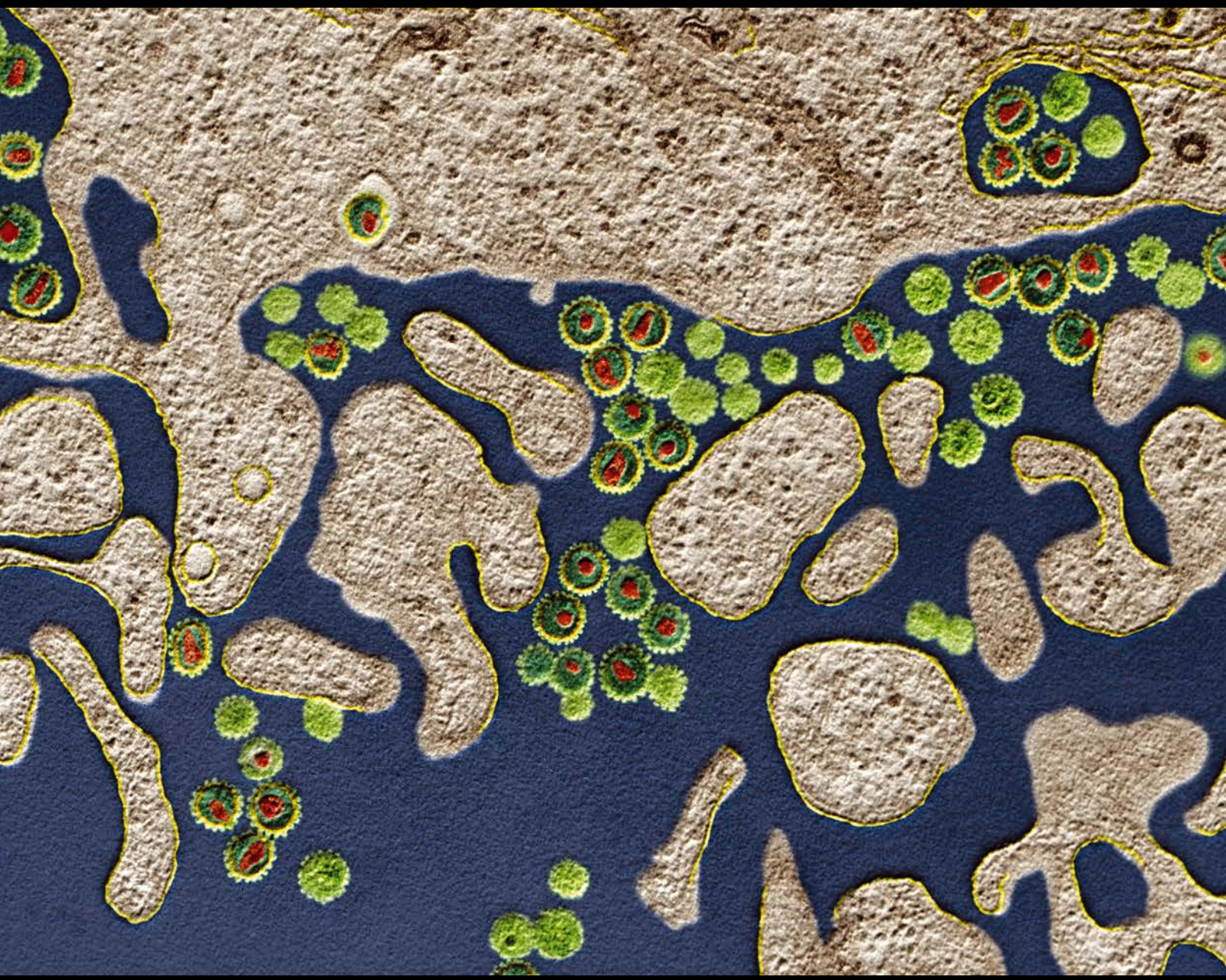
Journal of Immunology Research

The Role of HLA-Class Ib Molecules in Immune-Related Diseases, Tumors, and Infections 2016

Guest Editors: Fabio Morandi, Enrico Fainardi, Nathalie Rouas-Freiss, and Roberta Rizzo



The Role of HLA-Class Ib Molecules in Immune-Related Diseases, Tumors, and Infections 2016

Journal of Immunology Research

The Role of HLA-Class Ib Molecules in Immune-Related Diseases, Tumors, and Infections 2016

Guest Editors: Fabio Morandi, Enrico Fainardi, Nathalie Rouas-Freiss, and Roberta Rizzo

Editorial Board

Contents

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2017, Article ID 2309574, 2 pages
http://dx.doi.org/10.1155/2017/2309574

Editorial

The Role of HLA-Class Ib Molecules in Immune-Related Diseases, Tumors, and Infections 2016

Roberta Rizzo,[1] Enrico Fainardi,[2] Nathalie Rouas-Freiss,[3] and Fabio Morandi[4]

[1]*Department of Experimental and Diagnostic Medicine, Section of Microbiology, University of Ferrara, Ferrara, Italy*
[2]*Neuroradiology Unit, Department of Diagnostic Imaging, Azienda Ospedaliero-Universitaria Careggi, Florence, Italy*
[3]*CEA, Institut des Maladies Emergentes et des Thérapies Innovantes (iMETI), Service de Recherches en Hémato-Immunologie (SRHI), Hôpital Saint-Louis, Paris, France*
[4]*Laboratory of Oncology, G. Gaslini Scientific Institute, Genoa, Italy*

Correspondence should be addressed to Fabio Morandi; fabiomorandi@gaslini.org

Received 31 October 2016; Accepted 1 November 2016; Published 4 January 2017

HLA-class I family includes highly polymorphic HLA-class Ia molecules (HLA-A, HLA-B, and HLA-C), which play a central role in adaptive immunity, and "nonclassical" HLA-class Ib molecules (HLA-E, HLA-F, HLA-G, and HLA-H), characterized by a limited polymorphism and a few alleles that encode a small number of functional proteins. Both types of HLA-class I molecules can bind peptides generated from cytosolic antigens and present them to specific $CD8^+$ T lymphocytes. However, the main function of HLA-class Ib molecules is the modulation of immune responses, both in physiological and in pathological conditions. In contrast, nonclassical major histocompatibility complex (MHC) class I chain related (MIC) molecules show homology with classical human leukocyte antigen (HLA) molecules, but they do not bind beta-2 microglobulin and peptides. Expression of MIC proteins is upregulated on the cell surface in response to stress, and these molecules can interact with the activating natural killer cell receptor NKG2D, which is expressed by many cells of the immune system.

HLA-G, the best characterized HLA-Ib molecule, was firstly detected on fetal cytotrophoblast cells during pregnancy, where it abrogates maternal NK cell activity protecting fetal tissues. However, HLA-G expression and release can be detected in different cells and tissues in pathological conditions, such as tumors, transplantation, bacterial and viral infections, and inflamed tissues. HLA-G can interact with at least four specific receptors (ILT2, ILT4, KIR2DL4, and CD160) that are expressed on T and B lymphocytes, NK cells, neutrophils, and antigen presenting cells, thus inhibiting their functions.

HLA-E is expressed by all nucleated cells and binds peptides derived from the leader sequence of other HLA-class I molecules. In normal conditions, HLA-E interacts with CD94/NKG2A inhibitory receptor on NK cells, thus inhibiting their cytotoxicity against cells expressing HLA-class I molecules. Transformed or virus-infected cells, which usually downregulate HLA-class I expression, display a lower number of HLA-class I-derived peptides. This in turn leads to downregulation of HLA-E expression, thus allowing NK cells lo lyse these cells. However, different transformed cells can upregulate HLA-E expression to avoid NK cell mediated lysis. Finally, it has been demonstrated that HLA-E can also interact with CD94/NKG2C activatory receptor, thus leading to the activation of NK cell functions. This function is important for vascular remodeling during pregnancy.

HLA-F can act as chaperone for the β2-microglobulin-free heavy chain of other HLA-class I molecules, and its expression can be detected on the surface of activated lymphocytes. Moreover, it has been demonstrated that this molecule can be present in a peptide-free (open conformer) form and can cooperate with other HLA-class I open

conformers during cross-presentation. Finally, HLA-F open conformers can interact with different KIR on NK cells.

No functional HLA-H molecules encoded by HLA-H alleles have been yet characterized.

In this special issue dedicated to nonclassical HLA-class Ib molecules, we have discussed in a review novel findings obtained in the last two years, regarding the role of HLA-G in cancer, infectious diseases, autoimmune/inflammatory diseases, pregnancy, and transplantation.

One interesting paper by Y. Zhang et al. addressed the role of *MICB* in SLE patients. They have demonstrated that a single nucleotide polymorphism (SNP) called rs3828903 is associated with higher susceptibility to systemic lupus erythematosus (SLE), and a higher *MICB* gene expression was detected in SLE patients, thus suggesting a role of this molecule in the progression of the disease.

Three papers have addressed the role of HLA-E in pathological conditions. J. Di Cristofaro et al. have demonstrated that lung transplanted patients that displayed homozygosis for HLA-E*01:01 or HLA-E*01:03 alleles showed impaired overall survival as compared with patients displaying heterozygosis. Moreover, the presence of HLA-E*01:03 allele is correlated with a higher incidence of chronic rejection, as compared with patients with HLA-E*01:01 homozygosis. An interesting review by S. A. Joosten et al. discussed the role of pathogen-specific HLA-E restricted T-cell responses during infectious diseases. Finally, F. Morandi et al. have demonstrated that soluble HLA-E concentration was higher in BM plasma samples from neuroblastoma patients with metastatic disease than in those with localized tumors, thus suggesting a role of this molecule in the progression of the disease. The same results have been also obtained for soluble HLA-G.

Three papers have addressed the role of HLA-G in pathological conditions. G. Murdaca et al. have discussed in their review the role of HLA-G during allergic diseases, pointing out the role of this molecule in the suppression of the allergic reaction. R. Rizzo and coworkers have demonstrated that plasma sHLA-G levels were higher in women with primary human congenital cytomegalovirus (HCMV) infection than in nonprimary and uninfected pregnant women. Moreover, sHLA-G levels in amniotic fluid were higher in symptomatic than in asymptomatic fetuses. Collectively, these data suggested a role for HLA-G as a biomarker for HCMV infection. L. L. Olesen and T. V. F. Hviid have characterized the role of HLA-G in left ventricular systolic dysfunction (LVSD). They have demonstrated that soluble HLA-G was higher in patients with ejection fraction (EF) < 50% than in those with EF > 50%. However, sHLA-G was less specific as a biomarker of LVSD than other classic biomarkers. Moreover, a combined 14 bp ins-del/+3142SNP HLA-G haplotype was associated with EF < 40%.

Finally, an interesting review by L. L. Johansen and coworkers discussed the role of classical and nonclassical HLA-class I molecules in the progression of human melanoma.

Collectively, all these papers have added novel important findings on HLA-class Ib molecules in different settings, thus confirming their pivotal role in the control of the immune system both in physiological and in pathological conditions.

Roberta Rizzo
Enrico Fainardi
Nathalie Rouas-Freiss
Fabio Morandi

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 6829283, 11 pages
http://dx.doi.org/10.1155/2016/6829283

Review Article

The Pathophysiological Impact of HLA Class Ia and HLA-G Expression and Regulatory T Cells in Malignant Melanoma: A Review

Lasse Lindholm Johansen,[1,2] Jørgen Lock-Andersen,[2,3] and Thomas Vauvert F. Hviid[1,2]

[1]*Centre for Immune Regulation and Reproductive Immunology (CIRRI), Department of Clinical Biochemistry, Zealand University Hospital, Roskilde, Denmark*
[2]*Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark*
[3]*Department of Plastic Surgery, Zealand University Hospital, Roskilde, Denmark*

Correspondence should be addressed to Thomas Vauvert F. Hviid; tvh@regionsjaelland.dk

Received 20 May 2016; Revised 16 September 2016; Accepted 12 October 2016

Academic Editor: Fabio Morandi

Malignant melanoma, a very common type of cancer, is a rapidly growing cancer of the skin with an increase in incidence among the Caucasian population. The disease is seen through all age groups and is very common in the younger age groups. Several studies have examined the risk factors and pathophysiological mechanisms of malignant melanoma, which have enlightened our understanding of the development of the disease, but we have still to fully understand the complex immunological interactions. The examination of the interaction between the human leucocyte antigen (HLA) system and prognostic outcome has shown interesting results, and a correlation between the down- or upregulation of these antigens and prognosis has been seen through many different types of cancer. In malignant melanoma, HLA class Ia has been seen to influence the effects of pharmaceutical drug treatment as well as the overall prognosis, and the HLA class Ib and regulatory T cells have been correlated with tumor progression. Although there is still no standardized immunological treatment worldwide, the interaction between the human leucocyte antigen (HLA) system and tumor progression seems to be a promising focus in the way of optimizing the treatment of malignant melanoma.

1. Introduction

Cutaneous malignant melanoma is a type of cancer that develops in the melanocytes of the skin. The epidermis, which is the barrier of the body, that protects us from the outer environment, is made up of different types of cells, primarily squamous cells, basal cells, and melanocytes. In addition, the skin also contains important immune cells. Melanin is produced in the melanocytes and is the pigment that gives the skin its characteristic color, and it is in these cells that the malignant melanoma originates from; the tumors are frequently strongly pigmented. Another type of skin cancer is nonmelanoma skin cancer, which includes basal cell carcinoma and squamous cell carcinoma. These types of cancers are very common; however, they metastasize rarely. Unfortunately, there has been an annual increase in the incidence of malignant melanoma among different populations ranging from 3% to 7%, which corresponds to a doubling of rates every 10–20 years [1]. Worldwide, the highest incidence rates have been reported in Australia and New Zealand with incidence rates as high as 60 cases per 100,000 inhabitants per year [2]. Throughout Europe, age-standardized incidence rates in 2012 have been estimated to 11.4 per 100,000 for males and 11.0 per 100,000 for females. These have ranged from six new cases per 100,000 in Central and Eastern Europe, 10 cases in Southern Europe, and 19 cases in Northern Europe [3].

The median age is 62 years in the US, when the disease is detected for the first time. However, the disease also affects younger people under 30 and is one of the most common cancers among young people [4]. However, some studies from Australia, New Zealand, USA, several Western European, and Nordic countries have indicated a stabilization in the

incidence rates in both sexes mainly among young people, and an increase in the incidence of malignant melanoma in the age group > 60 years [5, 6]. Based on data from 39,000 patients, the Amercian Joint Committee on Cancer calculated the five- and ten-year survival rates according to the TNM classification system: these were for clinical stage I (histological tumor, thickness ≤ 1 mm and node negative) 97% and 93% (resp., five- and ten-year survival rates), stage II (tumor thickness >1 mm and node negative) 53% and 39%, and stage III (with regional lymph node metastases) 46% and 33%. Additional important tumor factors are ulceration and mitotic rate [7].

2. Clinical Evaluation and Risk Factors

Regarding anatomic localization, the back has traditionally been the predilection site in males and the legs in females, with a tendency in recent years to a change in female presentation towards a male pattern [8, 9]. Malignant melanoma can arise from normal skin, benign nevi, and dysplastic nevi, where dysplastic nevi can be considered as an intermediate stage.

Of other risk factors, in addition to dysplastic nevi, UV radiation plays a very important role in the incidence of malignant melanoma creating cellular lesions in the DNA, pyrimidine dimers of C-T mutations [10]. Recent research has shown that C-T mutations are frequently found in malignant melanomas. However, these mutations are also seen in pancreatic cancer; therefore, it is uncertain whether they are directly connected to UV-radiation [11, 12].

Melanoma is related to intermittent sun exposure as well as to accumulated sun exposure. Twice the risk of developing malignant melanoma has been seen in individuals with skin type I or II compared to skin type III or IV. Skin type I is defined as always sunburned, never tanned, and skin type IV as never sunburned, always tanned [13–15]. However, the relationship between the UV radiation and malignant melanoma is very complex, as it is pointed out that chronic exposure to UV radiation in subjects with outdoor work has a protective effect against the development of malignant melanoma [13–17]. This complex relationship is exposed further, as malignant melanomas develop not only on the more UV-exposed areas of the body, but also on the trunk, most often in men, and on the lower extremities, most often in women. This may suggest that it is not an accumulated amount of UV radiation that is important for the malignant development and, in addition, it may indicate that there is a significant genetic factor involved [8, 18]. Individuals with many nevi may have a greater lifetime risk for the development of malignant melanoma.

3. Diagnosis, Treatment, and Prognostic Evaluation

The principles of treatment of localized malignant melanoma consist primarily of surgical intervention. However, medications are also in use when treating advanced disease. The efficacy of interferon-α has been investigated in several studies, and it was shown that high doses of interferon-α could delay the time of the first distal metastasis; however, that delay did not have a positive effect on overall survival [19]. In a more recent prospective study, the role of high-dose interferon-α2b therapy, or completion lymph node dissection, for patients with melanoma, staged by sentinel lymph node biopsy, was evaluated in patients, enrolled between 1997 and 2003, with 71 months' follow-up. No positive effect on disease-free survival or overall survival was identified for adjuvant therapy with high-dose interferon-α in patients with a single tumor-positive sentinel lymph node [20]. Other studies have found adjuvant treatment with interferon to have an overall positive effect on survival. Garbe et al. found that low dose (3 MU) interferon-α2a, administrated subcutaneously, given three times a week for two years improved overall survival and disease-free survival in melanoma patients with first manifestation of metastasis in regional lymph nodes when compared to treatment with interferon combined with dacarbazine or observation alone [21]. Another study has found that one-year maintenance treatment with intermediate-dose adjuvant interferon-α2b improved relapse-free survival, but this was not the case with two-year maintenance therapy [22]. Due to the high toxicity of interferon-α this drug is not considered standard adjuvant therapy in some countries [23]. In a retrospective study by Hughes et al., treatment with high-dose interleukin-2 of 305 patients with either malignant melanoma or metastatic renal cell carcinoma was investigated. Twenty-five per cent of the patients with metastatic melanoma or renal cell carcinoma achieved stable disease, defined as less than 20% progression in the disease, and not higher than 30% progression in the disease, after initial treatment with interleukin-2. This was associated with improved overall survival compared with patients, who had progressive disease. A disease control rate (DCR) was listed as the percentage of patients, whose disease did not progress after initial treatment. If the stable disease was taken into account, the treatment with interleukin had a DCR of 37.7%, and the study pointed out that this rate was more indicative than previously reported response rates of 15–20%, which underestimated the effect of the treatment [24]. Several medications, such as dacarbazine, vemurafenib, dabrafenib, and trametinib, are being used in the treatment of metastatic melanoma, but it seems that there is still a need for drugs with better long-term effects and less toxicity [25]. Ipilimumab, a recombinant, monoclonal antibody that interacts with and blocks the cytotoxic T lymphocyte associated protein 4 (CTLA-4) receptor in activated T cells, was the first systemic treatment that showed an improvement in survival in a phase III trial, treating patients with advanced melanoma. In 2010, a phase III trial consisting of 676 patients with stage III-/IV advanced melanoma showed a significantly better median survival when the treatment with ipilimumab (10.1 months) was compared with the tumor-associated antigen (TAA) glycoprotein (gp100) vaccine (6.4 months) [26]. Ipilimumab and dabarbazine, combined, have also been shown to have a positive effect on median survival when compared to dacarbazine combined with placebo [27]. The TAA gp100 was one of the first identified. The gp100 epitope peptide is restricted to HLA-A*2402 and has been tested in clinical trials to treat melanoma patients [28]. The positive results from

the treatment with ipilimumab lead to the approval in the US by the Food and Drug Administration in 2011 and later by the European Medicines Agency [26, 27]. Of other drugs that have shown promising results in the treatment of melanoma are the programmed cell death protein 1 (PD-1) inhibitors, nivolumab, and pembrolizumab. The PD-1 is a receptor located on T cells that, by binding to the PD-L1 and PD-L2 ligands, prevents the activation of the T cell. In different types of cancer, these ligands are upregulated and expressed in the tumor microenvironment, which suppress the activation of the T cells [29]. The recombinant monoclonal antibody, nivolumab, has in a recent study shown significant improvements in overall survival and progression-free survival, as compared with dacarbazine, among previously untreated patients with metastatic melanoma without a B-Raf protooncogene, serine/threonine kinase (BRAF) mutation [30]. Interestingly, in a study of melanoma cell lines and patient samples, Johnson et al. found evidence that melanoma-specific HLA class II expression may function as a marker for predicting response to anti-PD-1/PD-L1 therapy [31].

4. The Effect of UV Radiation

UV radiation, which damages the DNA, results in an increase of the production of melanin and blocks the cell cycle through microphthalmia associated transcription factor (MITF). This blockage of the cell cycle occurs to prevent unrestrained progression of melanocytes until the DNA is no longer being damaged [32]. Melanocortin-1 receptor (MC1R), a previous link to the activation of MITF, is activated by melanocyte-stimulating hormone (MSH) which is activated by UV radiation.

MC1R, a gene with high genetic polymorphism, has a large influence on the pigmentation of the skin in every individual. There are a number of recessive MC1R alleles, which have a high penetrance in individuals with red hair. These individuals have a reduced ability to increase the UV-induced pigmentation [33]. Moreover, these alleles are associated with malignant melanoma with an odds ratio (OR) of 1.4–2.4 [34]. Germline mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A), a kinase that plays a role in UV-induced melanin production by encoding two different tumor suppressor genes, p16INK4a and p14ARF, are associated with familial melanoma [35]. These mutations have been observed in 10% of families with two cases of malignant melanoma and 30–40% of families with three or more cases of malignant melanoma [36]. High-penetrance mutations in a kinase, cyclin-dependent kinase 4 (CDK4), which also plays a role in the melanogenesis by inducing the progression of the cell cycle, are also observed [37]. BRAF, a member of the raf family, is a protein kinase that is encoded by the BRAF gene. This protein kinase is a part of mitogen-activated protein kinase pathway (MAPK signaling pathway) of melanocyte proliferation, and through the MAPK signaling pathway, BRAF regulates a variety of cellular processes, such as growth, proliferation, and apoptosis. Mutations in BRAF result in an alteration of the activity of the protein kinase, which hereby acts as an oncogene. This oncogene can give rise to benign and malignant neoplasms. The vast majority of mutations in BRAF are V600E mutations, in which glutamic acid (E) is replaced with valine (V) at codon 600 and causes the protein to become overactive [38]. The benign and malignant changes this transformation may give rise to are due to an increase in cell proliferation and survival [32, 39, 40]. As previously mentioned, malignant melanomas are more commonly found in individuals with skin type I-II, compared to skin type III-IV. A higher incidence of malignant melanomas is also seen in red-haired compared to dark-haired individuals [41]. Overall, in epidemiological studies, a higher risk of malignant melanoma is observed by a reduction in pigmentation and an increased number of nevi [32, 41, 42].

5. Immunological Mechanisms in Malignant Melanoma

Since the 1950s, the physiological function of the adaptive immune system in relation to cancer, understood as the individual's own defense against the growth of transformed cells, has been increasingly explored. This function is known as *immune surveillance*. Growing tumors may present different molecules that are recognized as foreign antigens and are therefore defeated by the immune system. These tumor antigens are recognized by $CD8^+$ T cells that differentiate into cytotoxic lymphocytes (CTL) and fight the transformed cells. Tumor antigens are presented by the major histocompatibility complex (MHC) class I molecules on dendritic cells, known as antigen-presenting cells (APC). The major histocompatibility complex in humans is named the human leukocyte antigen (HLA) system (Figure 1). In order for the differentiation of naive $CD8^+$ T cells to the $CD8^+$ CTL to take place, there must also be a costimulation and/or help from $CD4^+$ T cells that bind to MHC class II molecules. By APC B7 costimulation secondary signals help the activation of the $CD8^+$ CTL. The binding of $CD4^+$ T cells to the MHC releases cytokines that contribute to the differentiation process.

However, it is not always the case that the foreign tumor cells are suppressed as they may prevent the presentation to the $CD8^+$ T cells. This phenomenon is known as *immune escape*, which may take place in different ways: lack of expression of the tumor antigen, lack of expression of the MHC class I molecules, production of inhibitory cell surface proteins, or production and secretion of cytokines (Figure 2). Since the tumor cells have been able to develop these escape mechanisms, the hypothesis of *immune editing* was established, which consists of three parts: elimination, steady state, and escape [43]. In the first step, the tumor cells are recognized and killed by the immune response, as described earlier. In case of tumor variants that are not eliminated by the immune response, the immune system retains the growth of these cells throughout the life of the host and thus achieves steady state. At the final stage, escape, tumor cells avoid elimination and achieve tumor escape [44]. In cases where the tumor cells do not express MHC class I, the natural killer (NK) cells play an important role. All healthy human, eukaryotic cells express MHC class I at the cell surface as well as ligands for activating NK cells. NK cell inhibitory receptors react with the expressed MHC class I molecule, thus avoiding the lysing of the healthy cells. As mentioned,

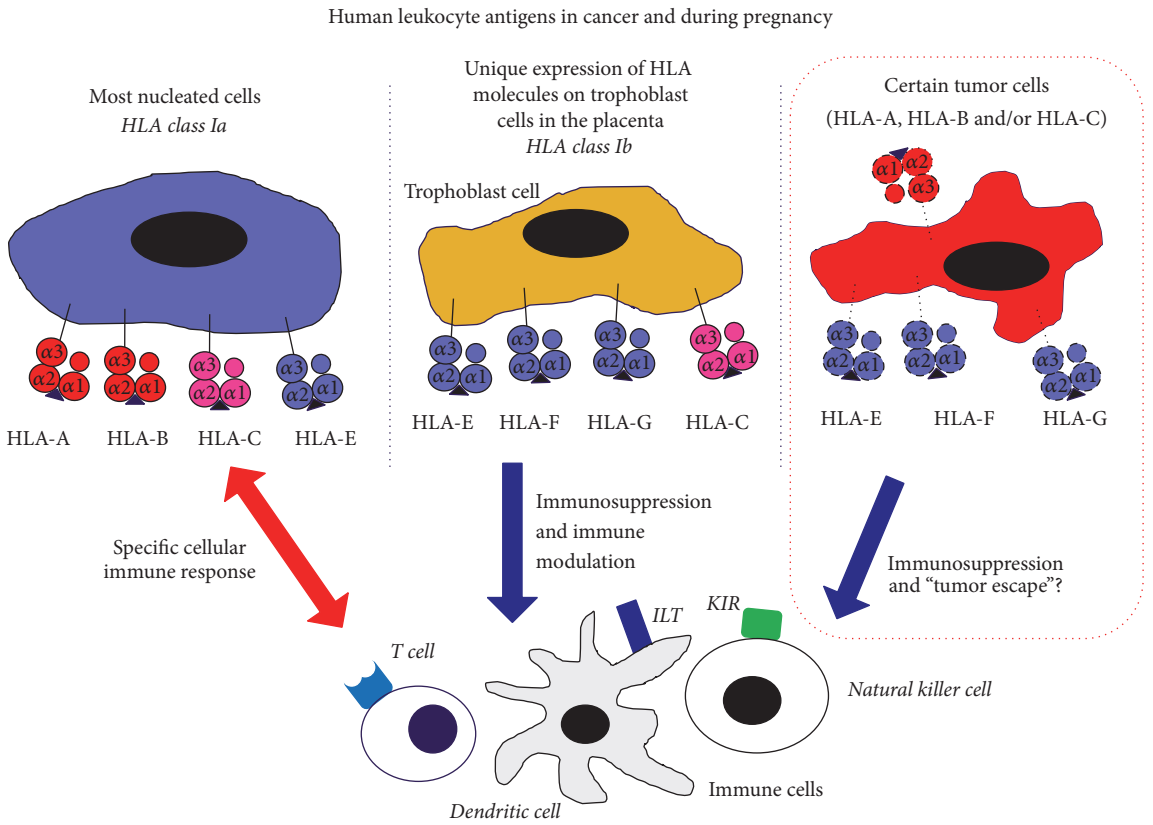


Figure 1: In some cases, tumor cells, for example, malignant melanoma cells, may obtain through selection processes an HLA expression profile that in varying degrees mimics the HLA expression on extra-villous trophoblast cells at the fetomaternal interface during pregnancy. The specific HLA expression profile on tumor cells involving one or several of the HLA class Ib molecules may be one mechanism leading to immunosuppression and immune escape (ILT: immunoglobulin-like transcript; KIR: killer-cell immunoglobulin-like receptors).

certain tumor cells downregulate their expression of MHC class I, which protects them from degradation by $CD8^+$ CTL. In the cases where MHC class I is downregulated, the NK cells are activated by the activating ligands on the surface of the tumor cell because they do not express MHC class I, which would otherwise react with the NK cell inhibitory receptors. In this way, the tumor cell is lysed even though it has achieved immune escape from the $CD8^+$ CTL. In the 1950s, it was shown in mice studies that the rejection of grafted tissue was coupled to the adaptive immune system. This was due to foreign antigens on the surface of the transplanted cells and foreign variants of surface proteins, especially MHC molecules, which mainly the T cells responded to, and a rejection of the transplanted tissue was mediated. MHC/HLA proteins are extremely polymorphic; there are a very high number of alleles, more than 1,000 for some of the genes. This makes it very unlikely that two randomly selected individuals may be able to function as donor and recipient, because the chance that they have two identical sets of MHC proteins is very small.

The tumor microenvironment has for many years been a point of interest in melanoma research. The melanoma cells interact with the microenvironment in many different ways, for example, through cell-matrix contact and through secreted growth factors and cytokines. In order for the melanoma cells to successfully migrate and invade, the cells need to activate growth factors that regulate cell-adhesion [45]. Basic fibroblast growth factor (bFGF), a growth factor that is produced and secreted in melanoma cells, which promotes proliferation and survival in human melanocytes in an autocrine manner, has been described to correlate with melanoma tumor progression. Furthermore, overexpression of bFGF enhances the proliferation of melanocytes and anchorage-independent growth [46]. The melanocytes that overexpress bFGF can, without the presence of insulin-like growth factor (IGF-1) and melanocyte-stimulating hormone (MSH), grow and proliferate, and the bFGF secreted by melanoma cells can stimulate proliferation of stromal cells in a paracrine manner [47]. Besides bFGF, other melanoma-secreted growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor- (TGF-) β, seem to induce proliferation and activation of fibroblasts and endothelial cells and in this way exercise paracrine functions in angiogenesis and stroma formation. The effects of PDGF stimulate the production of collagen and the glycoproteins, fibronectin and laminin, by neighboring fibroblasts [48]. PDGF, secreted by melanoma cells, furthermore stimulates the production and secretion of IGF-1, which stimulates the proliferation of melanoma cells. The promotion of melanoma growth is enhanced by the release of bFGF and endothelin by

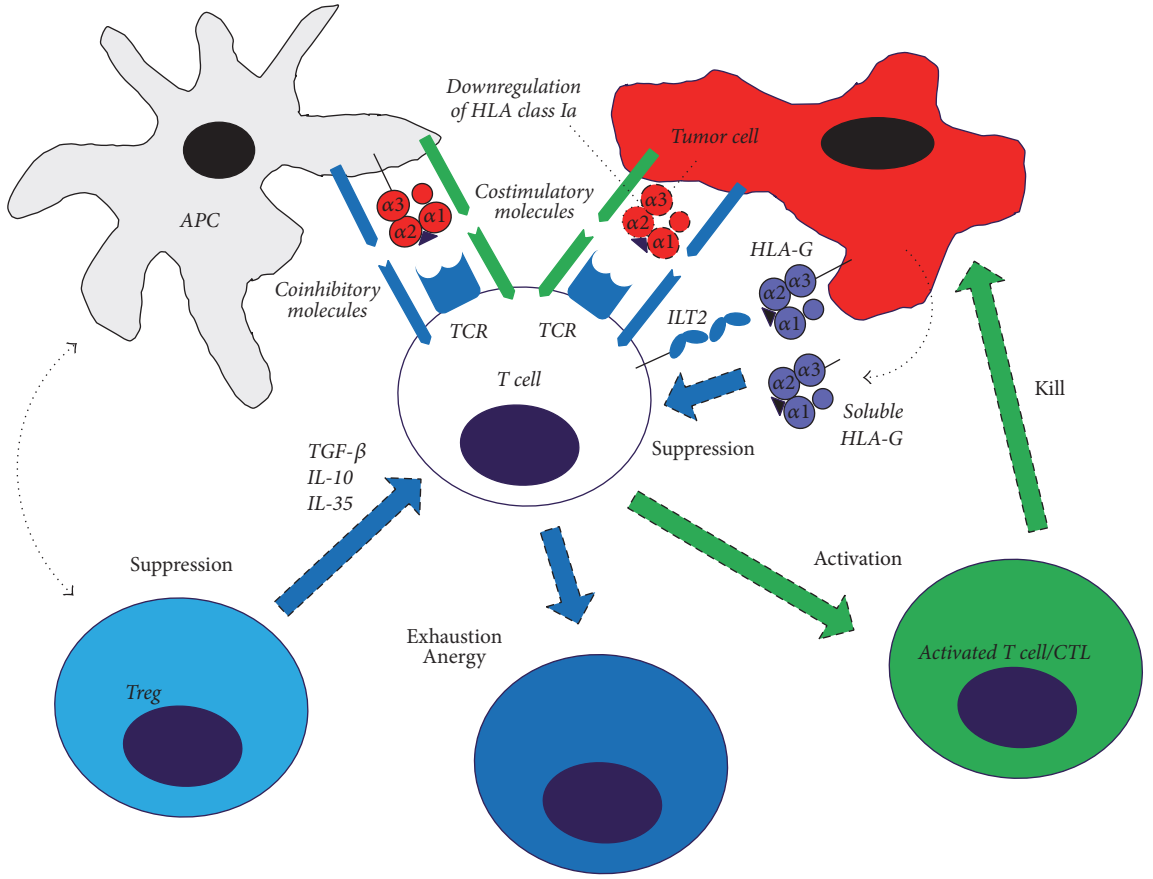


Figure 2: Schematic representation of immunological mechanisms discussed in the text that may lead to escape of malignant melanoma cells from immune surveillance. The expression of HLA class Ia molecules on the tumor cell surface may be compromised or downregulated. The tumor cell may begin, or may be selected, to express immunosuppressive HLA-G molecules that exist in both membrane-bound forms and soluble forms. Regulatory T cells in the tumor microenvironment secrete TGF-β, IL-10, and IL-35 that inhibit T cell functions. If the immune checkpoint balance is in favor of negative signals, it will result in inhibition of T cell responses with exhausted T cells and cytotoxic T lymphocytes in anergy (APC: antigen-presenting cell; CTL: cytotoxic T lymphocyte; IL-10: interleukin-10; IL-35: interleukin-35; ILT2: immunoglobulin-like transcript-2; TCR: T cell receptor; TGF-β: transforming growth factor-β; Treg: regulatory T cell).

activated fibroblasts [49]. A well-known mechanism in tumor progression is angiogenesis. In melanoma cells, this process is stimulated by both autocrine and paracrine growth factors, such as vascular endothelial growth factor (VEGF), bFGF, PDGF, and TGF-β, and the progression of the melanoma cells leads to increased levels of VEGF and bFGF [45, 50]. A member of the VEGF family produced by melanoma cells, placental growth factor (PIGF), binds to neuropilin-1 and neuropilin-2 receptors on endothelial cells, which, in synergy with VEGF, exert the angiogenic actions of the endothelial cells. The prognosis and tumor progression have been correlated with levels of PIGF, and a study by Fischer et al. has suggested that treatment against PIGF could be a potential target when developing novel anticancer therapies [51]. The secretion of interleukin-8 (IL-8) by endothelial cells has an effect on vascular permeability and stimulates the migration of melanoma and endothelial cells [50]. UV radiation (UVB), TGF-β1, and hypoxia can induce the expression of IL-8 in melanoma cells, potentially increasing their metastatic potential, and a study by Ugurel et al. suggested that increased levels of serum IL-8 in melanoma patients were correlated with advanced disease and poor overall survival [52]. Understanding the tumor microenvironment is essential in the ongoing development of treatments for melanoma, as well as other types of cancer, as it makes the foundation of knowledge required to target the many different molecular mechanisms in tumor progression.

6. HLA Class Ia Molecules in Malignant Melanoma

Major histocompatibility complex molecules are divided into two classes, classes I (which is subdivided into Ia and Ib) and II. The MHC class Ia, known as the classical class I molecules, is composed of HLA-A, HLA-B, and HLA-C and class Ib of HLA-E, HLA-F, and HLA-G [53].

HLA class Ia molecules consist of three subunits, a HLA class I heavy chain, β_2-microglobulin (β_2m), and a peptide [54]. In order for these molecules to be expressed on the cell surface, they have to undergo assembly in the

endoplasmatic reticulum followed by a transport to the cell surface. This process requires the transporter associated with antigen processing 1 (TAP1), TAP2, and different ER-resident chaperons such as calnexin, calreticulin, and tapasin [55, 56]. The heavy chain-β_2m heterodimer is stabilized through noncovalent protein-protein interactions by β_2m, which makes the binding of endogenous antigenic peptides possible with the help from TAP, and thereby allows for the assembled heavy chain-β_2m-peptide trimeric complexes to be transported to the cell surface, where they are recognized by CTL [57]. The expression of HLA class Ia molecules on the tumor cells may be modulated by cytokines in the tumor microenvironment. One example would be interferon-α (IFN-α) and interferon-β (IFN-β) that upregulate HLA class Ia expression.

It appears that low or no expression of HLA class Ia results in a poorer prognosis for patients with cervical cancer, rectal cancer, and melanoma patients with advanced disease [58–60]. However, it has also been shown that a total loss of HLA class Ia expression is correlated with improved survival in colorectal cancer patients [61]. The downregulation of HLA class Ia molecules has been shown to be correlated with a poorer response to immunotherapy of malignant melanoma patients in one study [62]. Tumors are known to downregulate the expression of HLA class Ia molecules, resulting in a significant tumor escape mechanism. This is due to a change in the interaction between tumor cells and specific T cells and NK cells [63–66]. In a study by Carretero et al., the amount of expressed HLA class Ia protein for ten metastatic lesions from a melanoma patient, who was under immunotherapy treatment, was measured. Two out of ten lesions showed tumor progression and had a low expression of HLA class Ia. The eight other lesions showed tumor regression and expressed a high amount of HLA class Ia [58]. In patients with rectal cancer, there has been demonstrated a higher amount of stage IV tumors in patients with a loss of HLA class Ia expression [60]. It has been suggested that immunotherapy leads to an alteration of the tumor microenvironment promoting a release of immune-stimulating factors by immune cells, which leads to an upregulation of HLA class Ia expression in tumor cells, eventually leading to the recognition and destruction of the tumor cells by antigen-specific T cells. However, if the tumor cells bear irreversible defects in the HLA class Ia genes, the antigen presentation remains defective after immunotherapy leading to tumor immune escape due to an impairment of the amplification of the immune response [67, 68].

Large series of tumor lesions from solid tumors of melanoma, colorectal, bladder, head and neck, breast, kidney, lung, prostate, and cervical carcinoma have shown a defect in HLA class Ia expression [69–72]. Depending on the tumor type, the downregulation, loss of HLA loci, and HLA class I allospecificities have ranged between 3.4% and 60%. The loss of heterozygosity at the HLA loci is frequent in tumors such as melanoma and colorectal carcinomas, but not in renal cell carcinoma, and this loss of heterozygosity could therefore contribute to the downregulation of HLA class Ia in specific types of tumors [73]. Total loss of HLA class Ia by tumor cells has been found to alter the immunological response against tumor cells due to resistance to recognition by CTL [74]. The formation of HLA class I heavy chain-β_2m-peptide complexes and the transport to the cell surface requires β_2m, and a loss of β_2m is frequently found in phenotypes, where loss of HLA class Ia is observed [75, 76]. The underlying mechanism for this loss of β_2m in malignant cells has been found to be due to loss of heterozygosity, mutations in one copy of the β_2m gene, and loss of the other copy [76]. In a study by Chang et al., where the molecular defects underlying HLA class I loss in five melanoma cells lines derived from recurrent metastases following initial clinical response to T cell-based immunotherapy were characterized, has suggested the emergence of mutations in the β_2m gene following strong T cell-mediated immune selection [77]. An interesting point was that the development of multiple escape mechanisms by melanoma cells to avoid T cell-mediated selective events might be reflected by multiple HLA class I defects within the tumor cell population [77].

7. HLA-G and Regulatory T Cells in Cancer and Pregnancy

Human leukocyte antigen-G, which is one of the nonclassical MHC class Ib molecules, can be detected in fetal tissue such as the amniotic sac, cell precursors, and cytotrophoblasts. HLA-G has basically a similar structure to HLA class Ia molecules but is, in contrast to these, known for a low genetic diversity [66]. In adults, HLA-G is found in the cornea, thymus, pancreas, endothelial cell precursors, and erythroblasts [78]. In addition, HLA-G is also expressed in antigen-presenting cells (APC) and macrophages. Overall, HLA-G is found in two different forms: membrane-bound HLA-G (G1–G4) and secreted isoforms (G5–G7) [79]. Human leukocyte antigen-G has an important tolerance-inducing function and can modulate the immune system by binding to inhibitory receptors on lymphoid cells, NK cells, dendritic cells, macrophages, and monocytes. HLA-G binds to three receptors: immunoglobulin-like transcript 2 (ILT2), immunoglobulin-like transcript 4 (ILT4), and killer immunoglobulin-like receptors 2DL4 (KIR2DL4). The ILTs are inhibitory, and KIR2DL4 possesses activating properties as well [80–83]. By binding to these inhibitory receptors, HLA-G may induce tolerance in various ways, such as differentiation, proliferation, cytokine secretion, and cytolysis of the normal immune response [79]. The complex between HLA-G and APCs has a certain inhibitory effect on $CD4^+$ T cells by inducing their differentiation into regulatory T cells [84]. Regulatory T cells are important to sustain immune tolerance and prevent autoimmune diseases. Using a process called trogocytose, wherein the plasma membrane and anchored proteins are transferred via cell-cell contact, the NK cells, dendritic cells, and T cells that receive membrane-bound HLA-G molecules from cancer cells, downregulate the immune response [85]. Thereby, HLA-G might contribute to a reduced immune response against the tumors of cancer patients [79]. Aberrant HLA-G expression has often been found in tumor lesions but is rare in adjacent "nontumor" tissue [73], and the expression of the nonclassical class Ib antigen in tumors has often been

associated with tumor progression and a poor prognosis for cancer patients [86–89]. Studies have shown that HLA-G is expressed in a variety of cancers, such as hepatocellular carcinoma, gastric cancer, and breast cancer, and is correlated with a poor survival [90–92]. HLA-G expression has, besides in cancer, also been found in other pathological situations such as transplantation and viral infections [89, 93–95]. Both soluble and membrane-bound HLA-G have the ability to upregulate inhibitory receptors [96].

A high frequency of HLA-G surface expression and high serum HLA-G concentration has been measured in both hematological and solid tumors, and it has been shown that the high expression of HLA-G and sHLA-G is correlated with a poorer prognosis in cancer patients. Therefore, it might indicate that HLA-G plays an important role in the development of tumors by inducing immune escape [97]. Soluble HLA-G is secreted by both tumor cells and cells of the immune system, such as monocytes, T cells, and dendritic cells, and it is conceivable that sHLA-G levels can be used as a diagnostic tool to distinguish benign from malignant tumors [79, 98]. The most abundant expression of HLA-G in normal conditions is on the surface of trophoblast cells. It is important to understand how the trophoblast cells and HLA-G-expressing cancer cells are partly similar to each other (Figure 1). The manner in which cancer cells avoid the immune system of the host by immune escape may be comparable to the fetomaternal tolerance observed between the mother and the fetus consisting of semiallogenic cells. Human leukocyte antigen-G molecules expressed on trophoblast cells can effectively suppress the local immune response in the uterus so that the fetus is not recognized as a foreign organism to be combated [53, 66]. This would provide a better understanding of how the tumor cells may avoid the host immune system. It may partly be by some of the same mechanisms as the semiallogenic fetus is accepted by the pregnant woman.

The regulatory T cells have an important task of promoting and maintaining immune tolerance by inhibiting other effectors, such as helper T cells and cytotoxic T cells, and prevent an excessive T cell response in chronic infections. It has been shown that $CD4^+$ and $CD8^+$ T cells that were stimulated in the presence of HLA-G lost their ability to respond to antigenic stimulation and developed into regulatory T cells with the ability to inhibit other T cells [84]. A study by Baumgartner et al. evaluated the amount of regulatory T cells in advanced melanoma disease and found that higher levels of regulatory T cells were correlated with a worse outcome in patients with advanced malignant melanoma, and this might probably be due to a negative effect on the antitumor response [99, 100]. Therefore, it can be speculated that high expression of HLA-G, as well as regulatory T cells, might contribute to a poor prognosis in patients with malignant melanoma. It has been difficult to characterize and study the regulatory T cells due to lack of biomarkers. However, it has been shown that these cells express the transcription factor Foxp3, which can both be used as a biomarker and also as an overall target for their development.

8. HLA-G in Malignant Melanoma: A Role for Cancer Immune Therapy Based on HLA-G?

A number of studies have investigated HLA-G protein expression in malignant melanoma. Controversies exist regarding the detection of HLA-G protein expression in melanoma tumor biopsies, while nearly all melanoma cell lines seem to be negative [101–105]. These controversies might be due to the use of different monoclonal antibodies, different technical procedures, and low number of samples in some studies. However, in general, approximately 30% of surgically removed lesions or biopsies are positive for HLA-G protein [102, 105, 106]. HLA-G expression seems to be correlated with malignant transformation and a worse prognosis with relapse or metastasis in some studies [104–106]. However, these are all small studies. Furthermore, soluble HLA-G serum levels have been reported to be elevated in melanoma patients [107].

Based on a mouse model it has been shown that HLA-G-positive tumor cells develop and tolerize the host antitumor immune response *in vivo* [108]. The xenotumor model involves the injection of human tumor cells (M8) transfected with HLA-G subcutaneously in immunocompetent mice. The model works because HLA-G can bind and mediate signals via the murine receptor paired immunoglobulin-like receptor-B (PIR-B), which is the homolog of human ILTs [108]. With the use of this model it was demonstrated that human tumor cells expressing HLA-G can grow in an immunocompetent host and that it affects both the innate and the adaptive immune system. The main mechanisms for the tumor escape mediated by HLA-G were an expansion of blood myeloid-derived suppressor cells (MDSCs), loss of peripheral $CD4^+$ and $CD8^+$ T cells, and a cytokine profile in favor of Th2 versus Th1/Th17 [108]. Interestingly, it was possible to inhibit the development of the tumor *in vivo* with the administration of a specific anti-HLA-G blocking antibody. This opens for the possibility for considering HLA-G as an immune checkpoint molecule, and blocking the function of HLA-G may be a new therapeutic strategy in cancer immunotherapy.

Results from an *in vitro* study of samples from renal cell cancer patients indicate that HLA-G-peptide-based cancer immunotherapy may be possible [109]. Several peptides derived from the HLA-G molecule were tested based on the binding motif to HLA-A24 alleles. One peptide, HLA-$G_{146–154}$, was observed to effectively induce peptide-specific CTLs and these exhibited cytotoxic activity against HLA-G-expressing HLA-A24-positive renal cell carcinoma cells [109]. Furthermore, a recent study showed that a MHC class II-binding peptide, HLA-$G_{26–40}$, was effective in eliciting a tumor-reactive $CD4^+$ T cell response [110]. It will be interesting in the future to explore the opportunities for modulating HLA-G expression in melanoma tumor cells and other tumors or induce an HLA-G peptide-specific immune response as new innovative cancer immunotherapy.

9. Conclusion

The incidence of malignant melanoma has been increasing through the years. Skin type, the number of nevi and

dysplastic nevi, and sun exposure are among the currently well-known risk factors in the development of this type of cancer. Several medications are being used in the treatment of the disease but have not yet been able to substitute for surgical excision as a stand-alone treatment. The effects of UV radiation are well known, and several mutations, such as CDKN2A and BRAF mutations, have been shown to correlate with malignant melanoma. The focus on and the exploration of the immunological side of the pathophysiology have, with an advance in medical technology, increased through the years, and correlations between the prognosis and the human leucocyte antigen (HLA) system have been described. A loss or downregulation of HLA class Ia has been seen to have a negative impact on the prognosis of malignant melanoma but has been seen to have a positive impact on the prognosis of other types of cancer. A high expression of HLA-G and regulatory T cells have both, separately, been shown to worsen the outcome of malignant melanoma. More studies are needed for a better understanding of the complex mechanisms behind the impact of HLA classes Ia and Ib on the prognosis in order to further advance the current diagnostic tools and treatment of the disease.

Competing Interests

The authors have no conflict of interests to declare.

Acknowledgments

Support for this work was generously provided by the Region Zealand Health Sciences Research Foundation.

References

[1] C. Garbe and U. Leiter, "Melanoma epidemiology and trends," *Clinics in Dermatology*, vol. 27, no. 1, pp. 3–9, 2009.

[2] R. MacLennan, A. C. Green, G. R. C. McLeod, and N. G. Martin, "Increasing incidence of cutaneous melanoma in Queensland, Australia," *Journal of the National Cancer Institute*, vol. 84, no. 18, pp. 1427–1432, 1992.

[3] J. Ferlay, E. Steliarova-Foucher, J. Lortet-Tieulent et al., "Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012," *European Journal of Cancer*, vol. 49, no. 6, pp. 1374–1403, 2013.

[4] 2016, http://www.cancer.org/cancer/skincancer-melanoma/detailedguide/melanoma-skin-cancer-key-statistics.

[5] I. Akushevich, J. Kravchenko, S. Ukraintseva, K. Arbeev, and A. I. Yashin, "Time trends of incidence of age-associated diseases in the US elderly population: medicare-based analysis," *Age and Ageing*, vol. 42, no. 4, pp. 494–500, 2013.

[6] F. Erdmann, J. Lortet-Tieulent, J. Schüz et al., "International trends in the incidence of malignant melanoma 1953–2008—are recent generations at higher or lower risk?" *International Journal of Cancer*, vol. 132, no. 2, pp. 385–400, 2013.

[7] C. M. Balch, J. E. Gershenwald, S. J. Soong et al., "Final version of 2009 AJCC melanoma staging and classification," *Journal of Clinical Oncology*, vol. 27, no. 36, pp. 6199–6206, 2009.

[8] J. Austoker, "Melanoma: prevention and early diagnosis," *British Medical Journal*, vol. 308, no. 6945, pp. 1682–1686, 1994.

[9] A. Klit, C. B. Lassen, C. H. Olsen, and J. Lock-Andersen, "Changing presentation of cutaneous malignant melanoma," *Danish Medical Journal*, vol. 62, no. 10, 2015.

[10] Y. Jiang, M. Rabbi, M. Kim et al., "UVA generates pyrimidine dimers in DNA directly," *Biophysical Journal*, vol. 96, no. 3, pp. 1151–1158, 2009.

[11] K. Dutton-Regester, H. Kakavand, L. G. Aoude et al., "Melanomas of unknown primary have a mutation profile consistent with cutaneous sun-exposed melanoma," *Pigment Cell and Melanoma Research*, vol. 26, no. 6, pp. 852–860, 2013.

[12] J. Wangari-Talbot and S. Chen, "Genetics of melanoma," *Frontiers in Genetics*, vol. 3, article 330, 2013.

[13] S. Gandini, F. Sera, M. S. Cattaruzza et al., "Meta-analysis of risk factors for cutaneous melanoma: I. Common and atypical naevi," *European Journal of Cancer*, vol. 41, no. 1, pp. 28–44, 2005.

[14] S. Gandini, F. Sera, M. S. Cattaruzza et al., "Meta-analysis of risk factors for cutaneous melanoma: II. Sun exposure," *European Journal of Cancer*, vol. 41, no. 1, pp. 45–60, 2005.

[15] S. Gandini, F. Sera, M. S. Cattaruzza et al., "Meta-analysis of risk factors for cutaneous melanoma: III. Family history, actinic damage and phenotypic factors," *European Journal of Cancer*, vol. 41, no. 14, pp. 2040–2059, 2005.

[16] V. Bataille, M. Boniol, E. De Vries et al., "A multicentre epidemiological study on sunbed use and cutaneous melanoma in Europe," *European Journal of Cancer*, vol. 41, no. 14, pp. 2141–2149, 2005.

[17] V. Bataille, A. Winnett, P. Sasieni, J. A. Newton Bishop, and J. Cuzick, "Exposure to the sun and sunbeds and the risk of cutaneous melanoma in the UK: a case-control study," *European Journal of Cancer*, vol. 40, no. 3, pp. 429–435, 2004.

[18] V. Bataille, "Sun exposure, sunbeds and sunscreens and melanoma. what are the controversies?" *Current Oncology Reports*, vol. 15, no. 6, pp. 526–532, 2013.

[19] A. M. M. Eggermont, "The role interferon-alpha in malignant melanoma remains to be defined," *European Journal of Cancer*, vol. 37, no. 17, pp. 2147–2153, 2001.

[20] K. M. McMasters, M. E. Egger, M. J. Edwards et al., "Final results of the sunbelt melanoma trial: a multi-institutional prospective randomized phase III study evaluating the role of adjuvant high-dose interferon α-2b and completion lymph node dissection for patients staged by sentinel lymph node biopsy," *Journal of Clinical Oncology*, vol. 34, no. 10, pp. 1079–1086, 2016.

[21] C. Garbe, P. Radny, R. Linse et al., "Adjuvant low-dose interferon α2a with or without dacarbazine compared with surgery alone: a prospective-randomized phase III DeCOG trial in melanoma patients with regional lymph node metastasis," *Annals of Oncology*, vol. 19, no. 6, pp. 1195–1201, 2008.

[22] J. Hansson, S. Aamdal, L. Bastholt et al., "Two different durations of adjuvant therapy with intermediate-dose interferon α-2b in patients with high-risk melanoma (Nordic IFN trial): a randomised phase 3 trial," *The Lancet Oncology*, vol. 12, no. 2, pp. 144–152, 2011.

[23] J. M. Kirkwood, C. Bender, S. Agarwala et al., "Mechanisms and management of toxicities associated with high-dose interferon α-2b therapy," *Journal of Clinical Oncology*, vol. 20, no. 17, pp. 3703–3718, 2002.

[24] T. Hughes, M. Klairmont, J. Broucek, G. Iodice, S. Basu, and H. L. Kaufman, "The prognostic significance of stable disease following high-dose interleukin-2 (IL-2) treatment in patients

with metastatic melanoma and renal cell carcinoma," *Cancer Immunology, Immunotherapy*, vol. 64, no. 4, pp. 459–465, 2015.

[25] D. M. Palathinkal, T. R. Sharma, H. B. Koon, and J. S. Bordeaux, "Current systemic therapies for melanoma," *Dermatologic Surgery*, vol. 40, no. 9, pp. 948–963, 2014.

[26] F. S. Hodi, S. J. O'Day, D. F. McDermott et al., "Improved survival with ipilimumab in patients with metastatic melanoma," *The New England Journal of Medicine*, vol. 363, no. 8, pp. 711–723, 2010.

[27] C. Robert, L. Thomas, I. Bondarenko et al., "Ipilimumab plus dacarbazine for previously untreated metastatic melanoma," *The New England Journal of Medicine*, vol. 364, no. 26, pp. 2517–2526, 2011.

[28] T. Baba, M. Sato-Matsushita, A. Kanamoto et al., "Phase I clinical trial of the vaccination for the patients with metastatic melanoma using gp100-derived epitope peptide restricted to HLA-A*2402," *Journal of Translational Medicine*, vol. 8, article 84, 2010.

[29] S. Suzuki, T. Ishida, K. Yoshikawa, and R. Ueda, "Current status of immunotherapy," *Japanese Journal of Clinical Oncology*, vol. 46, no. 3, pp. 191–203, 2016.

[30] C. Robert, G. V. Long, B. Brady et al., "Nivolumab in previously untreated melanoma without BRAF mutation," *The New England Journal of Medicine*, vol. 372, no. 4, pp. 320–330, 2015.

[31] D. B. Johnson, M. V. Estrada, R. Salgado et al., "Melanoma-specific MHC-II expression represents a tumour-autonomous phenotype and predicts response to anti-PD-1/PD-L1 therapy," *Nature Communications*, vol. 7, Article ID 10582, 2016.

