THE BIOLOGICAL AND CLINICAL ASPECTS OF HLA-G

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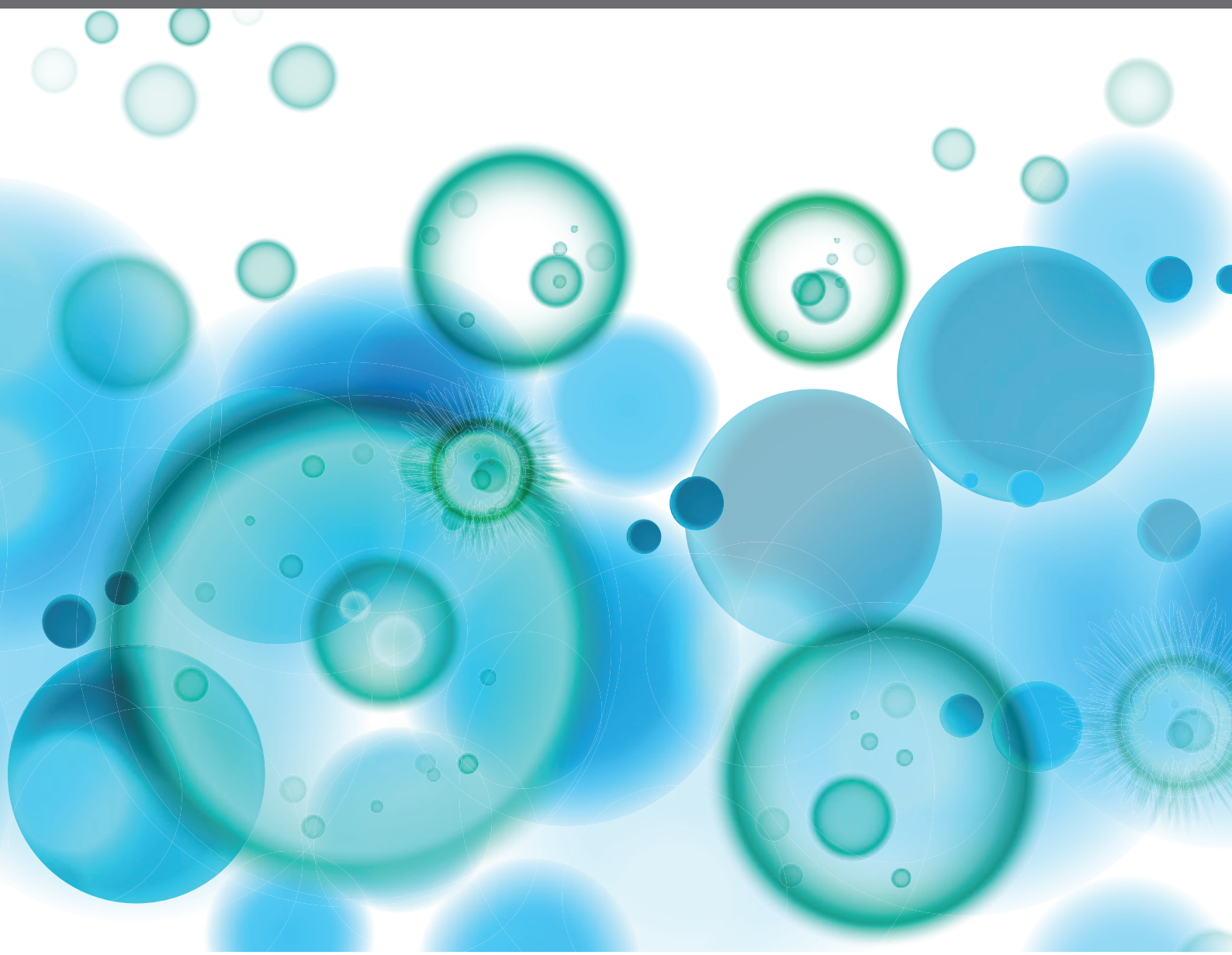

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THE BIOLOGICAL AND CLINICAL ASPECTS OF HLA-G

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Table of Contents

04 ***Editorial: The Biological and Clinical Aspects of HLA-G***
Joel LeMaoult and Wei-Hua Yan

06 ***The Role of HLA-G in Human Papillomavirus Infections and Cervical Carcinogenesis***
Hui-Hui Xu, Wei-Hua Yan and Aifen Lin

15 ***HLA-G Genotype/Expression/Disease Association Studies: Success, Hurdles, and Perspectives***
Giada Amodio and Silvia Gregori

24 ***HLA-G Neo-Expression on Tumors***
Maria Loustau, François Anna, Raphaelle Dréan, Martin Lecomte, Pierre Langlade-Demoyen and Julien Caumartin

39 ***Soluble HLA-G and HLA-G Bearing Extracellular Vesicles Affect ILT-2 Positive and ILT-2 Negative CD8 T Cells Complementary***
Esther Schwich, Gia-Gia T. Hò, Joel LeMaoult, Christina Bade-Döding, Edgardo D. Carosella, Peter A. Horn and Vera Rebmann

50 ***HLA-G Expressing Immune Cells in Immune Mediated Diseases***
P. Contini, Giuseppe Murdaca, Francesco Puppo and Simone Negrini

59 ***Roles of HLA-G in the Maternal-Fetal Immune Microenvironment***
Xiuxiu Xu, Yonggang Zhou and Haiming Wei

70 ***Intratumor Heterogeneity of HLA-G Expression in Cancer Lesions***
Xia Zhang, Aifen Lin, Qiu-Yue Han, Jian-Gang Zhang, Qiong-Yuan Chen, Yao-Han Ye, Wen-Jun Zhou, Hui-Hui Xu, Jun Gan and Wei-Hua Yan

79 ***Inhibition of iNKT Cells by the HLA-G-ILT2 Checkpoint and Poor Stimulation by HLA-G-Expressing Tolerogenic DC***
Ching-Lien Wu, Julien Caumartin, Giada Amodio, François Anna, Maria Loustau, Silvia Gregori, Pierre Langlade-Demoyen and Joel LeMaoult

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EDITORIAL
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Editorial: The Biological and Clinical Aspects of HLA-G

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Keywords: HLA-G, receptor, immune cells, pregnancy, cancer, tolerance

Editorial on the Research Topic

The Biological and Clinical Aspects of HLA-G

In this Research Topic, we hosted eight in-depth reviews, mini reviews, and original research articles on the biological and clinical aspects of HLA-G. This could be unattainable without the enthusiastic involvement of all contributing authors, participating reviewers and the assistance from the staff of Frontiers in Immunology.

HLA-G belongs to the non-classical HLA class I family. HLA-G features limited genetic variation, very restricted tissue expression, and immune tolerogenic functions, being a ligand of immune inhibitory receptors. HLA-G is now recognized as an important immune checkpoint. Moreover, at least seven isoforms (membrane-bound isoforms: HLA-G1∼HLA-G4; soluble isoforms: HLA-G5∼HLA-G7), can be generated due to its primary transcript alternative splicing. Since *HLA-G* gene had been identified in 1987 (1) and HLA-G protein expression first observed in extravillious cytotrophoblasts in 1990 (2), both genetic and molecular characteristics, and biological functions of HLA-G have been thoroughly investigated. Even though HLA-G main site of expression it the fetal cytotrphoblast, physiological expression in adults was reported in stem cells and some progenitor cells, somatic cells within immune provoleged tissues, and some immune cells. Furthermore, HLA-G ectopic expression is induced in a variety of pathological conditions. The immune suppressive functions of HLA-G are mediated by the signaling between HLA-G and the ILT-2 and ILT-4 receptors. The importance of this interaction has been well-described in a broad range of clinical settings such as reproduction, infection, autoimmune disease and cancer. HLA-G/ILT is a promising immune checkpoint, and the first phase I clinical trial for a new anti-HLA-G antibody started in 2020 in advanced solid cancer patients (3). In the context of pregnancy, HLA-G interacts with another receptor, KIR2DL4, that is principally expressed by uterine NK cells. Interaction of HLA-G with KIR2DL4 is clearly different from that with ILT-2 and ILT-4, and its role in pregnancy is currently emerging.

In this Research Topic, different aspects of the latest advances regarding HLA-G have been reviewed and explored including the significance of *HLA-G* genetic variability in HLA-G expression and disease predisposition, the roles of HLA-G in fetal-maternal immune tolerance, the neo- and heterogeneous expression of HLA-G in cancers, and the cellular and extracellular HLA-G expression in the regulation of various immune cell functions.

The regulation of HLA-G expression is multifactorial which can be affected by HLA-G genetic variability, post-transcriptional regulation and intracellular and extracellular microenvironmental signals. The predictive, diagnostic, and prognostic significance of HLA-G genotype and/or protein expression has been investigate in a wide range of clinical settings. Amodio and Gregori elaborate how HLA-G protein expression regulated by the polymorphisms in the 5′-upstream regulatory

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region (5′-URR), coding region and in the 3′-untranslated region (3′-UTR) through transcriptional and posttranscriptional regulation, and discussed the most studied HLA-G polymorphism 14-bp INS/DEL in association with diseases. They also recommend future approaches for the HLA-G polymorphism/protein expression/disease association studies. Xu et al. illustrate certain HLA-G polymorphisms as risk factors for the human papillomavirus infection and HLA-G expression in cervical cancer carcinogenesis. Signaling between HLA-G and its receptor engagement is the key requirement for the immune regulatory function of HLA-G. HLA-G and its receptor signaling can exert immune suppression with detrimental effects which favors cancer and virus infected cells by allowing them to escape from immune surveillance and attack, while beneficial in promoting immune tolerance for fetal-maternal or transplants acceptance. Contini et al. review the HLA-G expressing immune cells in physiological conditions, and both in autoimmune and non-autoimmune diseases, indicating potential roles of HLA-G positive immune subsets involvement in the pathogenesis of immune mediated diseases. Wu et al. report that HLA-G, and HLA-G-expressing tolerogenic DC-10 through ILT-2 pathway inhibit both human and murine invariant natural killer T (iNKT) cell activation. Schwich et al. explore the functions of two different soluble HLA-G forms, the purified sHLA-G1 protein and extracellular vesicles with or without HLA-G molecule, on the ILT-2 positive and negative CD8+ T cells. sHLA-G1 and HLA-G_{EV} differentially induce immune-exhausted or immune-suppressive phenotye in ILT-2 positive CD8+ T cells and ILT-2 negative CD8+ T cells, respectively. This finding indicate that sHLA-G1 and HLA-G_{EV} affect ILT-2 positive and ILT-2 negative CD8+ T cells complementary. Xu et al. showcase three important roles of extravillous trophoblast expressed HLA-G on the regualtion of immune cell subsets. First, HLA-G/ILT-2 and HLA-G/KIR2DL4 signaling induces immune cell producing proangiogenic cytokines to promote the spiral artery remodeling. Second, HLA-G/ILT-2/4 and HLA-G/KIR2DL4 signaling supresses the cytotoxicity of immune cells to maintain the fetal-maternal immune tolerance. Finally, HLA-G/ILT-2 and HLA-G/KIR2DL4 signaling induces the production of growth-promoting factors to favor fetal growth. Clinical significance of HLA-G neo-expression in cancers and its relation to advanced disease stage, tumor metastasis and poor prognosis in many tumors has been well-established, and a clinical trial with a monoclonal anti-HLA-G antibody to block the HLA-G/ILTs interaction was initiated recently (3). Zhang et al. demonstrate that intratumor heterogeneity of HLA-G expression is a common phenomenon in cancers, and that the degree of HLA-G expression detection varies dramatically with different antibodies used to probe. Loustau et al. discuss the advance of HLA-G neo-expression and clinical relevance in various tumor types, and point out the limitation such as more specific anti-HLA-G antibodies are extremely necessary for future in-depth studies on neo-expression of HLA-G in cancers.

In summary, this special issue highlights the current advances regarding the biological and clinical importance of HLA-G in various physio-pathological situations. Undoubtedly, HLA-G is a promising biomarker and therapeutic target for different diseases, even though isoform-specific antibodies are still lacking, which prevents advances in characterizing their clinical significance.

AUTHOR CONTRIBUTIONS

W-HY wrote the first draft of the manuscript and updated the last version. JL corrected the draft.

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REVIEW
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The Role of HLA-G in Human Papillomavirus Infections and Cervical Carcinogenesis

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Human leukocyte antigen (HLA)-G, a non-classical HLA-class I molecule, has a low polymorphism frequency, restricted tissue distribution and immunoinhibitory property. HLA-G expression in tumor cells and cells chronically infected with virus may enable them to escape from host immune surveillance. It is well-known that the HLA-G molecule is a novel biomarker and potential therapeutic target that is relevant in various types of cancers, but its role in cervical cancer has not been fully explored. In this review, we aim to summarize and discuss the immunologic role of the HLA-G molecule in the context of HPV infections and the process of cervical cancer carcinogenesis. A better understanding of the potential impact of HLA-G on the clinical course of persistent HPV infections, cervical epithelial cell transformation, tumor growth, recurrence and metastasis is needed to identify a novel diagnostic/prognostic biomarker for cervical cancer, which is critical for cervical cancer risk screening. In addition, it is also necessary to identify HLA-G-driven immune mechanisms involved in the interactions between host and virus to explore novel immunotherapy strategies that target HLA-G/immunoglobulin-like transcript (ILT) immune checkpoints.

Keywords: human leukocyte antigen G, human papillomavirus, viral infection, carcinogenesis, cervical cancer, immunotherapy

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INTRODUCTION

Cervical cancer ranks as the fourth most common female cancer worldwide, with an estimated 569,847 new cases and 311,365 deaths in 2018 (1). Persistent infection with high-risk human papillomavirus (hrHPV) is necessary but not sufficient to induce cervical cancer (2). Most HPV infections are transient and are cleared within months by host innate and adaptive immune responses (3). Failure to clear the virus leads to infection persistence, and only a minority of HPV-infected and transformed cells eventually avoid host immune surveillance, which leads to tumor growth and lymph node metastasis (4, 5). This host-dependent immunological status and HPV-induced immune escape are reflected in persistent infection and the subsequent progression of precancerous lesions to invasive cervical cancer, which indicates the complexity of host-virus interactions. Therefore, the roles of the immune system, not only in viral elimination but also in tumor antigen recognition, are extremely relevant in the process of cervical cancer carcinogenesis.

Accumulating evidence has supported the idea of a critical role for immunosuppressive mechanisms in promoting HPV-induced carcinogenesis, either by suppressing the capacity of the host to overcome HPV infection or by preventing the elimination of HPV-transformed epithelial

cells (3–7). Human leucocyte antigen (HLA) complex is located on chromosome 6p21.3. Several HLA molecules with different functions can be broadly divided into classical HLA-class I (HLA-A, -B, -C), non-classical HLA-class I (HLA-E, -F, -G), classical HLA-class II (HLA-DR, -DQ, -DP), and classical HLA-class III (8). The HLA system influences the host immune response by mediating antigen presentation (9). HLA-G has been termed "non-classical" due to its low frequency of polymorphisms, restricted tissue distribution and immunoinhibitory properties, which are different from the properties of classical HLA-class I molecules (10, 11). It has become increasingly evident that the HLA-G molecule is involved in modulating both innate and adaptive immune responses and in promoting immune escape in various types of cancers (10–13) and infectious diseases (14–16). However, to date, the possibility that *HLA-G* gene polymorphisms and/or protein expression affecting HPV infection persistence and cervical cancer risk remains to be explored.

