidic position, the site, the calculated molecular weight for the *N*-fragment, and the percentage of homology (similarity maxscore) with the sequence recognized by the protease (similarity maxsite) are indicated. *X* any amino acid; X_{Hy} hydrophobic amino acid

G1, which in turn might act as an immune-regulatory effector modulating the activity of NK cells, activated $CD8^+$ T lymphocytes, and T regulatory cells.

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