[32] M. H. Law, S. MacGregor, and N. K. Hayward, "Melanoma genetics: recent findings take us beyond well-traveled pathways," *Journal of Investigative Dermatology*, vol. 132, no. 7, pp. 1763–1774, 2012.

[33] D. L. Duffy, Z. Z. Zhao, R. A. Sturm, N. K. Hayward, N. G. Martin, and G. W. Montgomery, "Multiple pigmentation gene polymorphisms account for a substantial proportion of risk of cutaneous malignant melanoma," *Journal of Investigative Dermatology*, vol. 130, no. 2, pp. 520–528, 2010.

[34] S. Raimondi, F. Sera, S. Gandini et al., "MC1R variants, melanoma and red hair color phenotype: a meta-analysis," *International Journal of Cancer*, vol. 122, no. 12, pp. 2753–2760, 2008.

[35] A. M. Goldstein, M. Chan, M. Harland et al., "Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents," *Journal of Medical Genetics*, vol. 44, no. 2, pp. 99–106, 2007.

[36] F. A. de Snoo and N. K. Hayward, "Cutaneous melanoma susceptibility and progression genes," *Cancer Letters*, vol. 230, no. 2, pp. 153–186, 2005.

[37] T. Wiesner, A. C. Obenauf, R. Murali et al., "Germline mutations in BAP1 predispose to melanocytic tumors," *Nature Genetics*, vol. 43, no. 10, pp. 1018–1022, 2011.

[38] H. Davies, G. R. Bignell, C. Cox et al., "Mutations of the BRAF gene in human cancer," *Nature*, vol. 417, no. 6892, pp. 949–954, 2002.

[39] A. M. DeLuca, A. Srinivas, and R. M. Alani, "BRAF kinase in melanoma development and progression," *Expert Reviews in Molecular Medicine*, vol. 10, article e6, 2008.

[40] P. T. Wan, M. J. Garnett, S. M. Roe et al., "Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF," *Cell*, vol. 116, no. 6, pp. 855–867, 2004.

[41] V. Bataille, J. A. Newton Bishop, P. Sasieni et al., "Risk of cutaneous melanoma in relation to the numbers, types and sites of naevi: a case-control study," *British Journal of Cancer*, vol. 73, no. 12, pp. 1605–1611, 1996.

[42] A. E. Grulich, V. Bataille, A. J. Swerdlow et al., "Naevi and pigmentary characteristics as risk factors for melanoma in a high-risk population: a case-control study in New South Wales, Australia," *International Journal of Cancer*, vol. 67, no. 4, pp. 485–491, 1996.

[43] R. D. Schreiber, L. J. Old, and M. J. Smyth, "Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion," *Science*, vol. 331, no. 6024, pp. 1565–1570, 2011.

[44] J. F. A. P. Miller and M. Sadelain, "The journey from discoveries in fundamental immunology to cancer immunotherapy," *Cancer Cell*, vol. 27, no. 4, pp. 439–449, 2015.

[45] J. Villanueva and M. Herlyn, "Melanoma and the tumor microenvironment," *Current Oncology Reports*, vol. 10, no. 5, pp. 439–446, 2008.

[46] M. Nesbit, H. K. Nesbit, J. Bennett et al., "Basic fibroblast growth factor induces a transformed phenotype in normal human melanocytes," *Oncogene*, vol. 18, no. 47, pp. 6469–6476, 1999.

[47] E. Lázár-Molnár, H. Hegyesi, S. Tóth, and A. Falus, "Autocrine and paracrine regulation by cytokines and growth factors in melanoma," *Cytokine*, vol. 12, no. 6, pp. 547–554, 2000.

[48] K. S. M. Smalley, P. A. Brafford, and M. Herlyn, "Selective evolutionary pressure from the tissue microenvironment drives tumor progression," *Seminars in Cancer Biology*, vol. 15, no. 6, pp. 451–459, 2005.

[49] D. Ruiter, T. Bogenrieder, D. Elder, and M. Herlyn, "Melanoma-stroma interactions: structural and functional aspects," *The Lancet Oncology*, vol. 3, no. 1, pp. 35–43, 2002.

[50] G. H. Mahabeleshwar and T. V. Byzova, "Angiogenesis in melanoma," *Seminars in Oncology*, vol. 34, no. 6, pp. 555–565, 2007.

[51] C. Fischer, B. Jonckx, M. Mazzone et al., "Anti-PlGF Inhibits Growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels," *Cell*, vol. 131, no. 3, pp. 463–475, 2007.

[52] S. Ugurel, G. Rappl, W. Tilgen, and U. Reinhold, "Increased serum concentration of angiogenic factors in malignant melanoma patients correlates with tumor progression and survival," *Journal of Clinical Oncology*, vol. 19, no. 2, pp. 577–583, 2001.

[53] T. V. F. Hviid, "HLA-G in human reproduction: aspects of genetics, function and pregnancy complications," *Human Reproduction Update*, vol. 12, no. 3, pp. 209–232, 2006.

[54] M. A. Saper, P. J. Bjorkman, and D. C. Wiley, "Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 A resolution," *Journal of Molecular Biology*, vol. 219, no. 2, pp. 277–319, 1991.

[55] P. Cresswell, N. Bangia, T. Dick, and G. Diedrich, "The nature of the MHC class I peptide loading complex," *Immunological Reviews*, vol. 172, pp. 21–28, 1999.

[56] J. Yewdell, "To DRiP or not to DRiP: generating peptide ligands for MHC class I molecules from biosynthesized proteins," *Molecular Immunology*, vol. 39, no. 3-4, pp. 139–146, 2002.

[57] T. H. Hansen and D. R. Lee, "Mechanism of class I assembly with β2 microglobulin and loading with peptide," *Advances in Immunology*, vol. 64, pp. 105–137, 1997.

[58] R. Carretero, J. M. Romero, F. Ruiz-Cabello et al., "Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy," *Immunogenetics*, vol. 60, no. 8, pp. 439–447, 2008.

[59] E. S. Jordanova, A. Gorter, O. Ayachi et al., "Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients?" *Clinical Cancer Research*, vol. 14, no. 7, pp. 2028–2035, 2008.

[60] M. S. Reimers, C. C. Engels, H. Putter et al., "Prognostic value of HLA class I, HLA-E, HLA-G and Tregs in rectal cancer: A Retrospective Cohort Study," *BMC Cancer*, vol. 14, no. 1, article 486, 2014.

[61] E. C. M. Zeestraten, M. S. Reimers, S. Saadatmand et al., "Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients," *British Journal of Cancer*, vol. 110, no. 2, pp. 459–468, 2014.

[62] R. Benitez, D. Godelaine, M. A. Lopez-Nevot et al., "Mutations of the β2-microglobulin gene result in a lack of HLA class I molecules on melanoma cells of two patients immunized with MAGE peptides," *Tissue Antigens*, vol. 52, no. 6, pp. 520–529, 1998.

[63] I. Algarra, A. García-Lora, T. Cabrera, F. Ruiz-Cabello, and F. Garrido, "The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape," *Cancer Immunology, Immunotherapy*, vol. 53, no. 10, pp. 904–910, 2004.

[64] N. Aptsiauri, T. Cabrera, A. Garcia-Lora, M. A. Lopez-Nevot, F. Ruiz-Cabello, and F. Garrido, "MHC class I antigens and immune surveillance in transformed cells," *International Review of Cytology*, vol. 256, pp. 139–189, 2007.

[65] H. T. Khong and N. P. Restifo, "Natural selection of tumor variants in the generation of 'tumor escape' phenotypes," *Nature Immunology*, vol. 3, no. 11, pp. 999–1005, 2002.

[66] G. Kochan, D. Escors, K. Breckpot, and D. Guerrero-Setas, "Role of non-classical MHC class I molecules in cancer immunosuppression," *OncoImmunology*, vol. 2, no. 11, Article ID e26491, 2013.

[67] R. Carretero, T. Cabrera, H. Gil et al., "Bacillus Calmette-Guerin immunotherapy of bladder cancer induces selection of human leukocyte antigen class I-deficient tumor cells," *International Journal of Cancer*, vol. 129, no. 4, pp. 839–846, 2011.

[68] R. Carretero, E. Wang, A. I. Rodriguez et al., "Regression of melanoma metastases after immunotherapy is associated with activation of antigen presentation and interferon-mediated rejection genes," *International Journal of Cancer*, vol. 131, no. 2, pp. 387–395, 2012.

[69] H. P. Cathro, M. E. Smolkin, D. Theodorescu, V. Y. Jo, S. Ferrone, and H. F. Frierson Jr., "Relationship between HLA class I antigen processing machinery component expression and the clinicopathologic characteristics of bladder carcinomas," *Cancer Immunology, Immunotherapy*, vol. 59, no. 3, pp. 465–472, 2010.

[70] R. Geertsen, R. Böni, R. Blasczyk et al., "Loss of single HLA class I allospecificities in melanoma cells due to selective genomic abbreviations," *International Journal of Cancer*, vol. 99, no. 1, pp. 82–87, 2002.

[71] I. Maleno, N. Aptsiauri, T. Cabrera et al., "Frequent loss of heterozygosity in the β2-microglobulin region of chromosome 15 in primary human tumors," *Immunogenetics*, vol. 63, no. 2, pp. 65–71, 2011.

[72] B. Seliger, M. J. Maeurer, and S. Ferrone, "Tap off—tumors on," *Immunology Today*, vol. 18, no. 6, pp. 292–299, 1997.

[73] J. Bukur, S. Jasinski, and B. Seliger, "The role of classical and non-classical HLA class I antigens in human tumors," *Seminars in Cancer Biology*, vol. 22, no. 4, pp. 350–358, 2012.

[74] D. J. Hicklin, Z. Wang, F. Arienti, L. Rivoltini, G. Parmiani, and S. Ferrone, "Beta2-microglobulin mutations, HLA class I antigen loss, and tumor progression in melanoma," *The Journal of Clinical Investigation*, vol. 101, no. 12, pp. 2720–2729, 1998.

[75] B. Arce-Gomez, E. A. Jones, C. J. Barnstable, E. Solomon, and W. F. Bodmer, "The genetic control of HLA-A and B antigens in somatic cell hybrids: requirement for β2 microglobulin," *Tissue Antigens*, vol. 11, no. 2, pp. 96–112, 1978.

[76] B. Seliger, T. Cabrera, F. Garrido, and S. Ferrone, "HLA class I antigen abnormalities and immune escape by malignant cells," *Seminars in Cancer Biology*, vol. 12, no. 1, pp. 3–13, 2002.

[77] C.-C. Chang, M. Campoli, N. P. Restifo, X. Wang, and S. Ferrone, "Immune selection of hot-spot β2-Microglobulin gene mutations, HLA-A2 allospecificity loss, and antigen-processing machinery component down-regulation in melanoma cells derived from recurrent metastases following immunotherapy," *Journal of Immunology*, vol. 174, no. 3, pp. 1462–1471, 2005.

[78] E. D. Carosella, B. Favier, N. Rouas-Freiss, P. Moreau, and J. Lemaoult, "Beyond the increasing complexity of the immunomodulatory HLA-G molecule," *Blood*, vol. 111, no. 10, pp. 4862–4870, 2008.

[79] G. Curigliano, C. Criscitiello, L. Gelao, and A. Goldhirsch, "Molecular pathways: human leukocyte antigen G(HLA-G)," *Clinical Cancer Research*, vol. 19, no. 20, pp. 5564–5571, 2013.

[80] P. Fons, S. Chabot, J. E. Cartwright et al., "Soluble HLA-G1 inhibits angiogenesis through an apoptotic pathway and by direct binding to CD160 receptor expressed by endothelial cells," *Blood*, vol. 108, no. 8, pp. 2608–2615, 2006.

[81] G. F. Gao, B. E. Willcox, J. R. Wyer et al., "Classical and nonclassical class I major histocompatibility complex molecules exhibit subtle conformational differences that affect binding to CD8$\alpha\alpha$," *Journal of Biological Chemistry*, vol. 275, no. 20, pp. 15232–15238, 2000.

[82] M. López-Botet, F. Navarro, and M. Llano, "How do NK cells sense the expression of HLA-G class Ib molecules?" *Seminars in Cancer Biology*, vol. 9, no. 1, pp. 19–26, 1999.

[83] W. H. Yan and L. A. Fan, "Residues Met76 and Gln79 in HLA-G α1 domain involved in KIR2DL4 recognition," *Cell Research*, vol. 15, no. 3, pp. 176–182, 2005.

[84] J. LeMaoult, I. Krawice-Radanne, J. Dausset, and E. D. Carosella, "HLA-G1-expressing antigen-presenting cells induce immunosuppressive CD4+ T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 18, pp. 7064–7069, 2004.

[85] L. L. Nilsson, S. Djurisic, and T. V. F. Hviid, "Controlling the immunological crosstalk during conception and pregnancy: HLA-G in reproduction," *Frontiers in Immunology*, vol. 5, article 198, 2014.

[86] E. M. de Kruijf, A. Sajet, J. G. H. van Nes et al., "HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients," *Journal of Immunology*, vol. 185, no. 12, pp. 7452–7459, 2010.

[87] E. A. Donadi, E. C. Castelli, A. Arnaiz-Villena, M. Roger, D. Rey, and P. Moreau, "Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association," *Cellular and Molecular Life Sciences*, vol. 68, no. 3, pp. 369–395, 2011.

[88] R. Ferguson, A. V. Ramanakumar, A. Koushik, F. Coutlée, E. Franco, and M. Roger, "Human leukocyte antigen G polymorphism is associated with an increased risk of invasive cancer of

the uterine cervix," *International Journal of Cancer*, vol. 131, no. 3, pp. E312–E319, 2012.

[89] S.-M. Yie and Z. Hu, "Human leukocyte antigen-G (HLA-G) as a marker for diagnosis, prognosis and tumor immune escape in human malignancies," *Histology and Histopathology*, vol. 26, no. 3, pp. 409–420, 2011.

[90] M.-Y. Cai, Y.-F. Xu, S.-J. Qiu et al., "Human leukocyte antigen-G protein expression is an unfavorable prognostic predictor of hepatocellular carcinoma following curative resection," *Clinical Cancer Research*, vol. 15, no. 14, pp. 4686–4693, 2009.

[91] H.-X. Chen, A. Lin, C.-J. Shen et al., "Upregulation of human leukocyte antigen-G expression and its clinical significance in ductal breast cancer," *Human Immunology*, vol. 71, no. 9, pp. 892–898, 2010.

[92] L. Du, X. Xiao, C. Wang et al., "Human leukocyte antigen-G is closely associated with tumor immune escape in gastric cancer by increasing local regulatory T cells," *Cancer Science*, vol. 102, no. 7, pp. 1272–1280, 2011.

[93] A. Lin, X. Zhang, W.-J. Zhou et al., "Human leukocyte antigen-G expression is associated with a poor prognosis in patients with esophageal squamous cell carcinoma," *International Journal of Cancer*, vol. 129, no. 6, pp. 1382–1390, 2011.

[94] T. G. Silva, J. C. O. Crispim, F. A. Miranda et al., "Expression of the nonclassical HLA-G and HLA-E molecules in laryngeal lesions as biomarkers of tumor invasiveness," *Histology and Histopathology*, vol. 26, no. 12, pp. 1487–1497, 2011.

[95] I. Zidi and N. Ben Amor, "HLA-G regulators in cancer medicine: an outline of key requirements," *Tumour Biology*, vol. 32, no. 6, pp. 1071–1086, 2011.

[96] J. LeMaoult, K. Zafaranloo, C. Le Banff, and E. D. Carosella, "HLA-G up-regulates ILT2, ILT3, ILT4, and KIR2DL4 in antigen presenting cells, NK cells, and T cells," *FASEB Journal*, vol. 19, no. 6, pp. 662–664, 2005.

[97] P. Paul, N. Rouas-Freiss, I. Khalil-Daher et al., "HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 8, pp. 4510–4515, 1998.

[98] M. Cao, S.-M. Yie, J. Liu, S. R. Ye, D. Xia, and E. Gao, "Plasma soluble HLA-G is a potential biomarker for diagnosis of colorectal, gastric, esophageal and lung cancer," *Tissue Antigens*, vol. 78, no. 2, pp. 120–128, 2011.

[99] J. M. Baumgartner, R. Gonzalez, K. D. Lewis et al., "Increased survival from stage IV melanoma associated with fewer regulatory T cells," *Journal of Surgical Research*, vol. 154, no. 1, pp. 13–20, 2009.

[100] A. Correll, A. Tüettenberg, C. Becker, and H. Jonuleit, "Increased regulatory T-cell frequencies in patients with advanced melanoma correlate with a generally impaired T-cell responsiveness and are restored after dendritic cell-based vaccination," *Experimental Dermatology*, vol. 19, no. 8, pp. e213–e221, 2010.

[101] C.-C. Chang and S. Ferrone, "HLA-G in melanoma: can the current controversies be solved?" *Seminars in Cancer Biology*, vol. 13, no. 5, pp. 361–369, 2003.

[102] C.-C. Chang, S. P. Murphy, and S. Ferrone, "Differential in vivo and in vitro HLA-G expression in melanoma cells: potential mechanisms," *Human Immunology*, vol. 64, no. 11, pp. 1057–1063, 2003.

[103] G. Frumento, S. Franchello, G. L. Palmisano et al., "Melanomas and melanoma cell lines do not express HLA-G, and the expression cannot be induced by γIFN treatment," *Tissue Antigens*, vol. 56, no. 1, pp. 30–37, 2000.

[104] S. N. Wagner, V. Rebmann, C. P. Willers, H. Grosse-Wilde, and M. Goos, "Expression analysis of classic and non-classic HLA molecules before interferon alfa-2b treatment of melanoma," *The Lancet*, vol. 356, no. 9225, pp. 220–221, 2000.

[105] E. C. Ibrahim, S. Aractingi, Y. Allory et al., "Analysis of HLA antigen expression in benign and malignant melanocytic lesions reveals that upregulation of HLA-G expression correlates with malignant transformation, high inflammatory infiltration and HLA-A1 genotype," *International Journal of Cancer*, vol. 108, no. 2, pp. 243–250, 2004.

[106] M. Bezuhly, A. Howlett, P. Colp et al., "Quantitative HLA-G expression in metastasising and non-metastasising primary thin cutaneous melanomas," *Dermatology*, vol. 217, no. 3, pp. 281–283, 2008.

[107] S. Ugurel, V. Rebmann, S. Ferrone, W. Tilgen, H. Grosse-Wilde, and U. Reinhold, "Soluble human leukocyte antigen-G serum level is elevated in melanoma patients and is further increased by interferon-α immunotherapy," *Cancer*, vol. 92, no. 2, pp. 369–376, 2001.

[108] S. Agaugué, E. D. Carosella, and N. Rouas-Freiss, "Role of HLA-G in tumor escape through expansion of myeloid-derived suppressor cells and cytokinic balance in favor of Th2 versus Th1/Th17," *Blood*, vol. 117, no. 26, pp. 7021–7031, 2011.

[109] Y. Komohara, M. Harada, Y. Ishihara et al., "HLA-G as a target molecule in specific immunotherapy against renal cell carcinoma," *Oncology Reports*, vol. 18, no. 6, pp. 1463–1468, 2007.

[110] K. Ishibashi, T. Kumai, T. Ohkuri et al., "Epigenetic modification augments the immunogenicity of human leukocyte antigen G serving as a tumor antigen for T cell-based immunotherapy," *OncoImmunology*, vol. 5, no. 6, article e1169356, 2016.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 8417190, 10 pages
http://dx.doi.org/10.1155/2016/8417190

Research Article

Upregulation of Soluble HLA-G in Chronic Left Ventricular Systolic Dysfunction

Line Lisbeth Olesen[1] and Thomas Vauvert F. Hviid[2]

[1]*Department of Cardiology, Zealand University Hospital (Roskilde), 10 Sygehusvej, 4000 Roskilde, Denmark*
[2]*Department of Clinical Biochemistry, Centre for Immune Regulation and Reproductive Immunology (CIRRI), Zealand University Hospital (Roskilde) and Department of Clinical Medicine, University of Copenhagen, 10 Sygehusvej, 4000 Roskilde, Denmark*

Correspondence should be addressed to Thomas Vauvert F. Hviid; tvh@regionsjaelland.dk

Received 19 May 2016; Revised 20 July 2016; Accepted 25 August 2016

Academic Editor: Enrico Fainardi

Left ventricular systolic dysfunction (LVSD) defined by ejection fraction (EF) <40% is common, serious but treatable, and correct diagnosis is the cornerstone of effective treatment. Biomarkers may help to diagnose LVSD and give insight into the pathophysiology. The immune system is activated in LVSD, and the immunomodulatory molecule human leukocyte antigen-G (HLA-G) may be involved. The primary aim was to measure soluble HLA-G (sHLA-G) in the blood in different stages of LVSD (<30% and 30–40%), in the midrange EF 40–50%, and in preserved EF ≥ 50% and to validate sHLA-G as a LVSD biomarker. The secondary aim was to examine associations between HLA-G gene polymorphisms influencing expression levels and LVSD. The 260 study participants were ≥75 years old, many with risk factors for heart disease or with known heart disease. Soluble HLA-G was significantly and uniformly higher in the groups with EF < 50% (<30, 30–40, and 40–50%) compared to EF > 50% ($p < 0.0001$). N-terminal fragment-pro-B-type natriuretic peptide (NT-proBNP) and uric acid values were inversely related to EF. According to Receiver Operating Characteristic (ROC) curves NT-proBNP outperformed both sHLA-G and uric acid as biomarkers of LVSD. Soluble HLA-G in blood plasma was elevated in LVSD regardless of EF. A novel finding was that a combined 14 bp ins-del/+3142 SNP HLA-G haplotype was associated with EF < 40%.

1. Introduction

The new European guidelines for heart failure define three groups based on ejection fraction (EF): a group with reduced EF < 40% (left ventricular systolic dysfunction (LVSD)); a group in the grey zone with EF in the midrange 40–49%; and a group with preserved EF ≥ 50%. It is concluded that patients in the grey zone probably have mild systolic dysfunction and the reason for creating a separate group is to stimulate research into characteristics, pathology, and treatment of this group of patients [1]. A problem related to the grey zone is an uncertain definition of LVSD, as shown and described in the echocardiographic study of the present study [2].

Left ventricular systolic dysfunction affects about 2% of the population in the western world, including many with unrecognized LVSD. It accumulates in the elderly population because LVSD is the final stage in most cardiac diseases, mostly caused by atherosclerosis in the coronary arteries [3]. The prognosis is grave, but treatment can delay progression and reduce morbidity and mortality. Screening for systolic heart failure in high risk populations should be considered because correct diagnosis is the cornerstone of effective treatment.

Echocardiography is the gold standard to diagnose LVSD, but access is limited, and referral to echocardiography requires a well-founded suspicion of LVSD [3]. Thus it is pivotal to look for new biomarkers, which might also give a better insight into the pathophysiology because LVSD is a complex disorder with hemodynamic, metabolic, neurohormonal, inflammatory, and immunological changes [4]. New biomarkers should be validated against established biomarkers.

B-type natriuretic peptide (or brain natriuretic peptide (BNP)) and N-terminal fragment-proBNP (NT-proBNP) are well established as diagnostic and prognostic biomarkers in LVSD, and combination with ECG might increase the specificity and the ability to screen for LVSD in high-risk populations [5, 6]. NT-proBNP synthesis is initiated by LVSD via neurohormonal activation and increased wall stress in the heart, and it is a hemodynamic cardiac marker. Uric acid is an old biomarker that may be ready for a renaissance [7].

Almost every disease and any injury to the body are accompanied by inflammation and activation of the immune system. The inflammatory system is complex and crucial for survival, but it is a double-edged sword. In LVSD, proliferation of monocytes and macrophages is observed. There is an increase in harmful oxygen-free radicals, primarily produced by xanthine oxidase; uric acid reflects xanthine oxidase activity. Proinflammatory cytokines with detrimental effects on myocardial function include tumour necrosis factor-(TNF-) α, interleukin-1, and interleukin-6 [4, 8–10]. These evoke a counterbalance reaction with increased production of anti-inflammatory interleukin-10 and HLA-G [11, 12].

This study aims to evaluate HLA-G as a new biomarker for LVSD. Human leukocyte antigen (HLA)-G is an HLA class Ib molecule with immunomodulatory, immunosuppressive, and tolerance-inducing functions [13]. It is well described in pregnancy protecting the fetus from an immune response from the mother. It is associated with a lower risk of rejection of a transplanted organ, [13–16], and, in cases of heart transplantations, myocardial expression of HLA-G has been significantly correlated with low risk of rejection [17]. In contrast, in pathological conditions, like infections and cancer, in which a vigorous and maintained immune response is desirable, the expression of HLA-G is detrimental. In cancer, it has deleterious escape-effects and the expression of HLA-G by the tumour cells seems to accelerate relapse [18]. The HLA-G protein has under normal conditions a very restricted expression pattern [13]. It is expressed during pregnancy by extravillous cytotrophoblast cells at the feto-maternal interface and is important for inducing maternal tolerance to the semiallogenic fetus [19, 20]. Furthermore, HLA-G is expressed by certain monocytes, T cells, and dendritic cells [21]. Four membrane-bound HLA-G isoforms and three soluble HLA-G isoforms generated by alternative splicing have been reported [13, 22]. Membrane-bound full-length HLA-G1 can also be cleaved from the cell surface by metalloproteinases [23].

A single published study has indicated that soluble HLA-G (sHLA-G) in plasma is upregulated in patients with systolic heart failure, compared to healthy controls, and independent of NYHA class, EF, and other biomarkers [24]. The study included only ten control subjects who were markedly younger than the participants in the present study. The current study compares sHLA-G with the state-of-the-art biomarker NT-proBNP and with uric acid, both independent biomarkers, in order to clarify if sHLA-G in blood plasma can be used as a biomarker for LVSD in a group of high-risk elderly persons. For the first time, two polymorphisms in the HLA-G gene are investigated in relation to EF. Several studies have indicated that these polymorphisms modulate HLA-G expression.

2. Materials and Methods

2.1. Patients and Samples. Individuals ≥ 75 years old from the general population and from a heart failure clinic, with heart disease risk factors or with former or present cardiac disease, especially LVSD, as well as healthy persons, were invited to participate. Two hundred and sixty subjects were included in the study. All participants provided written informed consent and the study was carried out in accordance with the ethical standards of the Declaration of Helsinki and was approved by the local ethics committee of Region Zealand and the Danish Data Protection Agency. Baseline characteristics of the study subjects are shown in Table 1.

While resting in supine position at room temperature all of the 260 subjects had a blood sample taken. Blood samples were obtained as EDTA plasma and heparin plasma samples, whole blood (EDTA tubes), and serum, with rapid flow from a large antecubital vein using standard venipuncture techniques. For the plasma samples, subsequently, centrifugation was performed to obtain platelet-poor plasma. The plasma supernatant was separated and the aliquot was transferred to cryotubes. All blood aliquots were stored at −80°C until analyzed.

Within the same hour a transthoracic echocardiography was performed by an experienced level 3 echocardiographer using General Electric Vingmed Vivid 7 or 9 and MJS probe 1.5–4.0 MHz and following guidelines from the Danish Society of Cardiology. Based on EF the study subjects were divided into four groups: (1) ≥50% (preserved EF, considered as normal), (2) midrange EF 40–50%, (3) LVSD with EF of 30–40%, and (4) LVSD with EF < 30%. Presence of moderate or severe valvular dysfunction was registered.

2.2. ELISA for Full-Length sHLA-G (The sHLA-G1 and HLA-G5 Isoforms). The level of sHLA-G1/HLA-G5 molecules in blood plasma samples was determined by a commercially available sandwich enzyme immunoassay (ELISA) (EXBIO, Praha, Czech Republic) according to the manufacturer's instructions. This ELISA specifically detects sHLA-G1 and HLA-G5 in a β2-microglobulin- (β2m-) associated form. Samples were analyzed in duplicate on two independent assay plates, always with the same calibrators and positive and negative controls on each plate, and the first set of samples was also reanalyzed as the last samples to secure the reproducibility and precision of the assay. Blood plasma samples were diluted 1 + 3 with the provided Dilution Buffer (60 μL samples to 180 μL Dilution Buffer). Samples were thawed and mixed thoroughly and 100 μL of diluted plasma were loaded in duplicate onto microtiter plates precoated with the monoclonal antibody MEM-G/9 (anti-HLA-G1/G5). The plates were then incubated overnight at 4°C (with no shaking). Following five washing steps with 350 μL of the supplied washing buffer, 100 μL of conjugate solution was added and the plates were incubated at room temperature (RT) for one hour. The conjugate solution consisted of monoclonal anti-human β2-microglobulin antibody labeled with horseradish

Table 1: Baseline characteristics of the study population (LVSD, EF ≤ 40%) compared to the control group (EF > 40%).

Characteristics	Ejection fraction ≤ 40% LVSD: yes		Ejection fraction > 40% LVSD: no		All together	
	Number of individuals ($n = 61$)	Percentage	Number of individuals ($n = 199$)	Percentage	Number of individuals ($n = 260$)	Percentage
Age (years)	81		80		80 (75–92)	
Gender						
Females	14	23	119	60	134	52
Males	47	77	80	40	126	48
Body mass index (BMI) (kg/m^2)	26.1		25.4		25.6	
Smoking						
Present	8	13	18	9	26	10
Ex-smoker	35	57	95	48	130	50
Never smoker	18	30	86	43	104	40
History						
Angina pectoris	25	41	52	26	77	30
Acute Myocardial Infarction (AMI)	31	51	24	12	55	21
Percutaneous Coronary Intervention (PCI)	12	20	27	14	39	15
Coronary Artery Bypass Grafting (CABG)	15	25	15	8	30	12
Dilated cardiomyopathy	10	16	5	3	15	6
Valvular disorder	17	29	12	6	29	11
Valvular substitution	6	10	4	2	10	4
Arrhythmias	45	74	47	24	92	35
Atrial fibrillation P-P-P	38	62	37	19	75	29
Pacemaker	25	41	4	2	29	11
Hypertension	41	67	132	66	173	67
Diabetes mellitus	15	25	22	11	37	14
Hypercholesterolaemia	41	67	103	52	144	55
Thyroidal disease	6	10	25	13	31	12
Cerebrovascular disease	14	23	29	15	43	17
Peripheral arterial disease	10	16	13	7	23	9
Lung disease	27	44	40	20	67	26
Gastrointestinal disorder	33	54	91	46	124	48
Renal disorder (moderate-severe)	10	16	11	6	21	8
Musculoskeletal disease	41	67	119	60	160	62
Autoimmune disease	4	7	16	8	20	8
Anemia	10	16	11	6	21	8
Cancer	14	23	54	27	68	26
Charlson comorbidity index	3.7		1.5		2.0	
Corrected for age	7.3		4.9		5.5	
QRS-duration	138		94		104	

Table 1: Continued.

Characteristics	Ejection fraction ≤ 40% LVSD: yes		Ejection fraction > 40% LVSD: no		All together	
	Number of individuals (n = 61)	Percentage	Number of individuals (n = 199)	Percentage	Number of individuals (n = 260)	Percentage
Medical treatment						
Thrombocyte inhibitor	36	59	82	41	118	45
AK	23	38	30	15	53	20
Statin	35	57	74	37	109	42
Diuretics	46	75	74	37	120	46
Diuretics (Centyl)	10		49			
Diuretics (Furosemide)	32		23			
Aldosterone-antagonist	18	30	8	4	26	10
ACE-inhibitor	34	56	42	21	76	29
ATII	14	23	32	16	46	18
Beta-blockers	49	80	61	31	110	42
Clinical chemistry	Mean		Mean		Mean	
Hemoglobin (mmol/L)	8.1		8.2		8.2	
Potassium (mmol/L)	4.0		3.8		3.9	
Sodium (mmol/L)	139		139		139	
Creatinine (μmol/L)	116		84		92	
GFR	55		69		66	
C-reactive protein (CRP) (mg/L)	5.0		4.5		4.6	
Thyroid stimulating hormone (TSH) (mU/L)	1.3		1.6		1.5	
Albumin (g/L)	39		41		41	
Alanine aminotransferase (ALAT) (U/L)	20		21		21	
Uric acid (mmol/L)	0.49		0.35		0.38	
Glucose (mmol/L)	6.6		5.9		6.1	
NT-proBNP (pmol/L)	268		50		101	
Soluble HLA-G (U/mL)	71 (12–1144) (median, range)		52 (4–960) (median, range)		61 (4–1144) (median, range)	

Table 2: The distribution of soluble HLA-G (U/mL), NT-proBNP (pmol/L), and uric acid (mmol/L) according to the left ventricular ejection fraction (median, range).

Parameter	All study population	n	Left ventricular ejection fraction			
			>50%	40% to 50%	30% to 40%	<30%
Soluble HLA-G (U/mL)	61 (4–1144)	259	44 (4–960)	90 (11–758)	71 (12–1144)	71 (14–331)
NT-proBNP (pmol/L)	35 (4–1620)	259	24 (4–347)	41 (7–273)	134 (21–389)	307 (69–1620)
Uric acid (mmol/L)	0.37 (0.18–0.92)	257	0.34 (0.18–0.87)	0.37 (0.20–0.68)	0.44 (0.19–0.80)	0.44 (0.19–0.92)

peroxidase (HRP). After five additional washing steps, 100 μL of substrate solution with tetramethylbenzidine (TMB) were added to the plate, and the plate incubated once more at RT for 25 min with no shaking. Finally, 100 μL of acidic stop solution were added to the plates. The plates were then analyzed using a microplate reader. The assay was performed in a BEP2000 ELISA robot instrument (Siemens Healthcare Diagnostics, Germany).

2.3. Analysis of NT-proBNP and Uric Acid. NT-proBNP was measured on the Elecsys 2010 system (Roche Diagnostics). The assay is an electrochemiluminescent sandwich immunoassay that uses two polyclonal antibodies directed at residues 1–21 and 39–50 of the NT-proBNP molecule. The CV% of the assay is 3.2–2.4% from 20.7 to 585.5 pmol/L (175–4,962 ng/L) with an analytical range of 0.6–4138.6 pmol/L (5–35,000 ng/L). Plasma uric acid was measured on ARCHITECT ci8200 Integrated System (Abbott Diagnostics, North Chicago, IL, USA). One NT-proBNP test failed reducing the total number of study participants with a NT-proBNP test result to 259.

2.4. Genotyping of the 14 bp Insertion/Deletion Polymorphism (rs66554220) and the +3142 Single-Nucleotide Polymorphism (rs1063320) in the 3′-Untranslated Region of the HLA-G Gene. EDTA blood samples were carefully thawed and mixed thoroughly before processing. The DNA purification, using a Maxwell® 16 DNA Purification Kit, was performed in accordance with the manufacturer's instructions, and genomic DNA was stored at −20°C for further use. The real-time TaqMan PCR assay for genotyping of the HLA-G 14 bp insertion/deletion (ins/del) polymorphism in exon 8 (rs66554220) was performed using a LightCycler480 instrument (Roche Diagnostics, Switzerland) and performed as described by Djurisic et al. [25]. The genotyping of the +3142 SNP in the 3′UTR of the HLA-G gene (rs1063320) was performed as described by Bortolotti et al. [26].

2.5. Statistical Analysis. Specific *a priori* hypotheses were formulated. Each variable was tested for Gaussian (normal) distribution. In cases of a normal distribution, parametric tests were used (one-way ANOVA and unpaired t-test). Else, nonparametric tests were used (Kruskal-Wallis test, Mann–Whitney U test, and Jonckheere-Terpstra test). Receiver Operating Characteristic (ROC) curves were drawn for sHLA-G, proBNP, and uric acid. Charlson comorbidity index was calculated as described by Hall et al. [27]. Statistical analyses were made with the use of IBM SPSS version 22.0. Graphs were made in GraphPad Prism version 6 and IBM SPSS.

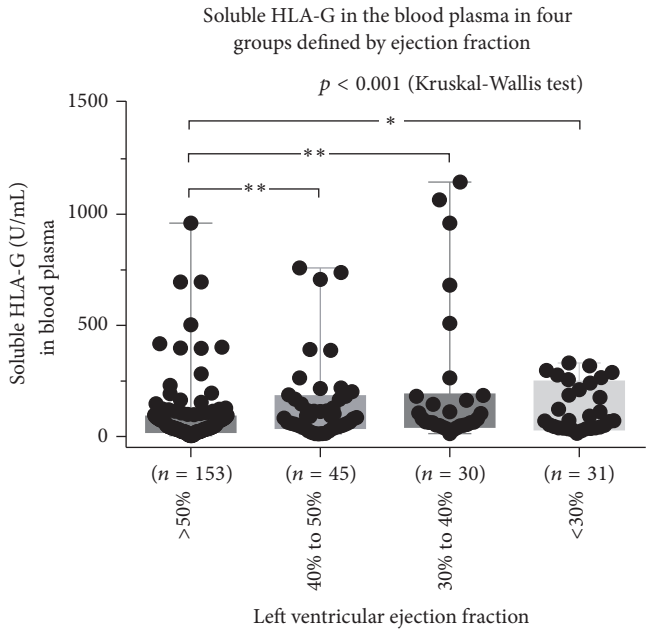


Figure 1: Soluble HLA-G in peripheral blood in relation to left ventricular ejection fraction (box and whiskers plot, min. to max., all points shown; $p < 0.001$, Kruskal-Wallis test; Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$) (one NT-proBNP test failed in the group with ejection fraction >50% reducing the number to 153).

3. Results

3.1. Characteristics of the Study Group. Table 1 shows the baseline characteristics of the study group (EF < 40%) versus the control group (EF ≥ 40%). According to echocardiography there were 154 (59.2%) subjects with EF ≥ 50%, 106 (40.8%) subjects with EF < 50%, 45 with EF 40–50%, and 61 (23.5%) with EF < 40% (30 with EF 30–40% and 31 with EF < 30%); 55 subjects had mitral or aortic valvular dysfunction to some degree, no one had severe valvular disease, and 20 patients with valvular disease also had LVSD.

3.2. Soluble HLA-G Levels in Blood Plasma Are Associated with Heart Failure. Soluble HLA-G in the blood plasma was significantly and uniformly higher in the two LVSD groups with EF < 30% and EF 30–40% and in the midrange group with EF 40–50%, compared to the group with preserved EF ≥ 50% ($p < 0.0001$, Kruskal-Wallis test (Figure 1 and Table 2)). The values of NT-proBNP and uric acid were increased with decreasing EF (Table 2). Furthermore, Receiver Operating Characteristic (ROC) curves showed that

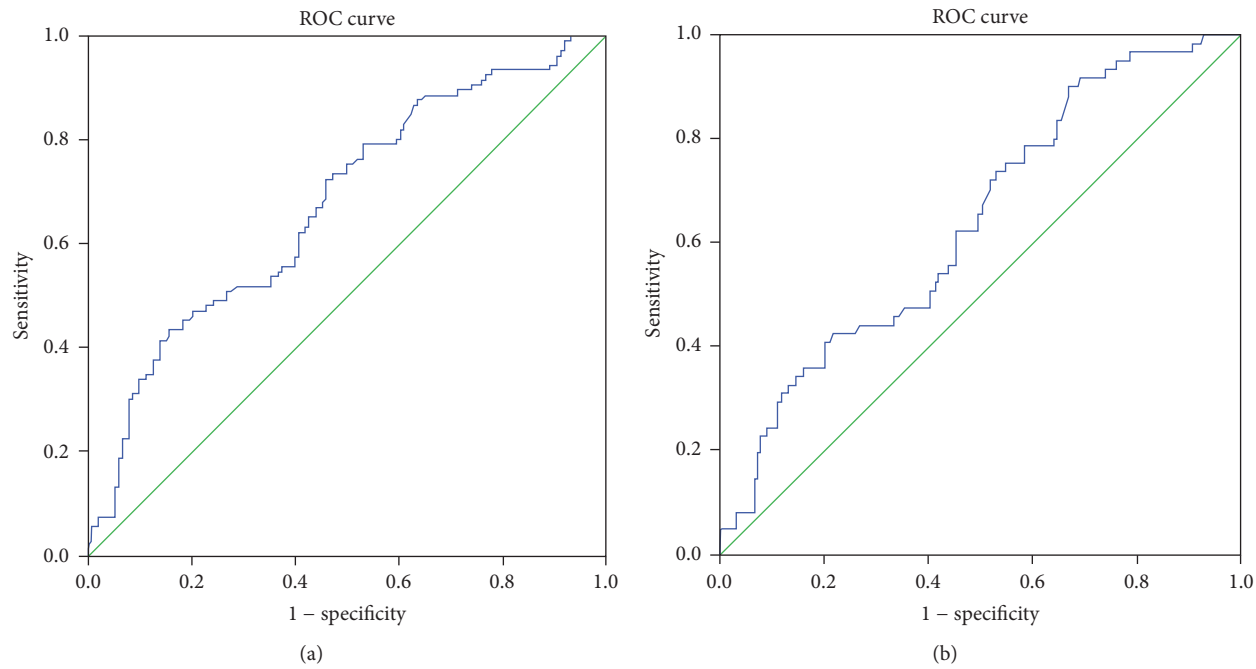


Figure 2: (a) Receiver Operating Characteristic (ROC) curve of soluble HLA-G with heart failure defined as ejection fraction <50%. Area under the curve is 0.676, $p < 0.001$. (b) Receiver Operating Characteristic (ROC) curve of soluble HLA-G with heart failure defined as ejection fraction <40%. Area under the curve is 0.639, $p = 0.001$.

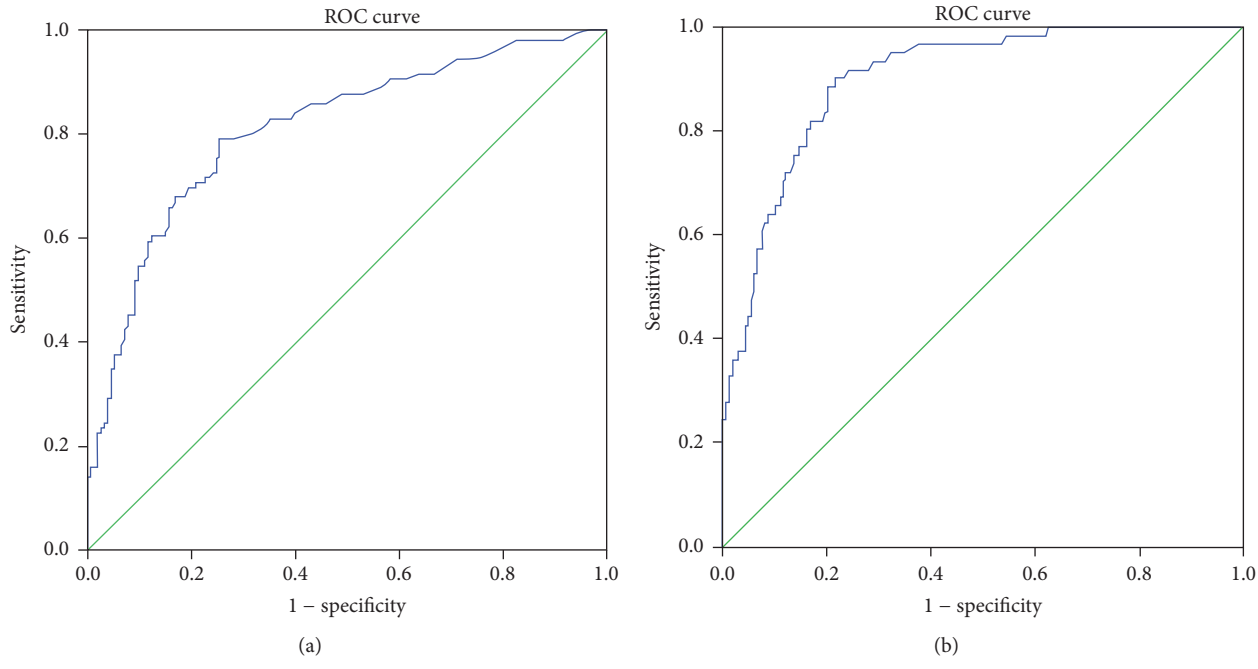


Figure 3: (a) Receiver Operating Characteristic (ROC) curve of NT-proBNP with heart failure defined as ejection fraction <50%. Area under the curve is 0.811, $p < 0.001$. (b) Receiver Operating Characteristic (ROC) curve of NT-proBNP with heart failure defined as ejection fraction <40%. Area under the curve is 0.902, $p < 0.001$.

NT-proBNP outperformed both sHLA-G and uric acid as a biomarker of LVSD (Figures 2–4).

There was no correlation between sHLA-G and uric acid, neither in the whole population nor in the patients with LVSD (Spearman, $p = 0.295$, $n = 256$; $p = 0.529$, $n = 105$).

There was a significant higher level of sHLA-G in cases of significant valvular heart disease for the whole study population of 260 subjects ($p = 0.002$, Mann–Whitney test Figure 5(a)). However, there was only a trend for the fraction of the population without LVSD ($p = 0.067$; Figure 5(b)).

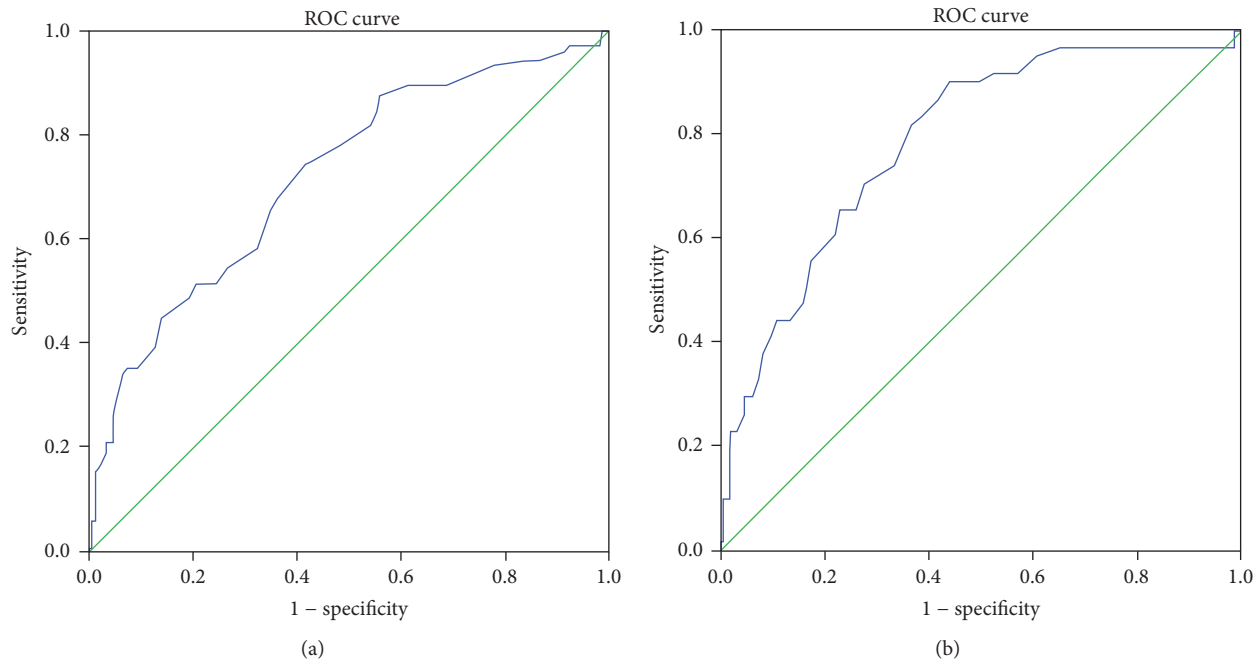


Figure 4: (a) Receiver Operating Characteristic (ROC) curve of uric acid with heart failure defined as ejection fraction <50%. Area under the curve is 0.721, $p < 0.001$. (b) Receiver Operating Characteristic (ROC) curve of uric acid with heart failure defined as ejection fraction <40%. Area under the curve is 0.788, $p < 0.001$.

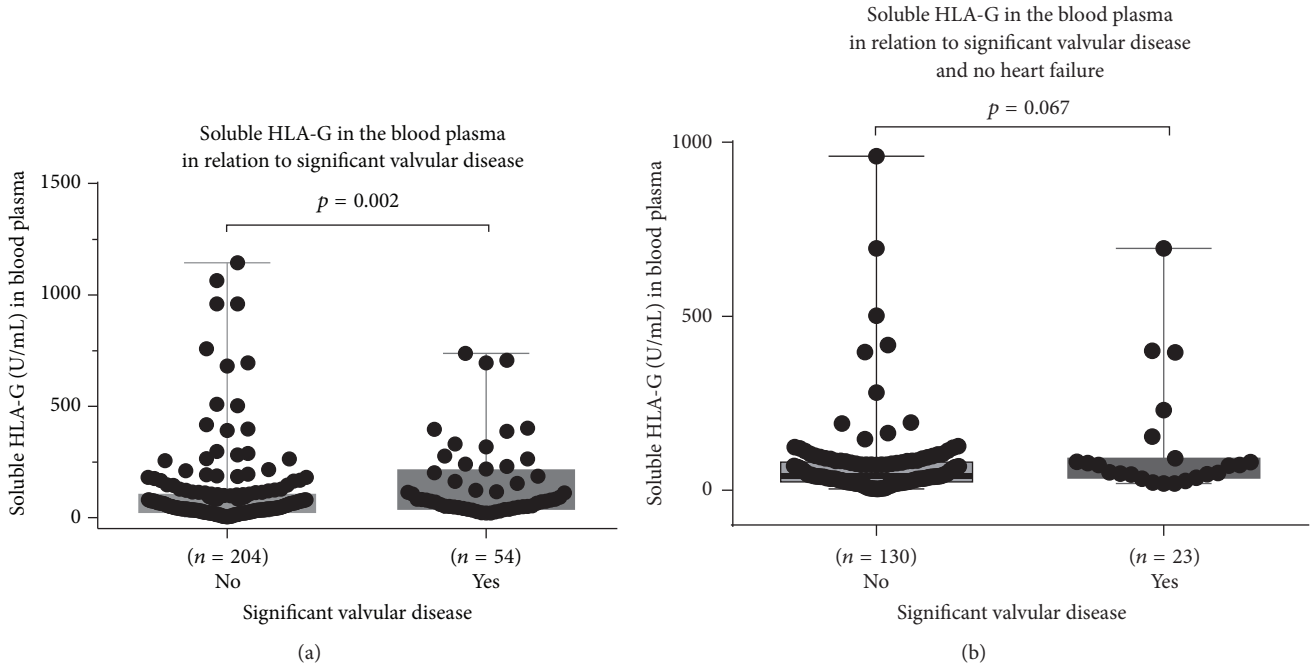


Figure 5: (a) Soluble HLA-G in peripheral blood in relation to significant valvular heart disease (box and whiskers plot, min. to max., all points shown; $p = 0.002$, Mann–Whitney test). (b) Soluble HLA-G in peripheral blood in relation to significant valvular heart disease in patients without heart failure (EF > 50%) (box and whiskers plot, min. to max., all points shown; $p = 0.067$, Mann–Whitney test).

3.3. HLA-G Gene Polymorphisms and Association with Risk of Heart Failure. There were no significant differences in the distributions of the 14 bp ins/del genotypes between the different EF groups (Table 3; $p = 0.82$, Chi-Square test). However, there was a tendency, when the group with EF > 40% was compared with the group with EF < 40% ($p = 0.10$, Chi-Square test). In the group with EF > 40%, the frequency of the Ins 14 bp/Ins 14 bp genotype

Table 3: Distribution of genotypes of the 14 bp insertion/deletion polymorphism in the 3′-untranslated region of the HLA-G gene in relation to left ventricular ejection fraction.

Ejection fraction	Del 14 bp/Del 14 bp	Del 14 bp/Ins 14 bp	Ins 14 bp/Ins 14 bp	Total
>50%[a]	61 (39.6%)	66 (42.9%)	27 (17.5%)	154 (100.0%)
40% to 50%	17 (37.8%)	17 (37.8%)	11 (24.4%)	45 (100.0%)
30% to 40%	12 (40.0%)	16 (53.3%)	2 (6.7%)	30 (100.0%)
<30%	12 (38.7%)	16 (51.6%)	3 (9.7%)	31 (100.0%)
>40%[b]	78 (39.2%)	83 (41.7%)	38 (19.1%)	199 (100.0%)
<40%[b]	24 (39.3%)	32 (52.5%)	5 (8.2%)	61 (100.0%)

[a] $p = 0.82$ (Chi-Square test, 0.40, df = 2); [b] $p = 0.10$ (Chi-Square test, 4.57, df = 2).

Table 4: Distribution of haplotypes of the 14 bp insertion/deletion polymorphism (Del/Ins) and the +3142 SNP (C/G) in the 3′-untranslated region of the HLA-G gene in relation to left ventricular ejection fraction.

Ejection fraction	DelC/DelC	DelC/DelG	DelC/InsC	DelC/InsG	DelG/DelG	DelG/InsG	InsG/InsG	Total
>40%[a]	62 (31.2%)	15 (7.5%)	1 (0.5%)	74 (37.2%)	1 (0.5%)	8 (4.0%)	38 (19.1%)	199 (100.0%)
<40%[a]	20 (32.8%)	4 (6.6%)	0 (0.0%)	23 (37.7%)	0 (0.0%)	9 (14.8%)	5 (8.2%)	61 (100.0%)

[a] $p = 0.033$ (Chi-Square test, 10.50, df = 4), for the Chi-Square test DelC/InsG was added to the DelC/DelC group and DelG/DelG was added to the DelG/InsG group.

was 19.1% and only 8.2% in the group with EF < 40% (Table 3).

This difference was statistically significant, when the combined haplotype of the two HLA-G gene polymorphisms, the 14 bp ins/del and the +3142 SNP, was analyzed (Table 4; $p = 0.033$, Chi-Square test). The combined genotype of the haplotype InsG/InsG was more frequent among subjects with EF > 40% than among patients with EF < 40%; the opposite was observed for the DelG/InsG combination of haplotypes.

In a separate analysis of the +3142 HLA-G SNP alone, no differences were observed in the distributions of the three genotypes between the group with EF < 40% and the group with EF > 40%. For the group with EF > 40%, the frequencies were C/C (32.2%), C/G (43.7%), and G/G (24.1%). For the group with EF < 40%, they were C/C (32.8%), C/G (44.3%), and G/G (23.0%).

4. Discussion

This study shows that sHLA-G is increased in patients with EF < 40 compared to patients with EF ≥ 40%. There was no difference in the concentration of sHLA-G in LVSD patients with EF < 30% and with EF between 30 and 40%. Thus, sHLA-G in the blood plasma does not indicate the severity of LVSD, and, in accordance with the study by Almasood et al., we conclude that sHLA-G is a very sensitive LVSD biomarker [8, 9, 24]. The assay used in the current study detects both soluble HLA-G5 and soluble HLA-G1 associated with β2-microglobulin. The source of sHLA-G in the blood from men and nonpregnant women is not well established but is probably derived from immune cells [21]. It can be speculated that the raise in sHLA-G associated with LVSD might originate from activated immune cells or from the heart.

Neither sHLA-G nor serum uric acid is comparable with NT-proBNP as LVSD-biomarker, primarily due to a poor specificity [5]. Soluble HLA-G is influenced by many other conditions, for example, cancer and autoimmune disease, which may confound the analysis and the results and reduce the suitability of sHLA-G as a biomarker for LVSD.

Specificity may be increased by genotyping for the combined haplotypes of the two tested HLA-G gene polymorphisms. An interesting novel finding in the current study was that the distribution of the combined haplotypes of the two tested HLA-G gene polymorphisms, the 14 bp ins/del and the +3142 SNP in the 3′UTR of the gene, was statistically significant between the subjects with EF > 40% and patients with EF < 40% (Table 4; $p = 0.033$). InsG/InsG was more frequent among subjects with EF > 40% than patients with EF < 40%; the opposite was observed for DelG/InsG. Interestingly, in healthy blood donors, the Ins 14 bp/Ins 14 bp HLA-G genotype has been significantly associated with low sHLA-G levels in the blood in several studies [28, 29]. Furthermore, the DelG/InsG combination has also been associated with a higher level of sHLA-G than the InsG/InsG combination, for example, in patients with multiple sclerosis; however, the DelC/DelC combination showed the highest concentrations of sHLA-G in the same study [30]. This is supported by the observations in the current study, which could indicate that specific HLA-G gene polymorphisms or haplotypes might influence the sHLA-G level in the blood and thereby the individual sHLA-G response in specific patients with systolic heart failure. It is not known whether this is due to genetic or epigenetic factors or if it is an adaptive mechanism triggered by the inflammatory process [19, 20].