MOLECULAR STRUCTURE OF HUMAN LEUKOCYTE ANTIGEN-G

The *HLA-G* gene consists of eight exons, seven introns, a 5′upstream regulatory region (URR) that extends at least 1,400 bp from the initial ATG start codon, and a 3′untranslated region (UTR), with a total length of 6,000 bp (12, 17). It is widely accepted that the *HLA-G* primary transcript is alternatively spliced into seven mRNAs, which encode four membrane-bound (HLA-G1, -G2, -G3, -G4) and three soluble (HLA-G5, -G6, -G7) protein isoforms (18, 19). Each unique HLA-G isoform contains one to three extracellular globular domains (α1, α2, α3) encoded by exon 2, exon 3, and exon 4, whereas the presence of intronic sequences are variable (IMGT/HLA Database).

The overall structure of HLA-G1 and that of its soluble counterpart HLA-G5 is similar to the structure of the classical HLA-class I antigens, which contain a heavy chain non-covalently bound to *β2*-microglobulin (*β2*m) (18). Peptide is bound in the antigen-binding cleft formed by the α1 and α2 domains (11, 20), whereas the α3 domain can bind co-receptors such as CD8 (21). Both HLA-G1 and HLA-G5 isoforms can also exist as *β2*m-free antigens (22). Other HLA-G isoforms lacking one or two extracellular globular domains (α2, or α3, or both) are smaller than HLA-G1/-G5 isoforms and are not associated with *β2*m (23). HLA-G1 to HLA-G4 are membrane-bound isoforms due to the presence of the transmembrane region encoded by exon 5 and a short cytoplasmic tail encoded by exon 6, which contains a stop codon. HLA-G5 and HLA-G6 are soluble isoforms due to the presence of intron 4, which contains a premature stop codon to prevent the translation of the transmembrane and cytoplasmic tail. HLA-G7 is a soluble isoform due to the presence of intron 2, which contains a premature stop codon and results in the expression of a soluble protein (18–20). All seven reported HLA-G isoforms contain the extracellular α1 domain.

In addition to the seven HLA-G monomers reported, the molecular structure of HLA-G is even more complex. A study on its crystal structure demonstrated that HLA-G can exist as a dimer with the intermolecular Cys42-Cys42 disulphide bond (24). *In vitro* and *in vivo* studies have shown that HLA-G dimers are observed for all isoforms except HLA-G3 (25). Moreover, *β2*m-associated and *β2*m-free dimers of HLA-G1 or HLA-G5 also exist (26–28). Dimer formation affects the specificity of receptor-HLA-G binding, as dimers exhibit a higher overall affinity to immunoglobulin-like transcript (ILT)2/4 receptors than monomers due to significant avidity effects (24, 28, 29).

Notably, unidentified HLA-G isoforms without an α1 domain were predicted based on RNA sequencing (RNA-seq), and several previously undescribed HLA-G isoforms have been identified in renal cancer samples (30). According to the nucleotide sequence of the *HLA-G* gene listed in the Ensembl database (ENST00000376828), this gene may possess a supplementary exon at the 5′-end, but this is absent from the sequence in the IMGT/HLA database. A novel HLA-G isoform named HLA-G1L was predicted by Tronik-Le Roux et al. (30); this isoform has five additional amino acids (MKTPR) located at the N-terminal end. Analysis of RNA-seq data indicates that some sequence reads may be initiated at exon 4, and thus could predict the existence of novel α1-deleted HLA-G isoforms that contain α2 and α3 domains or only the α3 domain. Other novel soluble HLA-G isoforms can be generated by the skipping of exon 6 coding for the transmembrane domain (30, 31). Lin et al. (32) indicated the existence of novel α1-deleted HLA-G isoforms containing intron 4 in 11.6% (44/379) of colorectal cancer lesions that exhibited negative staining with mAb 4H84 but that exhibited positive staining with mAb 5A6G7 ($4H84^{neg}5A6G7^{pos}$). Moreover, patients with $4H84^{neg}5A6G7^{pos}$ HLA-G isoforms had a better survival than patients with $4H84^{pos}5A6G7^{neg}$, and thus suggests a functional role for the novel α1-deleted HLA-G isoforms (31). However, the specific function of these novel HLA-G isoforms remains to be determined. The development of specific antibodies for these novel HLA-G isoforms is urgently needed and even inevitable (33).

HLA-G-MEDIATED IMMUNE SUPPRESSION

HLA-G expression was initially observed on cytotrophoblasts at the maternal-fetal interface (34), where HLA-G modulates the response of maternal immune cells that contribute to maintenance of tolerance to the fetus (35–37). HLA-G has a physiological tissue-restricted distribution property, as it is expressed by cytotrophoblasts (34), cornea (38), thymus (39), nail matrix (40), pancreatic islets (41), and erythroblasts (42). However, aberrant upregulated expression of HLA-G molecules has been detected in pathological conditions such as malignancies (43–45), infections and inflammatory diseases (14, 46–49), transplant grafts (50, 51), and autoimmune disorders (16, 52–54). In malignancies, aberrant HLA-G expression was preferentially detected in tumor tissues but was rarely detected in normal or adjacent non-tumorous tissues, which indicates that HLA-G might play a key role in tumor development (44).

Functionally, HLA-G has comprehensive immunosuppressive properties exerted in multiple steps to weaken anti-tumor immune responses by acting on immune cells through its inhibitory receptors: ILT2(CD85j/LILRB1), ILT4(CD85d/LILRB2), and KIR2DL4(CD158d) (11, 12, 55–59) (**Figure 1**). HLA-G inhibits the cytolytic function of natural killer (NK) cells (60, 61), cytotoxic T lymphocyte (CTL)-mediated cytolysis (62), macrophage-mediated cytotoxicity (63), allo-proliferative response of $CD4^+$ T cells (64, 65), maturation and function of dendritic cells (DCs) or B lymphocytes (66–69), stimulation of antigen-presenting cells (APCs) to secrete functional cytokines TGF-β and IL-10, and induction of apoptosis of $CD8^+$ T cells and $CD8^+$ NK cells (70, 71). In addition, HLA-G-receptor interactions could also exert long-term immunomodulatory effects by inducing immune suppressor/regulatory cells, such as regulatory T cells (Tregs) (72, 73), tolerogenic DCs (tDCs) (74, 75), mesenchymal stem cells (MSCs) (76), and myeloid-derived suppressor cells (MDSCs) (77, 78), among others. In addition to the interactions between HLA-G and its receptors, HLA-G-mediated immunosuppression by intercellular transfer mechanisms such as trogocytosis, exosomes, or tunneling nanotubes (TnTs) also represents another important complementary mechanism through which cancer cells escape destruction by the host immune system (11, 12, 79–81).

HLA-G POLYMORPHISMS IN THE CONTEXT OF HPV INFECTIONS

To date, 69 alleles that encode 19 proteins have been discovered (IMGT/HLA Database, February 2020). Polymorphic sites along the *HLA-G* gene may change the affinity of gene-targeted sequences for transcriptional or post-transcriptional factors (82, 83). In particular, the *14bp Insertion/Deletion (Ins/Del)* (rs66554220) in the 3′UTR is associated with *HLA-G* alternative splicing and mRNA stability (84, 85). The *+3142C/G* (rs1063320) located 167 bp downstream from the *14bp Ins/Del* polymorphic site may be a target for HLA-G-specific miRNAs (86), which could directly downregulate HLA-G expression through post-transcriptional regulation (87, 88).

Accumulating evidence has supported the concept that HLA-G polymorphisms are genetic susceptibility and/or protection-relevant factors for cervical HPV infections and viral persistence (89–101). Many studies have primarily focused on polymorphisms in the 3′UTR of the *HLA-G* gene (89–95), while few have assessed its promoter region (96). Studies by Xu et al. (89, 90) showed that *HLA-G 14bp Ins* or *+3142G* alleles are risk factors for HPV infections, especially hrHPV infections, compared with the alleles found in healthy women and that these alleles affect the progression of HPV18-associated cervical lesions in Chinese women. A similar finding was reported in a study performed in Brazilian women from São Paulo, Brazil; this study showed that the *HLA-G 14bp Ins/+3142G* haplotype was related to increased risk of high-grade cervical lesions, especially in smokers (91). Inconsistent results were obtained in Italy (92) and Taiwan (93), where increased risk for squamous cell carcinoma (SCC) was found to be associated with the *14bp del* or *+3142C* alleles, especially in SCC patients infected with the HPV16 genotype (93). Moreover, some have focused on the association between *HLA-G* 3′UTR polymorphisms and HPV infection among HIV-positive women who have a higher risk of developing HPV co-infection. The combination of the *+3142CX (CC or CG)* and *+3187AA* genotypes conferred the highest risk of HPV-induced aneuploidy in cervical cells among Brazilian women with HIV/HPV co-infections (94).A SNP (rs1633038) in the 3′UTR of the *HLA-G* gene was significantly related to higher HPV clearance rates among African-Americans with HIV/HPV co-infection, but this association was not observed in Hispanics or European-Americans (95).

Further evidence for the role of genetic factors in HPV infections and the carcinogenic process was provided by studies that showed an association with specific HLA-G coding region polymorphisms (96–104). Among the Canadian population, the *HLA-G*01:01:02* and *HLA-G*01:03* alleles were found to be related to an increased risk of HPV16 infection and persistent infections (96), while the *HLA-G*01:01:03* and *HLA-G*01:01:05* alleles were identified as significant predictors of cumulative coinfections over the follow-up period (97). In the same cohort, the *HLA-G*01:01:02*, *HLA-G*01:04:01* and *HLA-G*01:06* alleles were related to high-grade cervical intraepithelial neoplasia (HG-CIN) (98). The *HLA-G*01:01:02*, *HLA-G*01:06* and 3′UTR *14bp Ins* alleles were associated with disease progression from preinvasive to invasive cervical cancer among HPV-positive Canadian women (99). The homozygous *HLA-G*01:04:01* genotype was related to a significantly decreased risk of HPV infection (98), and the heterozygotic form of the *HLA-G*01:01:01* allele conferred significant protection against cancer (99). Among Brazilian women, the *HLA-G*01:04/14bp Ins* haplotype as well as HPV16 and HPV18 co-infection were preferentially related to HG-CIN, while the *HLA-G*01:03* allele was related to protection against HPV-related cervical lesions (100). Among HPV-positive pregnant women in Brazil, a protective effect of the *HLA-G*01:01:02* allele against the occurrence of CIN was observed in a cohort of HPV/HIV co-infected pregnant women (101). In a study that focused on the role of host factors in the vertical transmission of HPV infection from mother to offspring, the results showed that the *HLA-G∗01:01:01/01:04:01* genotype increased the risk of hrHPV infection in both cord blood and the infant's oral mucosa; moreover, the mother-child concordance of *HLA-G∗01:01:02/01:01:02* increased the risk of oral hrHPV infection both in the mother and offspring (102). In addition, a pilot analysis of HLA-G promoter methylation and HPV infection status showed no association between HLA-G methylation and HPV infections in healthy women (103, 104).

Overall, the discrepancy among these studies could be explained by differences in the study designs, ethnicity, sample sizes, and cancer types. Current data suggested that HLA-G gene polymorphisms (mainly located in the coding region or 3′UTR region) appear to be independent risk factors for HPV infection and cervical carcinogenesis, which supports the biological role of HLA-G molecules in shaping the tumor microenvironment (105).

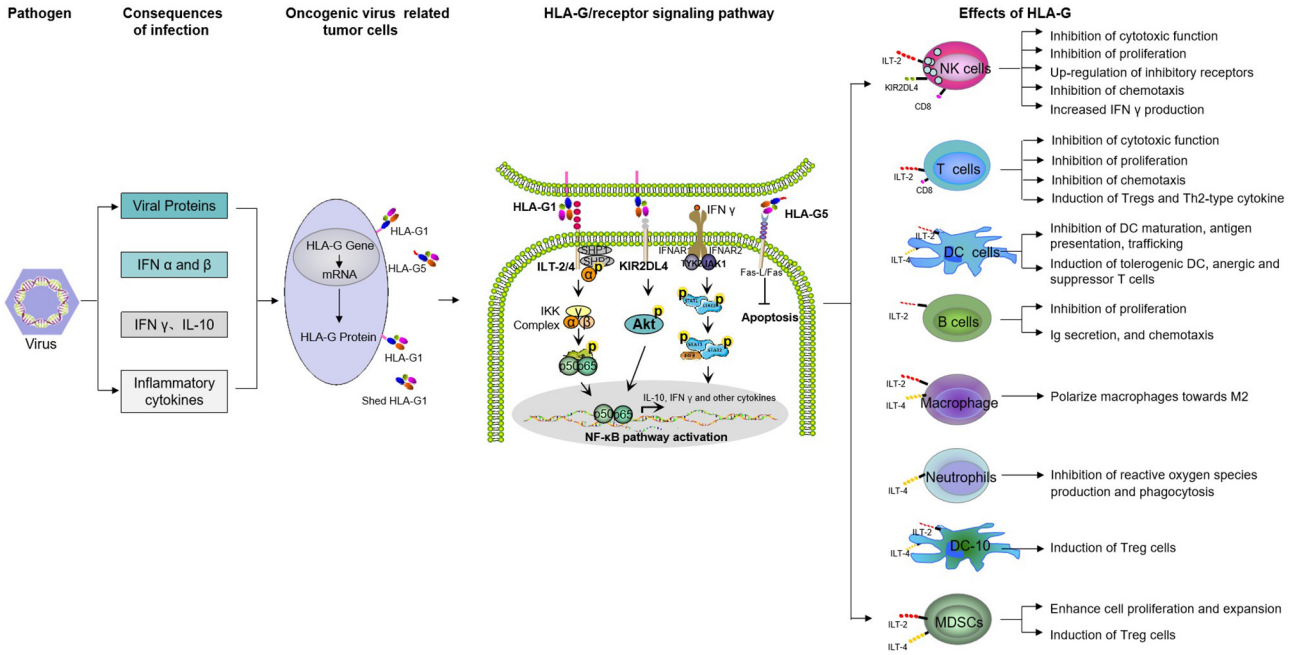

FIGURE 1 | Mechanisms of both membrane-bound and soluble HLA-G-mediated immune suppression in tumor immune evasion. NK, natural killer cells; DC, dendritic cells; MDSC, myeloid-derived suppressor cells; Tregs, regulatory T cells; DC-10, IL-10-differentiated dendritic cells.

HLA-G EXPRESSION IN CERVICAL CARCINOGENESIS

HLA-G expression may be induced after HPV infection, which leads to escape from host immunosurveillance. This evidence is derived from the results of a study that showed that HLA-G expression was significantly higher in CIN and cancer patients with HPV16/18 infections than in CIN patients without HPV infection (106). Several studies have investigated the relationship between HLA-G isoform expression and clinicopathologic features in patients with precancerous lesions and invasive cervical cancer (45, 90, 106–117).

Another study focused on HLA-G mRNA expression in cervical cancer in Korean patients using RT-PCR (15 normal tissues and 40 cervical cancer tissues) and found that high HLA-G mRNA expression was related to the early stages of cervical cancer (108). These results are consistent with the report by Rodriguez et al. (109), which showed upregulation of HLA-G protein expression in the early stages of cervical cancer in Colombian patients using immunohistochemistry (IHC) with mAb 4H84 (9 CIN III and 54 cervical cancer cases). Both studies supported a possible role for the HLA-G molecule in early cervical carcinogenesis (108, 109). The results of both studies further showed that Interleukin-10 (IL-10) expression was also significantly increased in cervical cancer tissues (108, 109), which supports a shift toward a Th2 cytokine microenvironment; this in turn may promote local immunosuppression by upregulating HLA-G expression (111, 118). Consistent with this, the results also revealed the inverse relationship among HLA-G expression levels and estimated numbers of tumor infiltrating lymphocytes (TILs) and CD57+ NK cells, which favors an escape from host anti-tumor activity (115). Moreover, three independent studies have reported evidence of a positive correlation between HLA-G expression and cervical carcinogenesis in a Chinese population (45, 106, 107). The results of all three studies indicated that HLA-G expression was negative in normal or adjacent non-tumorous tissues but was significantly increased along with CIN grade and cervical cancer metastasis. HLA-G expression may play an important role in determining the risk for cervical carcinogenesis. These clinical studies also analyzed clinicopathological parameters and demonstrated significant correlations between HLA-G expression and unfavorable prognosis, poor overall survival, and lymph node metastasis. However, inconsistent results were obtained in only two studies that showed that HLA-G expression was not related to cervical carcinogenesis (112, 113). Zhou et al. (112) described that in all normal epithelium, HLA-G expression was strong and uniform but was statistically down-regulated in CIN and SCC. Gonçalves et al. (113) reported that HLA-G was not expressed in any CIN or SCC. Futhermore, in experimental model of cervical cancer research, Real et al. (119) reported that low expression of HLA-G in Hela cell line (HPV18 infection). Thus, the role of HLA-G in malignancies has gained considerable clinical interest due to the possibility of exploiting it as a novel diagnostic/prognostic biomarker to identify cervical cancer and to monitor disease stage.

Additionally, three studies focused on soluble HLA-G (sHLA-G) isoform expression using different detection technologies (107, 110, 114). Guimarães et al. (110) analyzed sHLA-G expression in cervical cancer tissues from Brazilian patients using

IHC with the specific mAb 5A6G7 (27 with metastasis and 52 without metastasis). Low expression of sHLA-G isoforms was detected in all HPV-positive tissues, and the sHLA-G expression level was similar in both groups (110). Zheng et al. (107) investigated the sHLA-G expression level in the plasma of patients with cervical lesions using ELISA kit (sHLA-G, Exbio) with mAb MEM-G/9 (20 normal cervical tissues, 15 CIN I, 22 CIN II, 35 CIN III, and 80 cervical cancer tissues). sHLA-G expression levels in the plasma were significantly increased in CIN II-III and SCC patients, and their expression levels were also associated with differentiation and metastasis. Therefore, sHLA-G molecules may have significance in early cervical cancer screening (107). However, inconsistent results obtained in the Netherlands (366 cervical cancer) using ELISA kit (sHLA-G, Exbio) reported that sHLA-G levels were not associated with clinicopathological parameters or survival (114).

Overall, the discrepancies in these studies that examined HLA-G expression in cervical cancer patients are partly due to tumor heterogeneity (31). In the future, there will be a need for additional studies to obtain deeper insight into the association between HLA-G expression levels and advanced cervical cancer.

HLA-G AS A NOVEL TARGET FOR IMMUNOTHERAPIES

Cervical cancer accounts for 6.6% of all female cancers and is thus a major global health challenge, as ∼90% of cervical cancer deaths occur in less developed countries (1). High-risk HPV causes almost all invasive cervical cancers, and therefore, HPV screening and vaccination are needed to improve cervical cancer control (2). Despite significant advances in effective screening and preventive vaccination during the past decade, substantial regional and global disparities in the prognosis of cervical cancer patients still exist (120). Unfortunately, ∼30% of patients experience recurrence and metastasis after primary treatment, with an expected 5-year survival of < 10%. Few effective therapeutic strategies have been developed that specifically target recurrent or metastatic cervical cancer, particularly advanced-stage disease. Thus, novel therapeutic strategies, such as immunotherapy, are urgently needed in clinical settings (121, 122).

In recent years, an improved understanding of the molecular mechanisms of the interactions between HPV-associated cervical cancer and the host immune responses has driven the exploration of immunotherapy as one of the new therapeutics targeting immune checkpoints (123). HLA-G has comprehensive immunosuppressive properties that are exerted in multiple steps to weaken the anti-tumor immune responses by acting on immune cells through its inhibitory receptors. Fortunately, HLA-G expression can be downregulated through RNA interference or antibody blockade, which can allow recovery of the functions of immune effectors and prevent tumor reoccurrence. Thus, HLA-G could serve as a novel immune checkpoint molecule and play a key role in novel immunotherapy approaches that offer a promising perspective for tumor progression and advanced- stage cervical cancer.

It has been confirmed that miR-148a negatively regulates HLA-G expression by binding to the 3′UTR of the *HLA-G* gene (88). The long non-coding RNA HOX transcript antisense RNA (HOTAIR) may also serve as a competing endogenous RNAs (ceRNAs) to regulate HLA-G expression by sponging miR-148a in cervical cancer cells (116). Targeting the HOTAIR-miR-148a-HLA-G axis or HLA-G-specific miRs could represent a novel therapeutic strategy in cervical cancer. Intra-tumor heterogeneity of checkpoint molecule expression in cervical cancer is related to a poor chemo/radio-therapy response, lymph node metastasis and tumor recurrence. HLA-G has been identified as a cervical cancer stem cell (CCSC)-specific marker, and targeting HLA-G and its related signaling pathways may offer a novel strategy for CCSC-targeted therapy (124). Moreover, the HLA-G/ILTs axis has been recently recognized as a new immune checkpoint in addition to other immune checkpoints such as programmed cell death 1 (PD-1)/PD-L1 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4)/B7 (125). Different responses to checkpoint inhibitor therapy could be a consequence of heterogeneous intra- and inter-tumor expression of different types of checkpoint molecules, although data on the expression status of HLA-G, CTLA-4 and PD-L1 in cancers are rather limited (13, 125, 126). PD-1 is a major immunotherapeutic checkpoint target in various cancer types, but until now, few data have been available on the clinical efficacy of blocking this checkpoint protein in cervical cancer (2, 126). The expression of PD-1 was found to be heterogeneous in tumors and could be co-expressed with the immune checkpoint protein HLA-G (127). A recent study focused on tumor-infiltrating CD8+ T cells that express the HLA-G receptor ILT2 in renal-cell carcinoma (RCC), and the results emphasize the potential of therapeutically targeting the HLA-G/ILT2 checkpoint in HLA-G+ tumors (127). Overexpression of the immune checkpoint HLA-G molecule by tumor cells profoundly affects tumor-specific T cell immunity in the cancer microenvironment. In this regard, targeting multiple checkpoints, especially potential antagonists of the HLA-G/ILT-2/4 pathway, is urgently needed to target the entire tumor.

CONCLUSIONS

Considering the above studies that were reviewed, we propose that *HLA-G* gene polymorphisms have an impact on the immune response and likely determine those in specific populations who are at higher risk for cervical HPV infections and viral persistence. Aberrant HLA-G expression in cervical lesions could generate inhibitory signals in the cancer microenvironment, which would ultimately help tumor cells escape from immunosurveillance and reshape tumor progression and metastasis. The checkpoint molecule HLA-G with immune tolerance contribute to cervical carcinogenesis, but HLA-G could also represent a good immunotherapeutic target for cervical cancer treatment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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HLA-G Genotype/Expression/ Disease Association Studies: Success, Hurdles, and Perspectives

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The non-classical HLA-G is a well-known immune-modulatory molecule. In physiological condition, HLA-G surface expression is restricted to the maternal–fetal interface and to immune-privileged adult tissues, whereas soluble forms of HLA-G are detectable in various body fluids. HLA-G can be *de novo* expressed in pathological conditions including tumors, chronic infections, or after allogeneic transplantation. HLA-G exerts positive effects modulating innate and adaptive immune responses and promoting tolerance, or detrimental effects inducing immune escape mechanisms. HLA-G locus, in contrast to classical HLA class I gene, is highly polymorphic in the non-coding 3′ untranslated region (UTR) and in the 5′ upstream regulatory region (5′ URR). Variability in these regions influences HLA-G expression by modifying mRNA stability or allowing posttranscriptional regulation in the case of 3′ UTR or by sensing the microenvironment and responding to specific stimuli in the case of HLA-G promoter regions (5′ URR). The influence of genetic variations on the expression of HLA-G makes it an attractive biomarker to monitor disease predisposition and progression, or response to therapy. Here, we summarize the current knowledge, efforts, and obstacles to generate a general consensus on the correlation between HLA-G genetic variability, protein expression, and disease predisposition. Moreover, we discuss perspectives for future investigation on HLA-G genotype/expression in association with disease predisposition and progression.

Keywords: HLA-G, immune regulation, autoimmune diseases, pregnancy, cancer, HLA-G polymorphisms

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INTRODUCTION

HLA-G, a non-classical HLA class I molecule, was first described to play a critical role in maintaining fetal–maternal tolerance (1). Later, it has been shown that HLA-G modulates innate and adaptive immune responses and promotes tolerance in different clinical settings. HLA-G function is favorable during pregnancy or after transplantation since it protects from rejection, and in autoimmune disease as it prevents autoreactive responses, or it is detrimental when expressed by tumors or during chronic infections, inducing immune escape mechanisms (2).

Because the HLA-G gene has a limited number of polymorphisms within the coding region, relatively few distinct molecules are coded. Nevertheless, seven different HLA-G isoforms have been described: four membrane-bound (HLA-G1 to -G4), and three soluble (HLA-G5 to -G7) (3–5).

The magnitude of HLA-G gene and protein expression is controlled by polymorphisms in the promoter [5′-upstream regulatory region (5′ URR)] and in the 3′ untranslated region (3′ UTR), and several association studies between these polymorphic sites and disease predisposition, response to therapy, and/or HLA-G protein expression have been reported. However, results from these studies often have been weak and inconclusive (6–10). Here, we summarize efforts to generate a general consensus on the correlation between HLA-G genetic variability, protein expression, and disease predisposition. Moreover, we highlight and discuss limits hampering the possibility to define a unique framework in the correlation between HLA-G genetic and disease predisposition or HLA-G genetic and protein expression, or HLA-G genetic/protein expression and disease predisposition.

HLA-G HAPLOTYPES

The HLA-G gene has 74 alleles encoding for 24 different full-length proteins, and four null alleles encoding for truncated form of the protein (IPD78/IMGT/HLA; March 2020). HLA-G locus, similar to other classical HLA class I locus, is composed of eight exons and seven introns, but it presents a stop codon in exon 6, resulting in a short cytoplasmic tail (4) and in an extended 3′ UTR, mainly composed by the exon 8 (11). Since HLA-G discovery in 1987 (12), HLA-G locus has been accurately analyzed, and the variability detected at the HLA-G regulatory regions (e.g., 3′ UTR and 5′ URR) is relatively higher than that observed in the coding region (13, 14).

The first identified and most studied polymorphism of the HLA-G locus is a 14-base-pair insertion/deletion (14-bp INS/DEL) landing in the 3′ UTR (15). More detailed and large genetic studies identified 16 additional single-nucleotide polymorphisms (SNPs) in the HLA-G 3′ UTR, of which only nine—the 14-bp INS/DEL polymorphism, +3003 C/G, +3010 G/C, +3027 C/A, +3035 C/T, +3142 C/G, +3187 A/G, +3196 C/G, and +3227 G/A—were recognized as true polymorphisms (13). The discovery that some of these polymorphisms are in strong linkage disequilibrium allowed the identification of 41 3′ UTR haplotypes, designated from UTR-1 to UTR-41, with only nine UTRs accounting for more than 95% of all haplotypes worldwide (13, 14, 16, 17).

The HLA-G locus presents also several variations in the 5′ URR and in the coding region. SNPs in these regions are in linkage disequilibrium, and a limited number of haplotypes, clustering in few families, have been identified and studied, alone or in combination with 3′ UTR alleles (18–20). In detail, the analysis of 35 SNPs within the 5′ URR revealed 64 different haplotypes (named PROMO), of which only nine representing the 95% of alleles worldwide, clustering in four major groups (PROMO 010101, 010102, 0103, and 010104) (13, 20, 21). Similarly, 81 variations were identified in the coding region, the majority landing in introns, arranging in 93 different haplotypes, of which only 11 having a frequency higher than 1% (13), including a null allele G*0105N encoding for a non-functional protein (22, 23). Interestingly, when the 5′ URR, the coding region, and the 3′ UTR haplotypes have been combined in the "extended haplotypes," it was clear that, also in this case, among the 200 haplotypes identified, the majority of them was scarcely represented (13). Moreover, 5′ URR, coding region, and 3′ UTR haplotypes are in linkage disequilibrium; thus, a given PROMO haplotype is preferentially associated with one specific 3′ UTR and coding region haplotype (11, 20, 21).

HLA-G GENETIC FOOTPRINT AND CORRELATION WITH DISEASE COURSE

HLA-G protein levels can be associated with specific genotypes; thus, a number of studies have been performed trying to correlate HLA-G haplotypes with disease susceptibility and morbidity or to use them as a predictive factor for response to therapy or in transplantation outcome (24).

The most studied HLA-G variation is the 14-bp INS/DEL polymorphism. In the field of pregnancy, there is a quite good consensus on the association of the 14-bp INS/INS genotype with recurrent pregnancy loss (RPL); however, a number of caveats have been identified in these correlations, including the heterogeneity of the studies, the sample characteristics, and non-comparable measure of the genotypes (23–25). A recent meta-analysis considering only women of European countries not only corroborated the association of 14-bp INS/INS genotype and RPL, but also highlighted and confirmed that the discrepancies observed in previous studies could be due to ethnic diversity of the cohort analyzed (26).

In autoimmunity and cancer, results on the association of 14-bp INS/DEL genotypes with disease development or with response to therapy reported contradictory and/or inconclusive results. In the context of type 1 diabetes (T1D), the analysis revealed the association of the 14-bp DEL/DEL genotype with the development and the early onset of the disease (27, 28). Conversely, in Crohn's disease, a high frequency of the 14-bp INS allele has been associated with an increased risk of early disease onset (29). A meta-analysis performed considering 11 case–control studies from different autoimmune diseases showed the absence of a direct correlation between 14-bp INS/DEL genotypes and susceptibility to autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis multiple sclerosis, ulcerative colitis, Crohn's disease, idiopathic dilated cardiomyopathy, pemphigus vulgaris, and non-segmental vitiligo (7). The discrepancies observed might be attributed to the etiological mechanisms of the autoimmune disease in which gene-to-gene and gene-to-environment interactions are involved (see below), to the age of disease onset, and to the sample sizes.