There are certain limitations to the present study. One limitation of the current study is that the number of study participants is rather small; however, it is still the largest study until now regarding HLA-G in subjects with and without systolic heart failure. The study reflects clinical practice. The diagnosis of heart failure is uncertain for the small group with EF in the grey zone of 40–50%, but echocardiography is the standard diagnostic test and in this study performed by the most qualified [1, 2]. Important confounding factors

are morbidities other than systolic heart failure, and these are common and inevitable (Table 1).

One of the reactions of the body to injury is inflammation represented in this study by serum uric acid, which might trigger an immunologic response represented in this study by sHLA-G [10]. Both are biomarkers of LVSD, and thus inflammation and immune modulation seem to be involved in LVSD. This is in accordance with accumulating evidence that systemic and persistent inflammatory disorders predispose to cardiovascular diseases. This is the case in gout, rheumatoid arthritis, psoriasis, inflammatory bowel disease, lupus erythematosus, sclerosis disseminatus, and other autoimmune diseases and in chronic infections and cancers [4, 5, 18, 31]. It is also observed in conditions associated with long-lasting low grade inflammation and endothelial dysfunctions like atherosclerosis, diabetes mellitus, the metabolic syndrome, venous thromboembolism, smoking, and affective disorders and in chronic heart failure [4, 12]. Upregulation of HLA-G is present in most of these disorders, which nevertheless are dominated by inflammation [18]. Out-of-balance inflammation with persistent rise in inflammatory cytokines seems to be the common denominator for many potentially coherent diseases and disorders, and it acts self-reinforcing in a complex vicious circle [4]. The inflammatory triggers and mediators are poorly understood, but they promote and regulate the inflammatory cascade that predisposes to, for example, atherosclerosis and LVSD [31].

HLA-G is elevated in many conditions, and thus there is a lack of diagnostic precision and specificity, which may obscure evaluation of the significance of HLA-G as a biomarker of LVSD in a multimorbid ageing population like the one in the current study. Furthermore, it cannot be determined from the current study, which role sHLA-G might have in the pathogenesis and the clinical course and prognosis of LVSD and whether it is a simple marker or participate in LVSD.

Likewise, the pathophysiologic effect of uric acid in LVSD is unknown. Serum uric acid has a negative correlation with EF [8, 9]. It is an independent risk factor for LVSD, but it is not known if it is cause, consequence, or simply an epiphenomenon. The serum uric acid concentration is increased in patients with chronic LVSD, probably due to both reduced renal excretion and augmented production [8, 9]. Low-sodium diet, diuretics, and insulin resistance may increase reabsorption of uric acid. Cardiac and renal disorders are related and as cardiac function deteriorates with falling cardiac output, the glomerular filtration rate (GFR) falls, which leads to a reduction in renal uric acid excretion [10]. At the same time the inflammatory process associated with the chronic diseases accelerates, which contributes to an increase in serum uric acid, TNF, interleukin-1 and interleukin-6, and other cytokines and in sHLA-G [7]. The simultaneous elevation of uric acid and sHLA-G might represent different aspects of the same process, acting in negative feedback as proinflammatory and anti-inflammatory markers.

In LVSD there is an imbalance between proinflammatory and anti-inflammatory cytokines [32]. The clinical significance remains to be determined. There are indications that the prognosis may be improved by restraining the inflammatory process, and an increase in EF in LVSD has been observed following treatment with, for example, thalidomide, pentoxifylline, intravenous immunoglobulin, glucocorticoid, colchicines, methotrexate, biological agents, interleukin-10, influenza vaccination, HIV-therapy, antidiabetic sodium glucose cotransporters, and antidepressant drugs [4, 12, 32, 33]. However, no improvement was observed in a variety of antibiotic trials and in studies antagonizing TNF in patients with LVSD, and there have been mixed results in studies of the use of allopurinol, which inhibits xanthine oxidase [4, 8, 11, 12, 31]. In the future, therapies directed at downregulating or inhibiting inflammation may reduce atherosclerosis and its complications including heart failure [33]. Further studies are needed to elucidate the role of HLA-G in this scenario.

5. Conclusion

For the first time, it was shown in the current study that a combined haplotype (DelG/InsG) of two HLA-G gene polymorphisms, the 14 bp ins/del and the +3142 SNP, were more frequent among patients with EF < 40% than among subjects with EF ≥ 40%. The opposite was observed for the combined haplotype InsG/InsG. This probably influences the sHLA-G level in the blood, and this study also showed that sHLA-G was increased in patients with EF < 40 compared to patients with EF ≥ 40%.

Competing Interests

The authors declare that there were no financial or any other factors that may have led to a conflict of interests.

Acknowledgments

This work was supported by The Region Zealand Research Foundation of Cross-Sectional Health Projects. The authors would like to thank Anja S. Torbensen for helpful technical assistance regarding the measurements of sHLA-G and the HLA-G genotyping and Lili Hald Jensen for measurement of NT-proBNP. Furthermore, the authors would like to thank Roche Diagnostics for providing the diagnostic assays Elecsys 2010 system to measure NT-proBNP.

References

[1] P. Ponikowski, A. A. Voors, S. D. Anker et al., "2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: the Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) developed with the special contribution of the Heart Failure Association (HFA) of the ESC," *European Heart Journal*, vol. 37, no. 27, pp. 2129–2200, 2016.

[2] L. L. Olesen, A. Andersen, and S. Thaulow, "Hand-held echocardiography is useful for diagnosis of left systolic dysfunction in an elderly population," *Danish Medical Journal*, vol. 62, no. 7, Article ID A5100, pp. 1–5, 2015.

[3] A. Mosterd and A. W. Hoes, "Clinical epidemiology of heart failure," *Heart*, vol. 93, no. 9, pp. 1137–1146, 2007.

[4] L. Gullestad, T. Ueland, L. E. Vinge, A. Finsen, A. Yndestad, and P. Aukrust, "Inflammatory cytokines in heart failure: mediators and markers," *Cardiology*, vol. 122, no. 1, pp. 23–35, 2012.

[5] A. Maisel, C. Mueller, K. Adams Jr. et al., "State of the art: using natriuretic peptide levels in clinical practice," *European Journal of Heart Failure*, vol. 10, no. 9, pp. 824–839, 2008.

[6] L. L. Olesen and A. Andersen, "ECG as a first step in the detection of left ventricular systolic dysfunction in the elderly," *ESC Heart Failure*, vol. 3, no. 1, pp. 44–52, 2016.

[7] C. Ruggiero, A. Cherubini, A. Ble et al., "Uric acid and inflammatory markers," *European Heart Journal*, vol. 27, no. 10, pp. 1174–1181, 2006.

[8] G. S. Filippatos, M. I. Ahmed, J. D. Gladden et al., "Hyperuricaemia, chronic kidney disease, and outcomes in heart failure: potential mechanistic insights from epidemiological data," *European Heart Journal*, vol. 32, no. 6, pp. 712–720, 2011.

[9] G. Lippi, M. Montagnana, M. Franchini, E. J. Favaloro, and G. Targher, "The paradoxical relationship between serum uric acid and cardiovascular disease," *Clinica Chimica Acta*, vol. 392, no. 1-2, pp. 1–7, 2008.

[10] F. Leyva, S. D. Anker, I. F. Godsland et al., "Uric acid in chronic heart failure: a marker of chronic inflammation," *European Heart Journal*, vol. 19, no. 12, pp. 1814–1822, 1998.

[11] D. L. Mann, "Innate immunity and the failing heart. The cytokine hypothesis revisited," *Circulation Research*, vol. 116, no. 7, pp. 1254–1268, 2015.

[12] E. Oikonomou, D. Tousoulis, G. Siasos, M. Zaromitidou, A. G. Papavassiliou, and C. Stefanadis, "The role of inflammation in heart failure: new therapeutic approaches," *Hellenic Journal of Cardiology*, vol. 52, no. 1, pp. 30–40, 2011.

[13] T. V. F. Hviid, "HLA-G in human reproduction: aspects of genetics, function and pregnancy complications," *Human Reproduction Update*, vol. 12, no. 3, pp. 209–232, 2006.

[14] M. H. Larsen and T. V. F. Hviid, "Human leukocyte antigen-G polymorphism in relation to expression, function, and disease," *Human Immunology*, vol. 70, no. 12, pp. 1026–1034, 2009.

[15] E. A. Donadi, E. C. Castelli, A. Arnaiz-Villena, M. Roger, D. Rey, and P. Moreau, "Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association," *Cellular and Molecular Life Sciences*, vol. 68, no. 3, pp. 369–395, 2011.

[16] E. D. Carosella, N. Rouas-Freiss, D. T.-L. Roux, P. Moreau, and J. LeMaoult, "HLA-G. an immune checkpoint molecule," *Advances in Immunology*, vol. 127, pp. 33–144, 2015.

[17] R. Sheshgiri, N. Rouas-Freiss, V. Rao et al., "Myocardial HLA-G reliably indicates a low risk of acute cellular rejection in heart transplant recipients," *The Journal of Heart and Lung Transplantation*, vol. 27, no. 5, pp. 522–527, 2008.

[18] N. Rouas-Freiss, P. Moreau, J. LeMaoult, and E. D. Carosella, "The dual role of HLA-G in cancer," *Journal of Immunology Research*, vol. 2014, Article ID 359748, 10 pages, 2014.

[19] L. L. Nilsson, S. Djurisic, and T. V. F. Hviid, "Controlling the immunological crosstalk during conception and pregnancy: HLA-G in reproduction," *Frontiers in Immunology*, vol. 5, article 198, 2014.

[20] S. Djurisic and T. V. F. Hviid, "HLA class Ib molecules and immune cells in pregnancy and preeclampsia," *Frontiers in Immunology*, vol. 5, article 652, 2014.

[21] V. Rebmann, J. Regel, D. Stolke, and H. Grosse-Wilde, "Secretion of sHLA-G molecules in malignancies," *Seminars in Cancer Biology*, vol. 13, no. 5, pp. 371–377, 2003.

[22] A. Ishitani and D. E. Geraghty, "Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 9, pp. 3947–3951, 1992.

[23] G. M. Park, S. Lee, B. Park et al., "Soluble HLA-G generated by proteolytic shedding inhibits NK-mediated cell lysis," *Biochemical and Biophysical Research Communications*, vol. 313, no. 3, pp. 606–611, 2004.

[24] A. Almasood, R. Sheshgiri, J. M. Joseph et al., "Human leukocyte antigen-G is upregulated in heart failure patients: a potential novel biomarker," *Human Immunology*, vol. 72, no. 11, pp. 1064–1067, 2011.

[25] S. Djurisic, A. E. Sørensen, and T. V. F. Hviid, "A fast and easy real-time PCR genotyping method for the HLA-G 14-bp insertion/deletion polymorphism in the 3′ untranslated region," *Tissue Antigens*, vol. 79, no. 3, pp. 186–189, 2012.

[26] D. Bortolotti, V. Gentili, L. Melchiorri, A. Rotola, and R. Rizzo, "An accurate and reliable real time SNP genotyping assay for the HLA-G + 3142 bp C > G polymorphism," *Tissue Antigens*, vol. 80, pp. 259–262, 2012.

[27] W. H. Hall, R. Ramachandran, S. Narayan, A. B. Jani, and S. Vijayakumar, "An electronic application for rapidly calculating Charlson comorbidity score," *BMC Cancer*, vol. 4, article 94, pp. 1–8, 2004.

[28] T. V. F. Hviid, R. Rizzo, O. B. Christiansen, L. Melchiorri, A. Lindhard, and O. R. Baricordi, "HLA-G and IL-10 in serum in relation to HLA-G genotype and polymorphisms," *Immunogenetics*, vol. 56, no. 3, pp. 135–141, 2004.

[29] X.-Y. Chen, W.-H. Yan, A. Lin, H.-H. Xu, J.-G. Zhang, and X.-X. Wang, "The 14 bp deletion polymorphisms in HLA-G gene play an important role in the expression of soluble HLA-G in plasma," *Tissue Antigens*, vol. 72, no. 4, pp. 335–341, 2008.

[30] R. Rizzo, D. Bortolotti, N. Ben Fredj et al., "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*, vol. 73, no. 11, pp. 1140–1146, 2012.

[31] J. S. Zebrack and J. L. Anderson, "The role of inflammation and infection in the pathogenesis and evolution of coronary artery disease," *Current Cardiology Reports*, vol. 4, no. 4, pp. 278–288, 2002.

[32] C. Stumpf, K. Seybold, S. Petzi et al., "Interleukin-10 improves left ventricular function in rats with heart failure subsequent to myocardial infarction," *European Journal of Heart Failure*, vol. 10, no. 8, pp. 733–739, 2008.

[33] P. M. Ridker and T. F. Lüscher, "Anti-inflammatory therapies for cardiovascular disease," *European Heart Journal*, vol. 35, no. 27, pp. 1782–1791, 2014.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 3890306, 9 pages
http://dx.doi.org/10.1155/2016/3890306

Research Article

Study of Soluble HLA-G in Congenital Human Cytomegalovirus Infection

Roberta Rizzo,[1] Liliana Gabrielli,[2] Daria Bortolotti,[1] Valentina Gentili,[1] Giulia Piccirilli,[2] Angela Chiereghin,[2] Claudia Pavia,[2] Silvia Bolzani,[1] Brunella Guerra,[3] Giuliana Simonazzi,[3] Francesca Cervi,[3] Maria Grazia Capretti,[4] Enrico Fainardi,[5] Dario Di Luca,[1] Maria Paola Landini,[6] and Tiziana Lazzarotto[6]

[1]*Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Ferrara, Italy*
[2]*Operative Unit of Clinical Microbiology, St. Orsola-Malpighi University Hospital, Bologna, Italy*
[3]*Operative Unit of Obstetrics and Prenatal Medicine, Department of Medical and Surgical Sciences, St. Orsola-Malpighi University Hospital, University of Bologna, Bologna, Italy*
[4]*Operative Unit of Neonatology, St. Orsola-Malpighi University Hospital, Bologna, Italy*
[5]*Operative Unit of Neuroradiology, Careggi University Hospital, Firenze, Italy*
[6]*Operative Unit of Clinical Microbiology, Department of Specialised, Experimental, and Diagnostic Medicine, St. Orsola-Malpighi University Hospital, University of Bologna, Bologna, Italy*

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

Received 19 April 2016; Revised 9 August 2016; Accepted 16 August 2016

Academic Editor: Nejat K. Egilmez

Human leukocyte antigen-G (HLA-G) is a nonclassical HLA class I antigen that is expressed during pregnancy contributing to maternal-fetal tolerance. HLA-G can be expressed as membrane-bound and soluble forms. HLA-G expression increases strongly during viral infections such as congenital human cytomegalovirus (HCMV) infections, with functional consequences in immunoregulation. In this work we investigated the expression of soluble (s)HLA-G and beta-2 microglobulin (component of HLA) molecules in correlation with the risk of transmission and severity of congenital HCMV infection. We analyzed 182 blood samples from 130 pregnant women and 52 nonpregnant women and 56 amniotic fluid samples from women experiencing primary HCMV infection. The median levels of sHLA-G in maternal serum of women with primary HCMV infection were higher in comparison with nonprimary and uninfected pregnant women ($p < 0.001$). AF from HCMV symptomatic fetuses presented higher sHLA-G levels in comparison with infected asymptomatic fetuses ($p < 0.001$), presence of HLA-G free-heavy chain, and a concentration gradient from amniotic fluid to maternal blood. No significant statistical difference of beta-2 microglobulin median levels was observed between all different groups. Our results suggest the determination of sHLA-G molecules in both maternal blood and amniotic fluid as a promising biomarker of diagnosis of maternal HCMV primary infection and fetal HCMV disease.

1. 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 nonprimary infection (1%) [2, 3]. Nevertheless, it was estimated that, for all population seroprevalences, nonprimary maternal infections are responsible for the majority of congenital CMV infections [4].

Ten to fifteen percent of congenitally infected infants of primarily infected women will have symptoms at birth and around 10% of them will not survive. Moreover, 70–80% of surviving babies will suffer delayed sequelae such as sensorineural hearing loss, delay of psychomotor development, and visual impairment [5]. Most congenital infected infants

(85–90%) have no symptoms at birth, but 8% to 15% of them will develop delayed injury [3, 5].

The fetal compartment can be studied by invasive (amniocentesis) and noninvasive (ultrasound examination) techniques [6]. Ultrasonographic findings are helpful but not diagnostic findings since HCMV has features in common with other intrauterine infections and its sensitivity is poor [7]. 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 [8–10]. 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 upregulate specific HLA class I molecules binding to immune cell inhibitory receptors [11]. In this scenario, there is an interesting nonclassical 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 3 soluble (HLA-G5–G7) [12]. In addition, the HLA-G1 isoform can produce a soluble form called sHLA-G1, derived from membrane proteolytic shedding [13]. HLA-G is expressed at the maternal-fetal interface, on surface of trophoblasts [12], and the concentrations of soluble (s)HLA-G increase in the plasma samples of pregnant women during the first trimester of pregnancy [14]. HCMV infection modifies HLA-G expression in tissues and immune cells, with a downmodulation in infected cytotrophoblasts [15] and upregulation in infected peripheral blood cells [16]. Specific HCMV proteins modify HLA-G expression interacting with the HLA-G promoter, and affecting mRNA stability, protein translation, and the secretory pathway [17–19]. The increase in HLA-G expression is suggested as a mechanism for virus immune escape, due to the immune-inhibitory functions of HLA-G. Finally, it has been observed that another important component of HLA class I molecules, beta-2 microglobulin (b2M), has a diagnostic efficacy for differentiating symptomatic from asymptomatic HCMV congenital infection [20].

In order to explore the possible role of HLA-G molecules in congenital HCMV infection, we analyzed maternal and fetal sHLA-G and b2M expression in correlation with the risk of transmission and severity of HCMV infection.

2. Materials and Methods

2.1. Subjects. The study analyzed the serum samples of a cohort of 130 pregnant women who were referred to the Maternal-Fetal Medicine Unit, St. Orsola-Malpighi University Hospital, Bologna, between 2006 and 2011 for suspected primary maternal HCMV infection. Maternal primary HCMV infection was assessed at the Virology Unit of the same University Hospital. Written informed consent for the studies was obtained from all patients according to the protocol approved by the Scientific Ethical Committee of the Ferrara and Bologna Universities.

The women, aged between 18 and 40 years, were in the first or second trimester of pregnancy. They presented no previous autoimmune and inflammatory diseases and they were not on any anti-inflammatory or immune-modulatory drugs or hyperimmune globulin.

Primary infection was diagnosed based on clinical and laboratory history and HCMV IgM-positive and low/moderate HCMV IgG avidity results as well as positive DNAemia and/or seroconversion for HCMV. Nonprimary maternal HCMV infection was diagnosed, within the first 16 weeks of 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.

Fifty-two nonpregnant women with HCMV past infection (IgG positive and IgM negative) were recruited as healthy controls.

Moreover, 56 amniotic fluid samples were collected during amniocentesis (20-21 weeks of gestation) from those pregnant women with primary HCMV infection arising before the 14th week of gestation; HCMV detection on amniotic fluid samples was performed with virus isolation and real-time PCR.

2.2. Diagnosis of Congenital HCMV Infection in Fetuses and Infants. Infection status of the aborted fetuses was classified on the basis of histological and immunohistochemical tissue examination, whereas the infection status of infants was classified on the basis of viral isolation and real-time PCR from urine within the first 2 weeks of life.

Fetal symptomatic infection was defined as the presence of ultrasound abnormalities and histological and immunohistochemical findings in fetal organs with particular attention to the brain [21]. CMV disease in infected newborns was investigated through clinical, instrumental, and laboratory examination in the neonatal and subsequently monitored up to 6 years of age [22].

2.3. Anti-HCMV IgM and IgG Detection and IgG Avidity. Maternal serum samples were tested using the Enzygnost® HCMV IgM and Enzygnost HCMV IgG assays (Siemens Healthcare Diagnostics) and an in-house immunoblot for detection of HCMV-specific IgM [23]. HCMV IgG avidity was tested with the Radim® Cytomegalovirus IgG Avidity EIA WELL assay (Radim).

2.4. Virological Examinations. HCMV isolation from amniotic fluid was performed by shell-vial procedure as described elsewhere [24]. 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.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA) for Soluble HLA-G. sHLA-G levels in serum and amniotic fluid samples were assayed in triplicate as previously reported [25, 26] using the monoclonal antibody (MoAb) MEM-G9 (Exbio), which recognizes the HLA-G molecules, in b2M 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.

2.6. ELISA for Soluble Beta-2 Microglobulin and Albumin. b2M concentration was determined in triplicate using a commercial human beta-2 microglobulin 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 interassay CV of 3.5 to 6.4%.

2.7. Determination of sHLA-G Index. Fetal production of HLA-G was calculated with the following formula [27]:

$$\text{sHLA-G Index} = \frac{\text{amniotic fluid : serum sHLA-G}}{\text{amniotic fluid : serum albumin}}, \quad (1)$$

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

2.8. Western Blot Analysis. Serum samples and amniotic fluids were biotinylated with 0.2 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in pH 8.0 PBS 1x for 30 min at 4°C [26]. Samples were then immunoprecipitated for 2 hrs at room temperature with anti-HLA-G MoAb (MEM-G1, specific for HLA-G free-heavy chain, or MEM-G9, specific for b2M conjugated HLA-G, Exbio), washed twice in PBS 1x, and incubated overnight with protein G-Sepharose beads (Santa Cruz) at 4°C. The samples were washed twice and resuspended in 20 μL Laemmli Buffer (BioRad). We quantified protein concentration in immunoprecipitates by the Bradford assay (Bio-Rad Laboratories) using bovine albumin (Sigma-Aldrich) as standard. Total protein was denatured at 100°C for 5 min. Proteins were loaded with native or reducing buffers in 10% TGX-Precast gel (Biorad), with subsequent electroblotting transfer onto a PVDF membrane (Millipore) [28]. The membrane was incubated with a horseradish peroxidase- (HRP-) conjugated antimouse antibody (1 : 5000; Amersham Biosciences) and developed with the ECL kit (Amersham Biosciences). The images were acquired by Geliance 600 (Perkin Elmer).

2.9. Statistics. Statistical analysis was performed with Stat View software package (SAS Institute Inc). Given that the data, screened by Kolmogorov-Smirnov test, presented a normal distribution, statistical analyses were performed using Student's t-test. 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 analysis (JROCFIT software, John Hopkins University).

3. Results

3.1. sHLA-G and Beta-2 Microglobulin Levels in Maternal Serum Samples. We evaluated sHLA-G and b2M levels in sera of 130 pregnant women, 30 uninfected, 56 with primary HCMV infection, and 44 with nonprimary HCMV infection, and 52 nonpregnant women with HCMV past infection.

Detectable serum levels of sHLA-G were significantly more frequent in pregnant (130/130; 100%) than in nonpregnant women (31/52; 59.6%) ($p < 0.0001$). b2M molecules presented only slightly higher positive samples in pregnant women (65/100; 50%) than in nonpregnant women (20/52; 38.5%) ($p = 0.14$) (data not shown).

Regarding the median levels of sHLA-G, pregnant women showed higher levels of molecules in comparison with nonpregnant women (49 versus 21 ng/mL; range: 37.4–76.5 ng/mL versus 0.0–20.5 ng/mL) irrespective of HCMV infection ($p < 0.001$) (Figure 1(a)). In addition, we observed no statistical differences in sHLA-G serum median levels between actively HCMV infected (56 with primary and 44 with nonprimary infection) and 30 uninfected pregnant women (47 versus 50 ng/mL; range: 36.2–69.8 versus 37.4–76.5 ng/mL; $p = 0.43$) (data not shown).

Interestingly, subdividing the subjects according to the maternal HCMV infection status, primary infected pregnant women presented higher levels of sHLA-G median concentrations (62 ng/mL; 46.9–69.8 ng/mL) than nonprimary infected women (44 ng/mL; 36.2–57.2 ng/mL) ($p < 0.001$). Moreover, primary infected pregnant women presented higher sHLA-G median concentrations than uninfected women (50 ng/mL; 37.4–76.5 ng/mL) ($p = 0.006$) (Figure 1(a)).

The median levels of b2M were only slightly higher in actively HCMV infected (primary and nonprimary) than in uninfected pregnant women (1.8 versus 1.3 μg/mL; $p = 0.14$), in primary than in nonprimary HCMV infected (1.9 versus 1.4 μg/mL; $p = 0.12$), and, finally, in primary HCMV infected than in uninfected women (1.9 versus 1.3 μg/mL; $p = 0.06$) (Figure 1(b)).

3.2. sHLA-G and Beta-2 Microglobulin Levels in Amniotic Fluid. We evaluated sHLA-G and b2M levels in 56 amniotic fluid samples from women who were primarily HCMV infected before week 14 of gestation and accepted amniocentesis.

Out of the 56 amniotic fluids, 39 samples were HCMV negative with PCR and virus isolation and no congenitally infected newborns were found in this group.

Out of 17 amniotic fluids from mothers who transmitted the virus to their fetuses/babies, two were negative for both virus isolation and PCR. Despite these negative results, the 2 babies were congenitally infected, but asymptomatic at birth and during the follow-up period, as already described in the literature [9, 10]. The remaining 15 amniotic samples were positive for both virological tests (6×10^5 copies/mL median

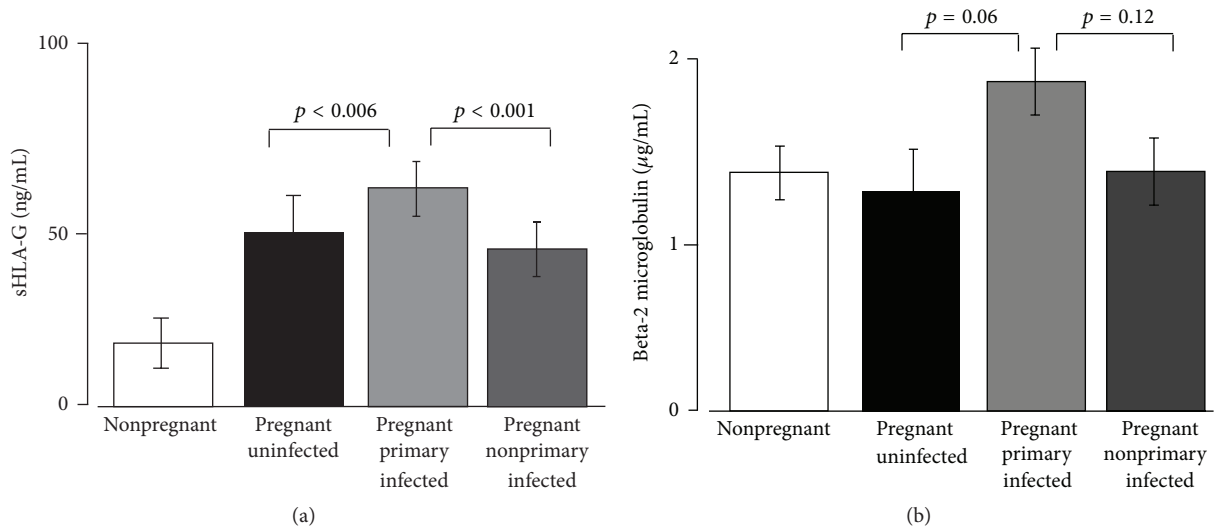


Figure 1: Maternal serum samples expression of (a) sHLA-G and (b) b2M molecules in nonpregnant and pregnant women. Pregnant women are classified as uninfected and primarily and nonprimarily HCMV infected. p values obtained by Student's t-test and mean ± standard deviation are reported.

viral load) except for one case where only HCMV-DNA was detected (10^3 copies/mL).

Overall, out of the 17 fetuses/babies infected with congenital HCMV, 5 newborns were asymptomatic at birth and during subsequent monitoring and 11 fetuses and 1 newborn were symptomatic.

The brains of all symptomatic fetuses were HCMV positive with severe histological brain damage and cerebral necrosis; 4 of the fetuses also showed pathological neurosonographic findings (periventricular hyperechogenicity and ventriculomegaly).

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

No statically significant difference in amniotic fluid HCMV load was observed between asymptomatic and symptomatic fetuses/newborns ($p = 0.88$, 95% CI: −3583021 to 3110188).

All the 56 amniotic fluid samples were positive for sHLA-G and b2M molecules (data not shown).

Median detectable levels of sHLA-G were significantly higher in amniotic fluids from infected symptomatic fetuses (73 ng/mL; 69–79 ng/mL) than in infected asymptomatic fetuses (32 ng/mL; 28–42 ng/mL) ($p < 0.001$) and in uninfected fetuses (31 ng/mL; 29–40.2 ng/mL) ($p < 0.001$) (Figure 2(a)).

b2M presented slightly higher median levels in amniotic fluids from infected symptomatic fetuses (4.5 μg/mL) than in infected asymptomatic fetuses (3.6 μg/mL) ($p = 0.039$) and uninfected fetuses (3.9 μg/mL) ($p = 0.042$) (Figure 2(b)).

When we considered maternal serum levels according to fetus infection status, we observed that sHLA-G concentrations were slightly higher in serum from women with symptomatic fetuses (51.2 ng/mL, 45–57 ng/mL) and in women with infected asymptomatic fetuses (49 ng/mL, 42–55 ng/mL) in comparison with uninfected fetuses (46 ng/mL, 44–52 ng/mL) ($p = 0.045$, $p = 0.042$, resp.) (Figure 2(c)).

b2M presented no differences in serum samples from women with infected symptomatic fetuses, infected asymptomatic fetuses, and uninfected fetuses (Figure 2(d)).

3.3. sHLA-G Concentration Gradient between Maternal Serum and Amniotic Fluid. The sHLA-G increase in amniotic fluids of infected symptomatic fetuses prompted the question of 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 3(a)). These results suggest a local fetal production of sHLA-G, increased only in fetuses with symptomatic HCMV infection.

3.4. sHLA-G Index. The association between fetal HCMV infection and increased sHLA-G expression in amniotic fluid was confirmed calculating the sHLA-G index in comparison with albumin. Albumin is the most prevalent serum protein which surrounds the embryo and is detected in amniotic fluids. Fainardi et al. [27] 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 sHLA-G,

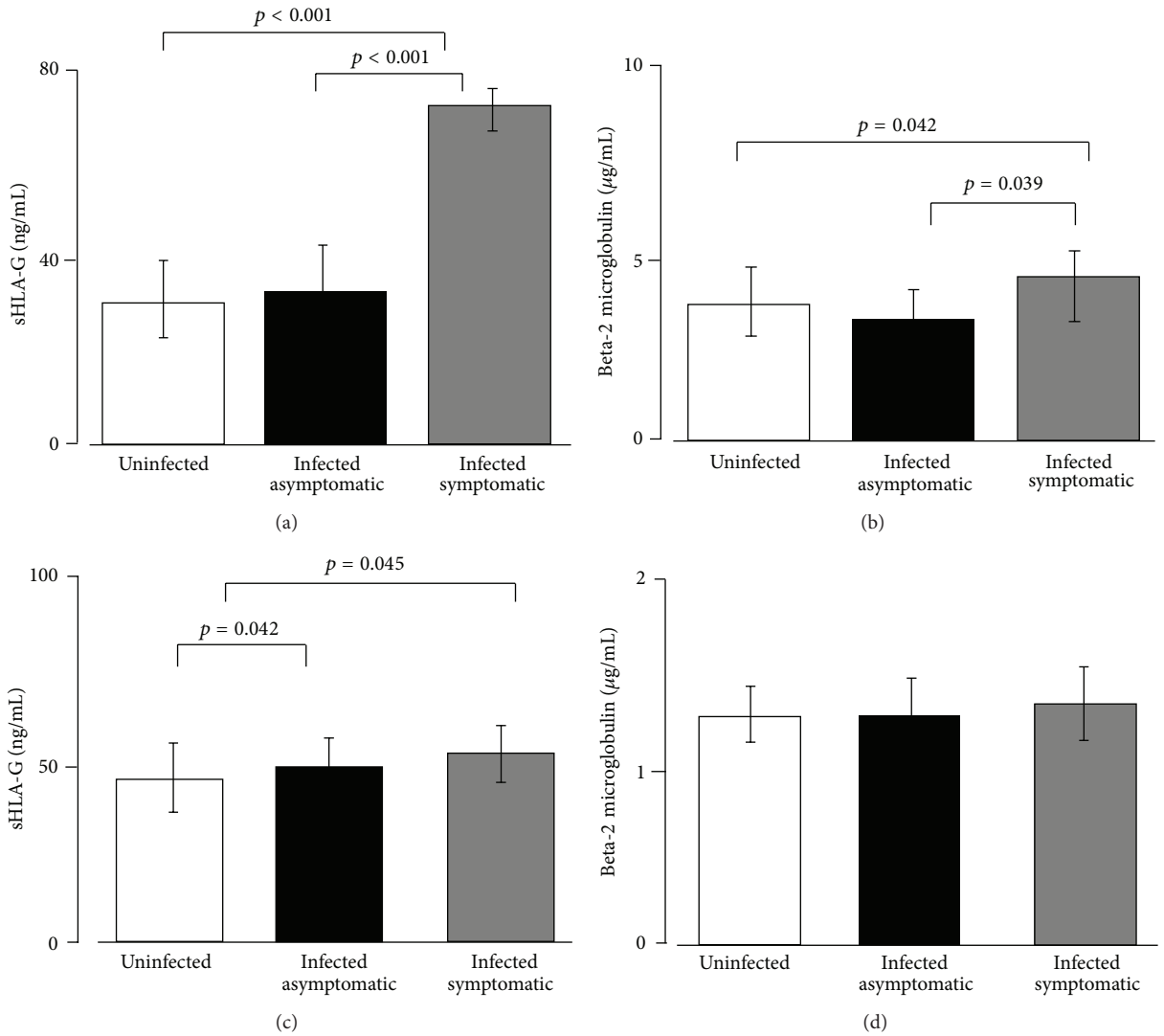


Figure 2: Amniotic fluid samples expression of (a) sHLA-G and (b) b2M molecules according to the fetal/neonatal outcome. Maternal serum samples expression of (c) sHLA-G and (d) b2M molecules according to the fetal/neonatal outcome. p values obtained by Student's t-test and mean ± standard deviation are reported.

evaluating the relative amount of amniotic sHLA-G and albumin compared with maternal serum levels. Any increase in the index could be ascribed to sHLA-G production in the fetal compartment. The highest sHLA-G indexes were detected in infected symptomatic fetuses (19.5%) compared to infected asymptomatic fetuses (6%) and uninfected (5.1%) ($p < 0.001$, $p < 0.001$, resp.) (Figure 3(b)).

3.5. HLA-G Free-Heavy Chain Analysis. HLA-G can be expressed as b2M associated or free-heavy chain. Previous studies documented a different distribution of these two conformations at the maternal-fetus interface [29]. We evaluated the presence of HLA-G free-heavy chain in both sera and in amniotic fluids from primary HCMV infected pregnant women with asymptomatic or symptomatic fetus. HLA-G free-heavy chain was detected with a tendency to be more frequent in amniotic fluids from symptomatic fetuses ($p = 0.074$). On the contrary, maternal sera did not present HLA-G free-heavy chain (Table 1).

Representative examples of maternal serum and AF reactivity to antigens on the Western blot are shown in Figure 3(c). In lines 1, 2, and 3, we reported HLA-G positive samples analyzed for b2M associated form (MEM-G9 detection), while the same samples were analyzed for HLA-G free-heavy chain (MEM-G1 detection) in lines 4, 5, and 6.

3.6. sHLA-G Predictive Efficacy. We analyzed serum and amniotic fluid samples for sHLA-G and selected different cut-offs to be used as differentiation values. ROC analysis showed serum values above 50 ng/mL and amniotic values above 30 ng/mL with the highest sensitivity and specificity and an Area Under an ROC Curve of 0.83 and 0.86, respectively

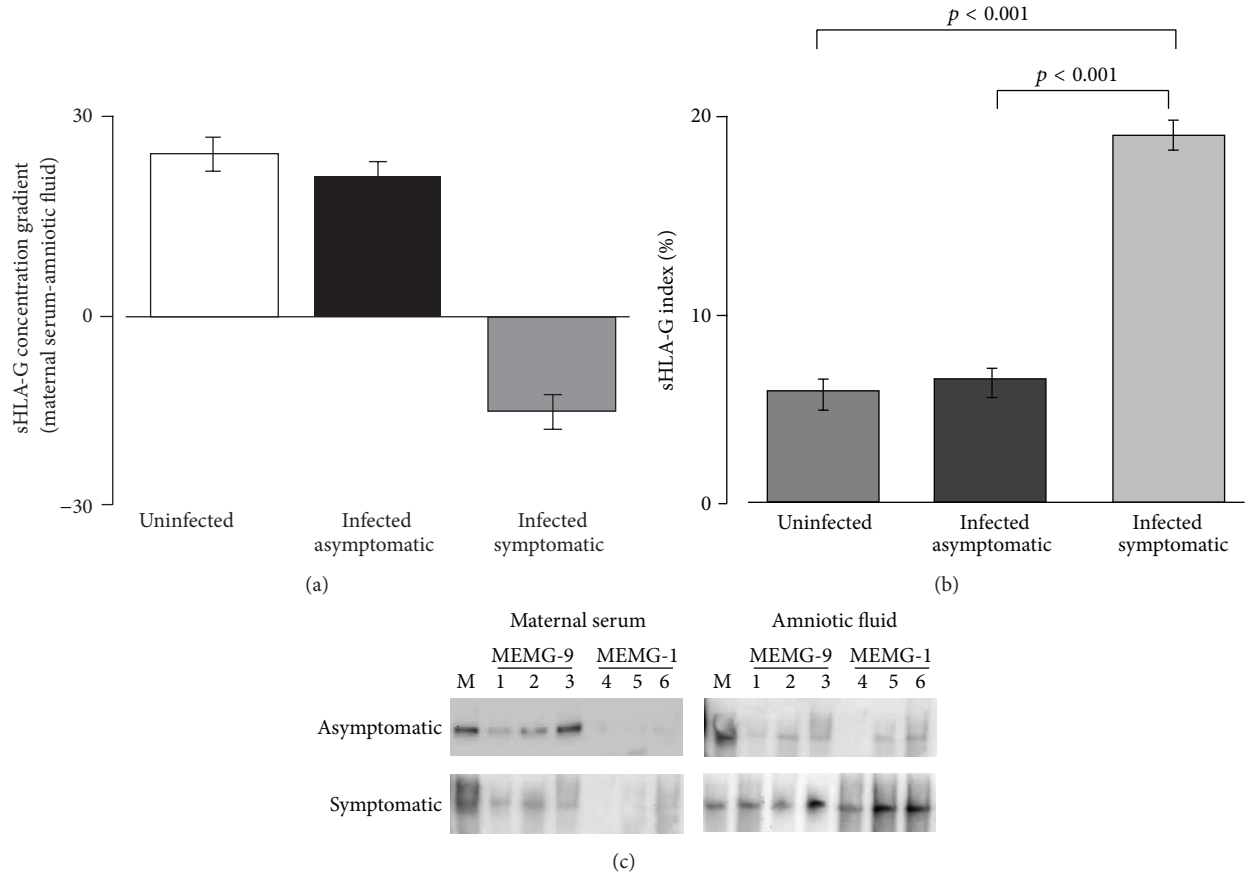


Figure 3: (a) sHLA-G concentration gradients according to the fetal/neonatal outcome. (b) sHLA-G indexes (%) according to the fetal/neonatal outcome. (c) Representative Western blot analysis of maternal serum and amniotic fluids samples from HCMV primary infected pregnancy subdivided according to the fetal/neonatal outcome. The analysis were performed after immunoprecipitation with anti-b2M associated HLA-G moAb (MEM-G9, Exbio) (lines 1 to 3) and anti-free HLA-G HC moAb (MEMG-1, Exbio) (lines 4 to 6). JEG3 cell line supernatants were used as positive control (M) and the positivity for HLA-G molecule was evidenced at 39 kD.

Table 1: Prognostic HLA-G biomarker of symptomatic congenital HCMV infection.

Parameters	Cutoff		Fetuses		Diagnostic accuracy 95% CI				AUA
			Sympt	Asympt	Sens%	Spec%	PPV%	NPV%	
Maternal serum samples									
HLA-G free-heavy chain		Presence	0	0	0	100	0	29.4	0.5
		Absence	12	5	0–26.7	47.9–100		10.4–55.9	
sHLA-G	50 ng/mL	Above	10	0	83.3	100	100	71.4	0.83
		Below	2	5	51.6–97.4	47.9–100	68.9–100	29.3–95.5	
Amniotic fluid samples									
HLA-G free-heavy chain		Presence	12	3	100	40	80	100	0.79
		Absence	0	2	73.3–100	6.4–84.6	51.9–95.4	19.2–100	
sHLA-G	30 ng/mL	Above	11	0	91.7	100	100	83.3	0.86
		Below	1	5	61.5–98.6	47.9–100	71.3–100	36.1–97.2	

Sens: sensitivity; Spec: specificity; PPV: positive predictive value; NPV: negative predictive value; AUA: Area Under an ROC Curve; Sympt: symptomatic; Asympt: asymptomatic.

(Table 1), in differentiating symptomatic from asymptomatic congenital infections. Similarly, the presence of HLA-G free-heavy chain in amniotic fluids shows a high sensitivity and specificity and an Area Under an ROC Curve of 0.79, in identifying symptomatic congenital infections. Logistic regression analysis excluded the presence of confounding variables.

4. Discussion

The prenatal diagnosis provides the optimal means for diagnosing HCMV fetal infection. The specificity is very good and the sensitivity depends on the kind of samples used (amniotic fluid > fetal blood), the technique used (Real-Time PCR-Polymerase Chain Reaction > viral culture), and the timing of the procedure with respect to the onset of maternal infection and the gestational age.

All literature data report that the amniotic fluid is the most appropriate material for the diagnosis of fetal HCMV infection. Positive results in amniotic fluid identify all HCMV infected fetuses (positive predictive value = 100%) but do not identify the infants who will have symptoms at birth [6].

Although the highest median values of HCMV-DNA in amniotic fluid tend to indicate an increased risk of severe infection, high viral loads may be associated with symptomatic or asymptomatic congenital infections. Indeed, a correlation between the high HCMV load in amniotic fluid and fetal/neonatal outcome has not been demonstrated [6].

More recently, some studies have evoked the prognostic value of fetal viremia/viral load and/or level of specific IgM; however this remains controversial. It has been proposed that platelet count gives a better indication. New data has demonstrated that the determination of multiple markers (haematological, biochemical, and virological markers) in fetal blood following virus detection in amniotic fluid is predictive of perinatal outcome in fetuses with HCMV infection [21]. Further studies in a larger number of symptomatic cases should be performed to verify the prognostic efficacy of determination of multiple parameters in fetal blood.

In this work, we analyzed the levels of sHLA-G and b2M molecules in the maternal serum samples and showed that sHLA-G median levels were significantly higher in maternal serum from pregnant women with primary HCMV infection than in nonprimary and uninfected, respectively ($p < 0.001$ and $p < 0.006$). On the contrary, beta-2 microglobulin levels were only slightly higher in maternal serum from pregnant women with primary HCMV infection than in nonprimary and uninfected, respectively ($p = 0.12$ and $p = 0.06$). The differences in immune competence towards HCMV in primary infected and in nonprimary infected mothers could explain the different production of sHLA-G that can act as viral immune escape mechanism or as a tentative reduction of immune activation in primary HCMV infection, which is not induced in nonprimary HCMV infected women.

Furthermore, we evaluated whether sHLA-G molecules could be considered prognostic biomarkers of symptomatic congenital HCMV infection.

We observed that sHLA-G levels in both maternal serum and amniotic fluid samples are significantly related to symptomatic HCMV fetal infections as supported by ROC analysis (Table 1). In fact, sHLA-G levels above 50 ng/mL in maternal blood and 30 ng/mL in amniotic fluid are correlated with symptomatic HCMV congenital infections. High levels of sHLA-G in maternal and fetal compartments show a specificity of 100% for symptomatic congenital infection and a sensitivity ranging between 83 and 92%.

The evidence that sHLA-G levels increase only in the presence of symptomatic fetuses suggests a specific fetal production of these molecules. We obtained confirmation of this hypothesis through (i) the evaluation of the concentration gradient, which is higher in the amniotic fluid versus the maternal blood in case of symptomatic infection; (ii) the sHLA-G indexes calculation, which support a fetal production; (iii) the HLA-G free-heavy chian, which is commonly expressed by distal trophoblasts [29], which is present only in amniotic fluid.

The increase of fetal HLA-G expression could be caused by HCMV-encoded proteins that are known to interact with HLA mRNAs and proteins, modifying their stability and secretory pathways [15–19]. The increase of HLA-G expression could enhance HCMV immune escape, increasing the risk of congenital infections and symptomatic sequelae. Our results suggest that serum and amniotic fluid sHLA-G might be an additional biomarker of congenital HCMV infection that could be considered in combination with currently used viral and biological markers [20, 30], providing the best key to the reliable identification of fetuses at risk of congenital disease as well as of fetuses with a favorable outcome.

5. Conclusions

To the best of our knowledge, this is the first observation that considers the possible use of serum and amniotic fluid sHLA-G as a biomarker to discriminate between symptomatic and asymptomatic HCMV congenital infection. However, future studies with larger cohort of fetuses should be performed in order to verify whether the addition of serum sHLA-G determination to virologic markers may be crucial in identifying fetuses at highest risk of severe pathologies.

Competing Interests

All authors reported no competing interests.

Acknowledgments

The technical expertise of Francesca Bellini from the Laboratory of Virology, St. Orsola-Malpighi University Hospital, Bologna, is greatly acknowledged. The authors thank Iva Pivanti for technical assistance. They also thank their Linguistic Consultant, Lucy Scioscia, for editing the English language text. This work was partially supported by grants (SA43GABR) from St. Orsola-Malpighi University Hospital Bologna (Italy), Ricerca Finalizzata 2006 from Health Care Ministry, Rome (Italy), Ricerca Finalizzata 2010 from Health Care Ministry, Rome (Italy), Young Scientists award "A. Liberati" 2012 and 2013, Regione Emilia Romagna (Italy).

References

[1] C. S. Peckham, "Cytomegalovirus infection: congenital and neonatal disease," *Scandinavian Journal of Infectious Diseases*, vol. 78, pp. 82–87, 1991.

[2] A. Kenneson and M. J. Cannon, "Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection," *Reviews in Medical Virology*, vol. 17, no. 4, pp. 253–276, 2007.

[3] K. B. Fowler, S. Stagno, R. F. Pass, W. J. Britt, T. J. Boll, and C. A. Alford, "The outcome of congenital cytomegalovirus infection in relation to maternal antibody status," *The New England Journal of Medicine*, vol. 326, no. 10, pp. 663–667, 1992.

[4] J. J. C. de Vries, E. W. van Zwet, F. W. Dekker, A. C. M. Kroes, P. H. Verkerk, and A. C. T. M. Vossen, "The apparent paradox of maternal seropositivity as a risk factor for congenital cytomegalovirus infection: a population-based prediction model," *Reviews in Medical Virology*, vol. 23, no. 4, pp. 241–249, 2013.

[5] S. B. Boppana, R. F. Pass, W. J. Britt, S. Stagno, and C. A. Alford, "Symptomatic congenital cytomegalovirus infection: neonatal morbidity and mortality," *The Pediatric Infectious Disease Journal*, vol. 11, no. 2, pp. 93–99, 1992.

[6] T. Lazzarotto, B. Guerra, L. Gabrielli, M. Lanari, and M. P. Landini, "Update on the prevention, diagnosis and management of cytomegalovirus infection during pregnancy," *Clinical Microbiology and Infection*, vol. 17, no. 9, pp. 1285–1293, 2011.

[7] B. Guerra, G. Simonazzi, C. Puccetti et al., "Ultrasound prediction of symptomatic congenital cytomegalovirus infection," *American Journal of Obstetrics and Gynecology*, vol. 198, no. 4, pp. 380–e7, 2008.

[8] T. Lazzarotto, B. Guerra, M. Lanari, L. Gabrielli, and M. P. Landini, "New advances in the diagnosis of congenital cytomegalovirus infection," *Journal of Clinical Virology*, vol. 41, no. 3, pp. 192–197, 2008.

[9] S. Gouarin, E. Gault, A. Vabret et al., "Real-time PCR quantification of human cytomegalovirus DNA in amniotic fluid samples from mothers with primary infection," *Journal of Clinical Microbiology*, vol. 40, no. 5, pp. 1767–1772, 2002.

[10] T. Goegebuer, B. Van Meensel, K. Beuselinck et al., "Clinical predictive value of real-time PCR quantification of human cytomegalovirus DNA in amniotic fluid samples," *Journal of Clinical Microbiology*, vol. 47, no. 3, pp. 660–665, 2009.

[11] A. Lin, H. Xu, and W. Yan, "Modulation of HLA expression in human cytomegalovirus immune evasion," *Cellular & Molecular Immunology*, vol. 4, no. 2, pp. 91–98, 2007.

[12] R. Rizzo, M. Vercammen, H. van de Velde, P. A. Horn, and V. Rebmann, "The importance of HLA-G expression in embryos, trophoblast cells, and embryonic stem cells," *Cellular and Molecular Life Sciences*, vol. 68, no. 3, pp. 341–352, 2011.

[13] R. Rizzo, A. Trentini, D. Bortolotti et al., "Matrix metalloproteinase-2 (MMP-2) generates soluble HLA-G1 by cell surface proteolytic shedding," *Molecular and Cellular Biochemistry*, vol. 381, no. 1-2, pp. 243–255, 2013.

[14] R. Rizzo, A. S. Andersen, M. R. Lassen et al., "Soluble human leukocyte antigen-G isoforms in maternal plasma in early and late pregnancy," *American Journal of Reproductive Immunology*, vol. 62, no. 5, pp. 320–338, 2009.

[15] Y. Jun, E. Kim, M. Jin et al., "Human cytomegalovirus gene products US3 and US6 down-regulate trophoblast class I MHC molecules," *Journal of Immunology*, vol. 164, no. 2, pp. 805–811, 2000.

[16] M. Onno, C. Pangault, G. Le Friec, V. Guilloux, P. André, and R. Fauchetz, "Modulation of HLA-G antigens expression by human cytomegalovirus: specific induction in activated macrophages harboring human cytomegalovirus infection," *The Journal of Immunology*, vol. 164, no. 12, pp. 6426–6434, 2000.

[17] B. Park, E. Spooner, B. L. Houser, J. L. Strominger, and H. L. Ploegh, "The HCMV membrane glycoprotein US10 selectively targets HLA-G for degradation," *The Journal of Experimental Medicine*, vol. 207, no. 9, pp. 2033–2041, 2010.

[18] M. T. Barel, M. Ressing, N. Pizzato et al., "Human cytomegalovirus-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane-bound, but not soluble HLA-G1 for degradation," *The Journal of Immunology*, vol. 171, no. 12, pp. 6757–6765, 2003.

[19] D. J. Schust, D. Tortorella, J. Seebach, C. Phan, and H. L. Ploegh, "Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11," *The Journal of Experimental Medicine*, vol. 188, no. 3, pp. 497–503, 1998.

[20] E. Fabbri, M. G. Revello, M. Furione et al., "Prognostic markers of symptomatic congenital human cytomegalovirus infection in fetal blood," *BJOG: An International Journal of Obstetrics and Gynaecology*, vol. 118, no. 4, pp. 448–456, 2011.

[21] L. Gabrielli, M. P. Bonasoni, D. Santini et al., "Congenital cytomegalovirus infection: patterns of fetal brain damage," *Clinical Microbiology and Infection*, vol. 18, no. 10, pp. E419–E427, 2012.

[22] M. Lanari, T. Lazzarotto, V. Venturi et al., "Neonatal cytomegalovirus blood load and risk of sequelae in symptomatic and asymptomatic congenitally infected newborns," *Pediatrics*, vol. 117, no. 1, pp. e76–e83, 2006.

[23] T. Lazzarotto, A. Ripalti, G. Bergamini et al., "Development of a new cytomegalovirus (CMV) immunoglobulin M (IgM) immunoblot for detection of CMV-specific IgM," *Journal of Clinical Microbiology*, vol. 36, no. 11, pp. 3337–3341, 1998.

[24] T. Lazzarotto, L. Gabrielli, M. P. Foschini et al., "Congenital cytomegalovirus infection in twin pregnancies: viral load in the amniotic fluid and pregnancy outcome," *Pediatrics*, vol. 112, no. 2, pp. e153–e157, 2003.

[25] R. Rizzo, B. Fuzzi, M. Stignani et al., "Soluble HLA-G molecules in follicular fluid: a tool for oocyte selection in IVF?" *Journal of Reproductive Immunology*, vol. 74, no. 1-2, pp. 133–142, 2007.

[26] E. Fainardi, R. Rizzo, L. Melchiorri et al., "Soluble HLA-G molecules are released as HLA-G5 and not as soluble HLA-G1 isoforms in CSF of patients with relapsing-remitting multiple sclerosis," *Journal of Neuroimmunology*, vol. 192, no. 1-2, pp. 219–225, 2007.

[27] E. Fainardi, R. Rizzo, L. Melchiorri et al., "Intrathecal synthesis of soluble HLA-G and HLA-I molecules are reciprocally associated to clinical and MRI activity in patients with multiple sclerosis," *Multiple Sclerosis*, vol. 12, no. 1, pp. 2–12, 2006.

[28] E. Fainardi, R. Rizzo, L. Melchiorri et al., "Presence of detectable levels of soluble HLA-G molecules in CSF of relapsing-remitting multiple sclerosis: relationship with CSF soluble HLA-I and IL-10 concentrations and MRI findings," *Journal of Neuroimmunology*, vol. 142, no. 1-2, pp. 149–158, 2003.

[29] P. J. Morales, J. L. Pace, J. S. Platt, D. K. Langat, and J. S. Hunt, "Synthesis of β(2)-microglobulin-free, disulphide-linked HLA-G5 homodimers in human placental villous cytotrophoblast cells," *Immunology*, vol. 122, no. 2, pp. 179–188, 2007.

[30] M. Leruez-Ville, J. Stirnemann, Y. Sellier et al., "Feasibility of predicting the outcome of fetal infection with cytomegalovirus at the time of prenatal diagnosis," *American Journal of Obstetrics and Gynecology*, 2016.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 2695396, 11 pages
http://dx.doi.org/10.1155/2016/2695396

Review Article

Characteristics of HLA-E Restricted T-Cell Responses and Their Role in Infectious Diseases

Simone A. Joosten,[1] Lucy C. Sullivan,[2] and Tom H. M. Ottenhoff[1]

[1]*Department of Infectious Diseases, Leiden University Medical Center, 2333 ZA Leiden, Netherlands*
[2]*Department of Microbiology and Immunology, The University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, VIC 3010, Australia*

Correspondence should be addressed to Simone A. Joosten; sajoosten@lumc.nl

Received 18 April 2016; Revised 28 June 2016; Accepted 10 July 2016

Academic Editor: Nathalie Rouas-Freiss

Human HLA-E can, in addition to self-antigens, also present pathogen-derived sequences, which elicit specific T-cell responses. T-cells recognize their antigen presented by HLA-E highly specifically and have unique functional and phenotypical properties. Pathogen specific HLA-E restricted $CD8^+$ T-cells are an interesting new player in the field of immunology. Future work should address their exact roles and relative contributions in the immune response against infectious diseases.

1. Introduction

T-cell activation requires specific recognition of antigen presented as small fragments (peptides) bound to major histocompatibility complex (MHC) molecules. The recognition of a particular peptide-MHC occurs through a highly specific T-cell receptor (TCR), which is selected in the thymus. Following TCR triggering, costimulation and the presence of polarizing cytokines together determine the T-cell activation pattern and guide ultimate T-cell differentiation. Classically, $CD4^+$T-cells recognize antigens scavenged extracellularly by the antigen presenting cell (APC) that are presented in MHC class II, whereas $CD8^+$ T-cells recognize endogenous antigens presented by MHC class I (MHC-I) [1, 2]. In spite of this widely held view, already decades ago it was shown that also antigens derived from intracellular pathogens such as viruses or intracellular bacteria can be presented in MHC-I [3]. More recently, cross-presentation by dendritic cells and autophagy have been elucidated as important mechanisms in this context [2, 4].