A meta-analysis carried out to solve the 14-bp INS/DEL genotype association with cancer, including a large sample size, a wide variety of cancer types, and a more diverse sample population, overall revealed that HLA-G 14-bp INS/DEL polymorphism is significantly associated with the cancer

susceptibility (30). However, these results are inconsistent with previous meta-analysis (8, 9, 24, 31), which concluded the absence of relationship between the HLA-G 14-bp INS/DEL polymorphism and the risk of cancer. Despite the positive correlation between 14-bp INS/DEL genotype and cancer susceptibility, some weaknesses of the latter study, linked to sample size, ethnicity, types of cancer, and sources of controls, have been reported. Indeed, stratified analysis accounting for the abovementioned variables failed to find a significant risk association (30).

In the context of allogeneic hematopoietic stem cell transplantation (HSCT), it has been shown that patients with better outcome carried 14-bp DEL/DEL and 14-bp INS/DEL genotypes, suggesting 14-bp INS/DEL genotype as potential biomarker of transplantation outcome (32, 33). Overall, the association between 14-bp INS/DEL genotypes and disease susceptibility provided some positive, but not conclusive, association. This might be attributed to a number of reasons, including high variability in the etiology of the diseases analyzed and to the relative limited number of patients enrolled in single correlation studies.

The discovery of 3′ UTR haplotypes and genotypes prompted investigators to reconcile the heterogeneity of the results obtained in studies on the association between 14-bp INS/DEL and diseases. We and other groups reported the protective role of specific UTRs in preventing RPL, with UTR-1, UTR-3, and UTR-4 present at low frequency in women with RPL (34–36). These studies indicate that analysis of 3′ UTR provided an improvement beyond the use of 14-bp INS/DEL genotypes in the association with pregnancy. UTR-1, UTR-3, and UTR-4 indeed contain 14-bp DEL, but they differ from other UTRs for additional specific SNPs (13, 14, 16, 17). Similar results were reported in T1D, showing that UTR-3 is present at low frequency in patients (37). In the context of allogeneic HSCT, only a weak association of the UTR-2, containing the 14-bp INS, with protection from acute graft-versus-host disease was reported, and the authors indicated that the study of the entire 3′ UTR, copamred to the sole analysis of the 14-bp INS/DEL, did not improve the prediction of transplant outcome (38).

Thus, far, few groups have investigated the impact of polymorphisms landing in the PROMO or the coding region of HLA-G with disease predisposition, and the most significant association has been found with the G*0105N null allele that is present with high frequency in RPL (19, 39, 40) and preeclampsia (41, 42). In celiac disease (CD), starting from the demonstration that 14-bp INS confers an increased risk factor for the disease in conjunction with the HLA-DQ2.5 (43), an extended analysis, including polymorphisms located in the PROMO and 3′ UTRs, showed the association of the haplotype containing PROMO 010102a and UTR-2 with CD susceptibility (44).

In conclusion, these results indicate that the correlation between HLA-G genotypes, and specifically those containing the 14-bp INS allele, and pregnancy loss or susceptibility to specific types of cancer has been achieved. Drawing conclusion on the association between HLA-G genotypes and susceptibility for other diseases is highly difficult because of the heterogeneity of the pathogenesis of the diseases analyzed, the age of disease onset, the cohorts of patients and controls used, and, in some cases, the limited number of case–control included in the study, disabling the statistical analysis.

ASSOCIATION HLA-G GENOTYPE/PHENOTYPE

Several groups integrated the knowledge on HLA-G genetic with the detection of the HLA-G protein, both as soluble (s) (HLA-G5 and shed-HLA-G1) and as membrane-bound (HLA-G1) form, with the aim to use HLA-G as biomarker of disease predisposition and progression, or response to therapy.

In 2001, Rebmann et al. (45) reported the first evidence that HLA-G genotype influences the amount of sHLA-G in circulation. The study performed in healthy individuals defined some alleles (G*01013 and G*0105N) being associated with low levels, and other (G*01041) with high levels of sHLA-G. Later, the identification of HLA-G 3′ UTR polymorphic sites prompted investigators to correlate HLA-G genetic variation at 3′ UTR and protein expression. The presence of the 14-bp INS allele has been associated with lower HLA-G production for most HLA-G5 and shed-HLA-G1 in plasma or serum, and HLA-G1 in trophoblast samples [reviewed in Carosella et al. (2)]. However, the presence of the 14-bp sequence leads to alternative splicing and the generation of a more stable mRNA associated with high HLA-G1 expression in trophoblast cell lines (46).

Correlation studies performed in autoinflammatory and autoimmune diseases revealed that the 14-bp INS allele was associated with low plasma levels of sHLA-G in Crohn's disease patients (29). In relapsing–remitting multiple sclerosis, the 14-bp DEL/DEL genotype correlated to high levels of HLA-G in cerebrospinal fluid; however, it did not associate with disease duration and clinical symptoms (47). On the same line, in chronic lymphocytic leukemia, the 14-bp DEL allele has been associated with high levels of sHLA-G and HLA-G1, but only sHLA-G correlated with patient survival, possibly as a consequence of the high metalloproteinase activity involved in shedding HLA-G1 (48). A comprehensive analysis, performed to correlate HLA-G genotype/phenotype and disease outcome, showed a link between the 14-bp INS/INS genotype with pretransplant low sHLA-G levels and severe adverse events after HSCT (49). Similarly, the 14-bp DEL haplotype correlated with high sHLA-G serum levels and reduced episodes of heart transplant rejection (50). Despite the variety of the disease investigated, 14-bp INS and DEL alleles have been confirmed to be associated with low and high HLA-G, respectively, and in most cases high HLA-G with positive outcome of the disease.

More recently, investigators have studied the correlation of HLA-G 3′ UTR and protein expression. Haplotypes and diplotypes containing UTR-1 have been associated with high levels of plasma sHLA-G, those containing UTR-5 and UTR-7 with low levels of sHLA-G, and finally, alleles containing UTR-2, UTR-3, UTR-4, and UTR-6 with medium levels of sHLA-G (51). These results were confirmed in other biological fluids as the highest levels of HLA-G in seminal plasma have been detected in the presence of homozygosis for UTR-1 and UTR-3

(52). Our group defined the association of specific UTRs with the expression of HLA-G1 in a specific subset of tolerogenic DC, termed DC-10, inducible *in vitro* in the presence of IL-10 (53) and present *in vivo* (54). We showed higher frequency of UTR-2, UTR-5, and UTR-7 haplotypes and diplotypes in donors with DC-10 expressing low HLA-G1 and of UTR-3 in donors expressing high HLA-G1 (55). More recently, we confirmed that the UTR-3 haplotype is associated with high levels of HLA-G1 on circulating DC-10 (Amodio et al., submitted).

In conclusion, these results indicate a general consensus on the association between 14-bp INS and DEL allele and low and high expression of HLA-G, either soluble or membrane-bound isoforms, respectively. However, the 14-bp INS allele encodes for a transcript with a 92-bp deletion leading to a more stable mRNA fragment than that generated by the 14-bp DEL (56), suggesting that 14-bp INS might be also associated with high levels of HLA-G expression. Correlation studies including additional variations in the 3′ UTR improved the correlation between HLA-G genetic and protein expression partially solving the mRNA stability issue. Moreover, HLA-G protein expression is driven by genetic variations in the 3′ UTRs, but also by those landing in the promoter region; thus, variability of the microenvironment associated with specific disease could affect the HLA-G protein expression.

INTRACELLULAR AND EXTRACELLULAR MECHANISMS REGULATING HLA-G EXPRESSION

Genetic variations in the 3′ UTR, which contain several target sites for microRNAs (miRNAs), regulate at post-transcriptional level the HLA-G expression. Being miRNA cell-specific, this regulation may affect the expression of HLA-G at cell and tissue levels. Six miRNAs have been reported to regulate HLA-G expression: miR-148a, miR-148b, miR-152, miR-133a, miR-628-5p, and miR-548q (57). The direct effect of these miRNAs in HLA-G protein expression has been mainly demonstrated *in vitro*, using cell lines (**Figure 1**). However, several indirect evidences prompted investigators to correlate the presence of specific miRNA with HLA-G protein expression *in vivo*. In placenta, miR-148a and miR-152 are poorly expressed, whereas the HLA-G mRNA levels are high (58). Since miR-148a and miR-152 down-regulate HLA-G1 protein expression in cell lines, a possible inverse relationship between these molecules in placenta has been postulated (58). Similarly, miR-133 reduced HLA-G protein expression in trophoblast cell lines (59), and its low expression in primary colorectal cancer samples, in which the HLA-G levels are high (60), suggested a possible inverse correlation of these molecules. As anticipated avobe, thus far direct evidence of the role of specific miRNA on the expression of HLA-G *in vivo* is scanty.

An additional layer of posttranscriptional regulation of HLA-G protein expression is mediated by a specific RNA-binding protein (RBP) (**Figure 1**), the heterogeneous nuclear ribonucleoprotein R (HNRNPR), which binds the 3′ UTR of the transcripts, stabilizes them, and allows HLA-G1 expression in transduced cell lines (61). More recently, a distinct and unique region in the 3′ UTR of HLA-G has been identified, but neither miRNAs nor RBPs seem to bind to this site and to control HLA-G1 expression in cell lines (62). Thus, additional studies are warranted to define the relevance of this sequence into the regulation of HLA-G protein expression *in vivo*.

The HLA-G protein expression is regulated not only by allelic variability and posttranscriptional regulation, but also by specific regulatory regions present in the HLA-G promoter. Nucleotide variability in the promoter region may indeed influence HLA-G protein levels by modifying transcription factor–binding affinity. In the HLA-G locus, the enhancer A (EnhA) region, which allows the interaction with nuclear factor k-light-chain-ehnancer of activated B cells (NF-κB) family of transcription factors, binds only p50/p50 homodimers (63). Moreover, the binding of interferon regulatory factor 1 and 2 (IRF-1 and IRF-2) to interferon-stimulated response element in the HLA-G promoter is not present in HLA-G (64, 65). The presence of this unique HLA-G promoter region indicates that HLA-G expression is not influenced by NF-κB or by interferon α (IFN-α), IFN-β, and IFN-γ (**Figure 1**).

Despite the unresponsiveness to interferons, HLA-G transcriptional rate is increased by a number of anti-inflammatory cytokine and mediators, as the HLA-G promoter region presents specific regulatory elements. The presence in the HLA-G promoter of a heat shock element allows response to heat shock proteins (HSP) (**Figure 1**), essential for regulating the state of intracellular folding, assembly, and translocation of proteins, potent modulators of the immune responses, and necessary for placental development. HLA-G transcription is induced upon heat shock in tumor cell lines, *via* the heat shock transcription factor 1; however, the consequent HLA-G protein expression has not been investigated (66).

Glucocorticoids (dexamethasone) and progesterone, a hormone fundamental for endometrium maintenance and embryo implantation, increase the secretion of soluble HLA-G5 and HLA-G6 by trophoblasts (67–69). The molecular mechanism underlying this protein expression was proposed to be induced *via* the interaction of progesterone receptor complex binding to a unique progesterone response element (PRE) sequence present in the HLA-G promoter (13) (**Figure 1**). Classical HLA class I genes have neither a classical PRE nor the unique PRE identified in HLA-G, thus suggesting that progesterone may be involved in cell-specific regulation of the HLA-G protein expression (70, 71).

Hypoxia modulates different processes, and it is associated with induction of HLA-G. Low oxygen increases HLA-G mRNA expression in trophoblasts (72) and in tumor cell lines (73), but its effect on the expression of HLA-G protein is still undefined.

Finally, the HLA-G protein expression can be modulated by granulocyte–macrophage colony-stimulating factor (GM-CSF) in cell lines (74) and by IL-10 in monocytes (75) and DC-10 (53, 55). Although the mechanisms underlying the induction of HLA-G protein expression by the above cytokines have not been completely elucidated (**Figure 1**), these evidences support the role of HLA-G in promoting an anti-inflammatory and in inducting a protolerogenic microenvironment.

Overall, HLA-G protein expression is driven by specific alleles, and it is regulated by intracellular and extracellular signals.

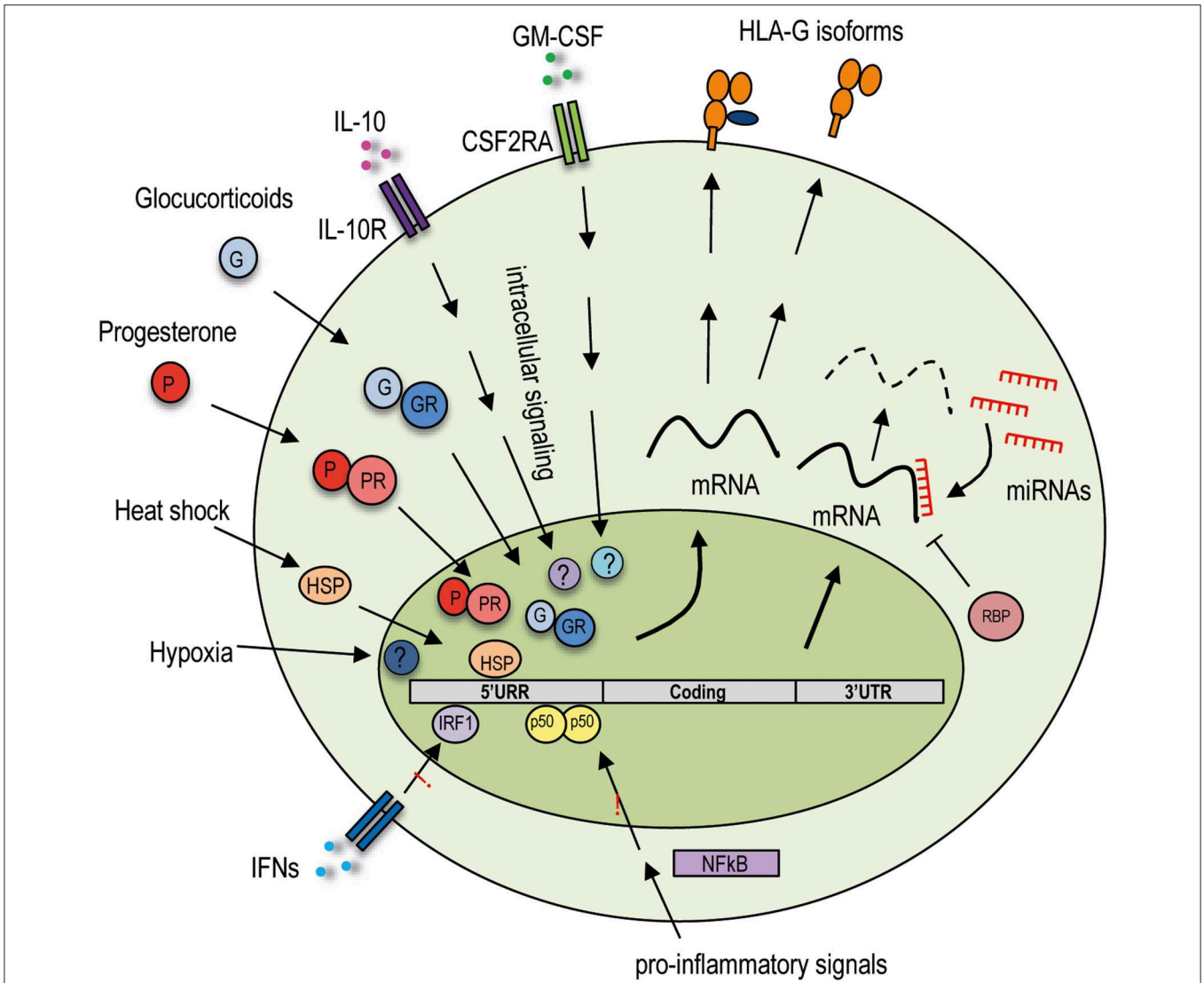

FIGURE 1 | Extracellular and intracellular regulatory mechanisms of HLA-G expression. Variability in the HLA-G promoter region influences HLA-G expression by sensing and responding to the extracellular signals. Variations in the 3′ UTR region may modify mRNA stability or allow posttranscriptional regulation. HLA-G is not responsive to proinflammatory signals acting on the NF-κB pathway and to IFN-mediated stimulation. The HLA-G promoter region is unique among the HLA class I genes as it interacts with specific transcription factors activated by extracellular stimuli induced by hypoxia and heat shock, hormones such as glucocorticoids and progesterone, and cytokines including IL-10 and GM-CSF. HLA-G expression is posttranscriptionally regulated by genetic variations in the 3′ UTR, which contain several target sites for miRNAs and can bind specific RNA-binding proteins. These different regulations concur in the induction or inhibition of the expression of the HLA-G protein, which by alternative splicing of the mRNA can be produced in different isoforms: membrane-bound or soluble. 5′ URR, 5′ upstream regulatory region; 3′ UTR, 3′ untranslated region; CSF2RA, colony-stimulating factor 2 receptor subunit alpha; IL-10R, IL-10 receptor; IFNs, interferons; GR, glucocorticoid receptor; PR, progesterone receptor; HSP, heat shock protein, IRF-1, interferon regulatory factor 1; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; RBP, RNA-binding proteins; miRNAs, microRNAs.

Despite results obtained in cell lines, only putative correlations between the presence of specific miRNAs and HLA-G protein levels *in vivo* have been suggested. Moreover, is has to be taken into account that the microenvironment (e.g., the presence of specific molecules, hormones and/or cytokines) might affect HLA-G protein expression. These considerations are particularly important when HLA-G genotype/protein association is studied in specific diseases.

CONCLUDING REMARKS

The discovery that HLA-G genetic variants represent target of gene expression regulation led to intensive research for the identification of HLA-G genetic association with disease predisposition or progression and HLA-G protein expression. Despite several good genotype/protein correlations have been reported using *in vitro* model, when these observations have

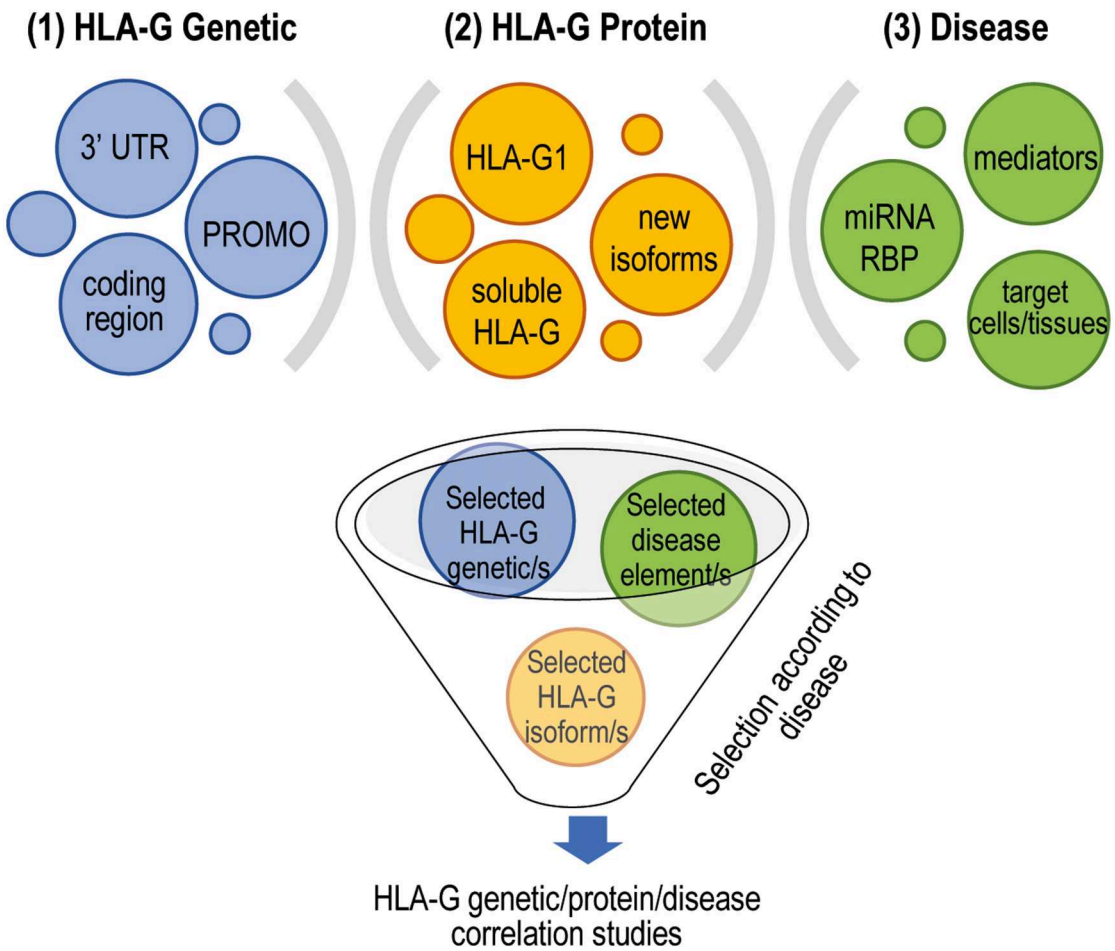

FIGURE 2 | Proper selection of targets to perform HLA-G genetic/protein and disease development association studies. Different layers of complexity, both cell specific and disease specific, have to be taken into account to reliably define the role of HLA-G in disease development. (1) To select the HLA-G polymorphisms to include in the analysis; (2) to include in the analysis the evaluation of disease-specific features such as the expression of specific miRNAs or RBP and the presence of proinflammatory and anti-inflammatory mediators that can modulate the expression of HLA-G, and the target cells or tissues to study that can be affected by the presence of HLA-G; (3) to identify the most appropriate HLA-G isoforms to be investigated. 3′ UTR, 3′ untranslated region; PROMO, variations in the promoter region; miRNAs, microRNAs; RBP, RNA-binding proteins.

been translated in disease setting, the diagnostic/prognostic relevance of these findings in some cases appeared weak. A number of issue should be considered: (i) in the majority of the studies, conclusions have been based on results from small patient cohorts and in subjects from different ethnicity; (ii) only few studies performed a complete assessment of HLA-G genotyping, protein expression, and diseases outcome; (iii) the mechanisms regulating HLA-G expression can be distinctly active in different diseases; (iv) excluding the well-studied 14-bp INS/DEL polymorphism, there is a high heterogeneity of the genetic variations investigated, hindering the possibility to claim univocal conclusions.

The specific intracellular signaling and the microenvironment characterizing a given disease have to be considered for a proper selection of the polymorphisms to be investigated. As an example, the use of the entire 3′ UTR could be relevant, but other regulatory elements (e.g., the expression of miRNAs) should be investigated in parallel, to have a comprehensive picture; otherwise, the analysis of 14-bp INS/DEL polymorphism could be sufficiently informative. Indeed, the expression of miRNAs can vary among different pathological conditions and in different cells, thus affecting the expression of HLA-G if present. Similarly, the observation that haplotypes comprising 3′ UTR, coding regions and PROMO are mainly defined by the 3′ UTR region suggested that the analysis of PROMO and coding regions of HLA-G gene should be considered for improving a better correlation between HLA-G genetic and disease predisposition if disease-specific mediators are also analyzed in parallel; otherwise, the sole analysis of the 3′ UTR might be sufficient. Finally, in several studies, the presence of HLA-G has been evaluated at mRNA but not at protein levels, being the most reliable indicator of activity. Another important aspect to consider in the analysis HLA-G proteins is the selection of the isoform, the site or cell population to be evaluated.

The recent discovery in renal carcinoma cells of new HLA-G transcripts, characterized by previously not described intron retention or exon skipping events, which cannot be recognized by the available antibodies (76) increases the complexity in the field. However, whether these transcripts encode for novel isoforms, are specifically produced by cancer cells, or have regulatory functions, remain to be defined. Based on these premises, it can be envisaged that a more sophisticated selection of the parameters to be investigated is critically important to improve the HLA-G genotype/expression/disease association studies. We propose that the following steps should be taken into account: the selection of the HLA-G genetic variations (e.g., 14-bp INS/DEL or 3′ UTRs, the coding region and/or the PROMO), which might depend on the type of diseases under consideration, on the tissue or the specific subset of cells analyzed; the possibility to analyze in parallel other specific parameters (e.g., the expression of miRNAs or RBP, soluble mediators including hormones or cytokines); the selection of the most relevant HLA-G isoform (e.g., soluble HLA-Gs or HLA-G1 or other isoforms) (**Figure 2**).

In conclusion, up to now a clear and univocal HLA-G genotype/expression/disease association has not been yet identified, with the exception for 14-bp IND/DEL allele. A more specific and "disease-oriented" analysis, meaning the selection of the relevant polymorphisms, isoforms, and regulatory factors that could impact on HLA-G expression, would be more helpful and affordable for better defining the interplay among HLA-G genetic variations, protein expression, and disease predisposition or response to therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article.

AUTHOR CONTRIBUTIONS

GA wrote the manuscript. SG designed and wrote the manuscript. All authors read and approve the final manuscript.

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REVIEW
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HLA-G Neo-Expression on Tumors

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HLA-G is known to modulate the immune system activity in tissues where physiological immune-tolerance is necessary (i.e., maternal-fetal interface, thymus, and cornea). However, the frequent neo-expression of HLA-G in many cancer types has been previously and extensively described and is correlated with a bad prognosis. Despite being an MHC class I molecule, HLA-G is highly present in tumor context and shows unique characteristics of tissue restriction of a Tumor Associated Antigen (TAA), and potent immunosuppressive activity of an Immune CheckPoint (ICP). Consequently, HLA-G appears to be an excellent molecular target for immunotherapy. Although the relevance of HLA-G in cancer incidence and development has been proven in numerous tumors, its neo-expression pattern is still difficult to determine. Indeed, the estimation of HLA-G's actual expression in tumor tissue is limited, particularly concerning the presence and percentage of the new non-canonical isoforms, for which detection antibodies are scarce or inexistent. Here, we summarize the current knowledge about HLA-G neo-expression and implication in various tumor types, pointing out the need for the development of new tools to analyze in-depth the HLA-G neo-expression patterns, opening the way for the generation of new monoclonal antibodies and cell-based immunotherapies.

Keywords: HLA-G, immune checkpoint, hematopoietic tumors, solid tumors, immunotherapy

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INTRODUCTION

Fetus and tumor development are closely related since they are both characterized by a rapid tissue proliferation, associated to a high expression of telomerase (1, 2) and expression of anti-apoptotic factors like survivin (3, 4). Placenta and tumor development is accompanied by angiogenesis induced by proteins of the VEGF family (5, 6) and favored by hypoxia (7). Strikingly, placenta and tumors are protected from the immune system through common immune escape mechanisms. Particularly, the induction of a tolerogenic microenvironment was demonstrated, involving the expression of inhibitory immune checkpoints inducing suppressive macrophages, dendritic cells (DCs) and regulatory T cells (T_{regs}). Among the pool of inhibitory checkpoints shared between the placentation process and the tumor development, HLA-G is emerging as a potent immune escape mechanism (**Figure 1**).

HLA-G is a non-classical MHC class I molecule first determined to be expressed on extravillous trophoblast that invade the decidua (8–11), similarly to the invasive growth process observed for tumors (12). Despite being restrictively expressed on healthy tissues, HLA-G was reported to be neo-expressed in several pathological contexts, especially during tumor development (13, 14). HLA-G neo-expression is always associated with a bad prognosis for patients.