Transplantation of hematopoietic cells as well as solid organs and detailed studies of viral infections provided the initial key information leading to the concept of genetic MHC restriction by autologous MHC molecules. This is currently often referred to as "conventional" or "donor-restricted" immunity [5]. However, numerous T-cell subsets have been identified that do not fulfil these criteria, including MHC class Ib restricted T-cells, CD1 restricted T-cells, MR1 restricted mucosal associated invariant T-cells (MAIT), NKT-cells, and $\gamma\delta$ T-cells, subsets that are collectively called "unconventional" or "donor-unrestricted T-cells" (DURT) [5]. Unconventional T-cells behave differently in terms of memory, kinetics, and ligands recognized compared to conventional T-cells as recently summarized [5].

An intriguing group of DURT family cells are the T-cells that are restricted by MHC class Ib molecules. These cells may share several critical properties with conventional T-cells but most importantly recognize antigens typically in the context of nonpolymorphic MHC-I molecules. The human MHC class Ib family, also called nonclassical HLA class I, is comprised of HLA-E, HLA-F, and HLA-G. The major difference with classical class Ia molecules is their very low level of allelic variation. Whereas HLA class Ia families are composed of several hundred family members for HLA-A, HLA-B, and HLA-C alleles, HLA-E, HLA-F, and HLA-G comprise only 3, 4, and 10 family members, respectively, and not all of these are actually expressed as functional proteins [6]. Immune cells express relatively high

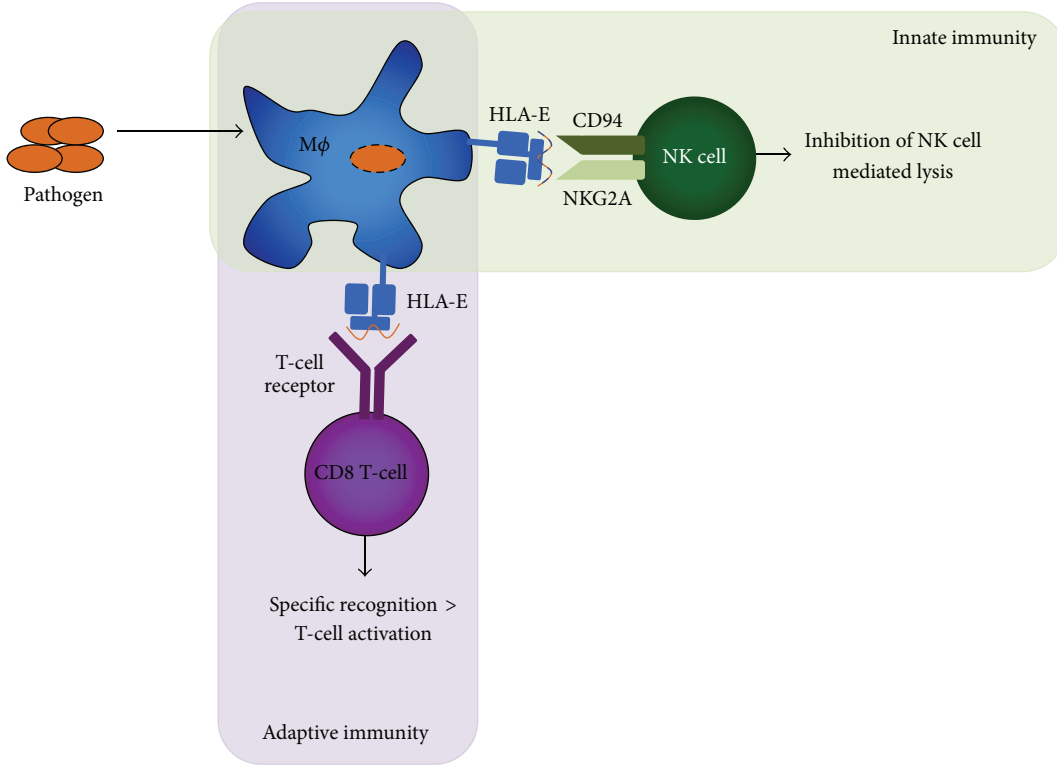


Figure 1: HLA-E serves a dual role in the immune system. HLA-E presents antigens, including pathogen-derived antigens on the cell surface of most cells. NK cells, as part of the innate immune system, will sense the presence of HLA-E presenting self or pathogen-derived peptides and thereby receive inhibitory signals from the CD94/NKG2A complex such that NK mediated lysis will be inhibited. In addition, $CD8^+$ T-cells may specifically recognize foreign peptide presented by HLA-E and become activated through their T-cell receptor, resulting in T-cell activation, expansion, and memory formation in the adaptive immune system.

levels of HLA-E protein, but also tissue cells can express the HLA-E protein (http://www.proteinatlas.org/). Although HLA-E was originally described to be broadly expressed by almost all cells that also express HLA class Ia molecules [7], other studies suggest HLA-E expression is restricted to lymphoid and endothelial cells [8]. Furthermore, pathogens can affect HLA-E cell surface expression; for example, human cytomegalovirus (CMV) can upregulate its expression [9]. HLA-E functions as ligand for CD94-NKG2 receptors and has a peptide-binding groove that is ideally suited for binding peptides derived from the leader sequences of other MHC-I molecules [10]. In this regard, the loss of leader-peptide loaded HLA-E expression is a marker for cells having lost expression of HLA class Ia molecules, which targets these cells for recognition and lysis by Natural Killer (NK) cells [10]. In contrast to HLA-E, HLA-F expression appears to be more restricted and is detected mostly in liver and bladder [10]. However, its expression is largely intracellular and in association with other MHC-I molecules, which has led to speculations that HLA-F might be involved in the intracellular stabilization of HLA class Ia molecules [10]. The third human MHC class Ib family member, HLA-G, has an even more narrow tissue distribution; its expression appears limited to trophoblasts in the placenta, and it has been associated with fetal-maternal tolerance [10]. HLA-G may function during pregnancy to inhibit NK mediated lysis as trophoblasts lack HLA-A and HLA-B expression [11].

Thus, given the intracellular expression of HLA-F and the placental restriction of HLA-G, limited information is available on T-cells interacting with these molecules, and their relevance to general immunity remains unclear. For this reason, the focus of this review will be on HLA-E restricted T-cells.

2. HLA-E

The role of HLA-E in the innate immune response is to present signal sequence-derived peptides of other HLA class I molecules to inhibit NK mediated lysis of cells via recognition by CD94/NKG2A [13]. However, HLA-E can also bind and present other peptide sequences, which can be self or pathogen derived and can be recognized by adaptive T-cells. HLA-E is thus considered to play a role in both innate and adaptive immunity, via interacting with both NK cells as well as presenting peptides to antigen specific $CD8^+$ T-cells (Figure 1).

Eleven alleles have been reported for HLA-E, only 3 of which can be translated into proteins, 2 of them being highly dominant, the $HLA\text{-}E^R$ ($E^*01{:}01$) and the $HLA\text{-}E^G$ ($E^*01{:}03$) variants, which differ only in a single amino acid at position

107, being arginine (E*01:01) or glycine (E*01:03). Position 107 is located on the loop between the β-strands outside of the α2 domain of the heavy chain, just outside the peptide-binding groove. The frequency of the HLA-E^R and HLA-E^G in the population is about equal, suggesting balanced selection in diverse populations [14, 15]. Whether HLA-E^R and HLA-E^G display functional differences has not been studied in detail [14, 16], but it has been demonstrated that HLA-E^G homozygous cells express higher levels of HLA-E and had higher peptide-binding affinity [16]. More recently, peptide elution studies revealed a different peptide-binding repertoire eluted from HLA-E^R versus HLA-E^G molecules, indicating that the F-pocket of HLA-E^G bound a smaller variety of peptides and had a stronger preference for a lysine at the pΩ position [17, 18].

2.1. HLA-E Peptide Binding. The structural basis of HLA-E's ability to bind signal sequence-derived peptides from HLA class Ia (HLA-I) molecules has been studied previously. Unlike HLA class Ia that typically contain 2 or 3 anchor residues, HLA-E contains 5 anchor residues in the peptide-binding groove that highly constrains the sequence of the bound peptide [19, 20]. However, HLA-E was additionally shown to bind peptides derived from viruses such as influenza M1 protein and EBV BZLF1 [21]. Moreover, a sequence from cytomegalovirus (CMV) glycoprotein UL40, which is identical to the HLA-C*03 leader sequence, can bind to HLA-E and is capable of preventing NK mediated lysis [9, 22]. The same was found for a HCV derived sequence, despite its sequence difference from signal peptides [23]. Peptide identification and characterization using random peptide approaches also revealed that a leucine on P9 is a critical anchor residue for HLA-E binding but did not identify methionine as critical P2 anchor for HLA-E binding and folding [24]. Identification of the motif within the HLA class Ia leader sequences critical for interaction with the CD94/NKG2 complex suggested anchor residues at positions 2, 6, 7, and 9, while solvent exposed residues at P5 and P8 were likely important for binding to CD94/NKG2 [25]. Thus, while P2 and P9 are anchor residues for binding to HLA-E and P5 and P8 are involved in the interaction with CD94/NKG2 [26], P8 is also the critical residue distinguishing self (signal sequence) from nonself (CMV UL40) and allowing for TCR recognition of CMV UL40 [12].

Recent peptide elution studies provided important insights in the types of peptides that are naturally presented by HLA-E and identified a larger array of peptides eluted from HLA-E than originally discovered [17, 18, 27]. Eluted peptides were generally short (8-9-10 mers), but occasionally also longer peptides were eluted including 11–17 mers [17, 18, 27]. This is in line with earlier studies, where also peptides greater than 8 amino acids could bind HLA-E [21]. Furthermore, eluted peptides were different from signal sequences, possessing hydrophobic amino acids on P2 and P9, consistent with a binding motif that was very similar to that of HLA-A2 [27]. Recent studies in rhesus macaques immunized with SIV-gag (Simian Immunodeficiency Virus), in specific CMV vectors, revealed a series of peptides that were recognized by $CD8^+$ T-cells but only a minority contained the canonical MHC class I antigen E (MHC-E) binding motif [28]. Structural analyses revealed that the peptide-binding cleft of HLA-E is rigid but relatively open compared to that of HLA class Ia family members. Peptides that lack the canonical residues can adopt a backbone structure that is similar to canonical peptides, allowing them to bind the HLA-E molecule [28]. These unique binding properties of HLA-E may explain the observed epitope diversity and breadth in SIV-gag in the rhesus macaque vaccination studies [28]. Furthermore, the authors suggested that the open structure of HLA-E may allow peptide exchange [28]; this may be in particular relevant for *Mycobacterium tuberculosis* (Mtb) as HLA-E expression is enriched in the Mtb phagosome [29]. Intriguingly, we have identified a large series of Mtb epitopes presented in HLA-E and recognized by mycobacteria exposed human donors, many of which lack the canonical residues [30].

Together, these data indicate that HLA-E binds signal sequence-derived peptides not only from MHC class Ia molecules but also from other self and even pathogen-derived sequences. Although many peptides contain canonical amino acids for binding HLA-E, clear examples exist for peptides which lack canonical residues and can still bind HLA-E. Presentation of nonself sequences, being absent during thymic selection, may elicit adaptive immune responses by $CD8^+$ T-cells.

2.2. HLA-E Restricted T-Cells. Specific recognition of pathogen-derived sequences presented by the unconventional presentation molecule HLA-E by the $CD8^+$ TCR could lead to specific activation of adaptive immune responses, independent of classically HLA restricted $CD4^+$ and $CD8^+$ T-cells. In several infectious disease models, evidence for such responses has been reported recently.

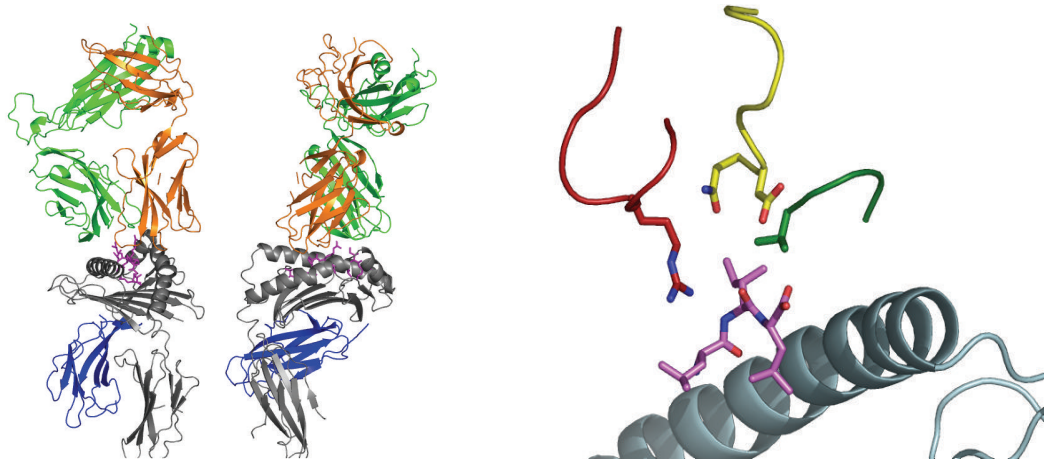
2.2.1. Viral Antigens. As viruses require the human host to survive and therefore reside within host cells, their proteins are presented by HLA class I molecules, including HLA class Ib. Peptides from *Epstein Barr Virus* (EBV) [34–36], *Cytomegalovirus* (CMV) [12, 37–41], and *Hepatitis C Virus* (HCV) [44] can be presented by HLA-E and are recognized by virus specific T-cells (Table 1). The peptide epitopes studied from CMV UL40 are highly similar to the HLA class Ia signal sequences, whereas the sequences from EBV BZLF1 and HCV appear more different. The functional and phenotypical description of these T-cells is rather limited, but they all express $CD8^+$ as expected for HLA class I restricted cells. In many studies, HLA-E restricted T-cells have been identified and enumerated using HLA-E tetramers whereas functional analyses were limited to the demonstration of target cell lysis. Likewise, phenotypical characterizations were very limited in scope but when performed showed a cytolytic T-cell phenotype (perforin, granzyme A/B) and IFNγ production in some studies [38, 41].

(1) HLA-E and CMV. Initial studies on possible recognition of HLA-E peptide complexes by T-cells in a TCR dependent manner were performed using signal sequences from HLA

Table 1: Antigen types/characteristics presented in HLA-E and recognized by T-cells.

Sequence	Origin	Description of antigen	Patients/donors	Recognition by T-cells	Reference
?	*Mycobacterium tuberculosis*	Glycopeptide from Mpt32**	T-cell clones derived from latently infected individuals	IFNγ production	[31, 32]
SLELGDSAL LLLGPGSGL	Human	TCR Vβ1 TCR Vβ2	Healthy donors, *in vitro* priming of CD8$^+$ T-cells	Target cell lysis	[33]
SQAPLPCVL	*Epstein Barr Virus*	BZLF1	T-cells from healthy donor; MS patients	T-cell proliferation, target cell lysis, TM staining	[34–36]
VTAPRTVLL VMAPRALLL VMAPRTLFL	Human Human Human	HLA-B15 HLA-Cw7 HLA-G1	T-cell lines from healthy donors	T-cell proliferation, target cell lysis	[34]
VMAPRTLIL VMAPRTLVL VMAPRTLLL	*Cytomegalovirus*	UL40	T-cell lines from healthy donors; lung transplant recipients	NK-CTL proliferation, target cell lysis, TM staining	[12, 34, 35, 37–42]
GMQFDRGYL AMLQDIATL KMLRGVNVL VEGEALATL AAVEELKAL AVAKAGKPL KLQERVAKL	*Salmonella enterica serovar typhi*	*S. typhimurium* GroEL	Healthy volunteers vaccinated with *S. typhi* strain Ty21a	Target cell lysis	[43]
YLLPRRGPRL	*Hepatitis C Virus*	Peptide HCV core aa 35–44	Chronic hepatitis C patients	IFNγ production	[44]
69 peptides	*Mycobacterium tuberculosis*	Multiple proteins expressed by Mtb	Latently infected individuals, BCG vaccinated newborns	CD8$^+$ T-cell proliferation	[30]
QMRPVSRVL	Human	Hsp60sp	Healthy donors, patients with type I diabetes	T-cell proliferation, target cell lysis	[45]
?	*Salmonella enterica serovar typhi*	?	Healthy volunteers vaccinated with *S. typhi* strain Ty21a	Cytokine producing T-cells	[46]
RMPPLGHEL VLRPGGHFL	*Mycobacterium tuberculosis* *Mycobacterium tuberculosis*	Rv2997, alanine rich dehydrogenase Rv1523, methyltransferase	T-cell clones derived from latently infected individuals	CD137 expression, ZAP70 phosphorylation; CD8$^+$ T-cell proliferation, TNFα production, TM staining	[47, 48]
VMTTVLATL RLPAKAPLL VMATRRNVL VLRPGGHFL	*Mycobacterium tuberculosis* *Mycobacterium tuberculosis* *Mycobacterium tuberculosis* *Mycobacterium tuberculosis*	Rv1734c, conserved hypothetical protein Rv1484, NADH-dependent enoyl reductase InhA Rv1518, unknown, possibly glycosyl transferase Rv1523, methyltransferase	Patients with pulmonary TB	CD8$^+$ T-cell proliferation, TNFα production, TM staining	[48]
?	*Salmonella typhi*	?	Healthy donors challenged with *Salmonella typhi*	Cytokine production	[49]

**D. Lewinsohn, personal communication, manuscript in preparation.



(a) (b)

Figure 2: Crystal structure of the HLA-E/KK50.4 TCR complex [12]. (a) Overview of the HLA-E/KK50.4 TCR structure showing two orthogonal views. The HLA-E heavy chain is shown in grey ribbons, the UL40 peptide in pink sticks, and β2m in blue ribbons. The KK50.4 alpha and beta chains are shown as orange and green ribbons, respectively. (b) The convergence of all 3 CDR β loops onto P8 Ile of the peptide bound to HLA-E. The HLA-E heavy chain is grey, peptide is pink, CDR1β is yellow, CDR2β is green, and CDR3β is shown in red.

class Ia alleles [34, 35, 42] (Table 1). It is *a priori* not clear why healthy human subjects would mount T-cell responses towards signal sequences of conserved class Ia molecules. While some of these studies lack information on the HLA-typing of the donors, others have suggested that these cells are mostly reactivity against nonself target peptides of signal sequences [34, 35, 42]. Similarly, cytotoxic $CD8^+$ T-cells recognizing HLA-E binding sequences from TCR Vβ chains were detected in peripheral blood [33]. An alternative, nonexclusive, explanation may be that these T-cells are reactive with nonself virally derived antigens that share sequence homology with the MHC class Ia derived signal sequences used in these studies.

Involvement of the TCR in specific recognition of HLA-E was derived from "NK-CTL" clones. Although these T-cells were originally termed "NK-CTL" due to their ability to lyse a broad range of allogeneic targets, it was subsequently found that these T-cells were specific for the CMV UL40-encoded peptide (VMAPRTLIL) bound to HLA-E [35, 42]. The detected alloreactivity was due to target cells possessing HLA-C alleles encoding the same sequence as the UL40 peptide (e.g., HLA-C*03). UL40-specific T-cells can reach frequencies in the circulation similar to those restricted by classical HLA-I, indicating their potential to play a significant role in CMV immunity [50].

The TCR from an UL40-specific T-cell clone, KK50.4, was cloned, expressed, and analysed for its interaction with HLA-E in complex with the UL40-epitope VMAPRTLIL (Figure 2) [12]. Overall, the structural basis for recognition of HLA-E largely overlaps that of TCR recognition of HLA class Ia. However, in order for UL40-specific T-cells to recognize the UL40 antigen or allogeneic HLA-I peptides, the UL40-specific TCR need to distinguish between the UL40 epitope (VMAPRTLIL) and nearly identical self peptides which may differ by as little as a single methyl group (e.g., VMAPRTLVL). Analysis of TCR sequences from UL40 specific T-cell clones suggested there were a limited number of TCRs capable of such discrimination, as all of the clones isolated utilized TRBV14 (Vβ16) and there was a characteristic arginine residue present in the CDR3β (Figure 2) [12]. Structural analyses of the KK50.4 clone showed that the convergence of CDR1, CDR2, and CDR3 of the KK50.4 β chain onto P8 Ile determined self/nonself discrimination (Figure 2). Notably, the highly selected arginine present in the CDR3β made multiple contacts with both HLA-E and peptide.

As the CMV UL40 peptide is highly homologous to HLA class Ia derived signal sequences, typically these T-cells are only observed in individuals where the UL40 epitope differs from that found in self-HLA-C alleles (e.g., HLA-C*07 homozygotes) [12]. However, other pathogen-derived peptides that bind to HLA-E are more different from the class Ia signal sequences and may therefore depend less on the donors' HLA class Ia genotype.

(2) HLA-E and HIV. HIV-nef proteins interact with the intracellular domain of HLA-A and HLA-B molecules, resulting in downregulation of HLA class Ia molecules from the cell surface. In contrast, HLA-C and HLA class Ib molecules, particularly, HLA-E and HLA-G, lack these intracellular nef-interaction domains and thus remain expressed normally on the cell surface of HIV infected cells [51]. In addition, it has been shown that a peptide from the HIV-1 capsid protein p24 (AISPRTLNA) may further enhance HLA-E surface expression [52]. However, recently it was shown that this peptide presented in HLA-E is not recognized by CD94/NKG2A and that these cells thus were not protected against NK mediated T-cell lysis [53]. This is most likely due to lack of homology between the HIV p24 peptide and HLA leader peptides, which prevents ligation of CD94-NKG2A to the HLA-E peptide complex [53]. Inhibition of NK mediated lysis of HIV-1 infected T-cells rather appears to be

the result of HLA-C expression and recognition by NK cells that specifically express the NK cell receptors KIR2DL1/2/3 [53]. Interestingly, it has not yet been investigated whether the HIV-1 p24 peptide, presented in HLA-E, may be recognized by the host adaptive immune system and thus may result in specific $CD8^+$ T-cells. Induction of such $CD8^+$ T-cell responses would be interesting from a therapeutic as well as vaccination point of view.

In rhesus macaques, MHC-E (or Mamu-E) is the homologue of human HLA-E, which also showed upregulated expression in HIV/SIV infected animals [28]. Vaccination of rhesus macaques with a CMV-based vector, expressing HIV gag, revealed a very strong $CD8^+$ T-cell response ($\alpha\beta$ TCR) with a large variety of specific interactions, with an estimated induction of 4 distinct epitopes per 100 amino acids in all tested HIV/SIV derived antigens [28]. Although this massive MHC-E restricted response was due to the specific design of the viral vector, it clearly illustrates the abundance of potential HLA-E epitopes in a large array of antigens. Detailed characterization of the peptide-binding domain revealed a relatively open structure, exposing many side chains for interaction with the TCR [28]. Interestingly, next to inducing MHC-E restricted T-cells, these CMV recombinant vectors also induced a significant population of other unconventional $CD8^+$ T-cells, which were restricted by MHC class II molecules.

2.2.2. Bacterial Antigens. Bacteria like *Salmonella* and Mtb are intracellular pathogens that hijack host cells to promote their own survival. Intriguingly, the expression of HLA-E is enriched on Mtb phagosomes compared to classical HLA class Ia family members, thus presumably facilitating HLA-E loading by Mtb peptides in infected cells [29]. In 1998, Lewinsohn et al. identified Mtb specific $CD8^+$ T-cell clones that appeared to be restricted to MHC class Ib [31], two of which were HLA-E restricted [32]. However the Mtb derived peptide ligands were not identified. Unpublished recent data point to a peptide derived from the Mtb glycoprotein Mpt32 (David Lewinsohn, personal communication, manuscript in preparation). In an independent effort, we have screened the Mtb genome for the presence of peptides that could potentially be presented by HLA-E and selected 69 peptides based on 3 different prediction algorithms [30]. Many of these peptides were recognized by donors that had been previously sensitized by mycobacteria, suggesting *in vivo* priming and T-cell memory for several HLA-E epitopes [30]. We have shown that these peptides presented in HLA-E elicit $CD8^+$ T-cell activation through the TCR, as measured by both ZAP70 phosphorylation (the first downstream effect in TCR signalling), as well as CD137 expression (a molecule exclusively expressed following specific antigen recognition on $CD8^+$ T-cells) [47]. Moreover, HLA-E restricted T-cell lines specific for Mtb had strongly reduced cytokine production in the presence of blocking antibodies against the $\alpha\beta$TCR or HLA-E [48]. Altogether, these data support specific recognition of HLA-E peptide complexes by Mtb specific TCRs. HLA-E binding peptides from Mtb were not capable of preventing NK mediated lysis in a CD94/NKG2A dependent manner [48], similar to HIV p24 [53], indicating that only surface expression of peptide containing HLA-E may not be sufficient.

Detailed characterization of these T-cells revealed that they are cytolytic or suppressive, and that T-cells reactive against the same peptide can display different functional polarities, indicating that polarity is not determined by the peptide. Interestingly, T-cell clones with cytolytic activity were also capable of inhibiting intracellular outgrowth of Mtb, suggesting that they are potent antimycobacterial effector cells [47]. Many of the HLA-E restricted Mtb specific T-cells did not produce typical cytotoxic T-lymphocyte associated cytokines, nor did they produce classical Th1 cytokines (IFNγ, TNF, and IL2), but instead they produced an array of Th2 cytokines including IL-4, IL-5, IL-10, and IL-13, as well as the Th2 associated transcription factor GATA-3 (Table 2) [47, 48]. In patients with TB disease, HLA-E tetramers identified Mtb specific HLA-E restricted $CD8^+$ T-cells, with the highest frequencies at TB diagnosis and waning of the response during successful treatment [48]. Moreover, in line with the knowledge that HLA-E is not susceptible to downregulation by HIV, we were able to detect HLA-E specific T-cells in patients that concomitantly were infected with Mtb and HIV [48].

Salmonella peptides presented by HLA-E are also recognized by HLA-E restricted T-cells. Volunteers vaccinated with a *S. typhi* vaccine had a robust HLA-E restricted T-cell response, as measured by the cytolytic capacities of these cells, such as granzyme B activity (Table 1) [43]. Kinetic analysis of these responses in a similarly vaccinated cohort revealed that the HLA-E restricted T-cells are long-lasting, up to 2 years after vaccination, again suggesting immune memory [46]. Moreover, following challenge experiments with *Salmonella* in unvaccinated, healthy volunteers, multifunctional HLA-E restricted $CD8^+$ T-cells were detected and correlated with protection against typhoid disease development [49].

2.2.3. Tumor and Self-Antigens. Interestingly, T-cells reactive with self Hsp60sp presented by HLA-E have also been identified, both in healthy donors and in patients with type 1 diabetes [45]. These $CD8^+$ T-cell lines were involved in discriminating self from nonself in the periphery, and defective discrimination between self and nonself was detected in the majority of patients with type 1 diabetes [45]. While many viruses are known to interfere with antigen processing and presentation, resulting in peptide presentation in a TAP-independent manner, this is also the case in many tumors. As a consequence, tumor unique antigens may be presented also in the context of HLA-E [54, 55], which may subsequently be recognized by cytotoxic T-cells [55]. In contrast to what was expected, in humans the presence of CTLs was only beneficial in patients with lung carcinoma if HLA-E was not expressed by the tumor, indicating that HLA-E restricted CTLs may not directly contribute to tumor elimination in these patients [56].

2.2.4. Autoimmune Diseases. HLA-E restricted T-cell responses have been studied only to a very limited extent in autoimmune diseases; however they could potentially play an important role. In patients with multiple sclerosis (MS),

Table 2: T-cell properties of MHC class Ib restricted T-cells; phenotype, cytokine production, transcription factors, cytolytic molecules.

		T-cell phenotype																					Cytokines										Transcr. factors					Function				
	Antigen	CD3	CD8	TCR$\alpha\beta$	CD25	LAG3	CD39	CTLA4	CD45RA	CD45RO	CCR7	CD27	CD28	CD56	CD57	CD62L	CD94	Perforin	Granzyme A	Granzyme B	Granulysin	CD107a	TRAIL	TCR Vβ16	TCR Vβ22	IFNγ	TNFα	IL-2	IL-4	IL-5	IL-10	IL-13	IL-17	TGF-β	MIP-1β	T-bet	GATA3	RORC	FOXP3	EOMES	Spec. target lysis	Path. killing	T-cell suppr.	TM binding	Reference
Self	VMAPRTLVL		+	+																				+																+				[42]
Self	VTAPRTVLL		+																					+																+				[34]
	VMAPRTLVL		+																					+																+				
	VMAPRTLIL		+																					+																+				
	VMAPRALLL		+																					+																+				
	VMAPRTLFL		+																					+																+				
Self	VMAPRTLIL, VMAPRTLLL		+	+										+			+																							+			+	[35]
Self	QMRPVSRVL		+															+	+	+																				+				[45]
Self	SLELGDSAL		+																																					+				[33]
	LLLGPGSGL		+																																					+				
EBV	SQAPLPCVL		+																					+																+				[34]
EBV	SQAPLPCVL		+	+										+			+																							+			+	[35]
EBV	SQAPLPCVL		+																																								+	[36]
CMV	VMAPRTLIL		+	+									−																											+			+	[37, 39]
CMV	VMAPRTLIL		+						+	+	−	−	−	+				+						+		+		−												+			+	[38]
CMV	VMAPRTLIL, VMAPRTLVL		+																					+																			+	[12]
CMV	VMAPRTLLL	+	+	+					lo	hi	−	−	#	+	−	−		+	+	+					+	+	+														?			+	[41]
CMV	VMAPRTLIL								+			−						+																										+	[40]
HCV	YLLPRRGPRL		+																							+																		[44]
Salmonella	GMQFDRGYL	+	+											−						+						+														+				[43]
	AMLQDIATL	+	+											−						+						+														+				
	KMLRGVNVL	+	+											−						+						+														+				
	VEGEALATL	+	+											−						+						+														+				
	AAVEELKAL	+	+											−						+						+														+				
	AVAKAGKPL	+	+											−						+						+														+				
	KLQERVAKL	+	+											−						+						+														+				
Salmonella	?	+	+						#							−						+				+	+	#					+		+									[46, 49]
Mtb	?		+																							+																		[31, 32]
Mtb	69 peptides	+	+	+	+	+		−										#		+	+					#								∗				−			+		+		[30]
Mtb	RMPPLGHEL	+	+	+	+	+	+	+	−		+							#	+	+	+					#	+	#	#	+	#	+			−	+	−	−	#	+	+	+	+	[47]
	VLRPGGHFL	+	+	+	+	+	+	+	−		+							#	+	+	+					#	+	#	#	+	#	+			−	+	−	−	#	+	+	+	+	
Mtb	VMTTVLATL	+	+	+														−		#	−		#			−	+	−	#	−	−	+	−								+	−		+	[48]
	RLPAKAPLL	+	+	+														−		#	−		#			−	+	−	#	−	−	+	−								+	−		+	
	VMATRRNVL	+	+	+														−		#	−		#			−	+	−	#	−	−	+	−								+	−		+	
	RMPPLGHEL	+	+	+														−		#	−		#			−	+	−	#	−	−	+	−								+	−		+	
	VLRPGGHFL	+	+	+														−		#	−		#			−	+	−	#	−	−	+	−								+	−		+	

"+" = positive in most samples; "−" = hardly seen; #: observed in about half of the samples; ∗: membrane bound TGF-β, not secreted.

increased frequencies of EBV specific, HLA-E restricted $CD8^+$ T-cells have been found to be associated mostly with the relapsing remitting form of the disease rather than the progressive form [36]. Moreover, $CD8^+$ T-cells induced by glatiramer acetate vaccination appear to be restricted to HLA-E and have immunomodulatory capacities, resulting in amelioration of MS [57–59]. HLA-E restricted $CD8^+$ T-cells in patients with MS appeared phenotypically different from healthy controls; however these T-cells were selected based on the expression of NKG2C and thus may reflect only a small subset of HLA-E restricted T-cells [60]. In rheumatoid arthritis (RA), limited information is available, although HLA-E polymorphisms may be associated with disease susceptibility and treatment responsiveness [61]. Interestingly and similar to the studies in MS, RA like autoimmunity can be strongly inhibited by induction of (self Hsp60) peptide specific Qa-1 restricted suppressor T-cells in mice [62]. Furthermore, there is a defect in $CD8^+$ T-cell recognition of HLA-E/Hsp60sp in patients with type I diabetes [45]. Thus, MHC-E restricted $CD8^+$ T-cells with immunoregulatory properties may be critical in amelioration of autoimmune diseases and deserve further detailed characterization.

2.3. Properties of HLA-E Restricted Human T-Cells. Surprisingly, little information is available on the phenotype and function of human $CD8^+$T-cells recognizing peptides presented by HLA-E. In many studies, HLA-E restricted T-cells have only been enumerated using tetramer staining, or the presence of HLA-E reactivity was demonstrated using cytolytic assays (Table 2). Analyses of the TCR composition were only performed in a limited number of studies and, as mentioned above, found consistent selection of TRAV14 ($V\beta16$) in CMV specific TCRs [12]. Additional studies have also determined $V\beta22$ usage in CMV restricted TCRs [41].

Basic descriptive information of HLA-E restricted T-cells, such as memory phenotype, is also largely lacking. The limited data that have been published do not suggest specific memory stages to be overrepresented among HLA-E restricted $CD8^+$T-cell populations, since both CD45RA positive and negative populations were identified, as was also reported for CCR7 (Table 2). Generally, the T-cells reported expressed cytolytic molecules, but in some cases these were weakly expressed or even undetectable (Table 2). Specific target cell lysis was frequently used as read out to demonstrate HLA-E restriction of $CD8^+$ T-cells. Recently, we demonstrated for the first time that HLA-E restricted $CD8^+$ T-cell clones had antibacterial activity against Mtb, considered to be an important property in immune control of intracellular pathogens (Table 2). Other studies have not assessed or reported viral or bacterial inhibition following HLA-E restricted $CD8^+$ T-cell activation or downstream target cell lysis. It will be of interest to identify the mechanism of control of intracellular outgrowth of Mtb, as this could be a yet unknown component of the immune system that could be harnessed for preventive or therapeutic interventions.

Cytokine production has been analysed in detail only in the most recent series of papers. Originally, studies focused on the production of classical Th1 cytokines, such as IFNγ and TNFα, and although sometimes detected in HLA-E restricted T-cells, not all produced IFNγ in response to specific peptide stimulation (Table 2). As mentioned above, we recently found Mtb specific HLA-E restricted $CD8^+$ T-cells to produce Th2 rather than Th1 cytokines and demonstrated that these cells utilize IL-4 to activate B-cells (Table 2). It would be interesting and relevant to investigate Th2 cytokine production also in HLA-E restricted $CD8^+$ T-cells in response to other ligands. Moreover, an analysis of transcription factor expression in HLA-E restricted $CD8^+$ T-cells has been limited thus far to our description of Mtb specific T-cells expressing GATA-3 (Table 2).

One of the major differences between innate and adaptive immunity is the formation of immunological memory during adaptive immune responses. As HLA-E restricted $CD8^+$ T-cells are activated through their TCR, in an antigen specific manner, it is likely that they also differentiate into memory cells. The formation of memory cells following HLA-E mediated antigen presentation would be an important prerequisite for successful application of these peptides in future vaccination strategies. However, information on T-cell memory development is limited. Phenotypically, effector memory [30] and effector memory recently activated [38, 41] have been described as indicators of memory. Moreover, screening for recognition of HLA-E restricted Mtb peptides by human donors showed recognition only in donors that had been sensitized by mycobacteria (as measured by PPD recognition), suggesting that *in vivo* priming, and thus memory induction, was critical [30]. Vaccination of healthy volunteers with a single dose of *S. typhi* strain Ty21a resulted in antigen specific, HLA-E restricted $CD8^+$ T-cells that were detectable up to 2 years after vaccination, suggesting that HLA-E restricted memory T-cells had been induced [46]. Also, individuals with latent TB and individuals successfully treated for TB disease still had circulating $CD8^+$ T-cells binding to HLA-E tetramers (TM) loaded with Mtb peptides (notwithstanding that the frequencies detected were highest in patients with active TB disease); the persistence of responses after microbiological cure thus also suggests immune memory [48].

2.4. Qa-1^b: The Murine HLA-E Homologue. The murine homologue of human HLA-E is Qa-1^b, which also presents signal sequences from MHC class I proteins that are called Qdm (Qa-1 determinant modifier) [54]. Qa-1^b can also bind CD94/NKG2A complexes to silence NK cell activity. However, also specific recognition of Qa-1^b peptide complexes by T-cells has been described [54]. Interestingly, similar to HLA-E, Qa-1^b restricted T-cells have been isolated that are able to detect differences in leader-sequenced derived peptides between mouse strains [63], although there is no indication they are involved in immunity to mouse CMV. Also, similar to HLA-E, Qa-1^b restricted T-cells have been implicated in immunity to pathogens, including *Listeria monocytogenes* [64] and *Salmonella typhimurium* [65].

Qa-1^b restricted *S. typhimurium* specific T-cells are curiously cross-reactive to self Hsp-60 derived peptides and thus have been implicated in autoimmune conditions [66]. Furthermore, there is a large and older body of research focusing on Qa-1^b restricted suppressor $CD8^+$ T-cells. These T-cells

reportedly recognize Qa-1^b in a TCR dependent manner and act to suppress autoreactive $CD4^+$ T-cells, thereby attenuating the development of autoimmune encephalomyelitis (a mouse model of multiple sclerosis), very similar to the role of HLA-E restricted $CD8^+$ T-cells evoked by vaccination in human MS [67–69]. In this model, Qa-1^b deficient mice developed exaggerated secondary CD4 responses, as a result of the lack of a population of regulatory $CD8^+$ T-cells, demonstrating the *in vivo* significance of suppressor Qa-1^b restricted T-cells. Qa-1^b restricted T-cells appear critical for the maintenance of self-tolerance. Their role in infectious diseases and in particular in the elimination of pathogens has not been studied in great detail thus far. Moreover, a detailed description of the phenotype and function of these cells is lacking and warrants further investigation.

3. Summary and Implications

HLA-E plays a dual role in the innate and adaptive immune system (Figure 1). The low polymorphism in HLA-E in conjunction with its relative insensitivity to downregulation by, for example, HIV, makes HLA-E an interesting target for vaccination strategies against infectious diseases and tumors. Small sets of peptides should suffice to induce T-cells recognizing foreign antigens and mount effector responses. The quality of these T-cell responses needs further investigation, however, given the diversity in functions that have been described thus far, ranging from cytotoxicity and pathogen control to immune suppression. Various pathogen-derived antigens can bind and stabilize HLA-E at the cell surface, for some of which this may be an essential mechanism to prevent NK mediated target cell lysis. However, many of these antigens can also be recognized in a TCR dependent manner by $CD8^+$ T-cells. These T-cells may suppress bystander T-cells or lyse (infected) target cells and inhibit intracellular bacteria, indicating an important functional contribution to the immune response. Nevertheless, the relative frequency of HLA-E restricted T-cells and their *in vivo* relevance in many cases remains unknown and unstudied. Although these T-cells are donor-unrestricted, they in many aspects display similar functionalities to classical, conventional T-cells.

Pathogen specific HLA-E restricted $CD8^+$ T-cells are an interesting new player in the field of immunology. Future work should address their exact roles and relative contributions in the immune response against infectious diseases.

Competing Interests

The authors declare that they have no competing interests.

References

[1] H. D. Moreau and P. Bousso, "Visualizing how T cells collect activation signals in vivo," *Current Opinion in Immunology*, vol. 26, no. 1, pp. 56–62, 2014.

[2] F. Benvenuti, "The dendritic cell synapse: a life dedicated to t cell activation," *Frontiers in Immunology*, vol. 7, article 70, 2016.

[3] J. G. P. Sissons and M. B. A. Oldstone, "Killing of virus-infected cells by cytotoxic lymphocytes," *Journal of Infectious Diseases*, vol. 142, no. 1, pp. 114–119, 1980.

[4] P. Escoll, M. Rolando, and C. Buchrieser, "Modulation of host autophagy during bacterial infection: sabotaging host munitions for pathogen nutrition," *Frontiers in Immunology*, vol. 7, article 81, 2016.

[5] D. I. Godfrey, A. P. Uldrich, J. Mccluskey, J. Rossjohn, and D. B. Moody, "The burgeoning family of unconventional T cells," *Nature Immunology*, vol. 16, no. 11, pp. 1114–1123, 2015.

[6] Anthony Nolan Research Institute, *HLA Class I and Class II Sequence Alignments, June 2009 Updates*, 2009, http://www.ebi.ac.uk/ipd/imgt/hla/.

[7] M. Ulbrecht, T. Honka, S. Person, J. P. Johnson, and E. H. Weiss, "The HLA-E gene encodes two differentially regulated transcripts and a cell surface protein," *Journal of Immunology*, vol. 149, no. 9, pp. 2945–2953, 1992.

[8] S. Coupel, A. Moreau, M. Hamidou, V. Horejsi, J.-P. Soulillou, and B. Charreau, "Expression and release of soluble HLA-E is an immunoregulatory feature of endothelial cell activation," *Blood*, vol. 109, no. 7, pp. 2806–2814, 2007.

[9] P. Tomasec, V. M. Braud, C. Rickards et al., "Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40," *Science*, vol. 287, no. 5455, pp. 1031–1033, 2000.

[10] I. Foroni, A. R. Couto, B. F. Bettencourt, M. Santos, M. Lima, and J. Bruges-Armas, "HLA-E, HLA-F and HLA-G—the non-classical side of the MHC cluster," in *HLA and Associated Important Diseases*, Y. Xi, Ed., chapter 3, pp. 61–109, InTech, Rijeka, Croatia, 2014.

[11] R. Apps, S. P. Murphy, R. Fernando, L. Gardner, T. Ahad, and A. Moffett, "Human leucocyte antigen (HLA) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies," *Immunology*, vol. 127, no. 1, pp. 26–39, 2009.

[12] H. L. Hoare, L. C. Sullivan, G. Pietra et al., "Structural basis for a major histocompatibility complex class Ib-restricted T cell response," *Nature Immunology*, vol. 7, no. 3, pp. 256–264, 2006.

[13] V. M. Braud, D. S. J. Allan, C. A. O'Callaghan et al., "HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C," *Nature*, vol. 391, no. 6669, pp. 795–799, 1998.

[14] C. Grimsley and C. Ober, "Population genetic studies of HLA-E: evidence for selection," *Human Immunology*, vol. 52, no. 1, pp. 33–40, 1997.

[15] T. Kraemer, R. Blasczyk, and C. Bade-Doeding, "HLA-E: a novel player for histocompatibility," *Journal of Immunology Research*, vol. 2014, Article ID 352160, 7 pages, 2014.

[16] R. K. Strong, M. A. Holmes, P. Li, L. Braun, N. Lee, and D. E. Geraghty, "HLA-E allelic variants: correlating differential expression, peptide affinities, crystal structures, and thermal stabilities," *Journal of Biological Chemistry*, vol. 278, no. 7, pp. 5082–5090, 2003.

[17] T. Kraemer, A. A. Celik, T. Huyton, H. Kunze-Schumacher, R. Blasczyk, and C. Bade-Döding, "HLA-E: presentation of a broader peptide repertoire impacts the cellular immune response—implications on HSCT outcome," *Stem Cells International*, vol. 2015, Article ID 346714, 12 pages, 2015.

[18] A. A. Celik, T. Kraemer, T. Huyton, R. Blasczyk, and C. Bade-Döding, "The diversity of the HLA-E-restricted peptide repertoire explains the immunological impact of the Arg107Gly mismatch," *Immunogenetics*, vol. 68, no. 1, pp. 29–41, 2016.

[19] C. A. O'Callaghan, J. Tormo, B. E. Willcox et al., "Structural features impose tight peptide binding specificity in the nonclassical MHC molecule HLA-E," *Molecular Cell*, vol. 1, no. 4, pp. 531–541, 1998.

[20] H. L. Hoare, L. C. Sullivan, C. S. Clements et al., "Subtle changes in peptide conformation profoundly affect recognition of the non-classical MHC class I molecule HLA-E by the CD94-NKG2 natural killer cell receptors," *Journal of Molecular Biology*, vol. 377, no. 5, pp. 1297–1303, 2008.

[21] M. Ulbrecht, S. Modrow, R. Srivastava, P. A. Peterson, and E. H. Weiss, "Interaction of HLA-E with peptides and the peptide transporter in vitro: implications for its function in antigen presentation," *Journal of Immunology*, vol. 160, no. 9, pp. 4375–4385, 1998.

[22] M. Ulbrecht, S. Martinozzi, M. Grzeschik et al., "Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis," *The Journal of Immunology*, vol. 164, no. 10, pp. 5019–5022, 2000.

[23] J. Nattermann, H. D. Nischalke, V. Hofmeister et al., "The HLA-A2 restricted T cell epitope HCV core$_{35–44}$ stabilizes HLA-E expression and inhibits cytolysis mediated by natural killer cells," *The American Journal of Pathology*, vol. 166, no. 2, pp. 443–453, 2005.

[24] J. Stevens, E. Joly, J. Trowsdale, and G. W. Butcher, "Peptide binding characteristics of the non-classical class Ib MHC molecule HLA-E assessed by a recombinant random peptide approach," *BMC Immunology*, vol. 2, no. 1, article 5, 2001.

[25] J. D. Miller, D. A. Weber, C. Ibegbu, J. Pohl, J. D. Altman, and P. E. Jensen, "Analysis of HLA-E peptide-binding specificity and contact residues in bound peptide required for recognition by CD94/NKG2," *Journal of Immunology*, vol. 171, no. 3, pp. 1369–1375, 2003.

[26] V. Braud, E. Y. Jones, and A. McMichael, "The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9," *European Journal of Immunology*, vol. 27, no. 5, pp. 1164–1169, 1997.

[27] M. H. Lampen, C. Hassan, M. Sluijter et al., "Alternative peptide repertoire of HLA-E reveals a binding motif that is strikingly similar to HLA-A2," *Molecular Immunology*, vol. 53, no. 1-2, pp. 126–131, 2013.

[28] S. G. Hansen, H. L. Wu, B. J. Burwitz et al., "Broadly targeted CD8$^+$ T cell responses restricted by major histocompatibility complex E," *Science*, vol. 351, no. 6274, pp. 714–720, 2016.

[29] J. E. Grotzke, M. J. Harriff, A. C. Siler et al., "The *Mycobacterium tuberculosis* phagosome is a HLA-I processing competent organelle," *PLoS Pathogens*, vol. 5, no. 4, Article ID e1000374, 2009.

[30] S. A. Joosten, K. E. van Meijgaarden, P. C. van Weeren et al., "*Mycobacterium tuberculosis* peptides presented by HLA-E molecules are targets for human CD8$^+$ T-cells with cytotoxic as well as regulatory activity," *PLoS Pathogens*, vol. 6, no. 2, Article ID e1000782, 2010.

[31] D. M. Lewinsohn, M. R. Alderson, A. L. Briden, S. R. Riddell, S. G. Reed, and K. H. Grabstein, "Characterization of human CD8$^+$ T cells reactive with Mycobacterium tuberculosis-infected antigen-presenting cells," *Journal of Experimental Medicine*, vol. 187, no. 10, pp. 1633–1640, 1998.

[32] A. S. Heinzel, J. E. Grotzke, R. A. Lines et al., "HLA-E-dependent presentation of Mtb-derived antigen to human CD8$^+$ T cells," *The Journal of Experimental Medicine*, vol. 196, no. 11, pp. 1473–1481, 2002.

[33] J. Li, I. Goldstein, E. Glickman-Nir, H. Jiang, and L. Chess, "Induction of TCR Vβ-specific CD8+ CTLs by TCR Vβ-derived peptides bound to HLA-E," *Journal of Immunology*, vol. 167, no. 7, pp. 3800–3808, 2001.

[34] C. Romagnani, G. Pietra, M. Falco et al., "Identification of HLA-E-specific alloreactive T lymphocytes: a cell subset that undergoes preferential expansion in mixed lymphocyte culture and displays a broad cytolytic activity against allogeneic cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 17, pp. 11328–11333, 2002.

[35] P. García, M. Llano, A. B. de Heredia et al., "Human T cell receptor-mediated recognition of HLA-E," *European Journal of Immunology*, vol. 32, no. 4, pp. 936–944, 2002.

[36] P. B. Jørgensen, A. H. Livbjerg, H. J. Hansen, T. Petersen, and P. Höllsberg, "Epstein-Barr virus peptide presented by HLA-E is predominantly recognized by CD8^{bright} cells in multiple sclerosis patients," *PLoS ONE*, vol. 7, no. 9, Article ID e46120, 2012.

[37] G. Pietra, C. Romagnani, P. Mazzarino et al., "HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8$^+$ cytolytic T lymphocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 19, pp. 10896–10901, 2003.

[38] P. Mazzarino, G. Pietra, P. Vacca et al., "Identification of effector-memory CMV-specific T lymphocytes that kill CMV-infected target cells in an HLA-E-restricted fashion," *European Journal of Immunology*, vol. 35, no. 11, pp. 3240–3247, 2005.

[39] C. Romagnani, G. Pietra, M. Falco, P. Mazzarino, L. Moretta, and M. C. Mingari, "HLA-E-restricted recognition of human cytomegalovirus by a subset of cytolytic T lymphocytes," *Human Immunology*, vol. 65, no. 5, pp. 437–445, 2004.

[40] L. C. Sullivan, G. P. Westall, J. M. L. Widjaja et al., "The presence of HLA-E-restricted, CMV-specific CD8$^+$ T cells in the blood of lung transplant recipients correlates with chronic allograft rejection," *PLoS ONE*, vol. 10, no. 8, Article ID e0135972, 2015.

[41] M. Allard, P. Tonnerre, S. Nedellec et al., "HLA-E-restricted cross-recognition of allogeneic endothelial cells by CMV-associated CD8 T cells: a potential risk factor following transplantation," *PLoS ONE*, vol. 7, no. 11, Article ID e50951, 2012.

[42] G. Pietra, C. Romagnani, M. Falco et al., "The analysis of the natural killer-like activity of human cytolytic T lymphocytes revealed HLA-E as a novel target for TCR α/β-mediated recognition," *European Journal of Immunology*, vol. 31, no. 12, pp. 3687–3693, 2001.

[43] R. Salerno-Gonçalves, M. Fernandez-Viña, D. M. Lewinsohn, and M. B. Sztein, "Identification of a human HLA-E-restricted CD8$^+$ T cell subset in volunteers immunized with *Salmonella enterica* serovar Typhi strain Ty21a typhoid vaccine," *Journal of Immunology*, vol. 173, no. 9, pp. 5852–5862, 2004.

[44] D. Schulte, M. Vogel, B. Langhans et al., "The HLA-ER/HLA-ER genotype affects the natural course of hepatitis C virus (HCV) infection and is associated with HLA-E-Restricted recognition of an HCV-Derived peptide by interferon-γ-secreting human CD8$^+$ T cells," *Journal of Infectious Diseases*, vol. 200, no. 9, pp. 1397–1401, 2009.

[45] H. Jiang, S. M. Canfield, M. P. Gallagher et al., "HLA-E-restricted regulatory CD8$^+$ T cells are involved in development and control of human autoimmune type 1 diabetes," *The Journal of Clinical Investigation*, vol. 120, no. 10, pp. 3641–3650, 2010.

[46] R. Salerno-Goncalves, R. Wahid, and M. B. Sztein, "Ex vivo kinetics of early and long-term multifunctional human leukocyte antigen E-specific CD8$^+$ cells in volunteers immunized

with the Ty21a typhoid vaccine," *Clinical and Vaccine Immunology*, vol. 17, no. 9, pp. 1305–1314, 2010.

[47] K. E. van Meijgaarden, M. C. Haks, N. Caccamo, F. Dieli, T. H. M. Ottenhoff, and S. A. Joosten, "Human $CD8^+$ T-cells recognizing peptides from *Mycobacterium tuberculosis* (*Mtb*) presented by HLA-E have an unorthodox Th2-like, multifunctional, *Mtb* inhibitory phenotype and represent a novel human T-cell subset," *PLoS Pathogens*, vol. 11, no. 3, Article ID e1004671, pp. 1–24, 2015.

[48] N. Caccamo, G. Pietra, L. C. Sullivan et al., "Human CD8 T lymphocytes recognize *Mycobacterium tuberculosis* antigens presented by HLA-E during active tuberculosis and express type 2 cytokines," *European Journal of Immunology*, vol. 45, no. 4, pp. 1069–1081, 2015.

[49] S. Fresnay, M. A. McArthur, L. Magder et al., "*Salmonella* Typhi-specific multifunctional CD8+ T cells play a dominant role in protection from typhoid fever in humans," *Journal of Translational Medicine*, vol. 14, article 62, 2016.

[50] L. C. Sullivan, C. S. Clements, J. Rossjohn, and A. G. Brooks, "The major histocompatibility complex class Ib molecule HLA-E at the interface between innate and adaptive immunity," *Tissue Antigens*, vol. 72, no. 5, pp. 415–424, 2008.

[51] G. B. Cohen, R. T. Gandhi, D. M. Davis et al., "The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells," *Immunity*, vol. 10, no. 6, pp. 661–671, 1999.

[52] J. Nattermann, H. D. Nischalke, V. Hofmeister et al., "HIV-1 infection leads to increased HLA-E expression resulting in impaired function of natural killer cells," *Antiviral Therapy*, vol. 10, no. 1, pp. 95–107, 2005.

[53] Z. B. Davis, A. Cogswell, H. Scott et al., "A conserved HIV-1-derived peptide presented by HLA-E renders infected T-cells highly susceptible to attack by NKG2A/CD94-bearing natural killer cells," *PLoS Pathogens*, vol. 12, no. 2, Article ID e1005421, 2016.

[54] T. van Hall, C. C. Oliveira, S. A. Joosten, and T. H. M. Ottenhoff, "The other Janus face of Qa-1 and HLA-E: diverse peptide repertoires in times of stress," *Microbes and Infection*, vol. 12, no. 12-13, pp. 910–918, 2010.

[55] C. C. Oliveira, P. A. van Veelen, B. Querido et al., "The non-polymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects," *The Journal of Experimental Medicine*, vol. 207, no. 1, pp. 207–221, 2010.

[56] M. Talebian Yazdi, S. van Riet, A. van Schadewijk et al., "The positive prognostic effect of stromal CD8+ tumor-infiltrating T cells is restrained by the expression of HLA-E in non-small cell lung carcinoma," *Oncotarget*, vol. 7, no. 3, pp. 3477–3488, 2016.

[57] A. F. Tyler, J. P. Mendoza, M. Firan, and N. J. Karandikar, "$CD8^+$ T cells are required for glatiramer acetate therapy in autoimmune demyelinating disease," *PLoS ONE*, vol. 8, no. 6, article e66772, 2013.

[58] S. Sinha, A. W. Boyden, F. R. Itani, M. P. Crawford, and N. J. Karandikar, "$CD8^+$ T-cells as immune regulators of multiple sclerosis," *Frontiers in Immunology*, vol. 6, article 619, 2015.

[59] D. K. Tennakoon, R. S. Mehta, S. B. Ortega, V. Bhoj, M. K. Racke, and N. J. Karandikar, "Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis," *The Journal of Immunology*, vol. 176, no. 11, pp. 7119–7129, 2006.

[60] K. Pannemans, B. Broux, A. Goris et al., "HLA-E restricted $CD8^+$ T cell subsets are phenotypically altered in multiple sclerosis patients," *Multiple Sclerosis Journal*, vol. 20, no. 7, pp. 790–801, 2014.

[61] M. Iwaszko, J. Świerkot, K. Kolossa, S. Jeka, P. Wiland, and K. Bogunia-Kubik, "Polymorphisms within the human leucocyte antigen-E gene and their associations with susceptibility to rheumatoid arthritis as well as clinical outcome of anti-tumour necrosis factor therapy," *Clinical and Experimental Immunology*, vol. 182, no. 3, pp. 270–277, 2015.

[62] J. W. Leavenworth, X. Tang, H.-J. Kim, X. Wang, and H. Cantor, "Amelioration of arthritis through mobilization of peptide-specific $CD8^+$ regulatory T cells," *Journal of Clinical Investigation*, vol. 123, no. 3, pp. 1382–1389, 2013.

[63] C. J. Aldrich, A. DeCloux, A. S. Woods, R. J. Cotter, M. J. Soloski, and J. Forman, "Identification of a tap-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen," *Cell*, vol. 79, no. 4, pp. 649–658, 1994.