Contrary to the classical MHC, HLA-G is characterized by a low polymorphism and tolerogenic functions. HLA-G can be expressed under, at least, seven isoforms. These are the product of

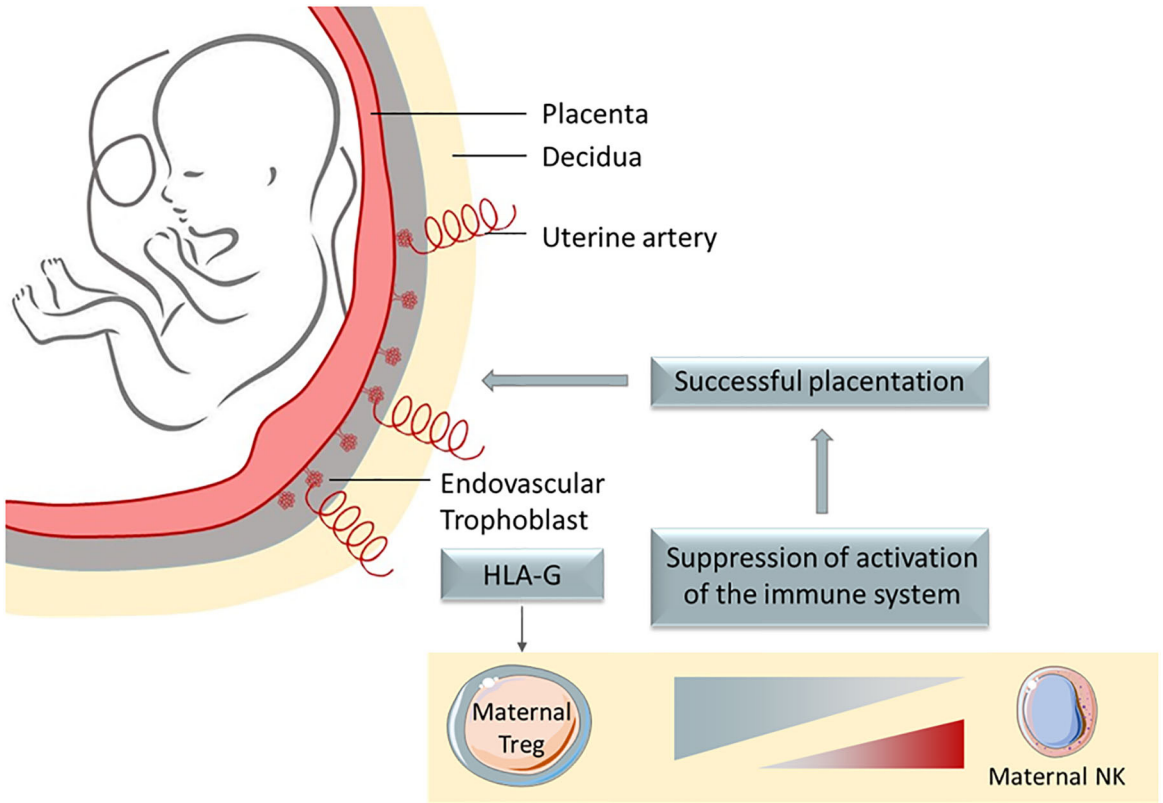

FIGURE 1 | HLA-G is a potent immune escape mechanism, whose main physiological role is to protect the semi-allogenic fetus from mother' immune system, being expressed on extravillous trophoblast that invades the decidua.

alternative splicing of a single primary transcript of RNA (15): four membrane isoforms (HLA-G1, HLA-G2, HLA-G3, and HLA-G4) and three soluble isoforms (HLA-G5, HLA-G6 and HLA-G7). HLA-G1 and HLA-G5 isoforms present the typical structure of MHC classical class I molecules: one heavy chain composed of three globular domains, associated or not to β-2-microglobulin (β2M). The other isoforms are shorter, with one or two globular domains and none is associated with the β2M (16–18). HLA-G exert its biologic tolerogenic function as a ligand by binding its specific receptors: ILT2 (LILRB1, CD85j), ILT4 (LILRB2, CD85d) et KIR2DL4 (CD158d) (19). HLA-G is the ligand of highest affinity for ILT2 and ILT4 receptors. Concerning the KIR2DL4 receptor, it is mostly expressed in NK cells, but its interaction with HLA-G and its inhibitory function remain controversial (20, 21). ILT2 and ILT4 belong to the leukocyte immunoglobulin (Ig)-like receptor family (LILRs), particularly to the inhibitory group: LILRBs, composed of 2 to 4 extra-cellular globular domains and 2 to 4 cytoplasmic inhibitory domains "ITIM" (Immunoreceptor tyrosine-based inhibitory motifs). ILT2 is expressed in all immune cell subsets (22), whereas ILT4 expression is limited to antigen presenting cells (APCs) like monocytes, neutrophils, DCs or macrophages. PIR-B is the ortholog of LILRBs in mice, expressed in B cells, DCs, granulocytes and macrophages, exerting the same inhibitory functions (22). ILT2 binds the HLA-G α3 domain, associated with β2M simultaneously (23) while ILT4 binds the α3 domain independently of β2M. Also, HLA-G can form dimers, which increase the avidity of the receptors ILT2 and ILT4 for this molecule (23).

In physiological conditions HLA-G expression has been described in the cytotrophoblast where it plays a major role by protecting the semi-allogenic tissues of the fetus from the maternal immune system. Otherwise, HLA-G is constitutively expressed in immune-privileged tissues like thymic epithelial cells (24, 25), cornea (26), pancreatic islets (27), mesenchymal stem cells (28, 29), erythroblasts or endothelial precursors (30, 31), and some peripheral tolerogenic T and dendritic cells (DC) subsets (32–34). Soluble isoforms have been detected in thymus (24), human first trimester and term placentas *in situ* and *in vitro* (35), plasma (36, 37), cerebrospinal fluid (CSF) (38, 39), in the male reproductive system, in seminal plasma (40), and in the cell culture supernatant of embryos (41–43). However, HLA-G expression can be induced or up-regulated in pathological contexts like (i) cancer (44–46), (ii) auto-immune and inflammatory diseases (47–49), (iii) viral infections (50–52), and (iv) allo-transplantations (53, 54). Indeed, many publications showed the high frequency of HLA-G expression in tumor cells, correlated with clinical background associated

with tumor immune escape and bad prognosis (45, 55). HLA-G expression, then, seems to be key for tumors to evade the immune system, even at low rates of expression. Although most of HLA-G immunosuppression function and role in tumor escape studies were performed *in vitro*, HLA-G involvement in tumor escape mechanism was studied and demonstrated *in vivo* in immunocompetent mice through the induction of MDSC (56). Furthermore, Lin et al. evidenced *in vivo* that HLA-G expression was associated with tumor metastasis and with poor survival (57). Inhibition of immune response by soluble HLA-G was also demonstrated *in vivo* (58). Noteworthy, HLA-G expression is induced by hypoxia, typical of solid tumor microenvironment (59). Because HLA-G is found on tumor cells and is rarely observed in healthy tissue, it appears to be an excellent tumor associated-antigen (TAA) to target in immune therapy. Furthermore, HLA-G has been recently defined as a major immune checkpoint (ICP). This molecule is capable of inhibiting not only cytolytic uterine NK cells in the context of pregnancy, but also: (i) cytolytic functions of peripheral NK (60, 61), (ii) cytolytic functions of antigen-specific cytotoxic T lymphocytes (CTL) (62), (iii) alloproliferative response of T $CD4^+$ cells (63), (iv) peripheric NK and T cell proliferation (64, 65), (v) B cell maturation and antibody production (66), phagocytic function of neutrophils (67), chemotaxis of NK, T and B cells (66, 68, 69), and (vi) maturation and function of DCs (70). Also, HLA-G was shown to induce the generation of suppressive immune cell subsets (64, 71, 72) (see **Figure 2**).

Although HLA-G is an MHC class I, it presents rare characteristics combining features of both TAA and ICP, playing a major role in the fine tuning of the immune system equilibrium into a tolerogenic or suppressive microenvironment. HLA-G turns to be a major advantage for tumor cell survival and development. In fact, HLA-G expression has been reported in numerous types of cancer, always associated with more advanced stage and aggressive development of the tumor.

HLA-G NEO-EXPRESSION IN HEMATOPOIETIC TUMORS

The role of HLA-G in hematopoietic malignancies is complex and remains unclear since HLA-G and its inhibitory receptors

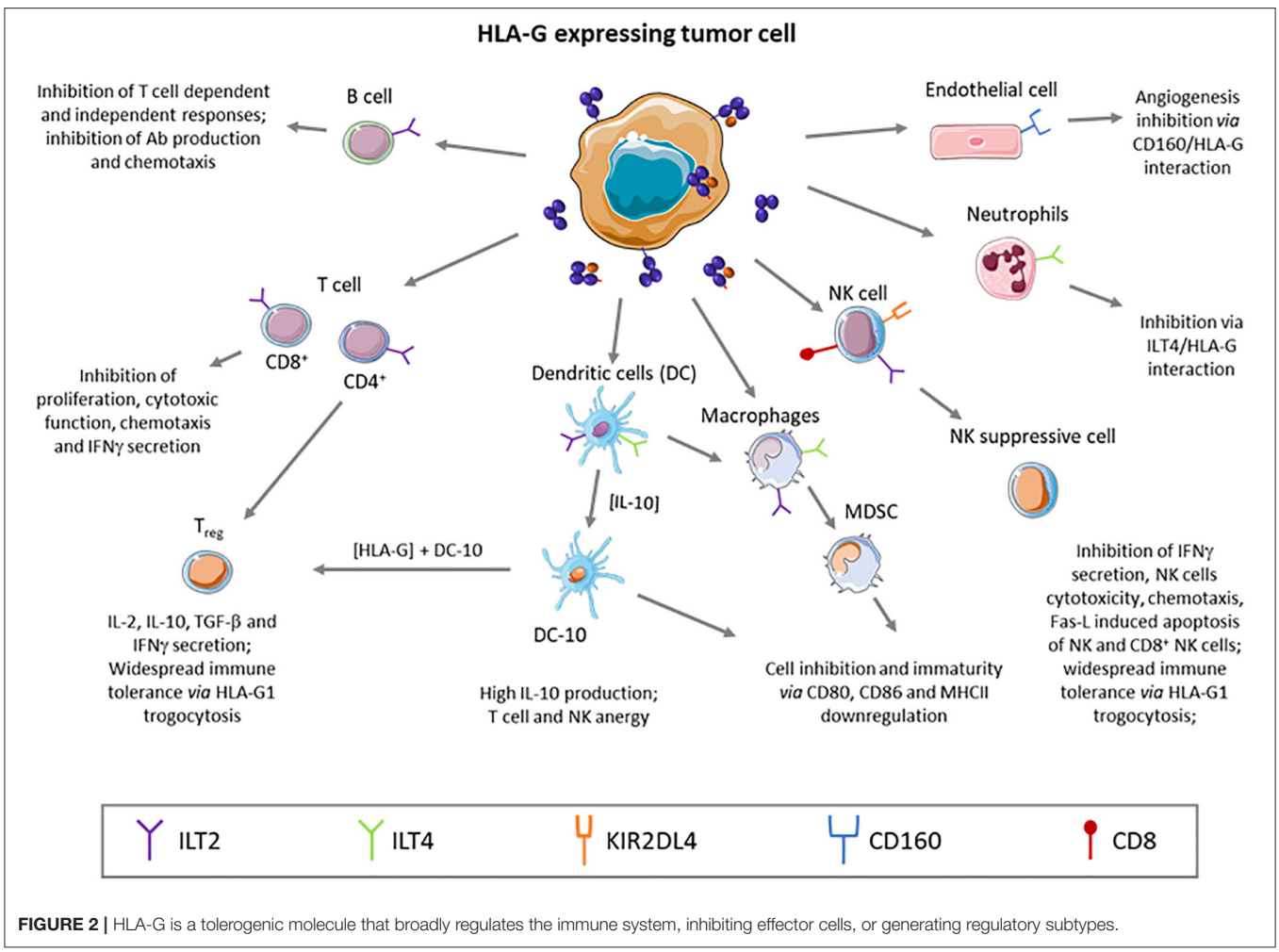

FIGURE 2 | HLA-G is a tolerogenic molecule that broadly regulates the immune system, inhibiting effector cells, or generating regulatory subtypes.

could be expressed on hematopoietic tumor cells and could inhibit proliferation in such tumors (45). Non-Hodgkin lymphomas (NHL) is a large group of cancers of lymphocytes. There are many different types of NHL which can be divided into aggressive (fast-growing) and indolent (slow-growing) types, composed by either B-cells or T-cells. In NHL, classical MHC molecules and HLA-G expression patterns were shown to be completely altered and correlated to a tumor relapse or transformation (73). It has been postulated that this phenomenon was associated to a deep genetic disorder and rearrangement, inducing HLA-G neo-expression in tumor cells. Chronic lymphocytic leukemia (B-CLL) is a mature lymphoid neoplasm currently categorized as an indolent type of malignant lymphoma. Nuckel et al. reported HLA-G expression on 1–54% of leukemic cells in B-CLL. They determined that patients with 23% or fewer HLA-G-positive cells had a significantly longer progression-free survival (PFS) time than patients with more than 23% of positive cells. Indeed, patients with a weak HLA-G expression showed a higher survival rate (120 months) than those with high HLA-G expression with a survival average of 23 months. Furthermore, humoral and cellular immunosuppression were significantly more prominent in the HLA-G-positive patients' group in comparison to the HLA-G-negative group. Indeed, the survival rate decrease was associated to an immune response deficiency, a CD4/CD8 T cells ratio and immunoglobulins (IgG) reduction and to an increase of secreted soluble HLA-G proteins (74–76).

B-CLL can progress slowly over years, but it eventually transforms into a more aggressive lymphoma such as the diffuse large B-cell (DLBCL) type. The diffuse large B-cell lymphoma (DLBCL) is a B cells cancer and is the most common type of NHL. DLBCL is characterized by its aggressiveness, which can be developed in the lymph nodes or in extranodal sites. DLBCL is the most frequent lymphoma and the most severe. In this type of lymphoma, the expression of HLA-G was determined to be relatively weak. However, the survival rate was directly correlated to the HLA-G expression, increasing from 47.5%, when HLA-G is expressed, to 73.3% in absence of HLA-G expression (77). Expression of HLA-G in classical Hodgkin lymphoma was also independently determined by the groups of Diepstra and Caocci. They both determined a relatively high expression of HLA-G (>54% of expressing tumor cells) in the Reed-Stenberg cells, with particular higher expression in nodular sclerosis (78, 79). However, their results on the HLA-G expression levels were different in the tumor microenvironment (TME).

Cutaneous lymphomas represent the second most frequent extranodal lymphomas and are cancers of lymphocytes primarily involving the skin. Cutaneous lymphomas are classified based on whether they are cancers of B or T lymphocytes, and, respectively, designated as cutaneous T cell lymphoma (CTCL) and cutaneous B cell lymphoma (CBCL). Although being mostly a benign disease, skin clonal lymphocytes can migrate to the nodes resulting in a more severe disease. These cells can persist mostly because of HLA-G and IL-10 secretion (80). All the T or B skin cells were determined to be HLA-G1 mRNA positives, but protein expression level was weaker. A strong correlation between IL-10 and HLA-G expressions was evidenced with a co-expression of these molecules in 73% of the cutaneous lymphoma investigated (81). Furthermore, for T cells, HLA-G protein expression was directly correlated with the tumor grade and stage.

HLA-G NEO-EXPRESSION IN SOLID TUMORS

Tumor development is dependent on its capability to escape from the immune response. According to Dr. Schreiber's 3E theory, three stages define the immune response and the interaction between tumor cells and their microenvironment: elimination, equilibrium and escape (82). The first phase of elimination is characterized by the production of new molecules, derived from oncogenic modifications of the brand-new tumor, and expression on their surface, known as neo-antigens, that are able to induce an efficient response by the immune system. In accordance with the classic immune surveillance theory, those new tumor cells that aren't destroyed in the initial stage, will proliferate, create a primitive tumor and will set up an equilibrium with the immune cells. This equilibrium phase can last months or years, until the tumor becomes able to engage the *escape* phase, where the plasticity of its genome allows it to evolve, change the environment, evade the immune control and spread. At the same time, the immune system might become tolerant or exhausted.

Actually, HLA-G can be involved in these three phases. During the elimination phase, HLA-G can inhibit T and B cells activation, proliferation, cytotoxic function of T and NK cells and can block the DCs and neutrophils functions (60, 62, 68, 76, 83, 84). Throughout the equilibrium phase, HLA-G can downregulate the MHC class II expression on DCs and induce suppressive myeloid cells, favoring the regulatory cell subsets (85). Finally, the escape phase is characterized by a high cell proliferation and, afterwards, a hypoxic environment (86). Hypoxia induces upregulation of V-EGF and HIF-1, and with the latter, HLA-G expression. Also, it was determined that immunosuppressive cytokines, such as IL-10 and TGF-β, are secreted and could favor HLA-G expression and maintenance by positive feedback (87).

HLA-G expression in multiple types of primary tumors has been demonstrated (88). HLA-G can be detected either on the cell-surface of tumor cells or on tumor infiltrating cells (TILs) particularly on lymphocytes, monocytes, macrophages and dendritic cells (DCs) (89–94). HLA-G was demonstrated to be crucial for the tumor development and its expression was specifically associated to malignant transformation (59). HLA-G expression in surrounding healthy tissue has never been detected but its expression in solid tumors has been described, particularly in advanced clinic stages (95, 96). Soluble HLA-G isoforms (sHLA-G) have been detected in patient's plasma with advanced stages and reserved prognostic (91, 95–99). Therefore, the role and functions of HLA-G in tumor immune escape and tumor development is beyond a hypothetical mechanism, its involvement and relevance has been widely documented. This tolerogenic molecule has been described in a plethora of solid tumors.

What brings another level of complexity in the detection of HLA-G and the understanding of its role in cancer progression is the existence of micro-vesicles bearing HLA-G, firstly described in the supernatant of HLA-G positive melanoma cells *in vitro* (100). Intercellular communication through extracellular vesicules (EV) released in the extracellular space or in body fluids is a known mechanism involved in healthy tissues as well as malignancies (101). These structures originate from the cell membrane or are exosomes, and can carry surface proteins, cytokines or growth factors (amongst others). Their role in the immune response modulation has been shown by Abusamra et al. by *in vitro* experiments that evidenced the induction of $CD8^+$ T-cells apoptosis by exosomes expressing Fas ligand (102). This observation and the known mechanism of action of EVs suggest that EVs harboring HLA-G could play a role in cancer immune escape, by inhibiting immune cells in the tumor microenvironment or at distal sites. The inhibition of monocyte differentiation and maturation into dendritic cells (DCs) by HLA-G1-bearing EVs originating from kidney cancer cells has been reported (103). Several clinical studies carried out with breast and ovarian cancer patients also support this hypothesis. High levels of HLA-G-EVs in breast cancers patients treated with neoadjuvant chemotherapy (NACT) correlates with a bad prognosis, whereas patients with high levels of free soluble HLA-G had better outcome. Moreover, the level of total circulating HLA-G molecules is not a predictable marker of patient's outcome (104). Similar findings were reported in a study with epithelial ovarian cancer (EOC) patients in which high levels of HLA-G EVs was a marker of inferior clinical outcome (105). Deciphering HLA-G EVs from free soluble HLA-G molecules seems thus to be of crucial importance to improve patient's diagnosis and the understanding of EVs mechanism of action, and investigate their relevance as immunotherapy target.

Renal cell carcinoma (RCC) affects 3% of occidental adults, with an increasing incidence in the last years. There are several subcategories of RCC, the principal being clear cell RCC (ccRCC) that represents 80% of RCC, followed by the papillary and chromophobe carcinoma, 10 and 5%, respectively. Frequently, this cancer is at an advanced stage presenting metastasis at the time of diagnosis, with a low rate of 5 years survival (<15%). In ccRCC, HLA-G mRNA and protein expressions have been strongly described (106–108). These expressions in patients seem to be age or sex independent but are highly related to the ccRCC sub-type. Frequently, there is no correlation between mRNA and protein expression which might be explained by a postranscriptional regulation that blocks translation (109). HLA-G loss of expression at the tumor cell-surface during cell culture could be explained by (i) the absence of transcription factors related to the hypoxic microenvironment or (ii) the lack of several cytokines such as IFN-γ, IFN-α, and IL-10 (110), but (iii) could also be related to a HLA-G isoform switch that could not be detected since antibodies detecting all the isoforms of HLA-G are missing, particularly for those that lack the α1 domain (111). Recently, a heterogeneous expression of immune checkpoints including PD-L1, B7H3, ILT2, and HLA-G in RCC was reported (112). This intratumor heterogeneity was found both at tumor cell and infiltrating immune cell levels in primary RCC (113). Interestingly, target cells' HLA-G expression specifically inhibited cytotoxicity of $CD8^+ILT2^+$ T-cells, but not their $CD8^+ILT2^-$ (PBMC) or $CD8^+PD\text{-}1^+$ (TIL) counterparts. HLA-G inhibition was counteracted by blocking the HLA-G/ILT2 interaction showing that $CD8^+ILT2^+$ TILs may therefore constitute a subset of fully differentiated cytotoxic T cells within the tumor microenvironment, independent of the $PD1^+$ TILs targeted by immune therapies, and specifically inhibited by HLA-G (114).

Colorectal cancer (CRC) is the 3rd most frequent cancer in the world and the 2nd mortality cause related to cancer. Most of them (96%) are present under the adenocarcinoma form associated to a transformation of luminal epithelial cells of the mucosa of the intestine. It affects mostly aged population (>50 years). It was determined that HLA-G expression was detected in 64% of the tumor samples in the primary site of the carcinoma but HLA-G expression was absent in the surrounding tissue, evidencing HLA-G as a malignant transformation marker (92, 96). HLA-G expression was correlated with advanced stages of the "tumor-node-metastasis" (TNM) classification and a diminution of the survival rate (<3 years) for HLA-G positive patients. However, HLA-G expression in CRC remains controversial. Due to the heterogeneity amongst techniques and technic tools, discordant results were obtained for HLA-G expression in CRC. Furthermore, HLA-G neo-expression was shown to depend on the microenvironment of the primary lesions, yet, absence of TME on metastases cannot be linked to HLA-G neo-expression (115). Thus, primary tumors are mostly associated to an active response expressing immunomodulatory molecules like HLA-G, whereas secondary or metastatic tumors favor a "hiding" strategy, avoiding being detected by the immune system. Co-expression of HLA-G and HLA-E has been described in CRC. Downregulation of expression of the classical MHC class I molecules allow tumor cells to escape from cytotoxic T cells (CTLs) despite rendering them sensitive to the NK response. Inhibition of the NK response is related to the upregulation of HLA-G and HLA-E which mediates the inhibition of NK cells through ILT2 and NKG2A receptors, respectively (83, 116).

Esophageal carcinoma is the 8th most frequent cancer in the world and the 4th cause of death related to cancer. The most frequent type is the esophageal squamous-cell carcinoma (ESCC) (90%). HLA-G expression extended from 65 to 90% of ESCC cases and was related to advanced stages of TNM (117–119). The lower survival rates were shown to be related to tumor cell infiltration in the lymph nodes and to the HLA-G expression. The mRNA studies performed indicated that the most frequent isoforms present were HLA-G1 and -G5 in the primary tissue, but were absent from surrounding tissue, and HLA-G5 was detected in patients sera (118).

Gastric cancer is the 5th most frequent cancer and causes more than 700 000 deaths per year in the world, being the 3rd cause of death in cancer. Usually, at the time of diagnosis this cancer is advanced and frequently presenting metastasis. 90–95%

of gastric cancer are adenocarcinoma with a gastric superficial mucosa cells origin. High HLA-G expression was reported with 73% of cases, from which 75% presented high expression levels (>50% of cells expressing HLA-G) (97). This expression is exclusive to the primary tumor, without no expression detected on the environmental tissue, and is related to the localization of the tumor, with higher expression in the cardia. Higher HLA-G expression is correlated with advance stage of the disease, the tumor lesion depth, the node invasion and the decrease of the survival rate. Several other groups have demonstrated corresponding results that HLA-G expression in tumor cells correlates with sHLA-G in patients' sera and higher infiltration of T_{reg} $CD4^+$ $CD25^+$ $FoxP3^+$ (120, 121). Also, Du et al. demonstrated that co-culture of PBMCs and SGC-7901 (a gastric cell line), transfected with HLA-G1, can induce an immune regulatory phenotype with an increase of IL-4 and IL-10 secretion and a decrease of IFN-γ secretion (121).

Pancreatic cancer is a relatively rare cancer (<2% of cancers). This adenocarcinoma is very aggressive, associated with a very bad prognosis. Pancreatic cancer is generally of endocrine origin, but it metastases easily, frequently to the liver, stomach and lungs. Zhou et al. have studied HLA-G expression in this cancer, and they have determined that 39.2% are HLA-G positive (122), depending on the tumor grade, increasing from T1-2 to T3 stages. HLA-G expression is correlated with a decrease of infiltrating T cells (TILs) $CD3^+$. Other groups confirmed the HLA-G expression in pancreatic adenocarcinoma ranging from 63 to 66% of tumors (123, 124). Xu et al. correlated the HLA-G expression with more aggressive characteristics, a more advanced stage (TNM II), an extra-pancreatic infiltration (T3 stage) and a lymphatic nodes engagement (124). Also, plasmatic sHLA-G was higher in pancreatic cancer patients in comparison to the controls and inversely proportional to $CD8^+$ $CD28^+$ peripheral T cells.

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults and the fourth most common cause of cancer-related death worldwide. HCC is usually caused by a chronic disease (infection or cirrhosis). HLA-G expression was determined to be present in 66.7% of the cases and correlates with a more advance TNM stage: 41.9% in stage II to 71.4% in stage III (125). HLA-G expression is associated with an increase of the $T_{reg}/CD8^+$ ratio and relapse occurs after ablation or resection. Other groups observed similar results (126, 127). Cai et al. indicated that the HLA-G expression remained diffuse and intracellular, detected HLA-G isoforms were essentially the HLA-G1 isoform (detection through WB) without the presence of the HLA-G5 isoform. Yet, sHLA-G was detected in patients' sera (125). This could be explained by the shedding of the HLA-G1 membrane isoform or by the expression of this molecule by other cells like monocytes as previously observed in melanoma and lung cancer. It was hypothesized that the expression of HLA-G could be sustained by the microenvironment of the primary tumor in agreement as observed in other type of cancers (107, 109, 115, 128) and with the 3E theory that points out that the metastatic sites of a cancer should present a totally different microenvironment from that of the primary tumor (82).

Thyroid nodes are cancers of the thyroid affecting 50 to 70% of the adult population which are mostly benign. This neoplasia presents a variable evolution and is constituted by 3 histologic sub-types: papillary thyroid carcinoma (PTC), follicular thyroid cancer (FTC) and anaplastic thyroid cancer (129). It has been demonstrated that HLA-G expression is crucial for the development of these cancers. Indeed, HLA-G expression is absent in non-pathological histologic tissue (130) whereas HLA-G expression is determined to be present in all thyroid tumors. A strong expression of HLA-G (>50% of HLA-G^+ cells) was observed in 80% of PTC and 79% of FTC but also in benign lesions. However, HLA-G expression was not correlated with cancer relapse, metastasis, node invasion or with mortality rate. It was proposed that HLA-G was necessary for cancer genesis given its pre-tumoral expression. Other groups confirmed this expression (131), although the different assays used to determine HLA-G expression, and the lack of a diagnostic methodology, induced some discrepancies between the results.

Melanoma is developed in melanocytes and its incidence is 11 in 100,000 (132). This cancer is not very aggressive, with a survival rate of 5 years in 81% of men and 87% of women (133). The expression of HLA-G in melanoma has been studied and demonstrated to be increased compared to melanocytic nevi (90), correlating HLA-G expression with cell transformation. HLA-G expression was also demonstrated to be increased on inflammatory infiltrating cells within the melanomas compared to nevus (134). sHLA-G was also increased in patients' sera seemingly being boosted by the IFN-α treatment applied (135). HLA-G expression was further shown to be associated to the malignant transformation and to bad prognosis, in case of metastasis or relapse, in different studies (128, 134, 136). Other groups demonstrated *in vitro* the immune-tolerogenic properties of HLA-G, protecting melanoma cell lines from the NK cells cytotoxicity (137), which were confirmed *in vivo* on xenogeneic melanoma models (56). Also, it was demonstrated that the tumor cells were able to modify their HLA-G isoform expression profile in order to modify their susceptibility against NK cells (44).

Gliomas represent 70% of the cerebral tumors, and their capability to modulate the immune response has been documented (138). The prognostic is usually bad since only 9.8% of the patients attain 5 years of survival after diagnosis. Wang et al. have reported that almost 70% of gliomas were HLA-G^+ independently of their nature: oligodendroglioma, astrocytoma or oligoastrocytoma (139). Those results were confirmed by other groups (140). Wiendl et al. have widely studied the expression of HLA-G in cell lines derived from glioblastomas. They have demonstrated that 4 of 12 tumor cell lines constitutively expressed HLA-G mRNA. Following IFN-γ treatment, the number of gliomas expressing HLA-G mRNA dramatically increased since 10 out of 12 tumor cell lines were then HLA-G positive. Similar observations were stated for the HLA-G cell-surface expression (141). Other groups confirmed such results on gliomas using the demethylating agent 5-Aza-2′-Deoxycytidine (140). Strikingly, it

was demonstrated that only 10% of glioblastoma cells expressing HLA-G were sufficient to inhibit the PBMCs alloresponse against the whole tumor (141). It has been widely demonstrated that an external stimulation is necessary to induce HLA-G expression at a transcriptional or translational level. In the context of glioblastomas, HLA-G expression was demonstrated to be influenced or regulated by environmental factors, particularly hypoxia or cytokines. Usually this environment is difficult to maintain *ex vivo*, where primary cells or cell lines loose rapidly their HLA-G expression, implying that the real expression levels of HLA-G in tumor cells are frequently underestimated (44).

Breast cancer represents 25.1% of female diagnosed cancers, and the 2nd most frequent of all cancers, with higher incidence in developed countries (142). There are three subtypes of breast cancer depending on the presence of 3 receptors for estrogen (ER), progesterone (PgR) and human epidermal growth factor (ERBB2/HER2). These different subtypes are mostly treated with chemotherapy or hormonal therapy. Ogiya et al. demonstrated that immune escape strategy of primary tumors is different from metastatic tumors in breast cancer. Primary tumors strategy involves higher infiltration of T cells expressing PD-L1 (143). Other studies indicate that secondary tumor focus present less immunoregulatory cells and weak expression of chemoattractants like CCL19/CCR7, CXCL9/ CXCR3, and IL15/IL15R (144). Nonetheless, the genomic and immune profile of a patient with triple-negative breast cancer that progressed during neoadjuvant chemotherapy plus PD-L1 blockade, showed a low level of expression of programmed cell death protein 1 (PD1) and a high level of expression of HLA-G at the time of diagnosis. This expression was associated with an immune evasive phenotype, increased cell motility and invasion, suggesting that HLA-G could be involved in tumor escape (145). Indeed, He et al. have studied the HLA-G expression in breast cancer and have determined that 66% of breast cancer cases are HLA-G positive, with a low HLA-G expression (<25% of tumor cells) in 64% of cases (146). This HLA-G expression on tumor cells is accompanied by the presence of sHLA-G in the sera and is associated with bad prognosis. It was also shown that there was an increase of circulating $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg} cells in HLA-G^+ patients compared with HLA-G^- patients (147). Other groups have shown that >60% were HLA-G^+, from which >23% of cases co-expressed HLA-E (148, 149). Besides the loss of classical MHC class I expression, HLA-G and HLA-E expressions remained protecting tumor cells against NK cells cytotoxic response. Ishibashi et al. have reported higher expression rates with 94.1% of HLA-G^+ tumor cells. They have demonstrated that a peptide derived from HLA-G1 (26–40 amino acid residues) was presented in the MHC class II context, inducing a CD4 response with consequent anti-HLA-G CTL detection. This was the first time that an anti-HLA-G cell response was ever reported (150). However, these results were never confirmed by other groups. Another study demonstrated that HLA-G expression was correlated to the double positive ER^+/PgR^+ tumors in 80% of cases (147). Previous studies had reported that HLA-G expression can be regulated by progesterone in mesenchymal cells, cytotrophoblast and choriocarcinoma cell line JEG3 (151, 152). Yet, the regulation via estrogens has never been reported.