[64] H. G. A. Bouwer, M. S. Seaman, J. Forman, and D. J. Hinrichs, "MHC class Ib-restricted cells contribute to antilisterial immunity: evidence for Qa-1b as a key restricting element for listeria-specific CTLs," *Journal of Immunology*, vol. 159, no. 6, pp. 2795–2801, 1997.

[65] W.-F. Lo, H. Ong, E. S. Metcalf, and M. J. Soloski, "T cell responses to gram-negative intracellular bacterial pathogens: a role for $CD8^+$ T cells in immunity to Salmonella infection and the involvement of MHC class Ib molecules," *Journal of Immunology*, vol. 162, no. 9, pp. 5398–5406, 1999.

[66] W.-F. Lo, A. S. Woods, A. DeCloux, R. J. Cotter, E. S. Metcalf, and M. J. Soloski, "Molecular mimicry mediated by MHC class Ib molecules after infection with gram-negative pathogens," *Nature Medicine*, vol. 6, no. 2, pp. 215–218, 2000.

[67] H. Jiang, S.-L. Zhang, and B. Pernis, "Role of $CD8^+$ T cells in murine experimental allergic encephalomyelitis," *Science*, vol. 256, no. 5060, pp. 1213–1215, 1992.

[68] H. Jiang, H. Kashleva, L.-X. Xu et al., "T cell vaccination induces T cell receptor Vβ-specific Qa-1-restricted regulatory $CD8^+$ T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 8, pp. 4533–4537, 1998.

[69] S. Sarantopoulos, L. Lu, and H. Cantor, "Qa-1 restriction of $CD8^+$ suppressor T cells," *Journal of Clinical Investigation*, vol. 114, no. 9, pp. 1218–1221, 2004.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 4326495, 14 pages
http://dx.doi.org/10.1155/2016/4326495

Review Article

Recent Advances in Our Understanding of HLA-G Biology: Lessons from a Wide Spectrum of Human Diseases

Fabio Morandi,[1] Roberta Rizzo,[2] Enrico Fainardi,[3] Nathalie Rouas-Freiss,[4] and Vito Pistoia[1]

[1]*Laboratory of Oncology, Istituto Giannina Gaslini, 16147 Genoa, Italy*
[2]*Department of Experimental and Diagnostic Medicine, Section of Microbiology, University of Ferrara, 44121 Ferrara, Italy*
[3]*Neuroradiology Unit, Department of Diagnostic Imaging, Azienda Ospedaliero-Universitaria Careggi, 50134 Florence, Italy*
[4]*CEA, Institut des Maladies Emergentes et des Therapies Innovantes (iMETI), Service de Recherche en Hemato-Immunologie (SRHI), Hôpital Saint-Louis, 75010 Paris, France*

Correspondence should be addressed to Fabio Morandi; fabiomorandi@ospedale-gaslini.ge.it

Received 6 May 2016; Accepted 23 July 2016

Academic Editor: Stuart Berzins

HLA-G is a HLA-class Ib molecule with potent immunomodulatory activities, which is expressed in physiological conditions, where modulation of the immune response is required to avoid allograft recognition (i.e., maternal-fetal interface or transplanted patients). However, HLA-G can be expressed *de novo* at high levels in several pathological conditions, including solid and hematological tumors and during microbial or viral infections, leading to the impairment of the immune response against tumor cells or pathogens, respectively. On the other hand, the loss of HLA-G mediated control of the immune responses may lead to the onset of autoimmune/inflammatory diseases, caused by an uncontrolled activation of the immune effector cells. Here, we have reviewed novel findings on HLA-G functions in different physiological and pathological settings, which have been published in the last two years. These studies further confirmed the important role of this molecule in the modulation of the immune system.

Dedicated to the memory of Professor Olavio Roberto Baricordi for his creative and groundbreaking work in the study of HLA-G implication in physiological and pathological conditions

1. Introduction

HLA-G is an important molecule with immunomodulatory properties, which belongs to "nonclassical" HLA-class Ib molecules, along with HLA-E, -F, and -H [1]. In contrast with "classical" HLA-class Ia molecules (HLA-A, -B, and -C), which are highly polymorphic, with a high number of alleles encoding a high number of functional proteins, HLA-Ib molecules display a very low polymorphism, with a small number of alleles encoding a limited number of proteins. Similarly to HLA-Ia molecules, also HLA-Ib molecules can bind peptides generated through a proteasome-driven degradation of cytosolic proteins and present them to specific subpopulations of $CD8^+$ T cells [2]. However, the main function of these molecules is to modulate the immune responses in both physiological and pathological conditions [3].

HLA-G molecule can be present in seven different isoforms encoded through alternative splicing of the same primary transcript. Membrane-bound isoforms (HLA-G1, -G2, -G3, and -G4) lack intron-4, which is retained in the soluble (s) isoforms (HLA-G5, -G6, and -G7), leading to the splicing of the transmembrane domain. In addition, sHLA-G1, which is structurally identical to sHLA-G5, can be generated by shedding of membrane-bound HLA-G1 isoform through metalloproteases-mediated cleavage [4]. Only HLA-G1 and -G5 present a full length heavy chain, which is associated with β2-microglobulin and binds small peptides, although it is unclear whether or not HLA-G can present them to $CD8^+$ T lymphocytes [5]. In this respect, Diehl et al. have

characterized specific motifs in HLA-G restricted peptides and they have concluded that HLA-G can present peptides similarly to classical HLA-class I molecules [6].

HLA-G can interact with immunoglobulin-like transcript 2 (ILT2), which is expressed by T and B lymphocytes, Natural Killer (NK) cells, monocytes/macrophages, and dendritic cells, and with ILT4, which is expressed only by myeloid cells (i.e., dendritic cells, monocytes, and macrophages and neutrophils) [7]. In addition, HLA-G can interact with KIR2DL4 expressed by NK cells [8] and CD160 expressed by T lymphocytes, NK cells, and endothelial cells [4]. Upon interaction with these receptors, HLA-G can affect the function of different cell populations. In particular, HLA-G (i) impairs T cell function, by inhibiting proliferation [9] and cytotoxicity [10] and by inducing apoptosis [11] and expansion of regulatory T cells [12, 13], (ii) inhibits differentiation, proliferation, and cytokine production in B lymphocytes [14], (iii) inhibits proliferation and cytotoxicity [15, 16] of peripheral blood NK cells and induces proliferation and release of proangiogenic factors in blood and uterine NK cells [17–19], (iv) inhibits chemotaxis of different T, B, and NK cell populations by downregulating chemokine receptors expression on their surface [20], (v) inhibits phagocytosis and production of reactive oxygen species in neutrophils [7], and (vi) dampens angiogenesis, by inhibiting endothelial cells proliferation [21].

In the last years, several papers have demonstrated that HLA-G can be present as a homodimeric molecule, which is generated through a disulfide bond [22–24]. Moreover, it has been observed that homodimer is more biologically active than the monomeric form [13, 25, 26].

2. HLA-G and Cancer

Overexpression of membrane-bound soluble and sHLA-G has been detected in different human solid and hematological tumors and might represent a mechanism performed by tumor cells to escape from the control of the immune system, by inhibiting NK and T cells mediated lysis [4].

In the last two years, several papers have addressed the role of HLA-G in tumor progression or have characterized this molecule as prognostic factor for the clinical outcome of cancer patients (Table 1).

Loumagne et al. have performed an interesting study using a murine model. In fact, HLA-G can interact with murine paired immunoglobulin-like receptor- (PIR-) B, ortholog of human ILT receptors, thus enabling the investigation of its role *in vivo*. Immunocompetent mice were injected either with syngeneic tumor cells coexpressing human HLA-G5 and β2-microglubulin (hβ2m) or with hβ2m$^+$ HLA-G5$^-$ tumor cells. Interestingly, hβ2m$^+$ HLA-G5$^-$ tumors were rejected, whereas hβ2m$^+$ HLA-G5$^+$ tumors secreted soluble HLA-G, which protected them from hβ2m-elicited immune rejection, and grew similarly to a poorly immunogenic tumor. They demonstrated that HLA-G5 tumor expression dampened anti-hβ2m B cell response through accumulation of myeloid-derived suppressor cells which inhibited T cell proliferation and reduced T and B cell tumor infiltrate [27].

Zheng et al. have demonstrated that HLA-G (mostly HLA-G1 and -G5) is expressed by tumor lesions in esophageal squamous cell carcinoma (ESCC) patients, but not in adjacent normal tissues or in healthy controls. Moreover, such expression positively correlated with lymph node metastasis and cancer cell differentiation. Accordingly, sHLA-G serum levels were higher in patients than in controls [28].

An interesting study has been performed by Reimers and colleagues on rectal cancer patients. They analyzed tumors by tissue microarray for the presence of Foxp3$^+$ cells (Tregs) and tumor expression of HLA-E and HLA-G. They observed that patients with rectal tumors characterized by loss of HLA class I expression, Foxp3$^+$ infiltration below median, and weak HLA-G expression displayed a worse overall survival (OS) and disease-free survival (DFS) [29]. In contrast, Guo et al. have demonstrated that the majority of colorectal cancer tissues tested positive for HLA-G or HLA-E expression, and half of them expressed both molecules. Moreover, expression levels of HLA-G or HLA-E and the combined expression of both molecules were all negatively correlated with OS of colorectal cancer patients. However, these authors found that only HLA-G expression can serve as independent factor for OS, whereas the expression of HLA-E was significantly correlated with tumor metastasis [30]. Yan et al. have analyzed the expression of soluble HLA-G5/G6 molecules in tumor samples from non-small-cell lung cancer (NSCLC) patients, using specific mAb 5A6G7. sHLA-G expression was observed in half of NSCLC lesions and was significantly higher in adenocarcinoma lesions than that in squamous cell carcinoma and adenosquamous carcinoma lesions. The authors concluded that sHLA-G could be a useful biomarker to discriminate adenocarcinoma from squamous cell carcinoma in NSCLC patients [31].

Another interesting study comes from Xu and colleagues. They analyzed HLA-G expression in a cohort of patients with pancreatic carcinoma and found high levels of HLA-G in the majority of tumor samples. Moreover, they demonstrated that HLA-G levels represented an independent predictor for patients' OS, and a positive correlation was found between HLA-G expression and tumor stage, extrapancreatic infiltration, lymph node involvement, and poor differentiation. Finally, they demonstrated that plasma levels of sHLA-G were higher in patients than in healthy controls, and sHLA-G levels inversely correlated to numbers of peripheral activated T cells, thus suggesting that sHLA-G promotes tumor immune escape through the inactivation T cell responses [32].

A study performed by Wang et al. described a relationship between HLA-G expression and the sharpness of low-grade glioma tumor borders in magnetic resonance images. In particular, high HLA-G expression was detected in larger tumors with blurred boundaries, which may be those prone to diffuse invasion. Therefore, patients with tumors that highly expressed HLA-G were less likely to have undergone complete resections [33].

Expression and function of HLA-G and regulatory microRNA (miR-152, -148A, -148B, and -133A) have been analyzed by Jasinski-Bergner and colleagues in renal cell carcinoma (RCC). They have observed an inverse correlation

Table 1: Novel findings on HLA-G and tumors.

Type of tumor	Observation	Correlation with clinical outcome	PB sHLA-G	Author
Murine model	HLA-G$^+$ tumors are protected from rejection	—	—	Loumagne et al. [27]
ESCC	Tumors but not adjacent tissues expressed HLA-G	Positive correlation with metastasis	Increased	Zheng et al. [28]
Rectal cancer	Tumors are HLA-G$^+$, FOXP3$^+$, and HLA-class I$^-$	Worse OS	—	Reimers et al. [29]
Colorectal cancer	Most tumors expressed HLA-G and/or HLA-E	Worse OS	—	Guo et al. [30]
NSCLC	HLA-G is expressed in adenocarcinoma but not in squamous cell carcinoma	—	—	Yan et al. [31]
Pancreatic cancer	Most tumors are HLA-G$^+$	Positive correlation with metastasis and worse OS	Increased	Xu et al. [32]
Glioma	HLA-G expression is higher in tumors with blurred boundaries	Positive correlation with invasiveness	—	Wang et al. [33]
Renal cell carcinoma	Expression of some miRNAs downregulates HLA-G expression and increased antitumor immune response	—	—	Jasinki-Bergner et al. [34]
Gastric cancer	TGF-β induced HLA-G expression through miR152 downregulation	—	—	Guan et al. [35]
Gastric cancer	HLA-G$^+$ DC-10 cells are increased in PB of patients	Worse prognosis	Increased	Xu et al. [36]
Murine model	LILRB1$^-$ NK cells are more effective against HLA-G$^+$ tumors	—	—	Wu et al. [37]
Pancreatic cancer	CD3$^+$ TIL are decreased in HLA-G$^+$ tumors	Worse OS	—	Zhou et al. [38]
Breast cancer	Opposite prognostic value of free sHLA-G and sHLA-G in MV after chemotherapy	sHLA-G in MV positively correlated with worse prognosis; free sHLA-G positively correlated with better outcome	—	König et al. [39]
Breast cancer	Higher sHLA-G levels in patients without previous pregnancy and breastfeeding history	Worse OS	Increased	Zidi et al. [40]
DLBCL	Different prognostic value of del/del polymorphisms of HLA-G gene	Worse OS	—	Bielska et al. [41]
NSCLC	SNP in *HLA-G* and *LILRB1* genes and expression of HLA-G and LILRB1 are related to clinical outcome	Positive correlation to high risk of tumor and tumor stage	—	Wiśniewski et al. [42]
Prostate cancer	Polymorphisms in 3′ UTR of *HLA-G* gene are related to clinical outcome	Positive correlation to higher susceptibility	—	Zambra et al. [43]
Gastric adenocarcinoma	Higher frequency of some polymorphisms of *HLA-G* gene in healthy controls than in patients	—	—	Khorrami et al. [44]
CLL	14 bp polymorphism in 3′ UTR of *HLA-G* gene is related to clinical outcome	Worse OS	—	Rizzo et al. [45]

between the expression of miR-148A and -133A and HLA-G protein *in situ* and *in vitro*. They concluded that a stable miRNA overexpression downregulated HLA-G expression, thus enhancing NK and LAK mediated cytotoxicity. This hypothesis has been confirmed by the analysis of immune cell infiltration [82]. They have also identified two additional miRNAs (miR-548q and miR-628-5p) which may regulate HLA-G expression in RCC, demonstrating a direct interaction of these miRNAs with the 3′ untranslated region (3′ UTR) of HLA-G. Stable overexpression of miR-548q and

miR-628-5p caused a downregulation of HLA-G mRNA and protein, leading to an enhanced NK cell-mediated cytotoxicity. In addition, they found an inverse correlation between the expression of miR-628-5p and HLA-G protein in primary RCC lesions and cell lines. The authors concluded that these miRNAs, which are able to tune HLA-G expression, might serve as future therapeutic targets [34]. The interactions between HLA-G and miR-152 (and TGF-β) have been also analyzed by Guan and colleagues in gastric cancer (GC). They observed a positive correlation between serum levels of TGF-β and HLA-G in GC patients and a direct TGF-β-mediated induction of HLA-G expression in GC cell lines *in vitro*. Furthermore, TGF-β also inhibited miR-152 expression, and HLA-G was posttranscriptionally regulated by miR-152. In addition, miR-152 overexpression repressed HLA-G upregulation induced by TGF-β. Finally, the authors observed that miR-152 expression levels were inversely correlated to both HLA-G and TGF-β levels in GC patients. They suggested a potential application of miR-152 as therapeutic target or potential biomarker for GC patients [35]. Also the study carried out by Xu et al. has focused on HLA-G expression in GC. The authors demonstrated that (i) human tolerogenic dendritic cells DC-10, which express HLA-G, are dramatically increased in the peripheral blood of GC patients as compared to healthy donors, (ii) the expression of HLA-G on these cells is significantly higher than in DC-10 cells from healthy donors, and (iii) concentration of plasma sHLA-G is higher in GC patients than controls. Finally, they demonstrated that these three parameters are worse prognostic factors for GC patients, thus suggesting an immunosuppressive role for HLA-G and DC-10 cells in GC [36].

A novel interesting study has been published by Wu et al., who have characterized the antitumor potential of NK cells genetically modified to downregulate the expression of LILRB1. They have demonstrated that LILRB1$^-$ NK cells proliferate upon stimulation signals, migrate, and eliminate HLA-G$^-$ targets cells as parental NK cells do. Moreover, LILRB1$^-$ NK cells exhibit higher proliferation, conjugate formation, degranulation, and killing activities compared to parent NK cells in the presence of HLA-G$^+$ target cells. Finally, LILRB1 silencing rescued NK cell antitumor activity in a xenograft cancer model [37].

The effect of HLA-G expression by tumor cells on tumor infiltrating lymphocytes (TIL) has been also addressed by Zhou et al. in pancreatic cancer (PC). They detected HLA-G overexpression in tumor samples from PC patients, but not in nontumor tissues. The number of CD3$^+$ TIL was significantly lower in HLA-G$^+$ than in HLA-G$^-$ tumors. More importantly, the authors demonstrated that HLA-G expression and low intratumoral CD3$^+$ staining represent independent prognostic factors pointing to worse OS of PC patients. These findings suggest that HLA-G expression impairs host antitumor immune response and predicts a poor prognosis in PC [38].

König and colleagues have demonstrated for the first time that HLA-G might also serve as prognostic marker to predict the clinical outcome of neoadjuvant chemotherapy (NACT). In fact, they analyzed total sHLA-G and HLA-G levels in extracellular vesicles (EV) in plasma samples from breast cancer (BC) patients, before and after NACT. They found that total and free sHLA-G levels were higher in treated patients than in healthy controls. More importantly, high sHLA-G in EV before NACT positively correlated to disease progression, whereas free sHLA-G levels were directly correlated to a better clinical outcome, thus demonstrating that different sHLA-G subcomponents may have different prognostic impacts on the clinical outcome of NACT treated BC patients [39]. Zidi et al. have also focused on HLA-G function in BC patients. They analyzed sHLA-G in plasma samples obtained from women with BC and correlated sHLA-G concentration with pregnancy and breastfeeding history. They reported significant differences in sHLA-G levels between BC patients with/without breastfeeding experience. Interestingly, BC patients without previous pregnancy experience display higher levels of sHLA-G, and patients without both pregnancy and breastfeeding history showed a significant enhancement in tumor size compared with patients who had both experiences. These data suggested that a previous pregnancy and breastfeeding experience may protect against advanced BC stages through reduced levels of sHLA-G [40].

Five novel and interesting studies have addressed the correlation of different polymorphism of *HLA-G* gene with tumor progression. Bielska et al. have investigated the influence of two HLA-G polymorphisms, HLA-G-725C/G/T and HLA-G 14-base pair, on the susceptibility to diffuse large B-cell lymphoma (DLBCL) and on the clinical course of the disease. They observed that frequencies of HLA-G-725GG or HLA-G-725GC genotype were lower, and those of the HLA-G ins/ins genotype were higher in the patients compared with controls. Moreover, patients carrying the HLA-G-725CC genotype presented a better OS than subjects with other HLA-G-725C/G/T polymorphisms. Patients with homozygous HLA-G del/del had a worse OS than subjects carrying the HLA-G del/ins or the HLA-G ins/ins genotype. On the basis of genotype distribution, authors defined two HLA-G genotype-based risk groups; a high-risk (HR) group presented a worse OS than low-risk (LR) patients [41]. Wiśniewski et al. have characterized several polymorphisms, three in *HLA-G* gene (-964A>G, -725C>G>T, and -716T>G in the promoter and a 14 bp ins/del in the 3′UTR), five in *LILRB1* gene (-5651G>A in intron 14, 5717C>T L622L, 5724G>A E625K, 5774 C>A P641P in exon 15, and 5806C>T in 3′UTR), and 9620 9A/10A polymorphisms in exon 7 of *KIR2DL* gene in NSCLC patients. They have observed that only one single nucleotide polymorphism (SNP) in *HLA-G* (-964A>G) and one in *LILRB1* (5724G>A) positively correlated with a higher risk of NSCLC. In addition, 5724G>A in *LILRB1* gene was associated with protection from tumor cell infiltration of regional lymph nodes. Finally, tumor tissue samples tested positive for HLA-G and LILRB1 protein expression, and those expression levels significantly correlated with tumor stage [42].

Zambra et al. have evaluated the impact of eight polymorphisms in the 3′UTR region on susceptibility to the development of prostate cancer (PCa). They identified the UTR-4 haplotype as a risk factor to PCa. Furthermore, the "non-14 bp Ins_+3142G_+3187A" haplotype, the +3003CT

genotype, and the +3003C allele are also related to a higher susceptibility to PCa. They conclude that HLA-G 3′UTR polymorphism has a great influence on PCa susceptibility [43].

Khorrami and colleagues investigated whether HLA-G polymorphisms might serve as a potential risk factor for clinical outcomes in gastric adenocarcinoma (GAC). They found that G*01:01:03:01 and G*01:01:08 allele distributions are significantly higher in healthy controls than in GAC patients and seem to have protective effect. Moreover, the G*01:01:03:01/G*01:04:01 and G*01:01:02:01/G*01:01:08 genotypes frequencies are higher in healthy controls than in patients. They conclude that *HLA-G* gene polymorphisms could affect GAC induction and its outcome [83].

In this line, Rizzo and coauthors showed the prognostic value of the 14-base pair deletion (del) polymorphism (rs66554220) in the 3′ untranslated region of HLA-G, with del/del patients showing reduced overall survival of patients with chronic lymphocytic leukemia, as compared to those with other genotypes [45].

In summary, novel findings in the last two years confirmed that the expression of HLA-G is generally detected in tumors but not in normal tissues, and such expression positively correlated with tumor progression, metastasis, invasiveness, and worse prognosis of patients. The role of HLA-G (through the interaction with LILRB1) in the control of antitumor immune response has also been confirmed both in cancer patients and in murine models of human tumors. Finally, several polymorphisms of *HLA-G* and *LILRB1* genes have been characterized in the last two years, with positive or negative correlation with tumor stage and susceptibility of patients to develop tumors of patients' survival, thus confirming the important role of HLA-G in tumorigenesis and tumor progression.

3. HLA-G and Pregnancy

The important role of HLA-G in human pregnancy has been fully characterized in the last years. Different HLA-G isoforms are expressed by trophoblast cells at maternal-fetal interface. HLA-G expressed and released by trophoblast cells can interact with cellular receptors expressed by immune (T cells, NK cells, macrophages, and dendritic cells) and nonimmune (endothelial cells) cells present in the decidua, triggering either inhibitory or activating signals. Such interactions allow us to (i) limit maternal immune response against semiallogeneic fetal tissues by impairment of decidual NK cell cytotoxicity, T and B cell proliferation, and induction of apoptosis of activated $CD8^+$ T cells, (ii) stimulate placental development through secretion of proangiogenic factors by decidual (d) NK cells and macrophages, and (iii) provide a protective effect for pregnancy outcome by stimulating IL-4 secretion by $CD4^+$ T cells [84]. In this view, some novel interesting data have been obtained by Tilburgs and coworkers, who have isolated and characterized invading HLA-G^+ extravillous trophoblasts (EVT) cells and HLA-G^- villous trophoblasts (VT). Using microarray and functional gene set enrichment analysis, they revealed a striking immune-activating potential for EVT, which was absent in VT. Moreover, they performed cocultures of HLA-G^+ EVT cells with matched decidual immune cells, and they found that EVT induced differentiation of $CD4^+$ T cells in $CD4^+CD25^{hi}FOXP3^+CD45RA^+$ regulatory T cells (Treg) and increased the expression of FOXP3 in these cells. Moreover, EVT did not enhance cytokine secretion in dNK (whereas stimulation of dNK with other *stimuli* confirmed the distinct cytokine secretion profiles of dNK compared to peripheral blood NK cells) [13]. The interaction between dNK and EVT at the maternal-fetal interface was further characterized in a second study, where the authors demonstrated that dNK interacts with filamentous projections from EVT enriched in HLA-G, which may represent the initial stage of synapse formation. Moreover, dNK in this area expressed HLA-G on the surface or in the cytoplasm. Activation of dNK downregulated HLA-G expression and restored cytotoxicity, whereas HLA-G expression was reacquired by incubation with EVT. These data suggested that HLA-G undergoes a cycle of trogocytosis, endocytosis, degradation, and finally reacquisition on dNK, upon interaction with EVT. However, dNK can be activated by cytokines and/or viral products to acquire the ability to control virus infection at the interface (i.e., in the presence of human cytomegalo virus-infected decidual stromal cells) [46]. Collectively, all these data confirmed that HLA-G^+ EVT cells have unique properties in maternal-fetal tolerance establishment.

Immune cells in the decidual compartment have been further characterized by Djurisic et al., who have analyzed sHLA-G and cells from placental bed and peripheral blood in first trimester. They found an increased number of T cells with low CD4 or CD8 expression in the placental bed. Soluble HLA-G was increased in "uterine blood" as compared to peripheral blood. Moreover, KIR2DL4 and LILRB1 expression was upregulated on uterine NK cells. Finally, a correlation was found between uterine sHLA-G and the fraction of KIR2DL4+ uterine NK cells. The authors hypothesize that the phenotype of uterine NK cells may be influenced by HLA-G on trophoblast cells and by sHLA-G in the uterus [47].

Ferreira and coworkers have performed an interesting study to analyze the genetic regulation of HLA-G expression in the placenta. They found a novel cis-regulatory enhancer element 12 kb upstream of *HLA-G* gene (Enhancer L). More importantly, deletion of this enhancer ablated HLA-G expression in JEG3 cells and in primary human trophoblast cells isolated from placenta. They demonstrated that this enhancer is cell-type specific and contains motifs for transcription factors of CEBP and GATA (which are essential for placentation), thus suggesting that these factors may also control HLA-G expression on trophoblast [48].

In the last two years, some studies have addressed interesting novel aspects of the role of HLA-G in pregnancy (Table 2). Klitkou et al. have performed the first large study simultaneously measuring sHLA-G in both maternal and umbilical cord blood to test whether there is a correlation between sHLA-G levels in maternal and fetal blood. Therefore, they have measured the levels of sHLA-G1/-G5 in maternal blood and paired umbilical cord blood samples from gestational week 20 (GW20) and at term. Soluble HLA-G

Table 2: Novel findings on HLA-G and pregnancy.

Observation	Correlation with outcome of pregnancy	Author
HLA-G$^+$ EVT but not HLA-G$^-$ VT have immune activating potential and induce Tregs	—	Tilburgs et al. [13]
dNK acquired HLA-G through interaction with EVT and can be reactivated by cytokine stimulation	—	Tilburgs et al. [46]
High HLA-G levels in placental bed correlated with high expression of KIR2DL4 and LILRB1 on dNK cells	—	Djurisic et al. [47]
Enhancer L on *HLA-G* gene is controlled by CEBP and GATA that are also essential for placentation	—	Ferreira et al. [48]
sHLA-G is higher in maternal blood at GW20 than at term and higher in maternal blood than in umbilical blood	—	Klitkou et al. [49]
Higher 14 bp alleles in fetus are related to higher sHLA-G in maternal blood at term	—	Dahl et al. [50]
sHLA-G in seminal plasma is related to 14 bp ins/del genotype and can predict the success of ART	Positive correlation with the success of ART	Dahl et al. [51]
Higher frequency of 14 bp ins alleles in *HLA-G* gene is related to the outcome of pregnancy	Positive correlation with RIF	Lashley et al. [52]
Higher sHLA-G and frequency of KIR2DL4$^+$ NK cells in uterine flushing samples from secondary infertile women than in primary infertile woman	Negative correlation with infertility	Rizzo et al. [53]
SNP in 3′ UTR of *HLA-G* gene is related to outcome of pregnancy	Positive correlation with high risk of preeclampsia	Quach et al. [54]
SNP in 5′ URR of *HLA-G* gene is related to outcome of pregnancy	Positive correlation with RSA	Agrawal et al. [55]
Lower sHLA-G and PAPP-A in women with GDM at third trimester	—	Beneventi et al. [56]
Higher sHLA-G in women with preexisting rheumatic disease at third trimester	Positive correlation with the success of pregnancy	Beneventi et al. [57]

levels were significantly lower in maternal blood at term than in GW20. At term, sHLA-G levels were significantly higher in maternal blood than in umbilical blood. They conclude that sHLA-G is not freely transferred over the placental barrier. Finally, they found a correlation between HLA-G levels in maternal blood in GW20 and at term and between HLA-G levels in maternal blood and umbilical cord blood at term. This might be due to shared genetic factors which affect sHLA-G production in mother and child, or it may support the theory that sHLA-G in the pregnant woman and the fetus is partly derived from the placenta, which is a shared organ between mother and child [49]. In this line, Dahl et al. have correlated *HLA-G* polymorphisms in the 3′UTR to sHLA-G levels in maternal blood plasma samples from GW20 and at term, as well as in fetal umbilical cord blood samples. They have observed that higher numbers of 14 bp ins alleles in the fetus were associated with higher maternal sHLA-G levels at term (restricting the analysis to 14 bp ins/del heterozygous mothers). Furthermore, they found that increasing numbers of fetal 14InsG alleles are related to significantly increased levels of sHLA-G in maternal blood plasma samples at term in heterozygous 14DelC/14InsG mothers. They conclude that combined fetomaternal HLA-G genotypes are related to sHLA-G levels in maternal blood plasma [50].

It has been previously demonstrated that human seminal plasma contains HLA-G [85]. In a novel study, Dahl et al. found that sHLA-G levels in seminal plasma samples correlated with *HLA-G* 14 bp ins/del genotype of the men, with the *del/del* genotype showing the highest level and the *ins/ins* genotype showing the lowest level of sHLA-G. Higher seminal plasma levels of sHLA-G were found in couples where the female partner became pregnant after assisted reproduction treatments (ART), compared with couples where no pregnancy was achieved, thus suggesting a possible role of seminal sHLA-G as an immunomodulatory factor in the female reproductive tract before and at the time of conception [51]. The role of HLA-G in the success of *in vitro* fertilization (IVF) has been also addressed by Lashley et al., who have performed genotyping of women with recurrent implantation failure (RIF) and their partners for HLA class I, HLA class II, HLA-G, and KIR alleles. Results were compared with those obtained from couples with successful embryo implantation after their first IVF and normal fertile couples. They found a higher frequency of HLA-C2 and 14 bp insertion in HLA-G in women with RIF than in controls, and they conclude that these two genetic features represent a risk factor which may affect the success of IVF [52]. In this view, Rizzo and coworkers have demonstrated that sHLA-G levels in uterine flushing samples were lower in primary infertile women than in women with secondary infertility. In addition, a lower number of endometrial CD56$^+$KIR2DL4$^+$ NK cells

were found in primary infertile than in secondary infertile women [53].

Two additional studies addressed the impact of HLA-G polymorphisms on the outcome of pregnancy. Quach and colleagues have addressed the correlation between SNP in the 3′UTR of the *HLA-G* gene and an increased risk of preeclampsia. They found that preeclamptic cases were associated with a G/G-genotype at SNP +3187. In addition, one SNP combination (+3027C/C and +3187G/G) was significantly more prevalent in preeclampsia cases. They hypothesized that HLA-G 3′UTR SNP-pair associations, and not individual SNPs, could be useful to predict susceptibility to preeclampsia [54]. In contrast, Agrawal et al. have addressed the correlation between SNP in the HLA-G 5′-upstream regulatory region (URR) and recurrent spontaneous abortion (RSA). They genotyped women with idiopathic RSA (and their partners) and control couples, and they found an increased risk for idiopathic RSA in women with mutant genotypes of -1179G>A, -725C>G/T, and -86A>C SNP. They observed a 3.5-fold increased risk for -1179G>A and 4.3-fold increased risk for -725C>G/T SNP among carriers of mutant parental genotypes in couples who have experienced idiopathic RSA. Finally, they demonstrated a downregulation of HLA-G expression in cases of idiopathic RSA with -1179G>A and -725C>G/T SNPs. They conclude that these SNP may affect pregnancy outcome through HLA-G downregulation [55].

Two interesting studies have been carried out by Beneventi and coworkers on gestational diseases. First, they analyzed plasma levels of pregnancy-associated plasma protein A (PAPP-A) and sHLA-G in women with gestational diabetes mellitus (GDM). They found that women with GDM had significantly lower first-trimester PAPP-A and sHLA-G concentrations than controls. In addition, sHLA-G levels increased during gestation in diabetic women, showing an opposite trend with respect to the controls. They conclude that PAPP-A and sHLA-G represent independent markers of GDM, and their variations may help to early unravel the onset of GDM [56]. In the second study, they analyzed sHLA-G levels in plasma and cord blood samples from pregnant women with preexisting rheumatic diseases and unaffected pregnant women. They found that third-trimester blood maternal sHLA-G concentrations were significantly higher in subjects with rheumatic diseases than in controls. Moreover, cord blood sHLA-G concentrations were significantly higher in rheumatic disease than in newborns from control mothers. In addition, they found a positive correlation between maternal and fetal (i) titers of ANA autoantibodies and (ii) sHLA-G circulating levels. These data suggested that autoimmune diseases prompt a maternal and fetal immune response which favors pregnancy immune tolerance through upregulation of sHLA-G in maternal and cord blood [57].

Collectively, these novel studies confirmed the important role of HLA-G expression on EVT in the remodeling of human deciduas and in the modulation of decidual NK cell functions. Moreover, recent observations suggested that maternal and fetal genotype can affect sHLA-G levels in maternal blood and consequently the success of pregnancy. Finally, a positive correlation between polymorphisms of *HLA-G* gene and infertility, preeclampsia, and abortion has been further confirmed.

4. HLA-G in Autoimmune/Inflammatory Diseases

Several studies in the last years have demonstrated that HLA-G plays an important role in the control of autoimmune/inflammatory diseases, such as multiple sclerosis (MS) [86], Crohn's disease (CD) [87], psoriasis [88], pemphigus [89], celiac disease [90], systemic lupus erythematosus (SLE) [91], asthma [92], juvenile idiopathic arthritis [93], and rheumatoid arthritis (RA) [94].

Novel data have been obtained by Catamo and coworkers, who have observed that five polymorphisms (namely, -477 C>G, -369 C>A, 14 bp del/ins, 3187 A>G, and 3196 C>G) and one haplotype (TCGGTACGAAITCCCGAG) of *HLA-G* gene were significantly more frequent in celiac patients than in healthy controls and were correlated with an increased susceptibility to the disease. Moreover, they found 5 additional polymorphisms of *HLA-G* gene (14 bp I/I, 3187 G/G, 3196 G/G, and 3003 C/C genotypes and TCGGTACGAAITCCCGAG haplotype) which were associated with increased disease susceptibility only considering patients and controls presenting the DQ2.5 or DQ8 HLA-DQ celiac disease risk haplotypes. These data confirmed that *HLA-G* gene polymorphisms correlated to susceptibility to celiac disease development, suggesting that HLA-G molecule is involved in disease pathogenesis [58]. In another study, the authors observed that 10 5′URR and 3 3′UTR polymorphisms in *HLA-G* gene and two haplotypes were associated with a higher risk for RA development, while one polymorphism in the 5′URR correlated with disease activity. These data suggested a possible association between *HLA-G* gene polymorphisms and susceptibility to develop RA disease and its severity [59]. Interestingly, Mariaselvam et al. have observed that the frequency of +3187A>G HLA-G polymorphism was higher in rheumatoid factor $(RF)^+$ than in RF^- patients, thus suggesting that this polymorphism might influence RF status [60].

In this regard, Veit and coworkers have observed that homozygosity for the +3142G allele was associated with an increased risk of RA in Brazil, and the presence of this allele in homozygosis could be responsible for a low HLA-G expression profile which favors the development of RA [61]. Moreover, they have also observed that sHLA-G is increased in RA patients with long-lasting chronic inflammation, and the percentage of patients showing specific binding of sHLA-G to LILRB1 was significantly decreased as compared to controls. Interestingly, RF^- patients were significantly overrepresented in the group of patients positive for LILRB1 binding. Furthermore, methotrexate treated patients had lower LILRB1 binding to sHLA-G molecules than nontreated patients. These results suggested that although increased levels of sHLA-G are observed, these molecules are not functional against inflammation, due to a low binding capacity to the receptor, thus highlighting the importance to also measure the binding capability of sHLA-G to LILRB1 [62].

Zhang et al. have observed a correlation between HLA-G 14 bp ins/del polymorphism and SLE susceptibility in Asian and Caucasian subjects [63]. In this line, Favoino et al. have analyzed serum sHLA-G and the HLA-G gene 14 bp ins/del polymorphism in patients with systemic sclerosis (SSc). They subdivided patients on the basis of sHLA-G levels in HLA-G high and low profile groups, and they detected a higher disease severity in HLA-G low than in HLA-G high group. Moreover, they detected a higher frequency of scleroderma in patients with HLA-G 14 bp del/del. These data suggested a modulatory effect of sHLA-G on SSc [64]. Similarly, Zidi and coworkers have observed a correlation between the 14 bp del/ins polymorphism and CD susceptibility in young-onset (but not in adult-onset) CD patients, concerning the genotype ins/ins. Moreover, they observed higher sHLA-G levels in CD patients than in controls, and they found that patients with 14 bp del/del and 14 bp del/ins genotypes are the high HLA-G producers. Dimers of sHLA-G were found in advanced disease stage, thus suggesting a role for sHLA-G as a prognostic marker for progressive disease in CD patients [65]. Dimers of sHLA-G have been also analyzed by Fainardi et al. in MS. They demonstrated that HLA-G dimers in cerebrospinal fluid were more frequent in MS patients than in controls and in magnetic resonance imaging (MRI) inactive than in MRI active MS patients, thus suggesting that HLA-G dimers may be implicated in termination of inflammatory response in MS [66]. Moreover, Mohammadi et al. have observed that 14 bp insertion in HLA-G could result in lower plasma HLA-G levels in patients, and they found a significant correlation of HLA-G genotype and its plasma levels with MS susceptibility, thus suggesting a role for HLA-G as a risk factor for MS [67].

de Albuquerque and coworkers have analyzed different polymorphism of 3′UTR of HLA-G gene in type 1 diabetes mellitus (T1D) patients. +3001 T allele was observed only in T1D patients, whereas the +3010 CC genotype and the UTR-3 haplotype (14 bp del/+3001C/+3003T/+3010C/+3027C/+3035C/+3142G/+3187A/+3196C), associated with low and moderate soluble HLA-G expression, respectively, were underrepresented in patients. They concluded that a decreased expression of HLA-G in pancreas should be detrimental in individuals genetically prone to produce less HLA-G [68].

Two interesting studies have evaluated the correlation between HLA-G and response to treatment.

Borghi et al. have analyzed the correlation between HLA-G 14-bp ins/del polymorphism and the response of psoriatic patients to systemic therapy (acitretin, cyclosporine, or anti-TNF-α). They found an increased frequency of HLA-G del allele and del/del genotype in responders only among patients treated with acitretin, and they proposed this HLA-G polymorphism as a potential marker of response to acitretin in psoriatic patients [69]. In contrast, Naidoo et al. have analyzed the relationship between HLA-G and statins treatment in patients with asthma. They demonstrated that statins upregulate mir-148b and -152, and these miRNAs can affect HLA-G expression. The binding of miRNAs to HLA-G is modulated by a SNP in the HLA-G 3′UTR that is associated with asthma risk (rs1063320). Finally, they observed that individuals with G allele of rs1063320 had reduced asthma-related exacerbations, thus suggesting that rs1063320 modifies the effect of statin benefit in asthma by modulating HLA-G expression through mir-148b and -152 [70].

We can conclude that in the last two years the ability of HLA-G to limit the progression of autoimmune/inflammatory diseases has been confirmed. In fact, sHLA-G levels and several HLA-G polymorphisms have been associated with higher susceptibility to disease or higher severity of the disease. Finally, HLA-G has been proposed also as predictive marker of response to treatment (Table 3).

5. HLA-G in Infectious Diseases

In the last years, the role of HLA-G in the progression of different infectious diseases has been fully described, for both microbial and viral infections [95]. Recent studies have addressed novel aspects of the role of HLA-G in bacterial infections. Han et al. have demonstrated that *Toxoplasma gondii* infection can upregulate sHLA-G release by trophoblast cells *in vitro*. Upon coculture with infected trophoblast cells, dNK cells undergo apoptosis through upregulation of caspase 3 and caspase 8. They demonstrated that apoptosis is induced by sHLA-G, since the expression of caspases is downregulated in the presence of HLA-G neutralizing antibody. The authors hypothesize that dNK cells apoptosis may contribute to the abnormal pregnancy outcomes with *T. gondii* infection [71].

Sadissou and coworkers firstly analyzed the possible association between sHLA-G and malaria or malaria related risk factors in pregnant women. They observed strong correlations between the maternal and cord plasma concentrations of sHLA-G. Moreover, high cord plasma levels of sHLA-G were independently associated with low birth weight and increased risk of *P. falciparum* infection in infancy. These results suggested a possible involvement of sHLA-G in generating immune tolerance during pregnancy-associated malaria. They concluded that sHLA-G may represent a useful marker of susceptibility to malaria in infants [72].

Bortolotti and coworkers carried out an interesting study on *Pseudomonas aeruginosa*, demonstrating that N-(3-oxododecanoyl)-l-homoserine lactone (3O-C12-HSL) produced by this bacteria is able to induce HLA-G expression in human monocytes and T cells, through p38/CREB and IL-10 induction. These data suggested that HLA-G may be a mechanism to create a protected niche for bacterial reservoir, similar to the role of HLA-G molecules during viral infections [73]. In this line, Rizzo et al. have demonstrated that sHLA-G plasmatic levels are normalized by antibiotic therapy in patients with cystic fibrosis and *Pseudomonas aeruginosa* infection, thus suggesting a systemic anti-inflammatory role. However, in the airway system, higher expression of HLA-G is associated with *P. aeruginosa* infection. Moreover, CF cell line and murine model expressed higher HLA-G molecules in the presence of *P. aeruginosa*, thus suggesting a role of HLA-G in reducing systemic inflammation, thus supporting *P. aeruginosa* infection [74].

Table 3: Novel findings on HLA-G in autoimmune/inflammatory disease.

Type of disease	Observation	Correlation with clinical outcome	Author
CD	Several polymorphisms and one haplotype of *HLA-G* gene are more frequent in patients than in controls	Positive correlation with susceptibility	Catamo et al. [58]
RA	Several polymorphisms and two haplotypes of *HLA-G* gene are more frequent in patients than in controls	Positive correlation with susceptibility	Catamo et al. [59]
RA	Two polymorphisms of *HLA-G* gene are more frequent in RF^+ than in RF^- patients	Positive correlation with disease severity	Mariaselvam et al. [60]
RA	Homozygosis of *3142G* allele is related to lower sHLA-G levels	Positive correlation with high risk of disease	Veit et al. [61]
RA	Higher sHLA-G levels in patients with chronic disease and higher LILRB1 binding in RF^- patients	Negative correlation with disease severity	Veit et al. [62]
SLE	*HLA-G* 14 bp ins/del polymorphisms are associated with susceptibility	Positive correlation with susceptibility	Zhang et al. [63]
SSc	Lower levels of sHLA-G in patients than in controls	Negative correlation with disease severity	Favoino et al. [64]
CD	Higher levels of sHLA-G in patients than in controls	Positive correlation with susceptibility	Zidi et al. [65]
MS	Higher sHLA-G dimers in MS than in controls and in MRI^- than in MRI^+	Positive correlation with termination of inflammatory response	Fainardi et al. [66]
MS	14 bp ins in *HLA-G* gene is related to lower sHLA-G levels	Positive correlation with high risk of disease	Mohammadi et al. [67]
T1D	Lower sHLA-G levels in pancreas are detrimental	Negative correlation with disease severity	de Albuquerque et al. [68]
Psoriasis	14 bp del/del genotype of *HLA-G* gene is more frequent in patients responding to therapy	Positive correlation with response to treatment	Borghi et al. [69]
Asthma	SNPs in 3′ UTR of HLA-G gene modulate the binding of mIRNAs	Positive correlation with high risk of disease	Naidoo et al. [70]

Oliveira Souza and coworkers have detected HLA-G expression in gastric tissue samples from patients harbouring *H. pylori*. Moreover, HLA-G expression was correlated with milder colonization by *H. pylori*, milder inflammatory activity, and location of bacteria in the gastric antrum. This pilot study explored for the first time HLA-G expression in the context of *H. pylori* infection, but the role of HLA-G remains to be defined [75].

Two important studies on viral infections have been carried out. Khorrami et al. have analyzed HLA-G in patients with Hepatitis C virus (HCV) treated with combined therapy (IFN-$\alpha 2\alpha$ and ribavirin), to evaluate possible difference between responder and nonresponder groups. They demonstrated that HLA-G and IL-10 levels in nonresponder group were higher than in responder and controls. Additionally, HLA-G and IL-10 were higher in patients at the beginning of treatment than in healthy individuals. These findings suggested that increase of HLA-G and IL-10 in HCV infected patients might affect the response to combined therapy in HCV patients [44]. Laaribi and coworkers have analyzed HLA-G 14 bp ins/del polymorphism at the 3′ UTR of HLA-G in patients with chronic Hepatitis B virus (HBV) infection, and they found an association between the 14 bp ins/del polymorphism and an enhanced HBV activity, with high HBV DNA levels. In particular, ins/ins genotype was associated with a 2.5-fold increased risk of susceptibility to high HBV replication compared with the del/del and ins/del genotypes. These results suggest a role for HLA-G polymorphisms as potential prognostic value for disease outcome evaluation [76].

In summary, these novel studies confirmed that upregulation of HLA-G expression and/or release is an immune escape mechanism performed by virus and bacteria during infection to avoid the recognition by immune effector cells and to reduce the inflammatory response. Also in this case, a predictive role of the success of therapy has been suggested for HLA-G (Table 4).

6. HLA-G in Transplantation

The important role of HLA-G as a tolerogenic molecule during allograft transplantation has been extensively characterized. HLA-G expression has been related to a better acceptance of the allograft, through its ability in shaping an allogeneic immune response into tolerance [96].

Misra et al. have evaluated the impact of HLA-G allele associated with UTR-haplotype in end stage renal disease (ESRD) and acute allograft rejection (AR) cases. The authors observed an increased risk for $G^*01{:}01{:}01{:}03$, $G^*01{:}01{:}02$, $G^*01{:}06$, and $G^*01{:}05{:}N$ haplotypes, while $G^*01{:}01{:}01{:}01$ and $G^*01{:}04{:}01$ haplotypes showed a protective effect in ESRD and AR cases. Moreover, they found (i) higher levels of

Table 4: Novel findings on HLA-G and infectious diseases.

Type of disease	Observation	Correlation with clinical outcome	Author
Toxoplasmosis	Toxoplasma increased the release of sHLA-G by trophoblast inducing apoptosis of dNK cells	Positive correlation with abnormal pregnancy	Han et al. [71]
Malaria	Higher sHLA-G levels in cord blood are related to low weight at birth and clinical outcome	Positive correlation with high risk of infection in infancy	Sadissou et al. [72]
Pseudomonas aeruginosa	*P. aeruginosa* induced HLA-G expression in monocytes and T cells protecting from immune response	—	Bortolotti et al. [73]
Pseudomonas aeruginosa	sHLA-G levels are decreased during antibiotic therapy in patients with cystic fibrosis	Negative correlation with inflammation	Rizzo et al. [74]
Helicobacter pylori	HLA-G expression correlated with milder colonization and milder inflammation	Negative correlation with inflammation	Oliveira Souza et al. [75]
HCV	Higher levels of sHLA-G and IL-10 in patients nonresponding to therapy with IFN	Negative correlation with response to therapy	Khorrami et al. [44]
HBV	Patients with *14 bp ins/ins* genotype have higher levels of HBV activity and HBV DNA copies	Positive correlation with worse clinical outcome	Laaribi et al. [76]

Table 5: Novel findings on HLA-G and transplantation.

Type of transplant	Observation	Correlation with clinical outcome	Author
Kidney	Higher HLA-G5/G6 levels in ESRD and lower levels of HLA-G5 in acute rejection	Negative correlation of HLA-G5 with rejection	Misra et al. [77]
Lung	Higher levels of sHLA-G in bronchoalveolar lavage from patients with acute rejection	Positive correlation with high risk of rejection	White et al. [78]
Lung	Expression of HLA-G in lung protected from CLAD and associated with graft acceptance	Positive correlation with graft acceptance	Brugière et al. [79]
Lung	Two haplotypes of HLA-G gene are associated with lower levels of sHLA-G	Positive correlation with worse long term survival	Di Cristofaro et al. [80]
Murine model	$CD4^+HLA\text{-}G^+$ regulatory T cells produce IL-10, sHLA-G, and IL-35 and ameliorated GvHD	—	Pankratz et al. [81]

soluble HLA-G5 and -G6 among ESRD cases and (ii) reduced levels of soluble HLA-G5 and increased levels of membrane-bound HLA-G1 and -G3 in AR cases. Decreased HLA-G expression was observed for $G^*01{:}01{:}01{:}03$ and $G^*01{:}05{:}N$ haplotypes in ESRD and AR cases. These results suggested that the variation of membrane-bound and soluble HLA-G isoforms correlated to the risk for ESRD and AR. Moreover, UTR-haplotypes are involved in different HLA-G expression patterns [77].

Three recent studies have analyzed the role of HLA-G in patients who undergo lung transplantation.

White and coworkers have demonstrated that lung soluble HLA-G concentrations were directly related to the presence of type A rejection but not to lymphocytic bronchiolitis (the principal complication of lung transplantation). They found that sHLA-G concentrations in bronchoalveolar lavage but not in serum positively correlated with the number of acute rejection episodes in the first 12 months after lung transplantation and thus may represent a novel marker of rejection risk [78]. Similarly, Brugière et al. have observed that lung HLA-G expression had a protective effect on chronic lung allograft dysfunction (CLAD), thus suggesting that early expression of HLA-G in the graft was positively correlated with graft acceptance in the long term [79]. An interesting study carried out by Di Cristofaro and coworkers observed that the presence of $HLA\text{-}G^*01{:}06$ UTR2 haplotype was associated with a worse evolution of cystic fibrosis, but not of long-term posttransplant survival. In contrast, $HLA\text{-}G^*01{:}04$ UTR3 haplotype was associated with lower levels of sHLA-G at day 0 and after three months, impaired long-term survival, increased CLAD occurrence, and the production of *de novo* donor-specific antibodies after three months. This study firstly observed that different HLA-G haplotypes can deeply affect clinical outcome of lung transplantation [80].

In the view of the treatment of transplant rejection, an interesting study comes from Pankratz and coworkers. They compared the features of two subsets of regulatory cells, $CD4^+HLA\text{-}G^+$ cells and $CD4^+CD25^+FoxP3^+$cells. They found that both cell populations display alterations in proximal-signaling pathways upon TCR stimulation and a hyperpolarization of the plasma membrane when compared to conventional $CD4^+$T cells. $CD4^+HLA\text{-}G^+$ cells secreted high levels of IL-10, sHLA-G, and IL-35, while $CD4^+CD25^+FoxP3^+$ cells expressed lower levels of these molecules and exerted their function in a contact-dependent manner via cAMP. More importantly, they demonstrated that $CD4^+HLA\text{-}G^+$ cells ameliorated graft-versus-host disease in a humanized mouse model, thus suggesting that these

cells can modulate polyclonal adaptive immune responses *in vivo*, representing a promising candidate for future clinical applications not only in transplanted patients but also for the treatment of autoimmune/inflammatory diseases [81].

In conclusion, these novel findings confirmed in general the important role of HLA-G in the acceptance of allograft and in the prevention of transplant rejection. Moreover, recent studies suggested a possible clinical application of $CD4^+HLA\text{-}G^+$ regulatory T cells in the prevention of GvHD (Table 5).

7. Conclusions

More than 280 papers on HLA-G have been published in the last two years confirming the important role of HLA-G in modulating the function of the immune system. Moreover, novel studies have highlighted the function of HLA-G gene (and corresponding molecule) as prognostic factor for patients' clinical outcome in different pathological settings. Finally, some authors have unraveled a role for HLA-G as a predictor of the response to treatments in patients with tumors or infections. The future goal of the scientific community will be to determine and standardize the clinical applications of HLA-G analysis, in order to introduce this molecule in the routine tests which might help disease diagnosis and patients' follow-up.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

The authors thank Mrs. Camilla Valentino for excellent secretarial assistance. This work has been supported by Fondazione Italiana per la Lotta al Neuroblastoma.

References

[1] P. Le Bouteiller and F. Lenfant, "Antigen-presenting function(s) of the non-classical HLA-E, -F and -G class I molecules: the beginning of a story," *Research in Immunology*, vol. 147, no. 5, pp. 301–313, 1996.

[2] F. Kievits, P. Ivanyi, P. Krimpenfort, A. Berns, and H. L. Ploegh, "HLA-restricted recognition of viral antigens in HLA transgenic mice," *Nature*, vol. 329, no. 6138, pp. 447–449, 1987.

[3] V. M. Braud, D. S. Allan, and A. J. McMichael, "Functions of nonclassical MHC and non-MHC-encoded class I molecules," *Current Opinion in Immunology*, vol. 11, no. 1, pp. 100–108, 1999.

[4] V. Pistoia, F. Morandi, X. Wang, and S. Ferrone, "Soluble HLA-G: are they clinically relevant?" *Seminars in Cancer Biology*, vol. 17, no. 6, pp. 469–479, 2007.

[5] C. Münz, S. Stevanović, and H. Rammensee, "Peptide presentation and NK inhibition by HLA-G," *Journal of Reproductive Immunology*, vol. 43, no. 2, pp. 139–155, 1999.

[6] M. Diehl, C. Münz, W. Keilholz et al., "Nonclassical HLA-G molecules are classical peptide presenters," *Current Biology*, vol. 6, no. 3, pp. 305–314, 1996.

[7] J. Baudhuin, J. Migraine, V. Faivre et al., "Exocytosis acts as a modulator of the ILT4-mediated inhibition of neutrophil functions," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 44, pp. 17957–17962, 2013.

[8] M. E. Le Page, J. P. Goodridge, E. John, F. T. Christiansen, and C. S. Witt, "Killer Ig-like receptor 2DL4 does not mediate NK cell IFN-γ responses to soluble HLA-G preparations," *Journal of Immunology*, vol. 192, no. 2, pp. 732–740, 2014.

[9] N. Lila, N. Rouas-Freiss, J. Dausset, A. Carpentier, and E. D. Carosella, "Soluble HLA-G protein secreted by allo-specific $CD4^+$ T cells suppresses the allo-proliferative response: a $CD4^+$ T cell regulatory mechanism," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 21, pp. 12150–12155, 2001.

[10] F.-A. Le Gal, B. Riteau, C. Sedlik et al., "HLA-G-mediated inhibition of antigen-specific cytotoxic T lymphocytes," *International Immunology*, vol. 11, no. 8, pp. 1351–1356, 1999.

[11] S. Fournel, M. Aguerre-Girr, X. Huc et al., "Cutting edge: Soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated $CD8^+$ cells by interacting with CD8," *Journal of Immunology*, vol. 164, no. 12, pp. 6100–6104, 2000.

[12] S. Le Rond, C. Azéma, I. Krawice-Radanne et al., "Evidence to support the role of HLA-G5 in allograft acceptance through induction of immunosuppressive/regulatory T cells," *Journal of Immunology*, vol. 176, no. 5, pp. 3266–3276, 2006.

[13] T. Tilburgs, Â. C. Crespo, A. Van Der Zwan et al., "Human HLA-G+ extravillous trophoblasts: immune-activating cells that interact with decidual leukocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 23, pp. 7219–7224, 2015.

[14] A. Naji, C. Menier, F. Morandi et al., "Binding of HLA-G to ITIM-bearing Ig-like transcript 2 receptor suppresses B cell responses," *Journal of Immunology*, vol. 192, no. 4, pp. 1536–1546, 2014.

[15] L. Pazmany, O. Mandelboim, M. Valés-Gómez, D. M. Davis, H. T. Reyburn, and J. L. Strominger, "Protection from natural killer cell-mediated lysis by HLA-G expression on target cells," *Science*, vol. 274, no. 5288, pp. 792–795, 1996.

[16] N. Rouas-Freiss, R. E. Marchal, M. Kirszenbaum, J. Dausset, and E. D. Carosella, "The α1 domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: is HLA-G the public ligand for natural killer cell inhibitory receptors?" *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 10, pp. 5249–5254, 1997.

[17] C. Li, B. L. Houser, M. L. Nicotra, and J. L. Strominger, "HLA-G homodimer-induced cytokine secretion through HLA-G receptors on human decidual macrophages and natural killer cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 14, pp. 5767–5772, 2009.

[18] A. van der Meer, H. G. M. Lukassen, M. J. C. van Lierop et al., "Membrane-bound HLA-G activates proliferation and interferon-γ production by uterine natural killer cells," *Molecular Human Reproduction*, vol. 10, no. 3, pp. 189–195, 2004.

[19] A. van der Meer, H. G. M. Lukassen, B. van Cranenbroek et al., "Soluble HLA-G promotes Th1-type cytokine production by cytokine-activated uterine and peripheral natural killer cells," *Molecular Human Reproduction*, vol. 13, no. 2, pp. 123–133, 2007.

[20] F. Morandi, N. Rouas-Freiss, and V. Pistoia, "The emerging role of soluble HLA-G in the control of chemotaxis," *Cytokine and Growth Factor Reviews*, vol. 25, no. 3, pp. 327–335, 2014.

[21] P. Fons, S. Chabot, J. E. Cartwright et al., "Soluble HLA-G1 inhibits angiogenesis through an apoptotic pathway and by

direct binding to CD160 receptor expressed by endothelial cells," *Blood*, vol. 108, no. 8, pp. 2608–2615, 2006.

[22] J. E. Boyson, R. Erskine, M. C. Whitman et al., "Disulfide bond-mediated dimerization of HLA-G on the cell surface," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 25, pp. 16180–16185, 2002.

[23] M. Shiroishi, K. Kuroki, L. Rasubala et al., "Structural basis for recognition of the nonclassical MHC molecule HLA-G by the leukocyte Ig-like receptor B2 (LILRB2/LIR2/ILT4/CD85d)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 44, pp. 16412–16417, 2006.

[24] M. Shiroishi, K. Kuroki, T. Ose et al., "Efficient leukocyte Ig-like receptor signaling and crystal structure of disulfide-linked HLA-G dimer," *Journal of Biological Chemistry*, vol. 281, no. 15, pp. 10439–10447, 2006.

[25] K. Kuroki and K. Maenaka, "Immune modulation of HLA-G dimer in maternal-fetal interface," *European Journal of Immunology*, vol. 37, no. 7, pp. 1727–1729, 2007.

[26] M. Zhong, X. Weng, Z. Liang et al., "Dimerization of soluble HLA-G by IgG-Fc fragment augments ILT2-mediated inhibition of T-cell alloresponse," *Transplantation*, vol. 87, no. 1, pp. 8–15, 2009.

[27] L. Loumagne, J. Baudhuin, B. Favier, F. Montespan, E. D. Carosella, and N. Rouas-Freiss, "*In vivo* evidence that secretion of HLA-G by immunogenic tumor cells allows their evasion from immunosurveillance," *International Journal of Cancer*, vol. 135, no. 9, pp. 2107–2117, 2014.

[28] J. Zheng, C. Xu, D. Chu et al., "Human leukocyte antigen G is associated with esophageal squamous cell carcinoma progression and poor prognosis," *Immunology Letters*, vol. 161, no. 1, pp. 13–19, 2014.

[29] M. S. Reimers, C. C. Engels, H. Putter et al., "Prognostic value of HLA class I, HLA-E, HLA-G and Tregs in rectal cancer: a retrospective cohort study," *BMC Cancer*, vol. 14, article 486, 2014.

[30] Z.-Y. Guo, Y.-G. Lv, L. Wang et al., "Predictive value of HLA-G and HLA-E in the prognosis of colorectal cancer patients," *Cellular Immunology*, vol. 293, no. 1, pp. 10–16, 2015.

[31] W.-H. Yan, D. Liu, H.-Y. Lu, Y.-Y. Li, X. Zhang, and A. Lin, "Significance of tumour cell HLA-G5/-G6 isoform expression in discrimination for adenocarcinoma from squamous cell carcinoma in lung cancer patients," *Journal of Cellular and Molecular Medicine*, vol. 19, no. 4, pp. 778–785, 2015.

[32] Y.-F. Xu, Y. Lu, H. Cheng et al., "High expression of human leukocyte antigen-G is associated with a poor prognosis in patients with PDAC," *Current Molecular Medicine*, vol. 15, no. 4, pp. 360–367, 2015.

[33] Y. Wang, X. Fan, H. Li et al., "Tumor border sharpness correlates with HLA-G expression in low-grade gliomas," *Journal of Neuroimmunology*, vol. 282, pp. 1–6, 2015.

[34] S. Jasinski-Bergner, A. Reches, C. Stoehr et al., "Identification of novel microRNAs regulating HLA-G expression and investigating their clinical relevance in renal cell carcinoma," *Oncotarget*, vol. 7, no. 18, pp. 26866–26878, 2016.

[35] Z. Guan, B. Song, F. Liu, D. Sun, K. Wang, and H. Qu, "TGF-β induces HLA-G expression through inhibiting miR-152 in gastric cancer cells," *Journal of Biomedical Science*, vol. 22, p. 107, 2015.

[36] D.-P. Xu, W.-W. Shi, T.-T. Zhang et al., "Elevation of HLA-G-expressing DC-10 cells in patients with gastric cancer," *Human Immunology*, vol. 77, no. 9, pp. 800–804, 2016.

[37] D. Wu, I. Kuiaste, P. Moreau, E. Carosella, and P. Yotnda, "Rescuing lymphocytes from HLA-G immunosuppressive effects mediated by the tumor microenvironment," *Oncotarget*, vol. 6, no. 35, pp. 37385–37397, 2015.

[38] L. Zhou, Z.-Y. Niu, Z.-Y. Liang et al., "HLA-G impairs host immune response and predicts poor prognosis in pancreatic cancer," *American Journal of Translational Research*, vol. 7, no. 10, pp. 2036–2044, 2015.

[39] L. König, S. Kasimir-Bauer, O. Hoffmann et al., "The prognostic impact of soluble and vesicular HLA-G and its relationship to circulating tumor cells in neoadjuvant treated breast cancer patients," *Human Immunology*, vol. 77, no. 9, pp. 791–799, 2016.

[40] I. Zidi, N. Kharrat, R. Sebai et al., "Pregnancy and breastfeeding: a new theory for sHLA-G in breast cancer patients?" *Immunologic Research*, vol. 64, no. 2, pp. 636–639, 2015.

[41] M. Bielska, M. Bojo, G. Klimkiewicz-Wojciechowska et al., "Human leukocyte antigen-G polymorphisms influence the clinical outcome in diffuse large B-cell lymphoma," *Genes Chromosomes and Cancer*, vol. 54, no. 3, pp. 185–193, 2015.

[42] A. Wiśniewski, A. Kowal, E. Wyrodek et al., "Genetic polymorphisms and expression of HLA-G and its receptors, KIR2DL4 and LILRB1, in non-small cell lung cancer," *Tissue Antigens*, vol. 85, no. 6, pp. 466–475, 2015.

[43] F. M. Zambra, V. Biolchi, C. C. de Cerqueira, I. S. Brum, E. C. Castelli, and J. A. Chies, "Immunogenetics of prostate cancer and benign hyperplasia—the potential use of an HLA-G variant as a tag SNP for prostate cancer risk," *HLA*, vol. 87, no. 2, pp. 79–88, 2016.

[44] S. Khorrami, H. Mohammadpour, K. Shahzamani et al., "The relationship between HLA-G and viral loads in non-responder HCV-infected patients after combined therapy with IFN-$\alpha 2\alpha$ and ribavirin," *Human Immunology*, vol. 76, no. 2-3, pp. 181–186, 2015.

[45] R. Rizzo, V. Audrito, P. Vacca et al., "HLA-G is a component of the chronic lymphocytic leukemia escape repertoire to generate immune suppression: impact of the HLA-G 14 base pair (rs66554220) polymorphism," *Haematologica*, vol. 99, no. 5, pp. 888–896, 2014.

[46] T. Tilburgs, J. H. Evans, Â. C. Crespo, and J. L. Strominger, "The HLA-G cycle provides for both NK tolerance and immunity at the maternal-fetal interface," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 43, pp. 13312–13317, 2015.

[47] S. Djurisic, L. Skibsted, and T. V. F. Hviid, "A phenotypic analysis of regulatory t cells and uterine NK cells from first trimester pregnancies and associations with HLA-G," *American Journal of Reproductive Immunology*, vol. 74, no. 5, pp. 427–444, 2015.

[48] L. M. Ferreira, T. B. Meissner, T. S. Mikkelsen et al., "A distant trophoblast-specific enhancer controls HLA-G expression at the maternal–fetal interface," *Proceedings of the National Academy of Sciences*, vol. 113, no. 19, pp. 5364–5369, 2016.

[49] L. Klitkou, M. Dahl, T. V. F. Hviid et al., "Human leukocyte antigen (HLA)-G during pregnancy part I: correlations between maternal soluble HLA-G at midterm, at term, and umbilical cord blood soluble HLA-G at term," *Human Immunology*, vol. 76, no. 4, pp. 254–259, 2015.

[50] M. Dahl, L. Klitkou, O. B. Christiansen et al., "Human leukocyte antigen (HLA)-G during pregnancy part II: associations between maternal and fetal HLA-G genotypes and soluble HLA-G," *Human Immunology*, vol. 76, no. 4, pp. 260–271, 2015.

[51] M. Dahl, T. L. Perin, S. Djurisic et al., "Soluble human leukocyte antigen-G in seminal plasma is associated with HLA-G

genotype: possible implications for fertility success," *American Journal of Reproductive Immunology*, vol. 72, no. 1, pp. 89–105, 2014.

[52] L. E. E. L. O. Lashley, L. A. J. van der Westerlaken, G. W. Haasnoot et al., "Maternal HLA-C2 and 14 bp insertion in HLA-G is associated with recurrent implantation failure after *in vitro* fertilization treatment," *Tissue Antigens*, vol. 84, no. 6, pp. 536–544, 2014.

[53] R. Rizzo, G. Lo Monte, D. Bortolotti et al., "Impact of soluble HLA-G levels and endometrial NK cells in uterine flushing samples from primary and secondary unexplained infertile women," *International Journal of Molecular Sciences*, vol. 16, no. 3, pp. 5510–5516, 2015.

[54] K. Quach, S. A. Grover, S. Kenigsberg, and C. L. Librach, "A combination of single nucleotide polymorphisms in the 3′untranslated region of HLA-G is associated with preeclampsia," *Human Immunology*, vol. 75, no. 12, pp. 1163–1170, 2014.

[55] D. Agrawal, S. Prakash, M. K. Misra, S. R. Phadke, and S. Agrawal, "Implication of *HLA-G* 5′ upstream regulatory region polymorphisms in idiopathic recurrent spontaneous abortions," *Reproductive BioMedicine Online*, vol. 30, no. 1, pp. 82–91, 2015.

[56] F. Beneventi, M. Simonetta, E. Locatelli et al., "Temporal variation in soluble human leukocyte antigen-G (sHLA-G) and pregnancy-associated plasma protein A (PAPP-A) in pregnancies complicated by gestational diabetes mellitus and in controls," *American Journal of Reproductive Immunology*, vol. 72, no. 4, pp. 413–421, 2014.

[57] F. Beneventi, C. Badulli, E. Locatelli et al., "Soluble HLA-G in pregnancies complicated by autoimmune rheumatic diseases," *Journal of Reproductive Immunology*, vol. 110, pp. 67–73, 2015.

[58] E. Catamo, L. Zupin, L. Segat, F. Celsi, and S. Crovella, "HLA-G and susceptibility to develop celiac disease," *Human Immunology*, vol. 76, no. 1, pp. 36–41, 2015.

[59] E. Catamo, C. Addobbati, L. Segat et al., "HLA-G gene polymorphisms associated with susceptibility to rheumatoid arthritis disease and its severity in Brazilian patients," *Tissue Antigens*, vol. 84, no. 3, pp. 308–315, 2014.

[60] C. M. Mariaselvam, A. B. Chaaben, S. Salah et al., "Human leukocyte antigen-G polymorphism influences the age of onset and autoantibody status in rheumatoid arthritis," *Tissue Antigens*, vol. 85, no. 3, pp. 182–189, 2015.

[61] T. D. Veit, C. P. S. de Lima, L. C. Cavalheiro et al., "HLA-G +3142 polymorphism as a susceptibility marker in two rheumatoid arthritis populations in Brazil," *Tissue Antigens*, vol. 83, no. 4, pp. 260–266, 2014.

[62] T. D. Veit, J. A. B. Chies, M. Switala et al., "The paradox of high availability and low recognition of soluble HLA-G by LILRB1 receptor in rheumatoid arthritis patients," *PLoS ONE*, vol. 10, no. 4, article e123838, 2015.

[63] X. Zhang, S. Li, Y. Zhang et al., "Meta-analysis of the relationship between 14bp insertion/deletion polymorphism of HLA-G gene and susceptibility to systemic lupus erythematosus," *Human Immunology*, vol. 75, no. 12, pp. 1171–1176, 2014.

[64] E. Favoino, I. E. Favia, S. Vettori et al., "Clinical correlates of human leucocyte antigen (HLA)-G in systemic sclerosis," *Clinical and Experimental Immunology*, vol. 181, no. 1, pp. 100–109, 2015.

[65] I. Zidi, H. Ben Yahia, D. Bortolotti et al., "Association between sHLA-G and HLA-G 14-bp deletion/insertion polymorphism in Crohn's disease," *International Immunology*, vol. 27, no. 6, pp. 289–296, 2015.

[66] E. Fainardi, D. Bortolotti, S. Bolzani et al., "Cerebrospinal fluid amounts of HLA-G in dimeric form are strongly associated to patients with MRI inactive multiple sclerosis," *Multiple Sclerosis Journal*, vol. 22, no. 2, pp. 245–249, 2016.

[67] N. Mohammadi, M. Adib, F. Alsahebfosoul, M. Kazemi, and M. Etemadifar, "An investigation into the association between HLA-G 14 bp insertion/deletion polymorphism and multiple sclerosis susceptibility," *Journal of Neuroimmunology*, vol. 290, pp. 115–118, 2016.

[68] R. S. de Albuquerque, C. T. Mendes-Junior, N. Lucena-Silva et al., "Association of HLA-G 3′ untranslated region variants with type 1 diabetes mellitus," *Human Immunology*, vol. 77, no. 4, pp. 358–364, 2016.

[69] A. Borghi, R. Rizzo, M. Corazza et al., "HLA-G 14-bp polymorphism: a possible marker of systemic treatment response in psoriasis vulgaris? Preliminary results of a retrospective study," *Dermatologic Therapy*, vol. 27, no. 5, pp. 284–289, 2014.

[70] D. Naidoo, A. C. Wu, M. H. Brilliant et al., "A polymorphism in HLA-G modifies statin benefit in asthma," *Pharmacogenomics Journal*, vol. 15, no. 3, pp. 272–277, 2015.

[71] M. Han, Y. Jiang, K. Lao et al., "sHLA-G involved in the apoptosis of decidual natural killer cells following toxoplasma gondii infection," *Inflammation*, vol. 37, no. 5, pp. 1718–1727, 2014.

[72] I. Sadissou, T. d'Almeida, G. Cottrell et al., "High plasma levels of HLA-G are associated with low birth weight and with an increased risk of malaria in infancy," *Malaria Journal*, vol. 13, no. 1, article 312, 2014.

[73] D. Bortolotti, J. LeMaoult, C. Trapella, D. D. Luca, E. D. Carosella, and R. Rizzo, "Pseudomonas aeruginosa quorum sensing molecule N-(3-oxododecanoyl)-L-homoserine-lactone induces HLA-G expression in human immune cells," *Infection and Immunity*, vol. 83, no. 10, pp. 3918–3925, 2015.

[74] R. Rizzo, G. Bergamini, D. Bortolotti et al., "HLA-G expression and regulation during *Pseudomonas aeruginosa* infection in cystic fibrosis patients," *Future Microbiology*, vol. 11, no. 3, pp. 363–373, 2016.

[75] D. M. B. Oliveira Souza, J. Genre, T. G. Alves Silva et al., "Upregulation of soluble HLA-G5 and HLA-G6 isoforms in the milder histopathological stages of *Helicobacter pylori* infection: a role for subverting immune responses?" *Scandinavian Journal of Immunology*, vol. 83, no. 1, pp. 38–43, 2016.

[76] A. B. Laaribi, I. Zidi, N. Hannachi et al., "Association of an HLA-G 14-bp Insertion/Deletion polymorphism with high HBV replication in chronic hepatitis," *Journal of Viral Hepatitis*, vol. 22, no. 10, pp. 835–841, 2015.

[77] M. K. Misra, S. K. Pandey, R. Kapoor et al., "HLA-G gene expression influenced at allelic level in association with end stage renal disease and acute allograft rejection," *Human Immunology*, vol. 75, no. 8, pp. 833–839, 2014.

[78] S. R. White, T. Floreth, C. Liao, and S. M. Bhorade, "Association of soluble HLA-G with acute rejection episodes and early development of bronchiolitis obliterans in lung transplantation," *PLoS ONE*, vol. 9, no. 7, Article ID e103643, 2014.

[79] O. Brugière, G. Thabut, I. Krawice-Radanne et al., "Role of HLA-G as a predictive marker of low risk of chronic rejection in lung transplant recipients: a clinical prospective study," *American Journal of Transplantation*, vol. 15, no. 2, pp. 461–471, 2015.

[80] J. Di Cristofaro, M. Reynaud-Gaubert, F. Carlini et al., "HLA-G*01:04~UTR3 recipient correlates with lower survival and

higher frequency of chronic rejection after lung transplantation," *American Journal of Transplantation*, vol. 15, no. 9, pp. 2413–2420, 2015.

[81] S. Pankratz, S. Bittner, A. M. Herrmann et al., "Human $CD4^+$ $HLA\text{-}G^+$ regulatory T cells are potent suppressors of graft-versus-host disease in vivo," *The FASEB Journal*, vol. 28, no. 8, pp. 3435–3445, 2014.

[82] S. Jasinski-Bergner, C. Stoehr, J. Bukur et al., "Clinical relevance of miR-mediated HLA-G regulation and the associated immune cell infiltration in renal cell carcinoma," *OncoImmunology*, vol. 4, no. 6, Article ID e1008805, 2015.

[83] S. Khorrami, R. Rahimi, H. Mohammadpour et al., "Association of HLA-G*01:01:02:01/G*01:04:01 polymorphism with gastric adenocarcinoma," *Human Immunology*, vol. 77, no. 2, pp. 153–157, 2016.

[84] P. Le Bouteiller, "HLA-G in human early pregnancy: control of uterine immune cell activation and likely vascular remodeling," *Biomedical Journal*, vol. 38, no. 1, pp. 32–38, 2015.

[85] M. H. Larsen, M. Bzorek, M. B. Pass et al., "Human leukocyte antigen-G in the male reproductive system and in seminal plasma," *Molecular Human Reproduction*, vol. 17, no. 12, pp. 727–738, 2011.

[86] R. Rizzo, D. Bortolotti, S. Bolzani, and E. Fainardi, "HLA-G molecules in autoimmune diseases and infections," *Frontiers in Immunology*, vol. 5, article 592, 2014.

[87] R. Rizzo, L. Melchiorri, L. Simone et al., "Different production of soluble HLA-G antigens by peripheral blood mononuclear cells in ulcerative colitis and Crohn's disease: a noninvasive diagnostic tool?" *Inflammatory Bowel Diseases*, vol. 14, no. 1, pp. 100–105, 2008.

[88] S. Aractingi, N. Briand, C. Le Danff et al., "HLA-G and NK receptor are expressed in psoriatic skin: a possible pathway for regulating infiltrating T cells?" *American Journal of Pathology*, vol. 159, no. 1, pp. 71–77, 2001.

[89] E. Gazit, Y. Slomov, I. Goldberg, S. Brenner, and R. Loewenthal, "HLA-G is associated with pemphigus vulgaris in Jewish patients," *Human Immunology*, vol. 65, no. 1, pp. 39–46, 2004.

[90] M. I. Torres, M. A. López-Casado, J. Luque, J. Peña, and A. Ríos, "New advances in coeliac disease: serum and intestinal expression of HLA-G," *International Immunology*, vol. 18, no. 5, pp. 713–718, 2006.

[91] R. Rizzo, T. V. F. Hviid, M. Govoni et al., "HLA-G genotype and HLA-G expression in systemic lupus erythematosus: HLA-G as a putative susceptibility gene in systemic lupus erythematosus," *Tissue Antigens*, vol. 71, no. 6, pp. 520–529, 2008.

[92] R. Rizzo, C. E. Mapp, L. Melchiorri et al., "Defective production of soluble HLA-G molecules by peripheral blood monocytes in patients with asthma," *The Journal of Allergy and Clinical Immunology*, vol. 115, no. 3, pp. 508–513, 2005.

[93] I. Prigione, F. Penco, A. Martini, M. Gattorno, V. Pistoia, and F. Morandi, "HLA-G and HLA-E in patients with juvenile idiopathic arthritis," *Rheumatology*, vol. 50, no. 5, pp. 966–972, 2011.

[94] R. Rizzo, I. Farina, D. Bortolotti et al., "HLA-G may predict the disease course in patients with early rheumatoid arthritis," *Human Immunology*, vol. 74, no. 4, pp. 425–432, 2013.

[95] L. Amiot, N. Vu, and M. Samson, "Immunomodulatory properties of HLA-G in infectious diseases," *Journal of Immunology Research*, vol. 2014, Article ID 298569, 14 pages, 2014.

[96] V. Rebmann, F. Da Silva Nardi, B. Wagner, and P. A. Horn, "HLA-G as a tolerogenic molecule in transplantation and pregnancy," *Journal of Immunology Research*, vol. 2014, Article ID 297073, 16 pages, 2014.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 7465741, 6 pages
http://dx.doi.org/10.1155/2016/7465741

Research Article

Soluble HLA-G and HLA-E Levels in Bone Marrow Plasma Samples Are Related to Disease Stage in Neuroblastoma Patients

Fabio Morandi,[1] Sarah Pozzi,[2] Barbara Carlini,[1] Loredana Amoroso,[3] Vito Pistoia,[1] and Maria Valeria Corrias[1]

[1]*Laboratorio di Oncologia, Istituto Giannina Gaslini, Via Gaslini 5, 16147 Genoa, Italy*
[2]*Centro Cellule Staminali, IRCCS AOU San Martino-IST, Largo R. Benzi 10, 16132 Genoa, Italy*
[3]*UOC Oncologia, Istituto Giannina Gaslini, Via Gaslini 5, 16147 Genoa, Italy*

Correspondence should be addressed to Fabio Morandi; fabiomorandi@gaslini.org

Received 20 May 2016; Revised 4 July 2016; Accepted 19 July 2016

Academic Editor: Ban-Hock Toh

The role of nonclassical HLA-class Ib molecules HLA-G and HLA-E in the progression of Neuroblastoma (NB), the most common pediatric extracranial solid tumor, has been characterized in the last years. Since BM infiltration by NB cells is an adverse prognostic factor, we have here analyzed for the first time the concentration of soluble (s)HLA-G and HLA-E in bone marrow (BM) plasma samples from NB patients at diagnosis and healthy donors. sHLA-G and sHLA-E are present in BM plasma samples, and their levels were similar between NB patients and controls, thus suggesting that these molecules are physiologically released by resident or stromal BM cell populations. This hypothesis was supported by the finding that sHLA-G and sHLA-E levels did not correlate with BM infiltration and other adverse prognostic factors (*MYCN* amplification and age at diagnosis). In contrast, BM plasma levels of both molecules were higher in patients with metastatic disease than in patients with localized NB, thus suggesting that concentration of these molecules might be correlated with disease progression. The prognostic role of sHLA-G and sHLA-E concentration in the BM plasma for NB patients will be evaluated in future studies, by analyzing the clinical outcome of the same NB patients at follow-up.

1. Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in children, with an incidence of 1 case per 100.000 children per year, and causes 15% of cancer deaths in pediatric age. NB originates from the sympathetic nervous system, most frequently in the adrenal medulla or the paraspinal ganglia. The causes are unknown, although 1-2% of NB may have a hereditary basis. Different genetic alterations have been characterized in NB, that is, gain-of-function of *ALK* gene, losses of 11q and 1p, gain of 17q, and amplification of the *MYCN* gene. NB is heterogeneous, as it may undergo spontaneous remission or evolve to progressive metastatic disease, with dissemination to lymph nodes, bone, bone marrow, liver, skin, and other organs [1]. In particular, BM infiltration is an indicator of poor outcome for NB patients [2]. The International Neuroblastoma Risk Group staging system takes into account genetic alterations, DNA ploidy, histological features, and clinical data, as criteria for defining the risk classes. The prognosis of low/intermediate risk NB patients is favorable, and tumors can be cured by surgery alone or minimal chemotherapy. In contrast, high-risk NB patients' prognosis is poor, in spite of aggressive treatment based on surgery, chemotherapy, radiation therapy, hematopoietic stem cell transplantation, and adjuvant therapy with retinoic acid. In fact, survival rates of these patients at 5 years are less than 50% [3].

In the last years, the role of HLA-class Ib molecules in the progression of NB has been characterized by our group [4–7] and by others [8]. HLA-Ib family includes HLA-G, HLA-E, HLA-F, and HLA-H. In contrast with high polymorphic HLA-Ia molecules (HLA-A, HLA-B, and HLA-C) all these molecules display a limited polymorphism, with few alleles encoding a limited number of functional proteins. Moreover, although HLA-class Ib molecules can bind small peptides and present them to specific $CD8^+$ T cell subsets (similarly

Table 1: Neuroblastoma patients' characteristics. First row indicates all the variables analyzed in NB patients, second row indicates the subgroups for each variable, and third row indicates the number of subjects in each group.

Age at diagnosis (months)		Sex		MYCN			BM infiltration			Stage				
<18	>18	M	F	s.c.	Ampl.	Neg.	1+	2+	3+	1	2	3	4	4s
19	12	12	19	22	9	19	5	4	3	6	1	10	12	2

s.c.: single copy; Ampl.: amplified; Neg.: negative.

to HLA-class Ia counterparts), their main function is the modulation of the immune response in both physiological and pathological conditions [9]. HLA-G and HLA-E are the best characterized among HLA-Ib molecules. HLA-G has seven different isoforms, four membrane bound (namely, HLA-G1, HLA-G2, HLA-G3, and HLA-G4) and three soluble (namely, HLA-G5, HLA-G6, and HLA-G7), that are generated by alternative splicing from the same primary transcript. HLA-G can interact with at least four receptors, namely, immunoglobulin-like transcript (ILT)2, ILT4, KIR2DL4, and CD160, thus affecting the function of different immune effector cells (T and B lymphocytes, natural killer NK cells, dendritic cells, granulocytes, and monocytes) [10]. In contrast, HLA-E can be expressed as membrane bound or soluble isoform (generated through metalloproteases cleavage) and can inhibit $CD8^+$ T cells or NK cells though interaction with CD94/NKG2A heterodimeric receptor. However, HLA-E can also interact with the activating receptor CD94/NKG2C, thus leading to NK cell activation. These interactions are crucial during trophoblast implantation to abrogate NK cell lysis of semiallogeneic fetal tissue and, on the other hand, to activate NK cell functions in the process of tissue remodeling [11].

We have previously demonstrated that soluble (s)HLA-G concentration is higher in plasma samples from NB patients than in controls, and sHLA-G can be released by NB cells themselves, or by monocytes (stimulated by soluble factors secreted by tumor cells). Moreover, high sHLA-G plasma levels correlated with NB patients' relapse [4]. Finally, we have assessed that HLA-G is expressed by metastatic NB cells in the bone marrow from NB patients [6].

Also soluble HLA-E levels are higher in NB patients than in healthy controls. However, we have demonstrated that high plasma levels of sHLA-E at diagnosis correlated with a better overall survival (OS) of NB patients at follow-up, in contrast with sHLA-G [5].

Here, we demonstrated for the first time that sHLA-G and sHLA-E are present also in BM plasma samples derived from either NB patients at diagnosis or healthy donors. Moreover, we have assessed that sHLA-G and sHLA-E levels in BM plasma samples are related to the stage of the disease. Analysis of these patients at follow-up will reveal whether sHLA-G and sHLA-E concentration in BM plasma may predict the clinical outcome of NB patients.

2. Materials and Methods

2.1. Patients and Controls. The study was approved by the Ethics Committee of the G. Gaslini Institute, Genoa, Italy.

Table 2: Healthy donors' characteristics. First row indicates all the variables analyzed in healthy controls, second row indicates the subgroups for each variable, and third row indicates the number of subjects in each group.

Age (years)		Sex	
Range	Mean ± SD	M	F
20–54	39.6 ± 13	8	5

NB patients ($n = 31$) were diagnosed during 2016 in AIEOP centers. Bone marrow (BM) samples were collected at diagnosis and centralized at Istituto Giannina Gaslini in Genoa, Italy. BM plasma samples were obtained after centrifugation ($3000g \times 10'$). NB patients were staged according to the International Neuroblastoma Staging System [12]. Patients' characteristics, that is, age at diagnosis, sex, *MYCN* amplification (single copy or amplified), BM infiltration, and stage, are summarized in Table 1.

As controls, BM aspirates were obtained from 13 healthy donors, selected according to the Transplant Unit Clinical Protocol of Ematologia 2 at the IRCCS San Martino-IST in Genoa, following a written informed consent at the time of donation. Samples were processed as described in [13], and an aliquot was taken at the end of processing to perform quality control tests, such as $CD34^+$ cell count, in vitro progenitors' cell growth, and sterility. The remaining BM blood sample from this aliquot was subjected to centrifugation ($3000g \times 10'$) to obtain BM plasma. Donor's characteristics are summarized in Table 2.

All BM plasma samples were stored at −80°C until use.

2.2. ELISA. Enzyme-Linked Immunosorbent Assay (ELISA) for sHLA-G and sHLA-E was performed as previously described [14]. Briefly, MaxiSorp Nunc-Immuno 96-microwell plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 1 μg/mL of MEM-G9, specific for HLA-G HC (Exbio, Prague), that recognizes sHLA-G1/G5, or 3D12 mAb, specific for HLA-E HC (eBioscience, Science Center Drive, San Diego, CA, USA). After three washes with PBS 0.05% Tween 20 (washing buffer), plates were saturated with 200 μL/w of PBS 2% BSA (Sigma, St. Louis, MO, USA) for 30 min at RT. One hundred μL of BM plasma samples and serial dilutions of 721.221.G1 cell line supernatant (for HLA-G) or total extract from normal peripheral blood mononuclear cells (standard) were added to each well and incubated at RT for 1 hour. After three washes, 100 μL of detection reagent (HRP-conjugated anti-β2 microglobulin mAb, Exbio, Vestec, CZ) was added,

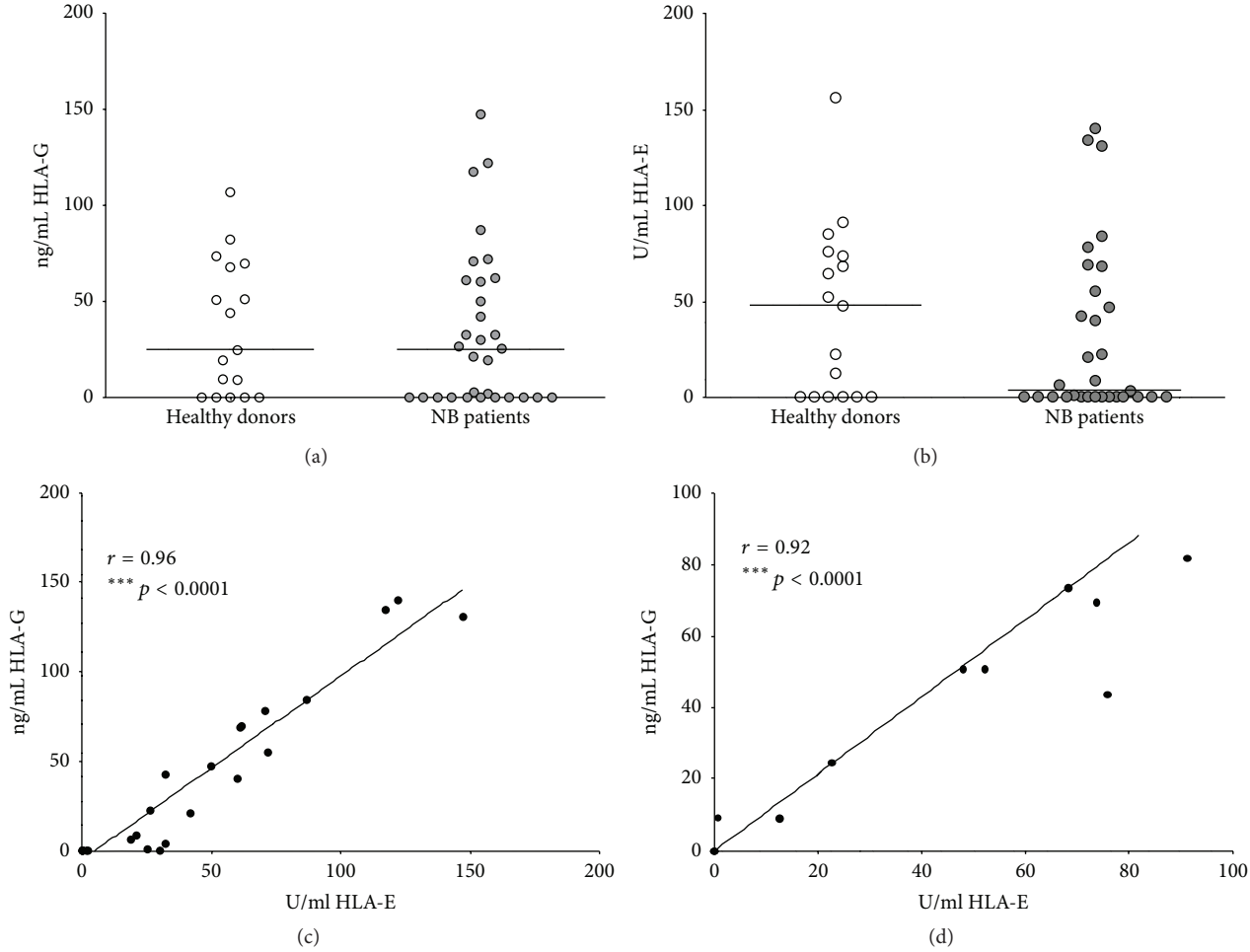


Figure 1: Levels of soluble (s)HLA-G (a) and sHLA-E (b) have been analyzed in BM plasma samples from NB patients (grey circles) and healthy BM donors (white circles). Horizontal lines indicated medians. Results are expressed as ng/ml (sHLA-G) or arbitrary units (U)/ml (sHLA-E). Correlation between BM plasma levels of sHLA-G and sHLA-E have been analyzed in NB patients (c) and controls (d). Linear regression of data and r and p values are indicated.

and plates were incubated for 1 hour at RT. After three washes, 100 μL of TMB (substrate for HRP, Sigma) was added, and reaction was stopped after approximately 30 minutes by adding H_2SO_4 5 N. Absorbance at 450 nm was measured using Infinite® 200 PRO spectrometer (Tecan Group Ltd., Seestrasse, Männedorf, Switzerland). Results are expressed as ng/mL sHLA-G and arbitrary units/mL sHLA-E (1 unit = quantity of sHLA-E in 1 μg of total extract).

2.3. Statistics. Normal distribution of data was tested using Kolmogorov-Smirnov test, using Prism software (GraphPad Software Inc., La Jolla, CA). Since data distribution was not normal, differences in plasma levels between (i) patients and controls or (ii) different groups of patients were compared by Mann-Whitney test, using Prism software. Correlations between plasma levels of sHLA-G and sHLA-E were calculated by Spearman's test using Prism software. A p value 0.05 was considered as statistically significant. Significance ranges are the following: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

3. Results

3.1. Soluble HLA-Ib Molecules Are Physiologically Present in BM Plasma Samples. First, we have tested sHLA-G and sHLA-E concentration in BM plasma samples from NB patients and healthy donors. As shown in Figure 1(a), sHLA-G concentration was similar between NB patients (median ± SE: 24.69 ± 8.45 ng/mL) and controls (25.16 ± 7.38 ng/mL). In contrast, sHLA-E levels were lower in NB patients (3.72 ± 7.89 U/mL) than in controls (48.01 ± 10.93 U/mL). However, such difference was not statistically significant, likely due to the wide distribution of the results in both groups (Figure 1(b)). Finally, sHLA-G and sHLA-E levels in BM plasma samples from NB patients ($r = 0.96$, $p < 0.0001$, Figure 1(c)) and healthy donors ($r = 0.92$, $p < 0.0001$, Figure 1(d)) strongly correlated with each other.

3.2. Soluble HLA-Ib Molecules Correlated with Disease Progression. We have next analyzed possible correlation between sHLA-G and sHLA-E levels in BM plasma samples

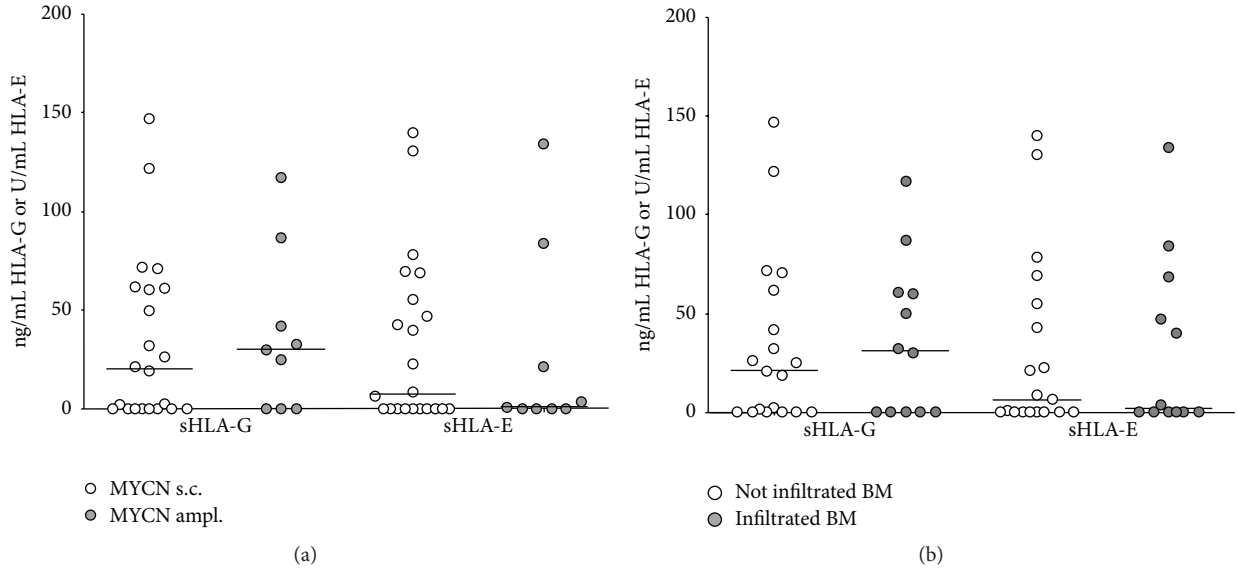


Figure 2: Levels of soluble (s)HLA-G and sHLA-E in BM plasma samples have been analyzed in patients presenting (grey circles) or not (white circles) MYCN amplification (a) or BM infiltration (b). Horizontal lines indicated medians. Results are expressed as ng/ml (sHLA-G) or arbitrary units (U)/ml (sHLA-E).

and patient's characteristics or clinical parameters. Accordingly, NB patients were divided into two groups on the basis of (i) *MYCN* amplification (single copy *versus* amplified), (ii) BM infiltration (not infiltrated *versus* infiltrated), (iii) age at diagnosis (<18 months *versus* >18 months), (iv) stage of the disease (stages 1-2 *versus* stages 3-4), and (v) sex (male *versus* female). Next, differences in sHLA-G and sHLA-E levels between these groups of NB patients have been evaluated.

No significant differences in sHLA-G levels have been detected between NB patients (i) carrying amplified (29.96 ± 13.57 ng/mL) or single-copy (23.65 ± 9.6 ng/mL) *MYCN* gene (Figure 2(a)) and (ii) presenting (31.17 ± 11.99 ng/mL) or not (21.05 ± 9.91 ng/mL) NB cells infiltrating the BM (Figure 2(b)). In contrast, sHLA-E levels were higher in (i) patients with single-copy *MYCN* (7.56 ± 9.17 U/mL) than in those with amplified *MYCN* (1.03 ± 16.2 U/mL) (Figure 2(a)) and in (ii) patients with infiltrated BM (6.45 ± 10.29 U/mL) than in those without BM infiltration (1.86 ± 12.43 U/mL) (Figure 2(b)). However, such differences were not statistically significant.

Furthermore, both sHLA-G and sHLA-E levels were similar between patients with an age below (21.05 ± 9.92 ng/mL sHLA-G and 6.45 ± 10.29 U/mL sHLA-E) or above (28.77 ± 11.36 ng/mL sHLA-G and 2.37 ± 12.81 U/mL sHLA-E) 18 months at diagnosis (Figure 3(a)). Notably, no correlation was found between age and sHLA-G or sHLA-E levels in BM plasma samples in healthy donors (data not shown).

Both sHLA-G and sHLA-E levels were significantly higher in patients with disease stages 3-4 (32.34 ± 8.08 ng/mL sHLA-G and 13.87 ± 9.42 U/mL sHLA-E) than in those with disease stages 1-2 (0 ± 4.32 ng/mL sHLA-G and 0 ± 3.27 U/mL sHLA-E, $p = 0.01$ and 0.03, resp.) (Figure 3(b)). Surprisingly, both sHLA-G and sHLA-E levels were found to be higher in male (45.87 ± 12.5 ng/mL sHLA-G and 34.19 ± 14.83 U/mL sHLA-E) than in female (2.52 ± 8.81 ng/mL sHLA-G and 0 ± 8.18 U/mL sHLA-E, $p = 0.05$ and 0.03, resp.) NB patients (Figure 3(c)). In contrast, healthy donors showed higher levels of sHLA-G and sHLA-E in female (50.74 ± 14.1 ng/mL sHLA-G and 52.25 ± 14.8 U/mL sHLA-E) than in male (12.35 ± 10.98 ng/mL sHLA-G and 11.33 ± 13.25 U/mL sHLA-E) subjects. However, such differences were not statistically significant (Figure 3(d)).

4. Discussion

To the best of our knowledge, this is the first demonstration of the presence of sHLA-class Ib molecules HLA-G and HLA-E in BM plasma samples. Previous studies have demonstrated that sHLA-G can be released by some cell populations that are present in the BM environment, such as erythroblasts [15] and mesenchymal stromal cells [16–19]. In contrast, no information is available regarding HLA-E expression and release in the BM. The strong correlation observed between the levels of these two molecules in BM samples either from NB patients or controls suggested that both molecules may be released by the same cell populations, or at least induced by similar *stimuli*.

We have previously demonstrated that metastatic NB cells in the BM expressed high levels of HLA-G on their surface, in contrast with primary tumors, that tested negative for HLA-G [6]. Here, we have demonstrated that both sHLA-G and sHLA-E are present at similar levels in NB patients and healthy donors, thus suggesting that malignant metastatic NB cells are unlikely involved in their release. This observation is further confirmed by the finding that BM infiltration by metastatic NB cells did not affect sHLA-G or sHLA-E levels in BM plasma samples. Moreover, *MYCN* amplification and age at diagnosis that represent important prognostic factors were

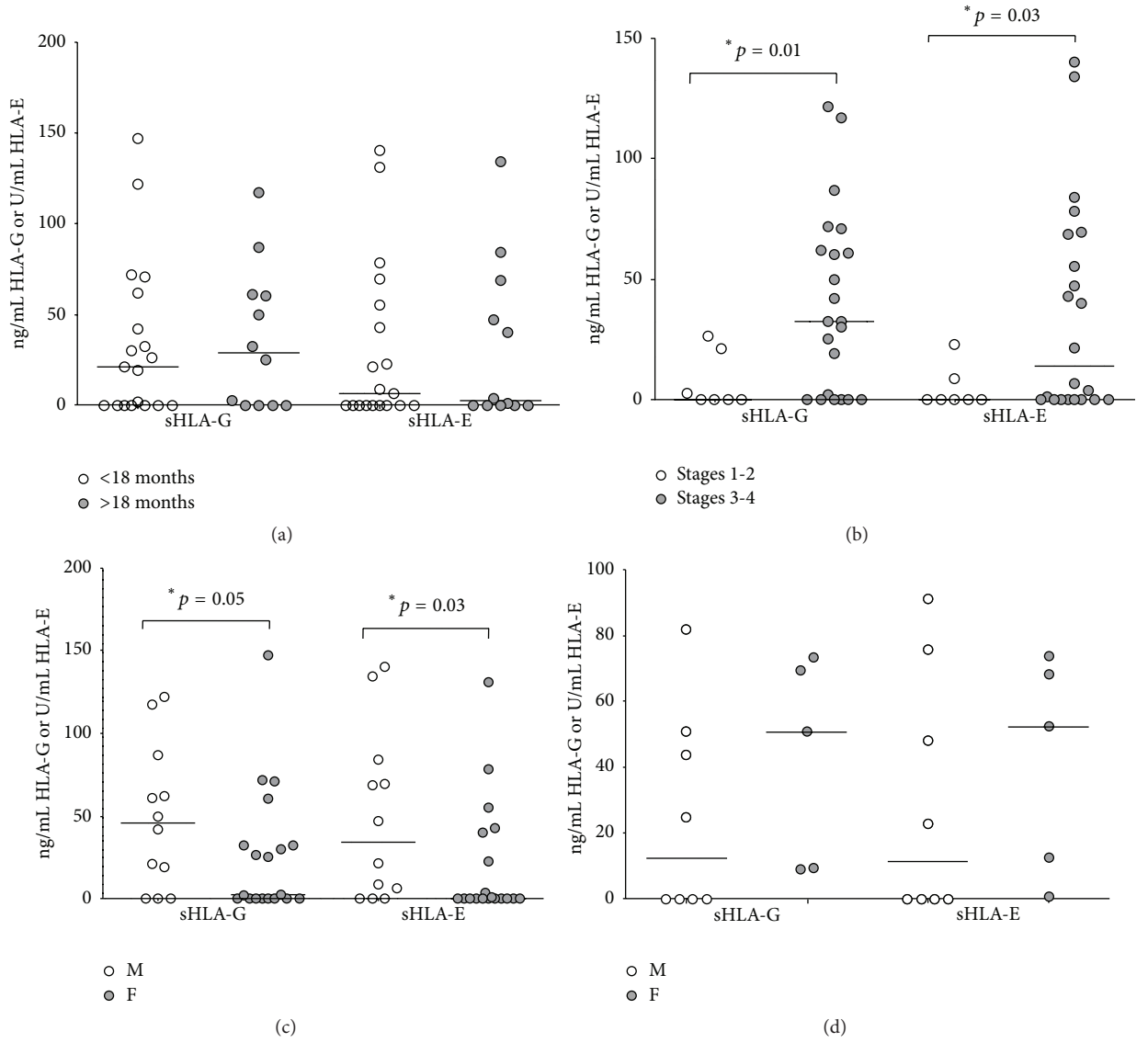


Figure 3: Levels of soluble (s)HLA-G and sHLA-E in BM plasma samples have been analyzed in patients with age at diagnosis above (grey circles) or below (white circles) 18 months (a) and in patients with stages 3-4 (grey circles) or 1-2 (white circles) disease (b). Differences between female (grey circles) or male (white circles) subjects have been analyzed in NB patients (c) and healthy donors (d). Horizontal lines indicated medians. Results are expressed as ng/ml (sHLA-G) or arbitrary units (U)/ml (sHLA-E). p values are indicated where differences are statistically significant.

not related to sHLA-G and sHLA-E levels in BM, thus further suggesting that these molecules might be released by BM stromal cells or BM resident cell populations instead of NB cells themselves, and may be present in the BM environment in physiological conditions. However, the increased tumor burden might be correlated to a higher release of tumor-derived factor(s) that, in turn, can upregulate HLA-G and HLA-E production by BM stromal cells.

The finding that sHLA-G and sHLA-E BM plasma levels are higher in male than in female patients is in line with a previous study on multiple sclerosis, where the authors demonstrated that sHLA-G levels in plasma samples were higher in male than in female patients [20]. However, this study has been carried out using peripheral blood plasma samples, and this is the first demonstration of this difference between male and female subjects in bone marrow plasma samples. Notably, such difference may be a prerogative of NB patients, since sHLA-G and sHLA-E levels were higher in female than in male normal subjects.

The most important finding of our study is the demonstration that sHLA-G and sHLA-E levels were significantly higher in BM plasma samples from patients with metastatic disease than in patients with localized NB. This data may suggest that the levels of these molecules in the BM at diagnosis might be associated with disease progression and might be predictive of the clinical course of NB patients. However, this hypothesis can be confirmed only by analyzing the clinical parameters of these patients at follow-up.

5. Conclusions

In conclusion, we demonstrated for the first time that soluble HLA-Ib molecules HLA-G and HLA-E are present in BM plasma samples in physiological and pathological conditions, and their concentration correlated with stage disease in NB patients. The prognostic value of sHLA-G and sHLA-E concentration in BM plasma samples from NB patients at diagnosis has to be confirmed in future studies.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Vito Pistoia and Maria Valeria Corrias equally contributed as last author.

Acknowledgments

This study has been supported by Fondazione Italiana per la Lotta al Neuroblastoma and Ministero del Lavoro, della Salute e delle Politiche Sociali (Progetti di Ricerca Corrente—5 per mille). The authors thank Mrs. Camilla Valentino for excellent secretarial assistance. They also thank Dr. Sebastiano Barco and Dr. Giuliana Cangemi for their collaboration.

References

[1] N. R. Pinto, M. A. Applebaum, S. L. Volchenboum et al., "Advances in risk classification and treatment strategies for neuroblastoma," *Journal of Clinical Oncology*, vol. 33, no. 27, pp. 3008–3017, 2015.

[2] F. Morandi, M. V. Corrias, and V. Pistoia, "Evaluation of bone marrow as a metastatic site of human neuroblastoma," *Annals of the New York Academy of Sciences*, vol. 1335, no. 1, pp. 23–31, 2015.

[3] K. T. Vo, K. K. Matthay, J. Neuhaus et al., "Clinical, biologic, and prognostic differences on the basis of primary tumor site in neuroblastoma: a report from the International Neuroblastoma Risk Group project," *Journal of Clinical Oncology*, vol. 32, no. 28, pp. 3169–3176, 2014.

[4] F. Morandi, I. Levreri, P. Bocca et al., "Human neuroblastoma cells trigger an immunosuppressive program in monocytes by stimulating soluble HLA-G release," *Cancer Research*, vol. 67, no. 13, pp. 6433–6441, 2007.

[5] F. Morandi, G. Cangemi, S. Barco et al., "Plasma levels of soluble HLA-E and HLA-F at diagnosis may predict overall survival of neuroblastoma patients," *BioMed Research International*, vol. 2013, Article ID 956878, 9 pages, 2013.

[6] F. Morandi, P. Scaruffi, F. Gallo et al., "Bone marrow-infiltrating human neuroblastoma cells express high levels of calprotectin and HLA-g proteins," *PLoS ONE*, vol. 7, no. 1, article e29922, 2012.

[7] V. Pistoia, F. Morandi, G. Bianchi, A. Pezzolo, I. Prigione, and L. Raffaghello, "Immunosuppressive microenvironment in neuroblastoma," *Frontiers in Oncology*, vol. 3, article 167, 2013.

[8] D. T. Lau, M. D. Norris, G. M. Marshall, M. Haber, and L. J. Ashton, "HLA-G polymorphisms, genetic susceptibility, and clinical outcome in childhood neuroblastoma," *Tissue Antigens*, vol. 78, no. 6, pp. 421–427, 2011.

[9] P. Le Bouteiller and F. Lenfant, "Antigen-presenting function(s) of the non-classical HLA-E, -F and -G class I molecules: the beginning of a story," *Research in Immunology*, vol. 147, no. 5, pp. 301–313, 1996.

[10] V. Pistoia, F. Morandi, X. Wang, and S. Ferrone, "Soluble HLA-G: are they clinically relevant?" *Seminars in Cancer Biology*, vol. 17, no. 6, pp. 469–479, 2007.

[11] F. Morandi and V. Pistoia, "Interactions between HLA-G and HLA-E in physiological and pathological conditions," *Frontiers in Immunology*, vol. 5, article 394, 2014.

[12] S. L. Cohn, A. D. J. Pearson, W. B. London et al., "The International Neuroblastoma Risk Group (INRG) classification system: an INRG task force report," *Journal of Clinical Oncology*, vol. 27, no. 2, pp. 289–297, 2009.

[13] D. Vicente, M. Podestà, A. Pitto et al., "Progenitor cells trapped in marrow filters can reduce GvHD and transplant mortality," *Bone Marrow Transplantation*, vol. 38, no. 2, pp. 111–117, 2006.

[14] F. Morandi, C. Venturi, R. Rizzo et al., "Intrathecal soluble HLA-E correlates with disease activity in patients with multiple sclerosis and may cooperate with soluble HLA-G in the resolution of neuroinflammation," *Journal of Neuroimmune Pharmacology*, vol. 8, no. 4, pp. 944–955, 2013.

[15] C. Menier, M. Rabreau, J.-C. Challier, M. Le Discorde, E. D. Carosella, and N. Rouas-Freiss, "Erythroblasts secrete the nonclassical HLA-G molecule from primitive to definitive hematopoiesis," *Blood*, vol. 104, no. 10, pp. 3153–3160, 2004.

[16] F. Montespan, F. Deschaseaux, L. Sensébé, E. D. Carosella, and N. Rouas-Freiss, "Osteodifferentiated mesenchymal stem cells from bone marrow and adipose tissue express HLA-G and display immunomodulatory properties in Hla-mismatched settings: implications in bone repair therapy," *Journal of Immunology Research*, vol. 2014, Article ID 230346, 10 pages, 2014.

[17] F. Morandi, L. Raffaghello, G. Bianchi et al., "Immunogenicity of human mesenchymal stem cells in HLA-class I-restricted T-cell responses against viral or tumor-associated antigens," *Stem Cells*, vol. 26, no. 5, pp. 1275–1287, 2008.

[18] R. Rizzo, D. Campioni, M. Stignani et al., "A functional role for soluble HLA-G antigens in immune modulation mediated by mesenchymal stromal cells," *Cytotherapy*, vol. 10, no. 4, pp. 364–375, 2008.

[19] Z. Selmani, A. Naji, I. Zidi et al., "Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+ CD25highFOXP3+ regulatory T cells," *Stem Cells*, vol. 26, no. 1, pp. 212–222, 2008.

[20] F. Alsahebfosoul, A. Zavaran Hosseini, R. Salehi, M. Etemadifar, N. Esmaeil, and A. Jamshidian, "Evaluation of soluble human leukocyte antigen-G (sHLA-G) isoforms and regulatory T cells in relapsing-remitting multiple sclerosis," *Iranian Journal of Allergy, Asthma and Immunology*, vol. 14, no. 3, pp. 298–305, 2015.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 1910852, 8 pages
http://dx.doi.org/10.1155/2016/1910852

Clinical Study

HLA-E*01:03 Allele in Lung Transplant Recipients Correlates with Higher Chronic Lung Allograft Dysfunction Occurrence

Julie Di Cristofaro,[1] Mathieu Pelardy,[2] Anderson Loundou,[3] Agnès Basire,[2] Carine Gomez,[4] Jacques Chiaroni,[1] Pascal Thomas,[5] Martine Reynaud-Gaubert,[4] and Christophe Picard[1,2]

[1]*CNRS, EFS, ADES UMR 7268, Aix-Marseille Université, 13916 Marseille, France*
[2]*Immunogenetics Laboratory, EFS-Alpes Méditerranée, 13005 Marseille, France*
[3]*Department of Public Health, EA 3279 Research Unit, Marseille University Hospital, Aix-Marseille University, Marseille, France*
[4]*Service de Pneumologie, Hôpital Nord, 13015 Marseille, France*
[5]*Service de Chirurgie Thoracique, Hôpital Nord, 13015 Marseille, France*

Correspondence should be addressed to Christophe Picard; christophe.picard@efs.sante.fr

Received 30 March 2016; Revised 8 May 2016; Accepted 19 May 2016

Academic Editor: Fabio Morandi

Lung transplantation (LTx) is a valid therapeutic option for selected patients with end-stage lung disease. HLA-E seems to play a major role in the immune response to different viral infections and to affect transplantation outcome, in Hematopoietic Stem Cell Transplantation, for example. Two nonsynonymous alleles, HLA-E*01:01 and HLA-E*01:03, have functional differences, involving relative peptide affinity, cell surface expression, and potential lytic activity of NK cells. The aim of this retrospective study was to determine the impact of these two alleles for LTx recipients on anti-HLA alloimmunization risk, overall survival, and chronic rejection (CLAD). HLA-E was genotyped in 119 recipients who underwent LTx from 1998 to 2010 in a single transplantation center. In univariate analysis, both HLA-E homozygous states were associated with impaired overall survival compared to heterozygous HLA-E alleles ($p = 0.01$). In multivariate analysis, HLA-E*01:03 allele showed increased CLAD occurrence when compared to homozygous HLA-E*01:01 status (HR: 3.563 (CI 95%, 1.016–12), $p = 0.047$). HLA-E allele did not affect pathogen infection or the production of *de novo* DSA. This retrospective study shows an uninvestigated, deleterious association of HLA-E alleles with LTx and requires verification using a larger cohort.

1. Introduction

Lung transplantation (LTx) is a valid therapeutic option for selected patients with end-stage lung disease. Unfortunately, posttransplant prognosis is hampered by the occurrence of chronic lung allograft dysfunction (CLAD) which is highly prevalent and remains the major limitation to long-term survival and functional outcome in LTx compared to other solid-organ transplants [1]. CLAD commonly reflects a bronchiolar obstruction defining a bronchiolitis obliterans syndrome (BOS). Recently, another phenotype of CLAD with a predominant restrictive pattern has been identified and called restrictive allograft syndrome (RAS) [2]. BOS and probably RAS are considered to be a multistep injury remodeling phenomenon targeted by recurrent immunologic events such as acute rejection and the development of *de novo* Donor Specific Antibodies (DSA). Several nonimmunological risk factors have been proposed, although not yet widely accepted as cytomegalovirus pneumonitis, bacterial/fungal/non-CMV viral infections, and persistent neutrophil influx and sequestration. Also, various genetic factors have been identified such as TGF- (Transforming Growth Factor-) B1, Toll-Like Receptor (TLR), and IL- (Interleukin-) 17. However, confirmation was not consistent across all studied cohorts [3].

Recently, we showed that HLA-G genetic polymorphism could be associated with LTx outcome, especially with CLAD occurrence [4]. HLA-G is a nonclassical HLA class I molecule, closely related to HLA-E: the HLA-E molecule also plays a crucial role in inflammatory and adaptive immune responses. HLA-E binds preferentially to the inhibitory

CD94/NKG2A and activating CD94/NKG2C (which lacks ITIM motif) receptors, selectively expressed on NK cells and a subset of CTL cells, modulating their cell-mediated activity [5]. Furthermore, HLA-E has also been showed to react with CD8 T cells expressing the conventional T Cell Receptor (TCR), suggesting that HLA-E involvement in the adaptive immune system responses is mediated by T cells [6].

HLA-E mRNA is expressed in most tissues [7], but its cell surface expression appears to be controlled by the binding of a range of different peptides, such as signal peptides derived from classical and nonclassical MHC class I molecules, stress protein peptides, and peptides derived from different pathogens [8, 9]. Thus, physiologically, HLA-E is expressed at the cell surface of endothelial cells, T and B lymphocytes, monocytes, and macrophages [10].

As HLA-G, HLA-E displays limited polymorphism with 21 alleles listed in the IMG/HLA database (release 3.23.0), including 9 proteins. HLA-E*01:01 and HLA-E*01:03 are the main alleles observed, with similar frequencies (~50%) in different populations [11–14]. They differ at codon 107 (R/G) and encode HLA-ER (E*01:01) and HLA-EG (E*01:03) proteins. Functional differences between these two isoforms involve relative peptide affinity, cell surface expression, and potential lytic activity of NK cells [15]. Indeed, HLA-E*01:03 protein is more thermally stable, exhibits higher cell surface expression, and is a potentially stronger inhibitor of the NK cell's lytic activity compared to HLA-E*01:01 [15].

A limited number of studies, with conflicted results, investigated into the influence of HLA-E genotype on transplantation outcomes. Although it is very difficult to show an impact of HLA-E polymorphism in HSCT, because of a limited amount of clinical data (cohort size, patient treatment differences), the majority of studies supported an association between the HLA-E*01:03 allele and a lower risk of graft-versus-host disease, decreased mortality, and greater disease-free survival suggesting graft-versus-leukemia (GVL) effect after Hematopoietic Stem Cell Transplantation (HSCT) [16–20].

Control of HLA-E cell surface expression by bacterial or viral infections and particularly human CMV is well established [21]. One study showed that CMV-associated HLA-E-restricted T cell alloreactivity was tightly regulated by NK receptors [22]. Furthermore, a recent study demonstrated that CMV-associated HLA-E-restricted T cells from a kidney transplant recipient recognize and lyse allogenic endothelial cells independently of their CMV status and HLA-E genotype, supporting a potentially detrimental HLA-E alloreactivity [23]. Interestingly, HLA-E molecules expressed in transgenic mice elicited an alloantigenic reaction indistinguishable from classical MHC class I molecules [24]. Finally, natural HLA antibodies directed against HLA-E have been detected in the sera of nonalloimmunized healthy male donors, probably induced by cross-reactive bacterial antigens and/or peptides derived from ingested food or allergens [25].

Since immunosuppressed lung transplant patients are particularly sensitive to infection and HLA sensitization, we speculated that HLA-E genotype could have an influence on the lung protection/allograft rejection balance.

Considering the immunotolerogenic properties of HLA-E, the main objectives of this study were to determine the impact of HLA-E alleles in a cohort of 138 adult LTx recipients on overall survival, disease-free survival (with and without CLAD), and viral and bacterial infection and on HLA sensitization.

2. Material and Methods

2.1. Study Design and Patient Characteristics. We conducted a retrospective single-center study based on adult patients who underwent LTx at the Marseille Lung Transplant Center between December 1998 and December 2010. Patients who had less than 90-day survival or who were lost to follow-up were excluded. There were 138 adult LTx recipients (mean (SD) age: 39.3 (16.3) years, 64 women and 55 men) eligible for analysis.

Patients received a first lung transplant (26 single LTx; 112 bilateral LTx) for an initial diagnosis of cystic fibrosis (43%), emphysema (25%), pulmonary fibrosis (20%), or another diagnosis (12%). The mean age at transplant procedure was 39.3 (13.3) years. The mean follow-up time was 37.2 months (CI: 10.8–63.2 months). HLA-A, HLA-B, HLA-DR, and HLA-DQ mismatching between donor and recipient were 56%, 82%, 68%, and 53%, respectively. *De novo* post-LTx DSA were detected in 51 recipients (38%) using Luminex single-antigen flow beads. Forty-two, 26, 11, and 11 post-LTx DSA were detected at Month 1, M3, M12, and M24, respectively [3].

2.2. Posttransplant Clinical Management. Immunosuppression and prophylaxis: all recipients received a similar standardized immunosuppressive regimen in accordance with our institutional protocols. Induction therapy consisted of intravenous administration of rabbit anti-thymocyte globulins (Pasteur Merieux, Lyon, France) given for the first 3 postoperative days (except when daily lymphocyte count was below 200/mm^3 and when there were cytomegalovirus (CMV) and/or EBV mismatches, i.e., seronegative recipient and seropositive donor). A high dose of methylprednisolone was additionally administered (6 mg/kg/d Day 1, 2 mg/kg/d Day 2 and Day 3, and 1 mg/kg/d thereafter). The standard triple maintenance immunosuppressive regimen consisted of cyclosporin (adjusted to maintain whole blood trough levels varying between 200 and 250 ng/mL) or tacrolimus after 2003 (adjusted to maintain whole blood trough levels varying between 8 and 12 ng/mL), azathioprine (1 mg/kg/d) or mycophenolate mofetil after 2003 in 5 patients (adjusted to a white blood cell count above 4000 mm^3), and steroids (prednisone) tapered to 0.25 mg/kg/d over the first 3 months and stopped if possible around 12 months after surgery.

Episodes of acute cellular allograft rejection were treated with intravenous methylprednisolone (5 mg/kg/d for 3 consecutive days) and then rapidly reduced. All CMV-positive recipients were treated with IV ganciclovir switched to oral valganciclovir as soon as possible for prophylaxis over the first 15 days after transplantation. All CMV-mismatched recipients (seronegative recipient and seropositive donor) were treated with IV ganciclovir or oral valganciclovir when

available for prophylaxis for the first 3 postoperative months. CMV-negative recipients who received a graft from a negative donor did not receive antiviral prophylaxis. All recipients were screened weekly for CMV infection with a polymerase chain reaction assay and pp65 antigenemia for the first 12 weeks, monthly thereafter, and when clinically indicated.

Pulmonary function tests (PFTs) were routinely conducted at our center on a monthly basis for the first 12 postoperative months, at 2-month intervals in the second year, and at 3-month intervals thereafter. In addition, PFTs were conducted when patients had clinical symptoms or a decline in home spirometry values of at least 10% on 2 consecutive days. Spirometry was measured in a constant volume (830 L) whole body plethysmograph (MasterLab, Jaeger, Wurzburg, Germany) and included measurement of forced vital capacity (FVC), FEV_1, residual volume (RV), and total lung capacity (TLC). Forced expiratory flow rate between 25% and 75% of FVC (FEF_{25-75}) was obtained from the best flow-curve volume. The baseline FEV_1 value was calculated as the average of the 2 best FEV_1 values at least 3 weeks apart. Baseline values of TLC and FEV_1/FVC were defined as the average of the 2 measurements obtained at the same time as the best 2 FEV_1 measurements.

The diagnosis of CLAD included both BOS and RAS phenotypes. BOS was defined according to the ISHLT guidelines [19]. RAS was defined as an irreversible decline in TLC to <90% of baseline for more than 3 weeks [2].

2.3. HLA-E Genotyping. A home-made primer extension method described in Julie et al., 2011, was used to simultaneously analyze 5 SNPs of the HLA-E gene in 119 patients to detect HLA-E*01:01, HLA-E*01:02, HLA-E*01:03, and HLA-E*01:04 alleles [11]. Nineteen acid nucleic samples were either lost (10) or degraded (9).

Briefly, the HLA-E multiplex PCR coamplified two PCR fragments (exons 1-2 by forward primer TGGTAGATGGAACCCTCCTTT and reverse primer GTGAATCTGGGACCCGAAG and exon 3 by forward primer GTGGGCGGGACTGACTAAG and reverse primer AGTAGCCCTGTGGACCCTCT) encompassing three and two SNPs, respectively. The multiplex PCR was performed on 200 and 75 ng of genomic DNA, respectively, in a final volume of 25 μL containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.1 units of Taq DNA-polymerase (Invitrogen, Cergy-Pontoise, France) and a defined concentration of each primer. Amplification was carried out as follows: 1 cycle at 95°C for 5 min; 30 cycles at 95°C for 30 s, 63°C for 45 s, and 72°C for 75 s; and 1 cycle at 72°C for 7 min. After control on 2% (w/v) agarose gel, 15 μL of PCR product was incubated with 5 units of shrimp alkaline phosphatase and 1 unit of exonuclease-I (Euromedex, Souffelweyersheim, France) for 1 h at 37°C followed by 15 min at 80°C to remove unincorporated primers and dNTPs.

The second step involved the incorporation of a fluorescent dNTP into extension-primers annealed upstream or downstream to each SNP. This multiplex extension reaction including five forward or reverse extension-primers (48T-AGTGTGGAAATACTTCAAGGAGTG to detect polymorphism C/G in codon 66, 10T-CCGCACAGATTTTCCGAGTGAA to detect polymorphism C/T in codon 77, 25T-CTGCGGACGCTGCG to detect polymorphism C/G in codon 83, 41T-CGCGGAGGAAGCGCC to detect polymorphism A/G in codon 107, and 41T-GCATGTGTCTTCCAGGTAGGCTC to detect polymorphism G/A in codon 157) was performed using the SNaPshot kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's protocol in a final volume of 10 μL containing 3 μL of the PCR product, 5 μL of SNaPshot mix, and extension-primers. The reaction was programed as follows: 25 cycles at 95°C for 10 s, 50°C for 5 s, and 60°C for 30 s. The product of the extension reaction (10 μL) was purified with 1 unit of SAP for 1 h at 37°C, followed by 15 min at 80°C for enzyme inactivation.

The fluorescence and size of the extended products were determined by capillary electrophoresis on an ABI PRISM 3130XL genetic analyzer (Applied Biosystems) using POP-7 polymer and a 36 cm capillary array. Capillary electrophoresis was performed according to the manufacturer's protocol.

Data were analyzed using GENEMAPPER v4.0 with specific detection parameters. Using an in-house computer program, output files (.txt) exported from GENEMAPPER 4.0 were automatically formatted into files readable by the "*Phenotype*" application of the GENE[RATE] computer tool package [26].

HLA-G and UTR genotyping data were from [4].

2.4. Studied Variables. Variables applied in univariate and multivariable analyses were grouped into four categories:

(1) Preoperative donor variables: donor age, gender, CMV status, and classical HLA type.

(2) Preoperative and postoperative recipient variables: recipient age, gender, initial diagnosis, HLA type and HLA Donor Specific Antibodies (DSA), CMV status and bacterial infection at M1 and M3, HLA-E, and HLA-G polymorphism.

(3) Preoperative donor-recipient matching: age, gender, CMV mismatch, and HLA mismatch.

(4) Intraoperative variables: ischemic time and type of procedure (single versus bilateral LTx).

2.5. Statistical Analyses. Missing data led to the exclusion of the concerned sample from further analyses. No multiple imputations were used in this study.

HLA-G and HLA-E Global Linkage Disequilibrium and HLA-E frequencies were estimated using an EM algorithm implemented in the GENE[RATE] computer tools. Deviations from Hardy-Weinberg equilibrium (HWE) were tested using a nested likelihood model.

Median values and ranges were used for continuous variables and percentages for categorical variables. For each continuous variable, the study cohort was initially split into quartiles and into two groups at the median.

Analysis using the Kruskal-Wallis test, Fisher's exact test, and Chi-square test was applied to determine clinical

Table 1: (a) Univariate analysis of death risk factors. (b) Univariate analysis of CLAD occurrence risk factors.

(a)

Variables	p value
Diseases other than cystic fibrosis	<0.001
HLA class II DSA	<0.001
HLA-G*01:04~UTR3	0.001
Older recipients	0.001
CLAD occurrence	0.002
HLA class I DSA	0.03
HLA-B mismatch	0.03

(b)

Variables	p value
HLA-DQ mismatch	0.002
HLA class I DSA	0.008
Diseases other than cystic fibrosis	0.01
HLA-DR mismatch	0.01
Single lung transplantation	0.03
HLA-G*01:04~UTR3	0.03
Older recipients	0.05

significance wherever required, in particular for any relevance between HLA-E alleles and the status of CMV infection.

The primary endpoints of this study were overall survival (OS) and disease-free survival (DFS). OS was defined as the interval between the date of transplantation and last follow-up visit or death. DFS was defined as the time interval from transplantation to the first event, either the diagnosis of CLAD or death without diagnosis of CLAD. The Kaplan-Meier method was used to estimate OS and freedom from CLAD. The log-rank test was used to assess the univariate effects on OS and DFS. For all analyses, $p < 0.05$ was considered statistically significant.

Multivariate analyses were performed using Fine and Gray's proportional hazards regression model.

All analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL) and the cmprsk package (developed by Gray, June 2001) on R2.3.0 software (http://www.R-project.org/).

3. Results

3.1. Conditions Associated with Overall Survival and Allograft Function. Overall survival of the LTx population was 74% and 68% at 12 and 24 months, respectively (median survival, 7 years). In univariate analysis, the conditions associated with better survival were an initial diagnosis of cystic fibrosis (CF) compared to other indications, HLA class II DSA detected at M3 and/or M12, HLA-G*01:04~UTR3 haplotype, the younger age of the recipient, absence of CLAD occurrence, the number of recipient/donor mismatches at the B locus, and HLA class I DSA detected at M3 and/or M12 (Table 1(a)). During the study period, 29 LTx recipients developed CLAD

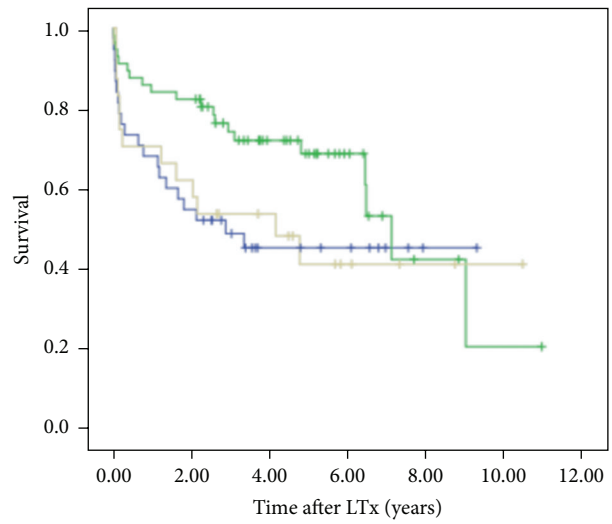


Figure 1: Survival curves in LTx recipients according to the presence of heterozygous HLA-E (in green) compared to homozygous HLA-E (homozygous HLA-E*01:01 in light grey, homozygous HLA-E*01:03 in blue) (log-rank test, $p = 0.01$).

(including 5 patients (17%) with RAS and 24 (83%) with BOS), corresponding to a proportion of 8%, 21%, and 30% of the cohort at 1, 2, and 3 years after LTx, respectively. The major risks for CLAD were HLA class I DSA detected at M3 and/or M12, recipient/donor mismatches at HLA-DR and HLA-DQ loci, the single lung transplant procedure, the non-CF population, and carrying the HLA-G*01:04~UTR3 haplotype (Table 1(b)).

3.2. HLA-E Allelic Frequencies. A total of 3 HLA-E genotypes were observed in these patients (Table 2). Homozygous HLA-E*01:01 was detected in 38/119 (32%) patients, homozygous HLA-E*01:03 was detected in 24/119 (20%), and heterozygous HLA-E was detected in 57/119 (48%). HLA-E frequency distribution was in Hardy-Weinberg equilibrium. HLA-E allele and genotype frequencies were also concordant with previously published data showing a relatively equal distribution between HLA-E*01:01 and HLA-E*01:03 in Western Europe [11]. Analysis of two-locus Global Linkage Disequilibrium (GLD) showed that the HLA-E alleles are not in significant GLD with HLA-G or UTR loci.

3.3. Impact of HLA-E Alleles and Genotypes on LTx Outcome. HLA-E genotype was not associated with patient characteristics (Table 2). HLA-E*01:01/01:03 heterozygous state was associated with survival advantage ($p = 0.01$) when compared to HLA-E*01:01 or HLA-E*01:03 homozygous states. The two-year survival rate was 80% ± 8% for HLA-E heterozygous genotypes and 50% ± 8% and 50% ± 10% for homozygous HLA-E*01:01 and homozygous HLA-E*01:03 states, respectively (Figure 1). HLA-E*01:03 allele was associated with CLAD occurrence (Figure 2). The two-year freedom from CLAD rate was 44%±25% for the HLA-E*01:03 allele and 73.4%±8% for the homozygous HLA-E*01:01 state.

Table 2: Baseline comparison of distribution and risk factors for 119 patients who underwent LTx according to HLA-E genotypes.

	E*01:01/*01:01	E*01:01/*01:03	E*01:03/*01:03	*p*	E*01:01/*01:01	E*01:03 allele	*p*
Genotypes frequency (*N*, %)	38 (31.9)	57 (47.9)	24 (20.2)		38 (31.9)	81 (68.1)	
Male (*N*, %)	15 (40)	28 (49)	12 (50)	*0.60*	15 (39.5)	40 (49)	*0.33*
Age of recipient (SD)	39.1 (14.2)	40.23 (13)	39.6 (12)	*0.92*	39.1 (14.2)	40 (13)	*0.72*
Initial disease				*0.33*			*0.91*
Emphysema (*N*, %)	10 (26.3)	11 (19.3)	10 (41.7)		10 (26.3)	21 (25.9)	
Fibrosis (*N*, %)	8 (21.1)	8 (14)	5 (20.8)		8 (21.1)	13 (16.1)	
Cystic fibrosis (*N*, %)	14 (36.8)	28 (49.1%)	6 (25%)		14 (36.8)	34 (42.1)	
Others (*N*, %)	6 (15.8)	10 (17.5%)	3 (12.5)		6 (15.8)	13 (16)	
Transplant procedure				*0.58*			*0.92*
Lung and heart transplantation (*N*, %)	1 (2.6)	0 (0)	1 (4.2)		1 (2.6)	1 (1.2)	
Single lung transplantation (*N*, %)	8 (21.1)	14 (24.6)	4 (16.7)		8 (21.1)	18 (22.2)	
Bilateral lung transplantation (*N*, %)	29 (76.3)	43 (75.4)	19 (79.2)		29 (76.3)	62 (76.5)	
CMV status							
D+/R− (*N*, %)	6 (16.2)	7 (12.3)	4 (17.4)	*0.83*	6 (16.2)	11 (13.8)	*0.78*
D+/R+ or − (*N*, %)	19 (51)	31 (54)	9 (40)	*0.46*	19 (51)	40 (50)	*0.88*
D+ or −/R+ (*N*, %)	22 (58)	36 (63)	12 (50)	*0.54*	22 (58)	48 (59)	*0.88*
Mismatch HLA/6 (*N*)	5.03	5	4.83	*0.9*	5.03	4.95	*0.90*
Infection at M1 (*N*, %)	8 (53.3)	16 (47.1%)	2 (25)	*0.49*	8 (53.3)	18 (42.9)	*0.48*
Infection at M3 (*N*, %)	6 (46.2)	7 (38.9)	5 (50)	*0.83*	6 (46.2)	12 (42.9)	*0.84*
Infection in first year (*N*, %)	16 (42)	32 (56)	11 (45.8)	*0.37*	16 (42.1)	43 (53.1)	*0.26*
DSA at M1 (*N*, %)	7 (63.6)	13 (81.3)	5 (83.3)	*0.51*	7 (63.5)	18 (81.8)	*0.25*
DSA at M3 (*N*, %)	6 (66.7)	8 (66)	3 (60)	*1*	6 (66.7)	11 (64.7)	*0.92*
CLAD occurrence (*N*, %)	5 (22.7)	20 (40.8)	7 (58.3)	***0.06***	5 (22.7)	27 (44.3)	***0.02***
BOS (*N*, %)	5 (23.8)	16 (32.7)	6 (22.2)	*0.31*	5 (23.8)	22 (36.1)	***0.02***
RAS (*N*, %)	0 (0)	4 (8.2)	1 (8.3)	*0.41*	0 (0)	5 (8.2)	*0.32*
Overall survival (median, year)	2.37	3.7	2.6	***0.01***			

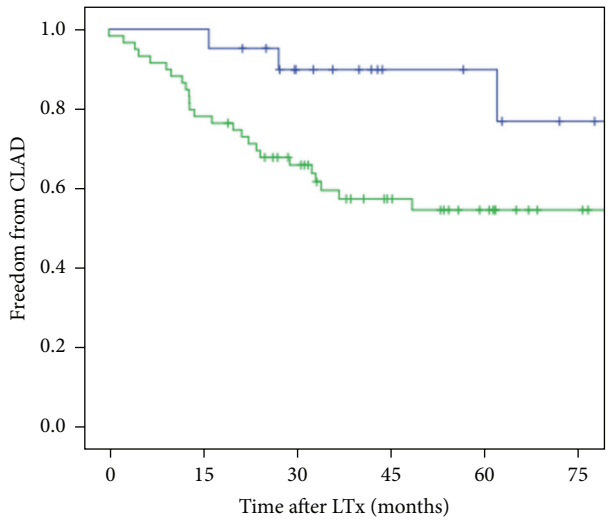


Figure 2: Freedom from CLAD in LTx recipients according to the presence of homozygous HLA-E*01:01 (in blue) compared to HLA-E*01:03 alleles (in green) (log-rank test, $p = 0.02$).

Cox proportional regression hazards modeling showed that the main risk factors for CLAD were a noncystic fibrosis initial diagnosis ($p < 0.001$), HLA-E*01:03 alleles carriers ($p = 0.047$), and HLA-G*01:04~UTR3 carriers ($p = 0.071$). The relative risks of CLAD were 8.612 for emphysema compared to cystic fibrosis patients (CI 95%, 3.13–23.824), 3.563 in recipients carrying HLA-E*01:03 allele compared to recipients who did not carry it (CI 95%, 1.016–12), and 3.037 in recipients carrying HLA-G*01:04~UTR3 compared to recipients who did not carry it (CI 95%, 0.889–7.412) (Table 3).

Table 3: Main risk factors for CLAD according to Cox regression multivariate analysis on recipient diagnosis, HLA-G*01:04~UTR3 haplotype, and HLA-E*01:03 allele (HR, hazard ratio; CI, confidence interval).

	Estimated HR (95% CI)	*p* value
Diagnosis		
Cystic fibrosis	Baseline	0.000
Emphysema	8.612 (3.113–23.824)	0.000
Fibrosis	1.515 (0.362–6.335)	0.569
Others	2.897 (0.822–10.214)	0.098
HLA-G*01:04~UTR3	2.567 (0.889–7.412)	0.071
HLA-E*01:03	3.563 (1.016–12.488)	0.047

None of the HLA-E alleles were significantly associated with the different clinical variables, such as bacterial infection at M1 or M3 and DSA detection at M1 and M3 (Table 2).

4. Discussion

This is the first study to show that HLA-E polymorphisms could be implicated in survival and CLAD occurrence in LTx. The two homozygous HLA-E states are associated with worse survival compared to the heterozygous state. These alleles however contribute differentially as HLA-E*01:03 allele is correlated to CLAD occurrence in multivariate analysis.

Although the exact pathogenesis of CLAD remains unknown, studies indicate that BOS begins with epithelial injury of the airways due to a variety of factors such as viral infection, autoimmune disease, and alloreactivity response, followed by an inflammatory reaction that leads to obliteration of the airways [3]. HLA-E is an immunomodulatory molecule that can function as both an immune-tolerogenic and immune-activating molecule and plays a dual role in natural and acquired immune responses.

The HLA-E-peptide complex can act as ligand for the CD94/NKG2 receptors expressed on the surface natural killer cells and represents a restriction element for the TCR. Although the two alleles only differ by a single amino acid in alpha 2 domain of the HLA-E heavy chain, HLA-E*01:03 is characterized by a stronger affinity for various peptides and a higher thermal stability than HLA-E*01:01, inducing its higher cell surface expression in PBMC or other cells [27]. These differences might influence the affinities for the different activator or inhibitor receptors, might induce different intracellular signaling, and might have an impact on the cellular immune response in the context of transplantation [28].

HLA-E has the ability to bind non-self-antigen and self-antigen, among which peptides derived from the leader sequences of classical and nonclassical HLA molecules. HLA-E and HLA-G, both categorized as nonclassical class I HLA, share immunosuppressive properties and immunological cell targets; furthermore, HLA-E has the highest affinity for the HLA-G leader peptide. These observations back quite a close relationship between these molecules, but this coordination remains unclear. For instance, in the context of pregnancy, HLA-E, binding an HLA-G peptide signal, interacts with CD94/NKG2C activating receptors to activate NK cells lysis of trophoblast cells during placental invasion, leading to tissue remodeling [29]. At a genetic level, the low number of coding SNPs in both HLA-E and HLA-G loci could suggest that amino acid modifications have serious functional consequences. However, HLA-G and HLA-E are not in GLD. Furthermore, the impact of HLA-G*01:04~UTR3 on CLAD occurrence seems to be independent of the HLA-E*01:03 allele; finally, contrary to HLA-G alleles, HLA-E*01:03 was not associated with an increase in anti-HLA alloimmunization, suggesting that HLA-E and HLA-G have both differential involvements and pathways. Thus, another hypothesis to explain the low level of coding modification in both HLA-E and HLA-G loci is that they lead to amino acid modifications with little functional consequences but that they are linked to SNPs in the regulatory region involved in quantitative expression.

Considering HLA-E and pathogen infections, HLA-E can bind to different viral peptides derived from CMV, the Epstein-Barr virus, the human immunodeficiency virus, the influenza virus, and Hepatitis C virus. A few studies have linked reactivation of CMV with organ rejection [30]. CMV infection in post-LTx is considered a risk factor for BOS [30, 31]. The CMV immune evasion protein, UL40, when complexed with HLA-E, can modulate NK cell functions via interactions with the CD94-NKG2A receptors, leading to viral evasion [31]. Recently, it has been suggested that latent CMV infection-mediated increase in the proportion of NKG2C+ NK cells may prime NK cell cytotoxicity and could be beneficial in preventing the progression and development of hematologic malignancies characterized by high HLA-E expression [32]. This effect, dependent potentially on HLA-E alleles, may be deleterious for transplantation occurrence.

Furthermore, the UL40-derived sequence can also be immunogenic, eliciting robust CD8+ T cell responses. Recently, CMV UL-40-specific T cells were detected in the peripheral blood of LTx recipients and were significantly associated with allograft dysfunction, such as BOS [33]. These cells were first identified between 6 and 12 months after transplant, a period that coincides with the cessation of antiviral prophylaxis and the highest risks for CMV reactivation, suggesting an antigen-driven expansion restricted preferentially by HLA-E. These cells could lyse a large array of allogenic target cells and directly damage the allograft [34]. In contrast, HLA-E can promote specific HLA-E-restricted CD8+ Treg cells that inhibit antiviral effector CD8+ T cells, diminishing virus control [35].

In this retrospective study, CMV reactivation data were unavailable. Mismatch CMV (D−/R− versus D+/R− or + and D+ or −/R+) were not associated with CLAD occurrence and overall survival ($p = 0.8$ and $p = 0.7$, resp.). None of the homozygous or heterozygous HLA-E alleles with a positive CMV recipient and/or donor showed a statistical difference on survival and CLAD occurrence compared to the same HLA-E alleles with a negative CMV recipient and donor ($p = 0.8$ and $p = 0.7$) (data not shown). The percentage of death (55%) was similar between positive CMV recipient and/or donor and negative CMV donor and recipient, whatever homozygous HLA-E alleles. These results can be explained by the small size of this retrospective monocentric cohort. Furthermore, reactivation of other viruses such as respiratory virus could be more relevant than CMV reactivation [36].

T cells are also able to recognize HLA-E binding peptides from bacteria such as *Mycobacterium tuberculosis*, *Salmonella*, and *Listeria* monocytogenes. In an unrelated allogenic stem cell transplantation study, homozygous HLA-E*01:01 was identified as a risk for the occurrence of severe bacterial but not viral infections [16]. None of the HLA-E alleles were associated with a higher risk of bacterial infection at M1 and M3.

Another hypothesis is that HLA-E*01:03 could lead to more efficient activation of CD8+ T cells alloreactivity. This mechanism has been suggested in a few related or unrelated stem cell allograft studies, showing that homozygous HLA-E*01:03 induced a significant graft-versus-leukemia effect [17–19, 37]. In this context, HLA-E*01:03 recipient may preferentially bind nonstandard minor histocompatibility antigen (mHag) peptides that can react with T cell activating

receptors [18, 19, 37]. Thus, as for the GVL effect, this HLA-E dependent, alloreactive cellular process could specifically generate lung tissue inflammation. Furthermore, it has been shown that HLA-E exhibits alloantigenic properties that are indistinguishable from classical HLA class I molecules when expressed in HLA-E*01:03 transgenic mice [24].

In summary, these data could be explained by the functional properties of the two HLA-E alleles in peptide affinity, cell surface expression, and potential lytic activity by NK cells or T cells. Therefore, a heterozygous status would offset the two homozygous states, each allele bringing benefits and risks to the overall survival of recipients via different mechanisms. For example, HLA-E*01:03 could promote cellular alloreactivity mechanisms, triggered or not by viral infection via different receptors. In contrast, HLA-E*01:01 could promote severe bacterial infection. Anyway, the impact of these two alleles in lung transplantation is concordant with the maintenance of these two alleles based on a balancing selection, meaning that there is a heterozygote advantage for individuals that are heterozygous at the HLA-E locus [38, 39].

Finally, the mechanism by which HLA-E alleles may promote CLAD and decrease long-term survival after LTx remains to be elucidated. The major limitation of this investigation is that it is a single-retrospective study and for certain analyses the patient cohort was small. It is possible that a few confounding factors not explored in this study may modify its interpretation. Furthermore, in the same way, a potential role of the donor's genotype and the possible interactions with the recipient's genotype could be studied. Thus, this association of HLA-E polymorphism with LTx occurrence needs verification using a larger cohort.

Abbreviations

LTx: Lung transplantation
CLAD: Chronic lung allograft dysfunction
FEV_1: Forced Expiratory Volume measured during the first second
FVC: Forced vital capacity
DSA: Donor Specific Antibodies
BOS: Bronchiolitis obliterans syndrome
RAS: Restrictive allograft syndrome
CTL: Cytotoxic T-Lymphocytes
OS: Overall survival
DFS: Disease-free survival
ITIM: ImmunoTyrosine Inhibitor Module
CT: Computed Tomography
PFTs: Pulmonary function tests
RV: Residual volume
TLC: Total lung capacity
CMV: Cytomegalovirus
SD: Standard Deviation
HSCT: Hematopoietic Stem Cell Transplantation
TGF: Transforming Growth Factor
TLR: Toll-Like Receptor
IL: Interleukin
D: Donor
R: Recipient.

Competing Interests

The authors have no conflict of interests to disclose.

Acknowledgments

This study was funded by the Etablissement Français du Sang, Marseille, France. This work was supported in part by "Vaincre la Mucoviscidose" through Funding "TP1008."

References

[1] R. D. Yusen, J. D. Christie, L. B. Edwards et al., "The registry of the international society for heart and lung transplantation: thirtieth adult lung and heart-lung transplant report—2013; focus theme: age," *Journal of Heart and Lung Transplantation*, vol. 32, no. 10, pp. 965–978, 2013.

[2] G. M. Verleden, G. Raghu, K. C. Meyer, A. R. Glanville, and P. Corris, "A new classification system for chronic lung allograft dysfunction," *Journal of Heart and Lung Transplantation*, vol. 33, no. 2, pp. 127–133, 2014.

[3] G. M. Verleden, R. Vos, B. Vanaudenaerde et al., "Current views on chronic rejection after lung transplantation," *Transplant International*, vol. 28, no. 10, pp. 1131–1139, 2015.

[4] J. Di Cristofaro, M. Reynaud-Gaubert, F. Carlini et al., "HLA-G*01:04~UTR3 recipient correlates with lower survival and higher frequency of chronic rejection after lung transplantation," *American Journal of Transplantation*, vol. 15, no. 9, pp. 2413–2420, 2015.

[5] V. M. Braud, D. S. J. Allan, C. A. O'Callaghan et al., "HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C," *Nature*, vol. 391, no. 6669, pp. 795–799, 1998.

[6] G. Pietra, C. Romagnani, M. Falco et al., "The analysis of the natural killer-like activity of human cytolytic T lymphocytes revealed HLA-E as a novel target for TCR alpha/beta-mediated recognition," *European Journal of Immunology*, vol. 31, no. 12, pp. 3687–3693, 2001.

[7] H. Heinrichs and H. T. Orr, "HLA non-A,B,C class I genes: their structure and expression," *Immunologic Research*, vol. 9, no. 4, pp. 265–274, 1990.

[8] N. Lee, M. Llano, M. Carretero et al., "HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 9, pp. 5199–5204, 1998.

[9] J. Michaëlsson, C. Teixeira de Matos, A. Achour, L. L. Lanier, K. Kärre, and K. Söderström, "A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition," *Journal of Experimental Medicine*, vol. 196, no. 11, pp. 1403–1414, 2002.

[10] G. Kochan, D. Escors, K. Breckpot, and D. Guerrero-Setas, "Role of non-classical MHC class I molecules in cancer immunosuppression," *OncoImmunology*, vol. 2, no. 11, Article ID e26491, 2013.

[11] D. C. Julie, S. Buhler, C. Frassati et al., "Linkage disequilibrium between HLA-G*0104 and HLA-E*0103 alleles in Tswa Pygmies," *Tissue Antigens*, vol. 77, no. 3, pp. 193–200, 2011.

[12] A. Antoun, S. Jobson, M. Cook, P. Moss, and D. Briggs, "Ethnic variability in human leukocyte antigen-E haplotypes," *Tissue Antigens*, vol. 73, no. 1, pp. 39–45, 2009.

[13] L. Carvalho dos Santos, L. V. Tureck, P. F. Wowk et al., "HLA-E polymorphisms in an Afro-descendant Southern Brazilian

population," *Human Immunology*, vol. 74, no. 2, pp. 199–202, 2013.

[14] E. C. Castelli, C. T. Mendes-Junior, A. Sabbagh et al., "HLA-E coding and 3' untranslated region variability determined by next-generation sequencing in two West-African population samples," *Human Immunology*, vol. 76, no. 12, pp. 945–953, 2015.

[15] L. C. Sullivan, C. S. Clements, J. Rossjohn, and A. G. Brooks, "The major histocompatibility complex class Ib molecule HLA-E at the interface between innate and adaptive immunity," *Tissue Antigens*, vol. 72, no. 5, pp. 415–424, 2008.

[16] R. Tamouza, V. Rocha, M. Busson et al., "Association of HLA-E polymorphism with severe bacterial infection and early transplant-related mortality in matched unrelated bone marrow transplantation," *Transplantation*, vol. 80, no. 1, pp. 140–144, 2005.

[17] R. Tamouza, M. Busson, V. Rocha et al., "Homozygous status for HLA-E*0103 confers protection from acute graft-versus-host disease and transplant-related mortality in HLA-matched sibling hematopoietic stem cell transplantation," *Transplantation*, vol. 82, no. 11, pp. 1436–1440, 2006.

[18] M. Danzer, H. Polin, J. Pröll et al., "Clinical significance of HLA-E*0103 homozygosity on survival after allogeneic hematopoietic stem-cell transplantation," *Transplantation*, vol. 88, no. 4, pp. 528–532, 2009.

[19] E. Hosseini, A. P. Schwarer, A. Jalali, and M. Ghasemzadeh, "The impact of HLA-E polymorphisms on relapse following allogeneic hematopoietic stem cell transplantation," *Leukemia Research*, vol. 37, no. 5, pp. 516–519, 2013.

[20] L. Wieten, N. M. Mahaweni, C. E. M. Voorter, G. M. J. Bos, and M. G. J. Tilanus, "Clinical and immunological significance of HLA-E in stem cell transplantation and cancer," *Tissue Antigens*, vol. 84, no. 6, pp. 523–535, 2014.

[21] M. Ulbrecht, S. Martinozzi, M. Grzeschik et al., "Cutting edge: the human cytomegalovirus *UL40* gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis," *The Journal of Immunology*, vol. 164, no. 10, pp. 5019–5022, 2000.

[22] G. Pietra, C. Romagnani, P. Mazzarino et al., "HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8+ cytolytic T lymphocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 19, pp. 10896–10901, 2003.

[23] M. Allard, P. Tonnerre, S. Nedellec et al., "HLA-E-restricted cross-recognition of allogeneic endothelial cells by CMV-associated CD8 T cells: a potential risk factor following transplantation," *PLoS ONE*, vol. 7, no. 11, article e50951, 2012.

[24] R. Pacasova, S. Martinozzi, H.-J. Boulouis et al., "Cell-surface expression and alloantigenic function of a human nonclassical class I molecule (HLA-E) in transgenic mice," *The Journal of Immunology*, vol. 162, no. 9, pp. 5190–5196, 1999.

[25] M. H. Ravindranath, T. Pham, M. Ozawa, and P. I. Terasaki, "Antibodies to HLA-E may account for the non-donor-specific anti-HLA class-Ia antibodies in renal and liver transplant recipients," *International Immunology*, vol. 24, no. 1, pp. 43–57, 2012.

[26] J. Nunes, *Generate: Tools for Analysis and Handling of Data with Ambiguities*, Laboratory of Anthropology, Genetics and Peopling History, University of Geneva, Geneva, Switzerland, 2006, http://geneva.unige.ch/generate/.

[27] N. Lauterbach, L. Wieten, H. E. Popeijus et al., "Peptide-induced HLA-E expression in human PBMCs is dependent on peptide sequence and the HLA-E genotype," *Tissue Antigens*, vol. 85, no. 4, pp. 242–251, 2015.

[28] M. A. Pabón, C. E. Navarro, J. C. Osorio et al., "Impact of human leukocyte antigen molecules E, F, and G on the outcome of transplantation," *Transplantation Proceedings*, vol. 46, no. 9, pp. 2957–2965, 2014.

[29] M. Llano, N. Lee, F. Navarro et al., "HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: Preferential response to an HLA-G-derived nonamer," *European Journal of Immunology*, vol. 28, no. 9, pp. 2854–2863, 1998.

[30] T. Martinu, D.-F. Chen, and S. M. Palmer, "Acute rejection and humoral sensitization in lung transplant recipients," *Proceedings of the American Thoracic Society*, vol. 6, no. 1, pp. 54–65, 2009.

[31] G. P. Westall, A. Michaelides, T. J. Williams, G. I. Snell, and T. C. Kotsimbos, "Bronchiolitis obliterans syndrome and early human cytomegalovirus DNAaemia dynamics after lung transplantation," *Transplantation*, vol. 75, no. 12, pp. 2064–2068, 2003.

[32] A. B. Bigley, K. Rezvani, N. Shah et al., "Latent CMV infection enhances anti-tumor cytotoxicity through accumulation of NKG2C+ NK-cells in healthy humans," *Clinical & Experimental Immunology*, 2016.

[33] L. C. Sullivan, G. P. Westall, J. M. L. Widjaja et al., "The presence of HLA-E-restricted, CMV-specific $CD8^+$ T cells in the blood of lung transplant recipients correlates with chronic allograft rejection," *PLoS ONE*, vol. 10, no. 8, Article ID e0135972, 2015.

[34] C. Romagnani, G. Pietra, M. Falco et al., "Identification of HLA-E-specific alloreactive T lymphocytes: a cell subset that undergoes preferential expansion in mixed lymphocyte culture and displays a broad cytolytic activity against allogeneic cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 17, pp. 11328–11333, 2002.

[35] T. A. W. Holderried, P. A. Lang, H.-J. Kim, and H. Cantor, "Genetic disruption of $CD8^+$ Treg activity enhances the immune response to viral infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 52, pp. 21089–21094, 2013.

[36] R. A. Vilchez, J. Dauber, and S. Kusne, "Infectious etiology of bronchiolitis obliterans: the respiratory viruses connection—myth or reality?" *American Journal of Transplantation*, vol. 3, no. 3, pp. 245–249, 2003.

[37] E. Hosseini, A. P. Schwarer, and M. Ghasemzadeh, "Do human leukocyte antigen E polymorphisms influence graft-versus-leukemia after allogeneic hematopoietic stem cell transplantation?" *Experimental Hematology*, vol. 43, no. 3, pp. 149–157, 2015.

[38] D. E. Geraghty, M. Stockschleader, A. Ishitani, and J. A. Hansen, "Polymorphism at the HLA-E locus predates most HLA-A and -B polymorphism," *Human Immunology*, vol. 33, no. 3, pp. 174–184, 1992.

[39] C. Grimsley and C. Ober, "Population genetic studies of HLA-E: evidence for selection," *Human Immunology*, vol. 52, no. 1, pp. 33–40, 1997.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 1343760, 6 pages
http://dx.doi.org/10.1155/2016/1343760

Research Article

Polymorphism rs3828903 within *MICB* Is Associated with Susceptibility to Systemic Lupus Erythematosus in a Northern Han Chinese Population

Yue-miao Zhang,[1,2,3,4] Xu-jie Zhou,[1,2,3,4] Fa-juan Cheng,[1,2,3,4] Yuan-yuan Qi,[1,2,3,4] Ping Hou,[1,2,3,4] Ming-hui Zhao,[1,2,3,4] and Hong Zhang[1,2,3,4]

[1]*Renal Division, Peking University First Hospital, Beijing 100034, China*
[2]*Peking University Institute of Nephrology, Beijing 100034, China*
[3]*Key Laboratory of Renal Disease, Ministry of Health of China, Beijing 100034, China*
[4]*Key Laboratory of Chronic Kidney Disease Prevention and Treatment, Peking University, Ministry of Education, Beijing 100034, China*

Correspondence should be addressed to Hong Zhang; hongzh@bjmu.edu.cn

Received 22 March 2016; Revised 5 June 2016; Accepted 9 June 2016

Academic Editor: Roberta Rizzo

Objectives. The variant rs3828903 within *MICB*, a nonclassical *MHC* class I chain-related gene, was detected to contribute to systemic lupus erythematosus (SLE) in a Caucasian population. This study aimed to investigate the association in a northern Han Chinese population. *Methods*. We recruited 1077 SLE patients and 793 controls for analysis. rs3828903 was genotyped by TaqMan allele discrimination assay. Using the public databases, its functional annotations and gene differential expression analysis of *MICB* were evaluated. *Results*. Significant association between the allele G of rs3828903 and risk susceptibility to SLE was observed after adjusting for sex and age ($P = 1.87 \times 10^{-2}$). *In silico* analyses predicted a higher affinity to transcription factors for allele G (risk) and *cis*-expression quantitative trait loci (*cis*-eQTL) effects of rs3828903 in multiple tissues (P ranging from 2.79×10^{-6} to 6.27×10^{-38}). Furthermore, higher mRNA expressions of *MICB* were observed in B cells, monocytes, and renal biopsies from SLE patients compared to controls. *Conclusion*. An association between rs3828903 and susceptibility to SLE has been detected in a Chinese population. This together with the functional annotations of rs3828903 converts *MICB* into a main candidate in the pathogenesis of SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by diverse clinical performances and outcomes [1]. Although its exact pathogenesis remains to be unclear, a number of studies have suggested the genetic component in the pathogenesis of SLE [2].

A significant association between major histocompatibility complex (*MHC*) locus and SLE susceptibility has been detected and validated in multiple populations [3–5]. However, compared with the classical *MHC* genes, the data is still limited about the roles of nonconventional *MHC* genes in SLE. A high-density single nucleotide polymorphism (SNP) screening of *MHC* in SLE demonstrated strong evidence for independent susceptibility regions, including rs3828903 within *MICB*, in a Caucasian population [6]. *MICB* belongs to a family of genes located in the *MHC* class I region, which encodes a stress-induced molecule involved in both innate and adaptive immunity. Its receptor NKG2D is mostly expressed on all natural killer (NK) cells and on subsets of NKT, CD8+ $\alpha\beta$, and $\gamma\delta$ T cells [7]. The NKG2D/MIC interaction was engaged in the pathogenesis of various autoimmune diseases [8–10] by altering their activity, including SLE.

MICB is known to be polymorphic. Significantly, several polymorphisms of *MICB* have been reported to modify the level of gene expression by altering the binding of transcription factors [11], suggesting that a profound dysregulation of *MICB* expression may cause autoreactive T-cell stimulation. This, in turn, underlies relevant differences in the natural immune response against infections or tumor transformation and autoimmune diseases [12]. rs3828903 is a regulatory variant within *MICB*. In spite of the fact that rs3828903 has been reported to be associated with the susceptibility to SLE [6], there is no information about its functionality or expression. Thus, further studies in different populations are warranted to confirm this finding. What is more important is that functional analyses are necessary in order to study the characteristics of rs3828903 and how it may affect the autoimmune response observed in SLE patients.

The present study was conducted to investigate whether there is also an association between *MICB* polymorphism rs3828903 and susceptibility to SLE in a northern Han Chinese population. Furthermore, using the public databases, the functional annotations of rs3828903 and gene differential expression analyses of *MICB* were evaluated.

2. Patients and Methods

2.1. Study Population. To identify the association of rs3828903 with SLE, a total of 1077 patients with SLE (31.55 ± 12.95 years, 883 females) who were of Han ethnicity living in north of China were enrolled in this study. The controls were 793 geographically and ethnically matched healthy blood donors (29.38 ± 13.15 years, 257 females).

All the patients met the revised SLE criteria of the American College of Rheumatology (ACR) [13]. The study was approved by the Ethic Review Committee of Peking University First Hospital. All subjects gave a written informed consent.

2.2. SNP Selection and Genotyping. The SNP rs3828903 within *MICB*, which was reported to be associated with SLE in a Caucasian population [6], was selected for association analysis. It was genotyped using a TaqMan allele discrimination assay (assay ID: AH0JEOO; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primers are as follows: forward 5′-GGTGGGATAGGGTGAGGAGATC-3′ and reverse 5′-GGAAACCATAGCTCCCACAATCTA-3′. The reporter sequences include VIC 5′-CACCACCTCCATTTC-3′ and FAM 5′-ACCACCCCCATTTC-3′.

2.3. Computational Assessment of rs3828903. The DNA features and regulatory elements of the regions that contain rs3828903 were identified by searching HaploReg v4.1 database (http://www.broadinstitute.org/mammals/haploreg/haploreg.php) and RegulomeDB database (http://regulome.stanford.edu/). Using HaploReg v4.1 database, the variant effect of rs3828903 on regulatory motifs was quantified as the difference of LOD (alt) − LOD (ref). A negative score suggested a relatively higher affinity for the reference sequence, while a positive score indicated a relatively higher affinity for the alternative. Besides, the *cis*-expression quantitative trait loci (*cis*-eQTL) effect of rs3828903 was summarized.

2.4. Gene Differential Expression Analysis of MICB. Using the ArrayExpress Archive database (http://www.ebi.ac.uk/arrayexpress/), gene differential expression analyses of *MICB* were checked in immune cell subsets and renal biopsies from SLE patients and healthy controls. In detail, the analysis data was derived from large-scale genome-wide gene expression analyses which were conducted in peripheral blood mononuclear cells (PBMCs) (E-GEOD-50772), B cells (E-GEOD-4588), CD3+ T cells (E-GEOD-13887), CD4+ T cells (E-GEOD-4588), monocytes (E-GEOD-46907), monocytes from healthy donors incubated with SLE sera (E-GEOD-46920), and tubulointerstitial and glomeruli samples (E-GEOD-32591).

2.5. Statistical Analyses. Significant deviation from the Hardy-Weinberg equilibrium in the controls ($P < 0.05$) was excluded. Statistical power was estimated using the software Power and Sample Size Calculations Version 3.0 (http://biostat.mc.vanderbilt.edu/PowerSampleSize) with a two-sided type I error rate of 0.05. To assess the possible association of rs3828903 with SLE, the allelic distribution between cases and controls was analyzed using the chi-square test. The odds ratio (OR) was provided with 95% confidence interval (95% CI). The age and sex were adjusted by logistic regression analysis. Quantitative variables with a normal distribution were expressed as means and standard deviations and the independent-samples t-test (2 groups) was used for analysis. Statistical analyses were performed with SPSS 16.0 software (SPSS Inc., Chicago, IL). A two-tailed P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Polymorphism rs3828903 Was Significantly Associated with SLE. The call rate for rs3828903 was 99.20% and the SNP was in the Hardy-Weinberg equilibrium in both cases and controls ($P > 0.05$). Taking into account the expected frequency of rs3828903 risk allele G (58.0%) in the general population, the combined set of 1,077 SLE cases and 793 controls provided a power of 96.0% to detect an association between SLE and the variant, with an OR of 1.4 at the 5% significance level.

The frequency of the risk allele G of rs3828903 was significantly higher in SLE patients as compared with healthy controls (62.26% versus 57.25%; OR = 1.23, 95% CI = 1.07 to 1.42, $P = 4.75 \times 10^{-3}$). And logistic regression analysis adjusting for sex and age also suggested a significant association between rs3828903 and SLE (OR = 1.30, 95% CI = 1.05 to 1.62, $P = 1.81 \times 10^{-2}$), indicating its potential role in the pathogenesis of SLE.

3.2. Various Regulatory Effects of rs3828903 Were Predicted. In HaploReg v4.1 database, rs3828903 was predicted to locate in promoter histone marks, enhancer histone marks, DNase,

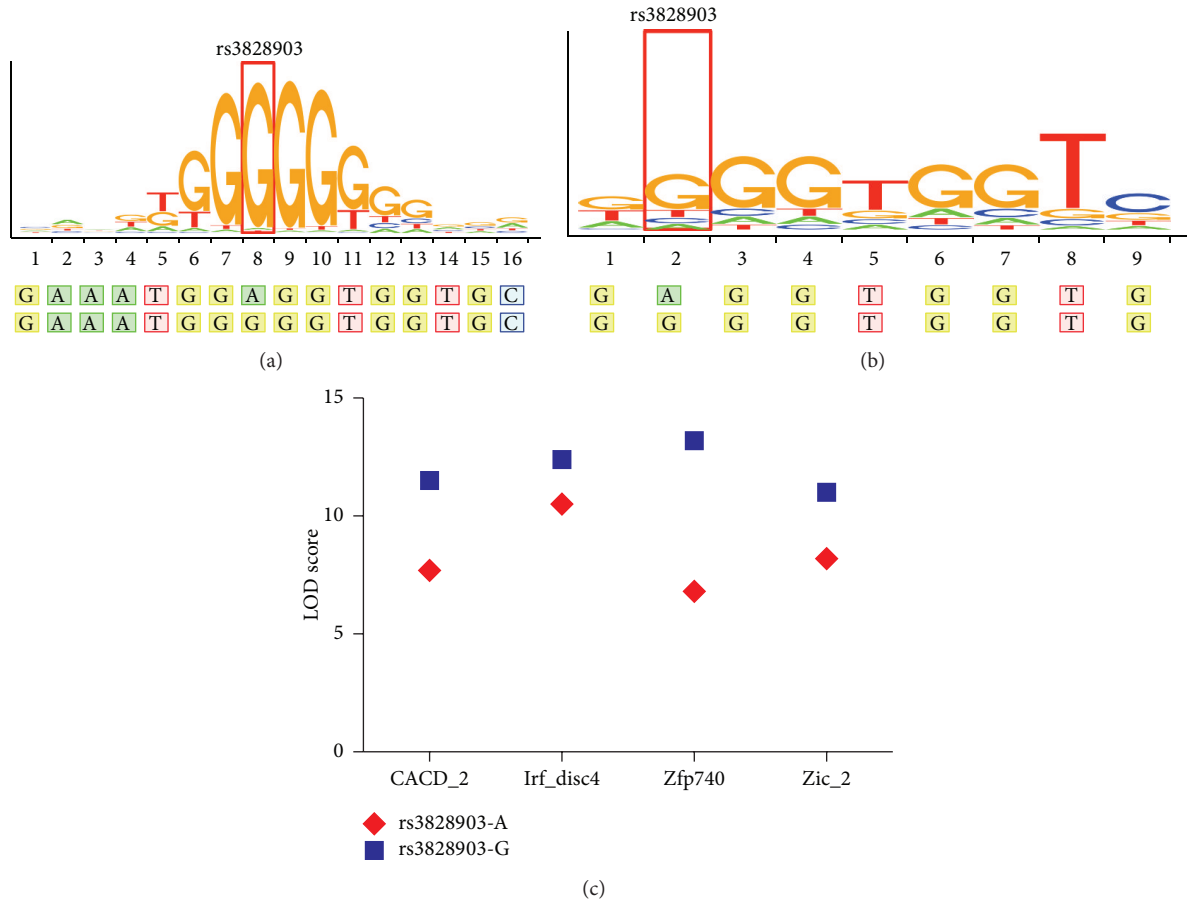


Figure 1: Systemic lupus erythematosus-associated rs3828903 predicted to be part of the motifs for Zfp740 and Zic_2 in both HaploReg v4.1 and RegulomeDB databases. (a, b) Degeneracy within the 16- and 9-base motifs is illustrated at all positions by the stacked letters at each position. The relative height of each letter is proportional to its overenrichment in the motif. A line is boxed around rs3828903-G; this systemic lupus erythematosus-associated risk allele G is predicted to form the 8th and the second nucleotide in the motifs. (c) Altering the rs3828903 allele from rs3828903-G to rs3828903-A decreases the binding affinity for transcription factors CACD_2, Irf_disc4, Zfp740, and Zic_2 in HaploReg v4.1 database.

proteins bound, and motifs changed regions within *MICB*. Four binding site motifs span the rs3828903 region for binding by the transcription factors (TFs) CACD_2, Irf_disc4, Zfp740, and Zic_2. The differences between the LOD scores for the alleles A and G (reference) were −3.8, −1.9, −6.4, and −2.8 for CACD_2, Irf_disc4, Zfp740, and Zic_2, respectively (Figure 1). Therefore, this model predicted a higher affinity to TFs for allele G (risk) relative to allele A. Also, in RegulomeDB database, rs3828903 showed a high score (1f, eQTL + TF binding/DNase peak). Consistent with the HaploReg v4.1 database, the motifs for binding by the TFs Zfp740 and Zic_2 have also been observed in RegulomeDB database (Figure 1), suggesting its potential role for gene expression regulation.

Considering the regulatory effects mentioned above, the *cis*-eQTL effect of rs3828903 has been validated in multiple tissues, including 12 tissues derived from a subset of 1641 samples across 43 sites from 175 individuals and nontransformed peripheral blood samples from 5311 and 1469 unrelated individuals (Table 1). The variant rs3828903 has been detected to affect the expression of *MICB* significantly (with P values ranging from 2.79×10^{-6} to 6.27×10^{-38}). Particularly, with an increase in sample size, the association fit was reinforced. This was particularly true for the study, which contained data from 5311 individuals.

3.3. Higher Expression Levels of MICB Were Observed in SLE. Using the ArrayExpress Archive database, we further ascertained whether *MICB* was expressed differently in SLE patients and healthy controls. As was shown in Figure 2, *MICB* mRNA expression was significantly or marginally significantly upregulated in SLE B cells (489.80 ± 95.50 versus 352.66 ± 96.13; $P = 1.31 \times 10^{-2}$; 7 SLE patients versus 9 controls), monocytes (1661.14 ± 532.87 versus 1065.38 ± 220.72; $P = 4.97 \times 10^{-2}$; 5 SLE patients versus 5 controls), tubulointerstitial samples (4.33 ± 0.22 versus 4.21 ± 0.16; $P = 7.45 \times 10^{-2}$; 32 SLE patients versus 15 controls), and glomeruli samples (7.92 ± 0.52 versus 6.77 ± 0.23; $P = 2.18 \times 10^{-13}$; 32 SLE patients versus 14 controls). Interestingly, although with a rather small sample size, a marginally significantly higher expression level of *MICB* has been observed in monocytes

Table 1: *cis*-eQTL effect of rs3828903 in multiple tissues in HaploReg v4.1 database.

Study	PMID	Tissue	Number	*P* value
GTEx2015_v6	25954001	Adipose_Subcutaneous	94	6.17×10^{-18}
GTEx2015_v6	25954001	Artery_Aorta	24	4.81×10^{-14}
GTEx2015_v6	25954001	Artery_Coronary	9	6.96×10^{-8}
GTEx2015_v6	25954001	Artery_Tibial	112	3.45×10^{-17}
GTEx2015_v6	25954001	Breast_Mammary_Tissue	27	1.16×10^{-9}
GTEx2015_v6	25954001	Cells_Transformed_fibroblasts	14	5.94×10^{-6}
GTEx2015_v6	25954001	Esophagus_Gastroesophageal_Junction	—	4.67×10^{-7}
GTEx2015_v6	25954001	Esophagus_Mucosa	18	2.05×10^{-14}
GTEx2015_v6	25954001	Esophagus_Muscularis	20	2.03×10^{-8}
GTEx2015_v6	25954001	Heart_Atrial_Appendage	25	2.79×10^{-6}
GTEx2015_v6	25954001	Nerve_Tibial	88	9.55×10^{-7}
GTEx2015_v6	25954001	Skin_Sun_Exposed_Lower_leg	96	2.00×10^{-10}
Westra2013	24013639	Whole_Blood	5311	6.27×10^{-38}
Fehrmann2011	21829388	Whole_Blood	1469	9.70×10^{-10}

from healthy donors incubated with SLE sera compared to those incubated with autologous serum (1047.50 ± 494.43 versus 300.40 ± 48.88; $P = 5.98 \times 10^{-2}$; 3 SLE patients versus 3 controls), while there was no difference of *MICB* mRNA expression in PBMC (1577.45 ± 488.74 versus 1610.67 ± 325.23; $P = 0.78$; 61 SLE patients versus 20 controls), CD3+ T cells (2195.09 ± 865.77 versus 1900.54 ± 715.70; $P = 0.35$; 10 SLE patients versus 17 controls), and CD4+ T cells (495.56 ± 144.59 versus 401.59 ± 94.80; $P = 0.12$; 8 SLE patients versus 10 controls) (Figure 2).

4. Discussion

In this study, a significant association between G allele of rs3828903 and the risk susceptibility to SLE has been detected. The risk G allele showed a higher affinity to the TFs, which significantly affects the expression level of *MICB*. Accordingly, a significantly higher expression level of *MICB* has been observed in SLE patients compared with controls, suggesting the important role of *MICB* in SLE.

SLE is a complex autoimmune disease with periods of waning disease activity and intermittent flares. Various external factors, such as infection, smoking, and ultraviolet light, were suggested to be involved in the disease pathogenesis. MICB belongs to a "stress-induced" family of MHC I-like proteins, which is generally expressed in normal tissues and monocytes. It can be induced by stress, such as heat shock [14], oxidative stress [15], viral and bacterial infections [16], DNA damage [17], and tumorigenesis [17], acting as danger signals to alert NK cells and the subsets of NKT, CD8+ $\alpha\beta$, and $\gamma\delta$ T cells through engagement of the NKG2D activating receptor [7]. As it has been widely accepted that NKG2D/MIC interaction is essential for NK cells and CD8+ T cells to sense the abnormal cell and subsequently eliminate it, MICB was regarded to play an important role in immune regulation in the pathogenesis of SLE. Epstein-Barr virus (EBV) is one of the most common infections in SLE, and suppression of *MICB* expression is employed by Epstein-Barr virus to escape NK cell recognition [18]. However, in the present study, it is a pity that we had no data about EBV positivity available for our patients cohort. In spite of the fact that no starting assumptions about disease pathogenesis are required except that genetic variation contributes to disease and that, starting from this recognition, the genes that are causally related to disease pathophysiology can be reliably identified, it should be of special interests for future pathogenesis studies to investigate if there are differences about EBV positivity between SLE and control subjects, since a huge amount of the world population is positive for EBV.

In the present study, we observed a significant association between the risk allele G of rs3828903 within *MICB* and SLE. The HaploReg v4.1 and RegulomeDB databases predicted a much higher affinity between the factor-binding site of rs3828903 risk allele G and the TFs, which could be the cause of its higher transcription level of *MICB* found in *in silico* analyses. Moreover, higher expressions in B cells, monocytes, and renal biopsies from SLE patients have been observed which may contribute to disease progression through activating NK cells and costimulating effector T cells. However, the differential gene expression of *MICB* was not observed in PBMC and T-cell subsets from SLE patients, which may be due to the fact that *MICB* was mainly expressed in normal tissues and monocytes.

To conclude, we have found that the allele G of rs3828903 was significantly associated with risk susceptibility to SLE in the current population. These data together with the functional annotations of rs3828903 convert *MICB* into a main candidate for being an additional *MHC* gene associated with SLE susceptibility.

Competing Interests

The authors declare no competing interests regarding the publication of this paper.

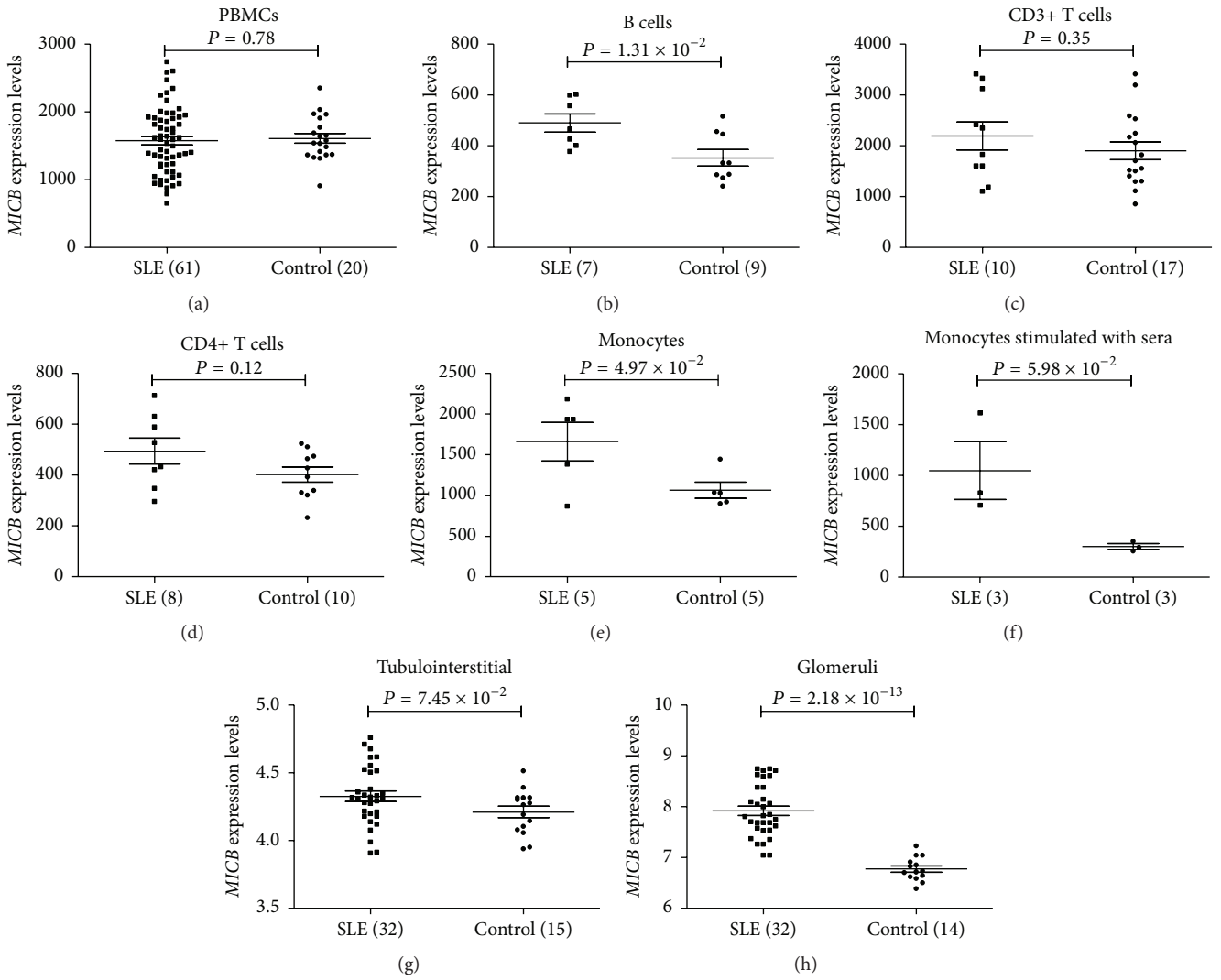


Figure 2: Gene differential expression analyses of *MICB* in immune cell subsets and renal biopsies from SLE patients and controls. (a)–(h) present the *MICB* expression levels in immune cell subsets and renal biopsy samples from SLE patients and normal donor controls. *MICB* mRNA expression was significantly or marginally significantly upregulated in SLE B cells (489.80 ± 95.50 versus 352.66 ± 96.13; $P = 1.31 \times 10^{-2}$; (b)), monocytes (1661.14 ± 532.87 versus 1065.38 ± 220.72; $P = 4.97 \times 10^{-2}$; (e)), tubulointerstitial samples (4.33 ± 0.22 versus 4.21 ± 0.16; $P = 7.45 \times 10^{-2}$; (g)), and glomeruli samples (7.92 ± 0.52 versus 6.77 ± 0.23; $P = 2.18 \times 10^{-13}$; (h)). Although with a rather small sample size, a marginally significantly higher expression level of *MICB* has been observed in monocytes from healthy donors incubated with SLE sera compared to those incubated with autologous serum (1047.50 ± 494.43 versus 300.40 ± 48.88; $P = 5.98 \times 10^{-2}$; (f)), while there was no difference of *MICB* mRNA expression in PBMC (1577.45 ± 488.74 versus 1610.67 ± 325.23; $P = 0.78$; (a)), CD3+ T cells (2195.09 ± 865.77 versus 1900.54 ± 715.70; $P = 0.35$; (c)), and CD4+ T cells (495.56 ± 144.59 versus 401.59 ± 94.80; $P = 0.12$; (d)). PBMCs: peripheral blood mononuclear cells; SLE: systemic lupus erythematosus. The expression data of *MICB* was captured from ArrayExpress Archive database (http://www.ebi.ac.uk/arrayexpress/).

Authors' Contributions

Dr. Yue-miao Zhang and Dr. Xu-jie Zhou contributed equally to this work.

Acknowledgments

This work was supported by grants from Beijing Natural Science Foundation (7152148), National Natural Science Foundation of China (81570629), Chinese Society of Nephrology (15020030591), Beijing Municipal Science and Technology for the Outstanding PhD Program (20121000110), Natural Science Fund of China to the Innovation Research Group (81021004), and National Key Technology R&D Program (2013BAI09B14).

References

[1] L. S. H. Lim, S. J. Lee, B. M. Feldman et al., "Systematic review of the quality of prognosis studies in systemic lupus

erythematosus," *Arthritis Care and Research*, vol. 66, no. 10, pp. 1536–1541, 2014.

[2] C.-F. Kuo, M. J. Grainge, A. M. Valdes et al., "Familial aggregation of systemic lupus erythematosus and coaggregation of autoimmune diseases in affected families," *JAMA Internal Medicine*, vol. 175, no. 9, pp. 1518–1526, 2015.

[3] J.-W. Han, H.-F. Zheng, Y. Cui et al., "Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus," *Nature Genetics*, vol. 41, no. 11, pp. 1234–1237, 2009.

[4] W. Yang, N. Shen, D.-Q. Ye et al., "Genome-wide association study in asian populations identifies variants in ETS1 and WDFY4 associated with systemic lupus erythematosus," *PLoS Genetics*, vol. 6, no. 2, Article ID e1000841, 2010.

[5] J. B. Harley, M. E. Alarcón-Riquelme, L. A. Criswell et al., "Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci," *Nature Genetics*, vol. 40, no. 2, pp. 204–210, 2008.

[6] L. F. Barcellos, S. L. May, P. P. Ramsay et al., "High-density SNP screening of the major histocompatibility complex in systemic lupus erythematosus demonstrates strong evidence for independent susceptibility regions," *PLoS Genetics*, vol. 5, no. 10, Article ID e1000696, 2009.

[7] R. A. Eagle and J. Trowsdale, "Promiscuity and the single receptor: NKG2D," *Nature Reviews Immunology*, vol. 7, no. 9, pp. 737–744, 2007.

[8] D. Yang, H. Wang, B. Ni et al., "Mutual activation of CD4+ T cells and monocytes mediated by NKG2D-MIC interaction requires IFN-gamma production in systemic lupus erythematosus," *Molecular Immunology*, vol. 46, no. 7, pp. 1432–1442, 2009.

[9] V. Groh, A. Brühl, H. El-Gabalawy, J. L. Nelson, and T. Spies, "Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 16, pp. 9452–9457, 2003.

[10] M. Allez, V. Tieng, A. Nakazawa et al., "$CD4^+NKG2D^+$ T cells in crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions," *Gastroenterology*, vol. 132, no. 7, pp. 2346–2358, 2007.

[11] S. Rodríguez-Rodero, S. González, L. Rodrigo et al., "Transcriptional regulation of MICA and MICB: a novel polymorphism in MICB promoter alters transcriptional regulation by Sp1," *European Journal of Immunology*, vol. 37, no. 7, pp. 1938–1953, 2007.

[12] S. Rodriguez-Rodero, L. Rodrigo, J. L. Fdez-Morera et al., "MHC class I chain-related gene B promoter polymorphisms and celiac disease," *Human Immunology*, vol. 67, no. 3, pp. 208–214, 2006.

[13] E. M. Tan, A. S. Cohen, J. F. Fries et al., "The 1982 revised criteria for the classification of systemic lupus erythrematosus," *Arthritis and Rheumatism*, vol. 25, no. 11, pp. 1271–1277, 1982.

[14] V. Groh, S. Bahram, S. Bauer, A. Herman, M. Beauchamp, and T. Spies, "Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 22, pp. 12445–12450, 1996.

[15] G. M. Venkataraman, D. Suciu, V. Groh, J. M. Boss, and T. Spies, "Promoter region architecture and transcriptional regulation of the genes for the MHC class I-related chain A and B ligands of NKG2D," *The Journal of Immunology*, vol. 178, no. 2, pp. 961–969, 2007.

[16] C. Watzl, "The NKG2D receptor and its ligands-recognition beyond the 'missing self'?" *Microbes and Infection*, vol. 5, no. 1, pp. 31–37, 2003.

[17] S. González, A. López-Soto, B. Suarez-Alvarez, A. López-Vázquez, and C. López-Larrea, "NKG2D ligands: key targets of the immune response," *Trends in Immunology*, vol. 29, no. 8, pp. 397–403, 2008.

[18] D. Nachmani, N. Stern-Ginossar, R. Sarid, and O. Mandelboim, "Diverse herpesvirus MicroRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells," *Cell Host and Microbe*, vol. 5, no. 4, pp. 376–385, 2009.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 6865758, 7 pages
http://dx.doi.org/10.1155/2016/6865758

Review Article

Immunoregulatory Role of HLA-G in Allergic Diseases

Giuseppe Murdaca,[1] Paola Contini,[1] Simone Negrini,[1] Giorgio Ciprandi,[2] and Francesco Puppo[1]

[1]*Department of Internal Medicine, Scleroderma Unit, Clinical Immunology Unit, University of Genova, 16132 Genova, Italy*
[2]*Department of Internal Medicine, Respiratory and Allergy Diseases Unit, University of Genova, 16132 Genova, Italy*

Correspondence should be addressed to Giuseppe Murdaca; giuseppe.murdaca@unige.it

Received 26 April 2016; Accepted 25 May 2016

Academic Editor: Nathalie Rouas-Freiss

Allergic diseases are sustained by a T-helper 2 polarization leading to interleukin-4 secretion, IgE-dependent inflammation, and mast cell and eosinophil activation. HLA-G molecules, both in membrane-bound and in soluble forms, play a central role in modulation of immune responses. Elevated levels of soluble HLA-G (sHLA-G) molecules are detected in serum of patients with allergic rhinitis to seasonal and perennial allergens and correlate with allergen-specific IgE levels, clinical severity, drug consumption, and response to allergen-specific immunotherapy. sHLA-G molecules are also found in airway epithelium of patients with allergic asthma and high levels of sHLA-G molecules are detectable in plasma and bronchoalveolar lavage of asthmatic patients correlating with allergen-specific IgE levels. Finally, HLA-G molecules are expressed by T cells, monocytes-macrophages, and Langerhans cells infiltrating the dermis of atopic dermatitis patients. Collectively, although at present it is difficult to completely define the role of HLA-G molecules in allergic diseases, it may be suggested that they are expressed and secreted by immune cells during the allergic reaction in an attempt to suppress allergic inflammation.

1. Introduction

The human major histocompatibility complex (HLA) encodes two sets of HLA class I molecules, which have been termed class Ia (or classical) and class Ib (or nonclassical) molecules. The class Ia antigens include the gene products of HLA-A, HLA-B, and HLA-C loci and are characterized by a broad tissue expression and by a high degree of polymorphism [1]. The class Ib antigens include the gene products of HLA-E, HLA-F, and HLA-G loci and are characterized by a restricted tissue distribution and by a limited polymorphism [2]. The encoding genes for classical and nonclassical HLA class I molecules are located on chromosome 6p21 [3]. Since its original description in the 1980s in choriocarcinoma cells and primary cytotrophoblast cells in the placenta [4–6], considerable evidence supports a central role for HLA-G in the suppression of immune responses, long-term immune escape, or tolerance and modulation of inflammation. Allergic diseases are considered as immunoregulatory disorders with decreased tolerance towards allergens [7]. In this review, we discuss the current knowledge and the potential role of HLA-G in allergic diseases.

2. HLA-G Structure

Seven HLA-G isoforms generated by alternative splicing of the primary HLA-G transcript have been characterized. Four of them, HLA-G1, HLA-G2, HLA-G3, and HLA-G4, are bound to the cell surface, while the remaining three, HLA-G5, HLA-G6, and HLA-G7, are detectable in soluble form (sHLA-G). The membrane HLA-G1 molecule, which is derived from the translation of the total HLA-G transcript, and its soluble counterpart HLA-G5 (sHLA-G) have a structure identical to that of classical HLA-A, HLA-B, and HLA-C antigens whereas the other isoforms are smaller lacking one or two domains [8–11]. Truncated isoforms are generated by excision of one or two exons encoding globular domains (G1–G4), while translation of either intron 4 or intron 2 yields sHLA-G5–G7 that lack the transmembrane domain [12]. Furthermore, sHLA-G1 isoform (shed G1 or sHLA-G1)

derives from the proteolytic cleavage of the membrane bound HLA-G1. Both HLA-G1 and HLA-G5 have an extracellular structure that is similar to other classical HLA class I molecules: a heavy chain of 3 globular domains that are linked noncovalently to β2-microglobulin. By contrast, the other truncated isoforms have a structure similar to HLA class II and do not bind β2-microglobulin. Both HLA-G1 and HLA-G5 can homodimerize either in association with β2-microglobulin or as free heavy chains. These dimers bind the LIL-RB1/2 (ILT-2/4) receptors for HLA-G with greater affinity as compared with monomeric HLA-G [13, 14]. HLA-G1 and HLA-G5 have been found outside the placenta. HLA-G generally plays immunosuppressive functions and does so by several mechanisms [15]. Membrane-bound HLA-G1 and soluble HLA-G1 and HLA-G5 inhibit uterine and peripheral NK cell activation, CD8+ T-cell mediated cytolysis, and CD4+ T-cell alloproliferative responses. Furthermore, HLA-G may also downregulate alloproliferative responses, induce immune tolerance by promoting the expansion of CD4+ CD25+ FoxP3+ T regulatory (Treg) lymphocytes, and trigger the differentiation of CD4+ T-cells in suppressor cells [15]. While originally described as "highly" restricted in its tissue expression, constitutive expression of HLA-G1 and/or HLA-G5 has been recognized in a number of tissues including thymic medulla, pancreatic islet cells, and peripheral CD14+ mononuclear cells [16, 17]. Both the promoter and the 3′ untranslated region (UTR) of HLA-G are highly polymorphic [5, 6]. Three variations in the 3′UTR, a 14 bp insertion/deletion [18–23], a single nucleotide polymorphism (SNP) substituting guanine at +3142 [24, 25], and a SNP substituting adenine at +3187 [24], can lead to differences in HLA-G expression [12, 25]. However, some polymorphic sites in the 3′UTR of HLA-G interfere with mRNA stability, alternative splicing, and binding of specific microRNAs (miRs) thereby modulating HLA-G mRNA and/or protein expression [26]. Indeed, overexpression of these miRs had also functional activities, since these miRs could directly downregulate HLA-G mRNA and/or HLA-G surface expression. Furthermore, cytokines such as interferon- (IFN-) γ and interleukin- (IL-) 10 trigger the expression of HLA-G by peripheral blood mononuclear cells (PBMCs) [27, 28]. IL-10 is a key regulator of immune and inflammatory responses and HLA-G plays an essential role in fetomaternal tolerance by inhibiting lysis by maternal NK cells. IL-10 enhances steady-state levels of HLA-G transcription in cultured trophoblast cells upregulating HLA-G cell surface expression in this cell type. Moreover, IL-10 is able to enhance HLA-G expression and to downregulate classical HLA class I and class II antigens on monocytes, thus regulating NK cells and T lymphocyte responses. These characteristics suggest that IL-10 could be proposed as immunosuppressor agent in the treatment of transplantation rejection and autoimmune diseases [27, 28].

3. HLA-G and Allergic Rhinitis

Allergic rhinitis (AR) is sustained by mucosal IgE-dependent inflammation which is promoted, maintained, and amplified by T-helper (Th) 2 cells [29]. The mucosal inflammation is characterized by mast cell and eosinophil activation. Interleukin- (IL-) 4 is a pivotal cytokine that orchestrates allergic inflammation, because it is the most important signal to induce Th2 polarization in allergic patients. IL-4 and IL-13 promote IgE synthesis, upregulate adhesion molecules selective for eosinophil recruitment, and cause increased mucous production and airway hyperreactivity [30–32]. Furthermore, peripheral blood mononuclear cells of AR patients predominantly produce IL-4 [33]. However, a defect in Treg lymphocytes has been demonstrated in allergic patients [7, 34]. Therefore, allergic diseases are considered as immunoregulatory disorders with reduced tolerance towards allergens [7]. These pathophysiologic events promote the production of allergen-specific IgE.

Our group investigated sHLA-A, sHLA-B, sHLA-C, and sHLA-G serum levels in AR patients allergic to *Betula alba*, *Parietaria judaica*, and Graminaceae [35]. sHLA-A, sHLA-B, and sHLA-C serum levels were significantly higher in AR patients as compared to healthy controls (1309 ± 73.3 ng/mL and 1001 ± 145.7 ng/mL, resp., $p = 0.011$). sHLA-G serum levels were also significantly higher in AR patients than in healthy controls (35.86 ± 2.7 ng/mL and 12.79 ± 2.4 ng/mL, resp., $p < 0.0001$). Moreover, we found a moderate but significant correlation between sHLA-G and sHLA-A, sHLA-B, and sHLA-C levels in AR patients ($r = 0.37$). Serum sHLA-G levels were also evaluated in patients with AR due to perennial allergens including house dust mite and cat and dog dandruff [36, 37]. Clinical severity was evaluated with a validated visual analogue scale (VAS) for quantifying the perception of nasal symptoms intensity and drug consumption at the end of the pollen season [38]. The serum levels of sHLA-G molecules resulted as significantly higher in patients with perennial AR than in healthy controls ($p < 0.0001$). Notably, there was a strong correlation between sHLA-G serum levels, VAS score ($r = 0.850$, $p < 0.001$), and drug use ($r = 0.793$, $p < 0.001$). Notably, a significant even though weak correlation between serum sHLA-A, sHLA-B, and sHLA-C levels and VAS was also observed ($r = 0.309$, $p = 0.016$) but not between serum sHLA-A, sHLA-B, and sHLA-C levels and drug consumption [37]. A further study showed that serum sHLA-G levels were significantly associated with allergen-specific IgE levels both in allergic rhinitis ($r = 0.468$ and $p = 0.003$) and in allergic asthma ($r = 0.479$ and $p = 0.006$) patients [39]. Finally, serum sHLA-G levels were higher in patients with seasonal allergy than in those with perennial allergy ($p = 0.0194$) [40]. Data from another group confirmed that serum sHLA-G levels are significantly higher also in children with allergic diseases [41].

AR management includes patient education, allergen avoidance, drug treatment, and, when appropriate, allergen-specific immunotherapy [42]. The aim of allergen-specific sublingual immunotherapy (SLIT) is to achieve clinical tolerance to the causal allergen through oral administration of high-dose allergens by shifting Th2 immune response, mainly mediated by IL-4, to Th1 response, mainly mediated by interferon- (IFN-) γ. We evaluated sHLA-G and sHLA-A, sHLA-B, and sHLA-C serum levels before and 3 months after the end of SLIT and correlated their values with IFN-γ production by peripheral blood mononuclear cells [43, 44]. sHLA-G levels decreased from 35.86 ± 2.7 ng/mL to

11.36 ± 1.6 ng/mL ($p < 0.0001$) and sHLA-A, sHLA-B, and sHLA-C levels decreased from 1309 ± 73.3 ng/mL to 695.3 ± 33.2 ng/mL ($p < 0.0001$). Notably, IFN-γ production increased from 44 ± 68 spots before SLIT to 181 ± 89 spots after SLIT ($p < 0.0001$) and significantly correlated with both sHLA-G ($r = -0.39$, $p = 0.023$) and sHLA-A, sHLA-B, and sHLA-C ($r = -0.38$, $p = 0.029$) changes. Furthermore, the percentage changes of sHLA-G and sHLA-A, sHLA-B, and sHLA-C levels were significantly correlated among themselves ($r = 0.84$) and with VAS score ($r = 0.63$ and $p = 0.60$, resp.).

4. HLA-G and Asthma

Persistent airway inflammation, structural remodelling, and bronchial hyperresponsiveness in lower airways are hallmarks of allergic asthma [45, 46]. Allergen-driven activation of Th2 CD4+ T-lymphocytes releasing IL-4, IL-5, and IL-13 perpetuate the inflammation via recruitment of other lymphocytes, eosinophils, and mononuclear cells [47–49]. Genetic factors play a central role in asthma pathogenesis. Indeed, over 100 genes have been implicated in asthma susceptibility [50]. One of such genes may be HLA-G. In fact, HLA-G genetic polymorphisms confer susceptibility to airway hyperresponsiveness and asthma development [3]. The G/G genotype at SNP-964 in the promoter region 4 is associated with asthma in the offspring of mothers with asthma or bronchial hyperresponsiveness (BHR), while the A/A genotype was associated with asthma in the offspring of asthma- and BHR-free mothers [37]. Tan et al. [51] demonstrated that the SNP-964G/A tagged two major promoter haplotype clades with evidence of longstanding balancing selection amongst African Americans, European Americans, and Han Chinese individuals. There is a strong linkage disequilibrium between SNPs in the HLA-G gene. The +3142 C/G SNP, which is located in the 3′UTR of the HLA-G gene, affects the targeting of miR-148a, miR-148b, and miR-152 and interacts with mother's asthma status to determine risk of asthma in the child [24]. Notably, the +3142 (rs10633320) G/G SNP resulted as protective against asthma among offspring of asthmatic mothers whereas the C allele was associated with asthma risk in the offspring of mothers without asthma. Statins upregulate miR-148b and miR-152 and, thus, affect HLA-G expression. It has been found that subjects carrying at least one copy of the G minor allele of the rs10633320 presented a decreased frequency of asthma-related exacerbations (emergency department visit, hospitalizations, or oral corticosteroid use). Moreover, there was no difference in exacerbation frequency between G/G and G/C genotypes [52]. A genome-wide association study (GWAS) confirmed in 6819 participants from the Framingham Heart Study the association of previously described genetic variants in FCERIA, STAT6, and IL-13 and identified potential susceptibility loci in the HLA-A, HLA-G, and HLA-DQA2 gene regions as risk factors for IgE dysregulation and atopy [53]. KIR2DL4 (CD158d) is a member of the killer cell immunoglobulin-like receptor family that is mainly expressed on natural killer (NK) cells and its ligand has been reported to be HLA-G [54–58]. Furthermore, NK-cell derived IFN-γ secretion has been reported to be critical for the generation of tolerogenic dendritic cell (DC) in the placenta [59]. It might be predicted that individuals with the functionally defective 9A allele of KIR2DL4 would not be able to secrete IFN-γ and might therefore be prone to producing Th2-biased immune responses and fewer tolerogenic DC. KIR2DL4 genotypes were analyzed in 2 cohorts of children at high risk for atopic disease [60]. However, there was neither significant relationship between KIR2DL4 genotype and the prevalence of atopy, as assessed by allergen skin prick testing in either cohort at any age, nor was there any significant relationship between KIR2DL4 genotype and the prevalence of wheeze, bronchial hyperreactivity, and asthma. A role for HLA-G in asthma pathogenesis was further suggested by the demonstration of the expression of the sHLA-G5 isoform in the airway epithelium and of increased circulating plasma levels of sHLA-G in children with atopic asthma [61]. Because airway inflammation in asthma involves a Th-2 skewing of lymphocytes similar to pregnancy, HLA-G is an attractive candidate molecule for promoting the immune profile characteristic of asthma. Localization of HLA-G in airway epithelium suggests that it could contribute to immune dysregulation and airway inflammation in chronic asthma. Tahan and Patiroglu [61] measured sHLA-G levels in bronchoalveolar lavage (BAL) of patients with mild persistent asthma and found increased levels as compared to controls ($p = 0.01$). Notably, there was no significant difference in sHLA-G BAL levels in Caucasian individuals as compared to African-American individuals. Furthermore, sHLA-G was present in the epithelium of endobronchial biopsies from 9 of 11 patients with asthma. These findings supported and confirmed a role for sHLA-G in asthma pathogenesis. Zheng et al. [62] confirmed that plasma sHLA-G levels were significantly higher in atopic asthmatic children than in healthy controls ($p < 0.001$). However, no significant association was observed between plasma sHLA-G, total IgE, and allergen-specific IgE levels. Moreover, sHLA-G levels were not significantly related to HLA-G 14 bp insertion/deletion polymorphism both in asthmatic children and in controls. On the contrary, we found a significant association between sHLA-G levels and allergen-specific IgE levels both in AR ($r = 0.468$ and $p = 0.003$) and in asthmatic ($r = 0.479$ and $p = 0.006$) patients [36]. Mapp et al. [63] demonstrated that baseline levels of IL-10 secretion by PBMC in patients with isocyanate-induced asthma and asymptomatic-exposed individuals are higher than those in nonoccupational allergic asthma and in healthy controls ($p < 0.0001$). Spontaneous production of sHLA-G by PBMCs resulted as significantly higher in patients with isocyanate-induced asthma than in the other groups ($p < 0.005$).

5. HLA-G, Allergy and Pregnancy

Rizzo et al. [64] evaluated the potential role of pregnancy and labor on plasma sHLA-G and IL-10 levels in women with AR or asthma and in healthy pregnant women. Plasma samples were obtained during the 3rd trimester of pregnancy,

at delivery, and at a nonpregnant state 2 years postpartum. The plasma levels of sHLA-G1 isoform and IL-10 resulted as significantly increased during labor in comparison with the levels detected during the 3rd trimester and 2 years after delivery ($p < 0.0001$). However, allergic women had lower plasma sHLA-G levels than nonallergic women during the 3rd trimester of pregnancy and at delivery ($p < 0.01$ and $p < 0.05$, resp.). Interestingly, no significant differences were found in samples obtained 2 years after pregnancy. Thymus-and-activation-regulated chemokine (TARC or CCL17) is one of the Th2-inducible chemokines produced by the thymus [65] and by trophoblasts and endometrial gland cells during pregnancy [65]. CCL17 is related to both allergy and pregnancy. Miyahara et al. [66] enrolled 70 paired full-term and normal-vaginal-delivery newborns and their mothers and reported that serum levels of CCL17 were higher in mothers with atopic dermatitis (AD) than in those without AD ($p < 0.001$). High umbilical cord serum levels of CCL17 were associated with infantile AD development ($p < 0.001$). Serum levels of CCL17 ($rs = 0.340$, $p < 0.001$) and sHLA-G ($rs = 0.600$, $p < 0.001$) showed high correlations between umbilical cord and maternal blood.

6. HLA-G and Atopic Dermatitis

Atopic dermatitis (AD) is a chronic disease usually beginning in childhood. AD is characterized by increased production of IL-4, IL-13, and IgE [67]. In AD biopsies, HLA-G positive cells were always found in the papillary and, less frequently, in the reticular dermis [64]. HLA-G was expressed mainly by infiltrating T cells but also, to a lesser extent and less frequently, by monocytes-macrophages or Langerhans cells [68].

7. What Is the Role of HLA-G in the Pathogenesis of Allergic Diseases?

HLA-G molecules have a complex immune regulatory role in transplantation, cancer, viral infections, chronic inflammatory diseases, and pregnancy [8, 9, 69–72]. In general, HLA-G is a tolerance-inducing molecule but it is also a stimulus for Th2 responses and Treg cells activation [73]. Allergic diseases are driven by a Th2-polarized inflammation [74] and allergic patients display a defect in Treg cells which may be restored by specific immunotherapy [34]. At present, it is difficult to completely clarify the role of HLA-G in allergic diseases. It may be suggested that they are expressed and secreted by immune cells during the allergic reaction and may represent a reactive attempt to suppress allergic inflammation. This hypothesis is supported by their increase during immunotherapy and is in keeping with the finding that antigen presenting cells and monocytes expressing HLA-G molecules are able to create a tolerogenic milieu enriched in IL-10 which, in turn, promotes Treg cells activity [75, 76]. HLA-G genetic polymorphisms confer susceptibility to airway hyperresponsiveness and asthma development. In particular, G/G genotype at SNP-964G/A in the promoter region is associated with asthma; the +3142 C/G SNP increases the risk of asthma in the child. By contrast, the +3142 (rs10633320) G/G SNP resulted as protective against asthma development. In conclusion, it could be postulated that increased HLA-G levels could be either compensatory or pathogenetic through mechanisms not yet completely known. Accordingly, it has been recently proposed that HLA-G should be no longer qualified as a "shield" to protect tissues and cells from immune destruction but, rather, as an "immune checkpoint" molecule [77].

Competing Interests

The authors do not have any conflict of interests in this paper.

References

[1] H. L. Ploegh, H. T. Orr, and J. L. Strominger, "Major histocompatibility antigens: the human (HLA-A,-B,-C) and murine (H-2K, H-2D) class I molecules," *Cell*, vol. 24, no. 2, pp. 287–299, 1981.

[2] C. A. O'Callaghan and J. I. Bell, "Structure and function of the human MHC class Ib molecules HLA-E, HLA-F and HLA-G," *Immunological Reviews*, vol. 163, pp. 129–138, 1998.

[3] D. Nicolae, N. J. Cox, L. A. Lester et al., "Fine mapping and positional candidate studies identify HLA-G as an asthma susceptibility gene on chromosome 6p21," *American Journal of Human Genetics*, vol. 76, no. 2, pp. 349–357, 2005.

[4] S. A. Ellis, I. L. Sargent, C. W. G. Redman, and A. J. McMichael, "Evidence for a novel HLA antigen found on human extravillous trophoblast and a choriocarcinoma cell line," *Immunology*, vol. 59, no. 4, pp. 595–601, 1986.

[5] S. A. Ellis, M. S. Palmer, and A. J. McMichael, "Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule," *The Journal of Immunology*, vol. 144, no. 2, pp. 731–735, 1990.

[6] D. E. Geraghty, B. H. Koller, and H. T. Orr, "A human major histocompatibility complex class I gene that encodes a protein with a shortened cytoplasmic segment," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 24, pp. 9145–9149, 1987.

[7] D. T. Umetsu and R. H. DeKruyff, "The regulation of allergy and asthma," *Immunological Reviews*, vol. 212, pp. 238–255, 2006.

[8] G. Murdaca, P. Contini, M. Setti et al., "Soluble human leukocyte antigen-G serum levels in patients with acquired immune deficiency syndrome affected by different disease-defining conditions before and after antiretroviral treatment," *Human Immunology*, vol. 72, no. 9, pp. 712–716, 2011.

[9] G. Murdaca, P. Contini, M. Setti et al., "Behavior of non-classical soluble HLA class G antigens in human immunodeficiency virus 1-infected patients before and after HAART: comparison with classical soluble HLA-A,-B,-C antigens and potential role in immune-reconstitution," *Clinical Immunology*, vol. 133, no. 2, pp. 238–244, 2009.

[10] B. Favier, J. LeMaoult, N. Rouas-Freiss, P. Moreau, C. Menier, and E. D. Carosella, "Research on HLA-G: an update," *Tissue Antigens*, vol. 69, no. 3, pp. 207–211, 2007.

[11] E. D. Carosella, B. Favier, N. Rouas-Freiss, P. Moreau, and J. Lemaoult, "Beyond the increasing complexity of the immunomodulatory HLA-G molecule," *Blood*, vol. 111, no. 10, pp. 4862–4870, 2008.

[12] M. H. Larsen and T. V. F. Hviid, "Human leukocyte antigen-G polymorphism in relation to expression, function, and disease," *Human Immunology*, vol. 70, no. 12, pp. 1026–1034, 2009.

[13] M. Shiroishi, K. Kuroki, T. Ose et al., "Efficient leukocyte Ig-like receptor signaling and crystal structure of disulfide-linked HLA-G dimer," *The Journal of Biological Chemistry*, vol. 281, no. 15, pp. 10439–10447, 2006.

[14] R. Apps, L. Gardner, A. M. Sharkey, N. Holmes, and A. Moffett, "A homodimeric complex of HLA-G on normal trophoblast cells modulates antigen-presenting cells via LILRB1," *European Journal of Immunology*, vol. 37, no. 7, pp. 1924–1937, 2007.

[15] S. R. White, "Human leucocyte antigen-G: expression and function in airway allergic disease," *Clinical and Experimental Allergy*, vol. 42, no. 2, pp. 208–217, 2012.

[16] E. D. Carosella, P. Moreau, J. Le Maoult, M. Le Discorde, J. Dausset, and N. Rouas-Freiss, "HLA-G molecules: from maternal-fetal tolerance to tissue acceptance," *Advances in Immunology*, vol. 81, pp. 199–252, 2003.

[17] V. Rebmann, A. Busemann, M. Lindemann, and H. Grosse-Wilde, "Detection of HLA-G5 secreting cells," *Human Immunology*, vol. 64, no. 11, pp. 1017–1024, 2003.

[18] M. J. Castro, P. Morales, J. Martínez-Laso et al., "Evolution of MHC-G in humans and primates based on three new 3'UT polymorphisms," *Human Immunology*, vol. 61, no. 11, pp. 1157–1163, 2000.

[19] G. A. Harrison, K. E. Humphrey, I. B. Jakobsen, and D. W. Cooper, "A 14 bp deletion polymorphism in the HLA-G gene," *Human Molecular Genetics*, vol. 2, no. 12, article 2200, 1993.

[20] X.-Y. Chen, W.-H. Yan, A. Lin, H.-H. Xu, J.-G. Zhang, and X.-X. Wang, "The 14 bp deletion polymorphisms in HLA-G gene play an important role in the expression of soluble HLA-G in plasma," *Tissue Antigens*, vol. 72, no. 4, pp. 335–341, 2008.

[21] D. Piancatelli, D. Maccarone, G. Liberatore et al., "HLA-G 14-bp insertion/deletion polymorphism in kidney transplant patients with metabolic complications," *Transplantation Proceedings*, vol. 41, no. 4, pp. 1187–1188, 2009.

[22] E. C. Castelli, C. T. Mendes-Junior, N. H. S. Deghaide et al., "The genetic structure of 3′untranslated region of the HLA-G gene: polymorphisms and haplotypes," *Genes and Immunity*, vol. 11, no. 2, pp. 134–141, 2010.

[23] P. Rousseau, M. Le Discorde, G. Mouillot, C. Marcou, E. D. Carosella, and P. Moreau, "The 14 bp deletion-insertion polymorphism in the 3′ UT region of the HLA-G gene influences HLA-G mRNA stability," *Human Immunology*, vol. 64, no. 11, pp. 1005–1010, 2003.

[24] S.-M. Yie, L.-H. Li, R. Xiao, and C. L. Librach, "A single base-pair mutation in the 3′-untranslated region of HLA-G mRNA is associated with pre-eclampsia," *Molecular Human Reproduction*, vol. 14, no. 11, pp. 649–653, 2008.

[25] Z. Tan, G. Randall, J. Fan et al., "Allele-specific targeting of microRNAs to HLA-G and risk of asthma," *American Journal of Human Genetics*, vol. 81, no. 4, pp. 829–834, 2007.

[26] E. A. Donadi, E. C. Castelli, A. Arnaiz-Villena, M. Roger, D. Rey, and P. Moreau, "Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association," *Cellular and Molecular Life Sciences*, vol. 68, no. 3, pp. 369–395, 2011.

[27] Y. Yang, W. Chu, D. E. Geraghty, and J. S. Hunt, "Expression of HLA-G in human mononuclear phagocytes and selective induction by IFN-γ," *The Journal of Immunology*, vol. 156, no. 11, pp. 4224–4231, 1996.

[28] P. Moreau, F. Adrian-Cabestre, C. Menier et al., "IL-10 selectively induces HLA-G expression in human trophoblasts and monocytes," *International Immunology*, vol. 11, no. 5, pp. 803–811, 1999.

[29] S. Romagnani, "Biology of human TH1 and TH2 cells," *Journal of Clinical Immunology*, vol. 15, no. 3, pp. 121–129, 1995.

[30] S. R. Durham, S. Ying, V. A. Varney et al., "Cytokine messenger RNA expression for IL-3, IL-4, IL-5, and granulocyte/macrophage-colony-stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophilia," *The Journal of Immunology*, vol. 148, no. 8, pp. 2390–2394, 1992.

[31] R. P. Schleimer, S. A. Sterbinsky, J. Kaiser et al., "IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium: association with expression of VCAM-1," *Journal of Immunology*, vol. 148, no. 4, pp. 1086–1092, 1992.

[32] O. M. Kon and A. Kay, "'T cells and chronic asthma," *International Archives of Allergy and Immunology*, vol. 118, no. 2–4, pp. 133–135, 1999.

[33] M. Imada, F. Estelle, R. Simons, F. T. Jay, and K. T. HayGlass, "Allergen-stimulated interleukin-4 and interferon-γ production in primary culture: responses of subjects with allergic rhinitis and normal controls," *Immunology*, vol. 85, no. 3, pp. 373–380, 1995.

[34] G. Ciprandi, D. Fenoglio, I. Cirillo et al., "Induction of interleukin 10 by sublingual immunotherapy for house dust mites: a preliminary report," *Annals of Allergy, Asthma and Immunology*, vol. 95, no. 1, pp. 38–44, 2005.

[35] G. Ciprandi, B. M. Colombo, P. Contini et al., "Soluble HLA-G and HLA-A,-B,-C serum levels in patients with allergic rhinitis," *Allergy*, vol. 63, no. 10, pp. 1335–1338, 2008.

[36] G. Ciprandi, P. Contini, G. Murdaca, A. M. Gallina, and F. Puppo, "Soluble HLA-G molecule in patients with perennial allergic rhinitis," *International Archives of Allergy and Immunology*, vol. 150, no. 3, pp. 278–281, 2009.

[37] G. Ciprandi, P. Contini, G. Murdaca, M. DeAmici, A. M. Gallina, and F. Puppo, "Soluble serum HLA-G and HLA-A, -B, -C molecules in patients with seasonal allergic rhinitis exposed to pollens," *International Immunopharmacology*, vol. 9, no. 9, pp. 1058–1062, 2009.

[38] P. J. Bousquet, C. Combescure, F. Neukirch et al., "Visual analog scales can assess the severity of rhinitis graded according to ARIA guidelines," *Allergy*, vol. 62, no. 4, pp. 367–372, 2007.

[39] G. Ciprandi, A. Corsico, and P. Pisati, "Serum-soluble HLA-G is associated with specific IgE in patients with allergic rhinitis and asthma," *Inflammation*, vol. 37, no. 5, pp. 1630–1634, 2014.

[40] G. Ciprandi and M. DeAmici, "Soluble HLA-G serum levels depend on allergy type and IgE levels," *Allergy & Rhinology*, vol. 5, no. 1, pp. 9–11, 2014.

[41] G. Ciprandi, M. De Amici, S. Caimmi et al., "Soluble serum HLA-G in children with allergic rhinitis and asthma," *Journal of Biological Regulators and Homeostatic Agents*, vol. 24, no. 2, pp. 221–224, 2010.

[42] G. Passalacqua and S. R. Durham, "Allergic rhinitis and its impact on asthma update: allergen immunotherapy," *Journal of Allergy and Clinical Immunology*, vol. 119, no. 4, pp. 881–891, 2007.

[43] G. Ciprandi, P. Continia, D. Fenoglio et al., "Relationship between soluble HLA-G and HLA-A,-B,-C serum levels, and interferon-γ production after sublingual immunotherapy in patients with allergic rhinitis," *Human Immunology*, vol. 69, no. 7, pp. 409–413, 2008.

[44] G. Ciprandi, P. Contini, A. Pistorio, G. Murdaca, and F. Puppo, "Sublingual immunotherapy reduces soluble HLA-G and HLA-A,-B,-C serum levels in patients with allergic rhinitis," *International Immunopharmacology*, vol. 9, no. 2, pp. 253–257, 2009.

[45] P. K. Jeffery, "Remodeling in asthma and chronic obstructive lung disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 164, no. 10, part 2, pp. S28–S38, 2001.

[46] P. K. Jeffery, A. J. Wardlaw, F. C. Nelson, J. V. Collins, and A. B. Kay, "Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity," *American Review of Respiratory Disease*, vol. 140, no. 6, pp. 1745–1753, 1989.

[47] B. L. Bradley, M. Azzawi, M. Jacobson et al., "Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness," *The Journal of Allergy and Clinical Immunology*, vol. 88, no. 4, pp. 661–674, 1991.

[48] S. T. Holgate, "The airway epithelium is central to the pathogenesis of asthma," *Allergology International*, vol. 57, no. 1, pp. 1–10, 2008.

[49] I. R. Mackay, F. S. Rosen, and A. B. Kay, "Advances in Immunology: allergy and allergic diseases: second of two parts," *The New England Journal of Medicine*, vol. 344, no. 2, pp. 109–113, 2001.

[50] C. Ober and S. Hoffjan, "Asthma genetics 2006: the long and winding road to gene discovery," *Genes and Immunity*, vol. 7, no. 2, pp. 95–100, 2006.

[51] Z. Tan, A. M. Shon, and C. Ober, "Evidence of balancing selection at the HLA-G promoter region," *Human Molecular Genetics*, vol. 14, no. 23, pp. 3619–3628, 2005.

[52] D. Naidoo, A. C. Wu, M. H. Brilliant et al., "A polymorphism in HLA-G modifies statin benefit in asthma," *Pharmacogenomics Journal*, vol. 15, no. 3, pp. 272–277, 2015.

[53] M. Granada, J. B. Wilk, M. Tuzova et al., "A genome-wide association study of plasma total IgE concentrations in the Framingham Heart Study," *Journal of Allergy and Clinical Immunology*, vol. 129, no. 3, pp. 840.e21–845.e21, 2012.

[54] S. Rajagopalan, J. Fu, and E. O. Long, "Cutting edge: induction of IFN-γ production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells," *The Journal of Immunology*, vol. 167, no. 4, pp. 1877–1881, 2001.

[55] W. H. Yan and L. A. Fan, "Residues Met76 and Gln79 in HLA-G α1 domain involved in KIR2DL4 recognition," *Cell Research*, vol. 15, no. 3, pp. 176–182, 2005.

[56] S. Rajagopalan, Y. T. Bryceson, S. P. Kuppusamy et al., "Activation of NK cells by an endocytosed receptor for soluble HLA-G," *PLoS Biology*, vol. 4, no. 1, article e9, 2006.

[57] C. Cantoni, S. Verdiani, M. Falco et al., "p49, a putative HLA class I-specific inhibitory NK receptor belonging to the immunoglobulin superfamily," *European Journal of Immunology*, vol. 28, no. 6, pp. 1980–1990, 1998.

[58] Y.-R. Yu, X.-H. Tian, Y. Wang, and M.-F. Feng, "Rapid production of human KIR2DL4 extracellular domain and verification of its interaction with HLA-G," *Biochemistry*, vol. 71, supplement 1, pp. S60–S65, 2006.

[59] P. Vacca, C. Cantoni, M. Vitale et al., "Crosstalk between decidual NK and $CD14^+$ myelomonocytic cells results in induction of Tregs and immunosuppression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 26, pp. 11918–11923, 2010.

[60] M. E. L. Le Page, J. P. Goodridge, G. Zhang, P. G. Holt, P. Sly, and C. S. Witt, "Genetic polymorphism of KIR2DL4 (CD158d), a putative NK cell receptor for HLA-G, does not influence susceptibility to asthma," *Tissue Antigens*, vol. 82, no. 4, pp. 276–279, 2013.

[61] F. Tahan and T. Patiroglu, "Plasma soluble human leukocyte antigen G levels in asthmatic children," *International Archives of Allergy and Immunology*, vol. 141, no. 3, pp. 213–216, 2006.

[62] X.-Q. Zheng, C.-C. Li, D.-P. Xu et al., "Analysis of the plasma soluble human leukocyte antigen-G and interleukin-10 levels in childhood atopic asthma," *Human Immunology*, vol. 71, no. 10, pp. 982–987, 2010.

[63] C. E. Mapp, S. Ferrazzoni, R. Rizzo et al., "Soluble human leucocyte antigen-G and interleukin-10 levels in isocyanate-induced asthma," *Clinical & Experimental Allergy*, vol. 39, no. 6, pp. 812–819, 2009.

[64] R. Rizzo, M. Stignani, P. Amoudruz et al., "Allergic women have reduced sHLA-G plasma levels at delivery," *American Journal of Reproductive Immunology*, vol. 61, no. 5, pp. 368–376, 2009.

[65] T. Imai, T. Yoshida, M. Baba, M. Nishimura, M. Kakizaki, and O. Yoshie, "Molecular cloning of a novel T cell-directed CC chemokine expressed in thymus by signal sequence trap using Epstein-Barr virus vector," *Journal of Biological Chemistry*, vol. 271, no. 35, pp. 21514–21521, 1996.

[66] H. Miyahara, N. Okazaki, T. Nagakura, S. Korematsu, and T. Izumi, "Elevated umbilical cord serum TARC/CCL17 levels predict the development of atopic dermatitis in infancy," *Clinical and Experimental Allergy*, vol. 41, no. 2, pp. 186–191, 2011.

[67] M. Akdis, C. A. Akdis, L. Weigl, R. Disch, and K. Blaser, "Skin-homing, CLA+ memory T Cells are activated in atopic dermatitis and regulate IgE by an IL-13-dominated ctokine pattern: IgG4 counter-regulation by CLA- memory T cells," *Journal of Immunology*, vol. 159, no. 9, pp. 4611–4619, 1997.

[68] K. Khosrotehrani, C. Le Danff, B. Reynaud-Mendel, L. Dubertret, E. D. Carosella, and S. Aractingi, "HLA-G expression in atopic dermatitis," *Journal of Investigative Dermatology*, vol. 117, no. 3, pp. 750–752, 2001.

[69] S. Aractingi, N. Briand, C. Le Danff et al., "HLA-G and NK receptor are expressed in psoriatic skin: a possible pathway for regulating infiltrating T cells?" *The American Journal of Pathology*, vol. 159, no. 1, pp. 71–77, 2001.

[70] C. Menier, N. Rouas-Freiss, B. Favier, J. Lemaoult, P. Moreau, and E. D. Carosella, "Recent advances on the non-classical major histocompatibility complex class i HLA-G molecule," *Tissue Antigens*, vol. 75, no. 3, pp. 201–206, 2010.

[71] G. Murdaca, P. Contini, M. Setti et al., "Behavior of serum human major histocompatibility complex class I antigen levels in human immunodeficiency virus-infected patients during antiretroviral therapy: correlation with clinical outcome," *Human Immunology*, vol. 68, no. 11, pp. 894–900, 2007.

[72] N. Rouas-Freiss, A. Naji, A. Durrbach, and E. D. Carosella, "Tolerogenic functions of human leukocyte antigen G: from pregnancy to organ and cell transplantation," *Transplantation*, vol. 84, no. 1, pp. S21–S25, 2007.

[73] E. D. Carosella, S. Gregori, N. Rouas-Freiss, J. Lemaoult, C. Menier, and B. Favier, "The role of HLA-G in immunity and hematopoiesis," *Cellular and Molecular Life Sciences*, vol. 68, no. 3, pp. 353–368, 2011.

[74] C. Bachert, M. van Kempen, and P. van Cauwenberge, "Regulation of proinflammatory cytokines in seasonal allergic rhinitis," *International Archives of Allergy and Immunology*, vol. 118, no. 2–4, pp. 375–379, 1999.

[75] E. D. Carosella, S. Gregori, and J. LeMaoult, "The tolerogenic interplay(s) among HLA-G, myeloid APCs, and regulatory cells," *Blood*, vol. 118, no. 25, pp. 6499–6505, 2011.

[76] J.-H. Lee, H.-H. Yu, L.-C. Wang, Y.-H. Yang, Y.-T. Lin, and B.-L. Chiang, "The levels of $CD4^+CD25^+$ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma," *Clinical & Experimental Immunology*, vol. 148, no. 1, pp. 53–63, 2007.

[77] E. D. Carosella, N. Rouas-Freiss, D. T.-L. Roux, P. Moreau, and J. LeMaoult, "HLA-G: an immune checkpoint molecule," *Advances in Immunology*, vol. 127, pp. 33–144, 2015.