Cervix cancer is the 2nd most frequent malign gynecologic cancer in the world representing 12% of female cancers (153). Pathogenesis is characterized by a progression of a cervical intraepithelial neoplasia (CIN) to cervix cancer (CC). Miranda et al. demonstrated that HLA-G is detected in 80.2% of CIN cases and in 64% of CC cases. Since the HLA-G expression level is higher in CC (48%) than in CIN (27%), it was suggested that HLA-G expression was correlated to the tumor development (154). HLA-G expression has also been correlated with IL-17 expression that could, on one hand, inhibit tumor progression by increasing the immune response, and on the other hand increase angiogenesis (155, 156). These results were confirmed by other groups who investigated HLA-G expression during the different stages of CIN. They concluded that HLA-G expression increased from 54% at CIN-I to 100% at CIN-IV, pointing out HLA-G as a good marker of the disease progression (157). Other groups also confirmed these results but percentages of HLA-G expression determined were weaker (158, 159). Guimaraes et al. demonstrated that HLA-G expression was highly correlated to human papilloma virus (HPV) in CC and inversely correlated to the MHC class I expression (158), confirmed by other group (159).

Serous epithelial ovarian cancer is the most common subtype of ovarian cancer (50–70% of ovarian cancer cases), followed by endometrioid carcinoma (10–25% of ovarian cancer cases). Diagnosis is frequently late given a mild symptomatology during first stages. This cancer is a serious carcinoma characterized by an aggressive development and bad prognosis. Endometrial carcinoma is the 3rd most frequent female cancer. This cancer is usually diagnosed at early stages and presents a favorable prognostic. HLA-G expression was demonstrated to be frequent in ovarian cancer (55%) with progression during disease development (160). HLA-G expression was evidenced at the transcriptional (qPCR) and translational levels (WB and IHC) with an increase from early stages (grade I/II) to late stages (grade III/IV) and a drop of survival rate of 5 years. Other groups have shown similar results, with higher HLA-G expression in serous carcinoma (161). HLA-G expression in endometrial cancer was studied by Barrier et al., who showed an expression of HLA-G mRNA in 55% of the cases of endometrioid cancers, mainly localized in the glandular epithelium with no expression was observed in the stromal tissue (162, 163), and the percentage of HLA-G^+ lesions was also correlated with an advanced stage of the cancer.

Lung cancer is the most frequent malign cancer in the world, with an average of 800 000 deaths per year. There are two categories of lung cancer, (i) the small cell lung cancer (SCLC) which represents 10–15% of the cases, and (ii) the non-small cell lung cancer (NSCLC) that represents 85–90% of lung cancer cases. Despite some improvements in treatment, NSCLC remains a disease with bad prognosis. Indeed, the survival rate of 5 years is <15%. The most significant criteria to define the gravity and advanced stage of this cancer is the TNM state. Clinical observations and markers are still variable,

so new markers are required to better define the stage of the disease. HLA-G expression has been proposed as one of such novel markers. Until now, all studies have been carried out in NSCLC for determining HLA-G expression. Yie et al. have demonstrated that 75% of the tumoral lesions they tested expressed HLA-G (164). HLA-G expression was considered as important (>50% of cells expressing HLA-G) in 80% of patients and was associated to the disease stage but independently of the histologic type lesion. HLA-G expression has also been correlated to a decrease in the survival rate. Other groups have confirmed these results not only by IHC, but also through sHLA-G dosage in patients' sera by ELISA (127). Western blot (WB) analysis demonstrated that the main HLA-G isoforms expressed were HLA-G1 and -G5. However, it seemed that sHLA-G origin was not from the tumor cell, but from peripheral blood monocytes (135, 165). Other authors demonstrated that sHLA-G was more frequently observed in adenocarcinoma (73%) than in epidermoid carcinomas (7%) or in adenosquamous carcinoma (10%). High HLA-G expression was determined in monocytes by in flow cytometry (166). In this context, Schütt et al proposed that membrane-bound HLA-G as well as sHLA-G were excellent progression markers (167) to be included as diagnosis markers.

TABLE 1 | Summary of the current available monoclonal antibodies raised against HLA-G isoforms.

Designation	Specificity	Immunogen	References
MEM-G/1	Denaturated heavy chain (α1 domain?)	Denaturated HLA-G1 heavy chain	(1–3)
MEM-G/2	Denaturated heavy chain (α1 domain?)	Denaturated HLA-G1 heavy chain	(4)
MEM-G/4	Denaturated heavy chain of HLA-G1, HLA-G2 and HLA-G5	Denaturated HLA-G1 heavy chain	(5)
MEM-G/9	Conformational HLA-G1/HLA-G5 isoforms associated with β2m	HLA-G recombinant protein refolded in presence of β2m and peptide	(5, 6)
G233	Conformational HLA-G1/HLA-G5 isoforms associated with β2m	Murine cells transfected with HLA-G1/β2m associated isoform	(7–9)
4H84	Denaturated heavy chain (α1 domain) of HLA-G1 to HLA-G7 isoforms	Peptide encompassing the amino acids 61-83 of HLA-G α1 domain	(4, 5, 10, 11)
5A6G7	Soluble isoforms HLA-G5 and HLA-G6	Peptide derived from intron 4 (SKEGDGGIM SVRESRSLSEDL) coupled with ovalbumin	(3, 12, 13)
2A12	Soluble isoforms HLA-G5 and HLA-G6	Peptide derived from intron 4 (SKEGDGGIM SVRESRSLSEDL) coupled with ovalbumin	(14, 15)
87G	Conformational HLA-G1/HLA-G5 isoforms associated with β2m and reported as blocking antibody	Murine cells transfected with HLA-G1/β2m associated isoform	(10, 16, 17)
HGY	Denaturated heavy chain (α1 domain?)	HLA-G purified proteins from placenta of pregnant women	(18, 19)

DISCUSSION

The expression pattern of HLA-G on tumors is difficult to determine. The detection of HLA-G expressing cells, the nature of HLA-G isoforms and their impact on the immune system remain uncertain and challenging. Indeed, specific monoclonal antibodies are insufficient to define the isoforms concerned in the different type of tumors and involved in their developments. Furthermore, HLA-G expression tends to disappear after surgical excision of tumor lesion requiring to develop new culture approaches to maintain HLA-G expression *ex vivo* (44).

HLA-G mRNA expression can be determined by RT-PCR as previously reported (59, 168). However, mRNA expression is not directly correlated or associated with HLA-G protein expression (109), limiting the estimation of HLA-G actual expression in tumor tissue, particularly concerning the presence and percentage of the non-canonical isoforms, for which the antibodies (169) are scarce or inexistent (111). Indeed, regarding the detection of membrane-bound or secreted HLA-G isoforms (respectively, HLA-G1 to -G4 and HLA-G5 to -G7), few antibodies against HLA-G have been generated (**Table 1**). To overcome this limitation, a workshop to establish and standardize anti-HLA-G *in vitro* detection assays was initiated by the group of ED Carosella et al. (170, 171) and a wet workshop was organized for quantification and identification of soluble HLA-G (172). This allowed to determine HLA-G expression by immunohistochemistry (IHC) western blot (WB), flow cytometry or ELISA assays in a more coordinated manner among laboratories. IHC and WB are essentially based on the utilization of the anti-HLA-G specific 4H84 and 5A6G7 monoclonal antibodies, whereas flow-cytometry and ELISA assays rely on 87G, MEM-G/9 and G233 monoclonal antibodies (mAbs). 4H84 mAb binds to the α1 domain (present in HLA-G1 to HLA-G7 isoforms) and the 5A6G7 mAb was raised against the intron 4 only present in secreted HLA-G isoforms (HLA-G5 to HLA-G7). Another antibody generated against denatured HLA-G is MEM-G/1, which can specifically detect denatured forms of HLA-G1 and -G2. Noteworthy, because MEM-G/1 targets an extracellular domain of native HLA-G which might be partially intrinsically disordered, this antibody not only can detect native forms of HLA-G2, but also competes with the LILRB2 binding of HLA-G2. These results provide novel insight into the functional characterization of HLA-G isoforms, pointing out its potential as ICP inhibitor (173). 87G, MEM-G/9 and G233 mAbs bind to conformational HLA-G α1 domain associated to the β2M (HLA-G1 and HLA-G5). However, immunoprecipitation assays on trophoblast surface demonstrated that G233 could detect a residual band of 39 kDa, either β2m-associated as well as a β2M-free heavy chain (174). Thus, determination of the HLA-G

isoforms expressed is dependent on the combination of these different techniques. It must be pointed out that none of the anti-HLA-G antibodies generated were raised against the α2 or α3 domains. HLA-G sequence is strongly homolog to classical HLA molecules, particularly for the α2 and the α3 domains, and less for the α1 domain (175). This explains the bias for the α1 specificity of anti-HLA-G antibodies. Furthermore, the limited mAb development is also related to the fact that murine B cells express the PIR-B receptor, homolog to ILT2 or ILT4 human receptors, which inhibits murine B cell maturation and Ab secretion upon binding to HLA-G protein (66). Due to these limitations, experiments related to HLA-G expression and functions on tumor cells are not trivial. We have to point out that HLA-G expression studies are mainly performed on transfected or transduced tumor cell lines since HLA-G expression is rapidly loss after *ex vivo* culture or primary tumors. Conformational anti-HLA-G mAbs are limited to the HLA-G1/β2M or HLA-G5/β2M associated isoforms and to date, no mAb specific for the other HLA-G isoforms is available. Furthermore, the single blocking mAb to date against HLA-G is the 87G that only inhibits the function of HLA-G1/β2M or HLA-G5/β2m through ILT2 receptor. HLA-G2 and HLA-G6 isoforms are of interest since they are demonstrated to be immunosuppressive. Indeed, HLA-G2 and HLA-G6 isoforms encompass the α3 domain of HLA-G that mediates the interaction with the ILT4 inhibitory receptor expressed by APCs. Beyond this expression by immune cells, ILT4 was described on breast, lung and kidney tumor cells (113, 176, 177). Such site of expression, quite unexpected for ILT4, is of great interest with respect to how it affects the phenotypic and functional characteristics of tumor cells that express it (178). Neo-expression of ILT4 in breast cancer and in non-small cell lung cancer (NSCLC) is associated with metastasis in lymphatic nodes and poor prognostic (179). ILT4 expression is associated with an increase of cell proliferation and motility *in vitro* of tumor cells and promotes metastasis *in vivo* (180). Indeed, even if ILT4 is an inhibitory receptor, expressed on cancer cells, ILT4 inhibits mechanisms that repress proliferation, growth, and spread of cancer cells. Upon binding to its ligand, the ILT4 receptor inhibits the pathways that represses proliferation, growth and dissemination of tumor cells (181, 182). Since HLA-G is the main ligand of ILT4, HLA-G binding to ILT4 expressing cells, either by soluble HLA-G6 or membrane-bound HLA-G2 isoforms, could promote tumor growth. This heterogeneous expression of different ICPs within tumors, showed in the context of RCC (113), emphasizes the redundant or cumulative mechanisms developed by tumor cells to promote their immune escape and their expansion. Yet, mAbs capable of binding and/or blocking the ILT4 interaction with the conformational HLA-G2 and HLA-G6 isoforms are strongly lacking. Due to these drawbacks, tumor cell lines are essentially transduced with either HLA-G1 or HLA-G5 isoforms. Several tumors downregulate their MHC class I molecules expression at their surface by inhibiting the β2M expression to escape from the immune system (183–185). HLA-G cell surface expression on such tumors, even HLA-G1, could be unaffected by the loss of β2M association through the formation of HLA-G multimers as determined during pregnancy (186). Resulting β2M-free HLA-G isoforms could still be immunosuppressive and inhibit the immune response, particularly the NK immune response that should lyse MHC class I negative tumor cells. However, mAbs raised specifically against β2M-free HLA-G isoforms are lacking. In consequence, determination of the panel of HLA-G isoforms expressed by tumor cells is severely limited and the implications of β2M-free HLA-G isoforms in the tumor immune escape mechanisms are misestimated. Furthermore, Tronik-Le Roux et al. recently reported the expression of new HLA-G isoforms, devoid of α1 domain, but encompassing α2-α3 or α3 domains. As a fact, these new isoforms cannot be detected by the existing anti-HLA-G antibodies (111). Although HLA-G neo-expression has been proven in numerous tumors, it remains underestimated in most of the cancer lesions.

Here we emphasize the requirement of new tool development to analyze the HLA-G expression by tumor cells, especially the generation of new anti-HLA-G monoclonal antibodies to determine the expression pattern of HLA-G isoforms expressed by tumor cells.

It was suggested that HLA-G2/G6 may comprise an adequate substitute in women carrying the null allele (G*0105N) (187, 188). Also, it was demonstrated that melanoma cells can rapidly switch from cell-surface HLA-G1 to intra-cellular HLA-G2 expression, restoring tumor sensitivity to NK lysis (189). One can hypothesize that a switch between HLA-G isoforms expressed occurred following the development of the tumor. At the initial development stages, tumor cells would inhibit APC maturation and functions through ILT4 receptors by expressing HLA-G2 and HLA-G6 isoforms. Then, following angiogenesis and the tumor vascularization, effector cells that infiltrate the tumor would be inhibited by HLA-G1 and HLA-G5 isoforms through ILT2 receptors expressed on effector cells. This implies that depending on the stage of the tumor, different immunotherapies against HLA-G should be applied.

Since HLA-G/ILT2 and HLA-G/ILT4 are ICPs, inhibiting the interaction between immunosuppressive HLA-G isoforms and its receptors should restore the immune response as demonstrated for anti PD-1 and anti-PD-L1 monoclonal antibodies. Therefore, developing blocking antibodies against HLA-G/ILT4 and/or HLA-G/ILT2 interaction would restore the immune response. Wiendl et al. demonstrated that only 10% of tumor cells expressing HLA-G were enough to protect the whole tumor against the immune response (141). Thus, even if HLA-G expression is weak or diffuse within the tumor, the administration of anti-HLA-G blocking antibodies should dampen the immune-protective effects of HLA-G. LeMaoult et al. recently demonstrated that ccRCC tumors strongly expressed HLA-G and that the cytotoxic effector TILs were $ILT2^{+}$ and $PD\text{-}1^{-}$ (114). In this context, inhibiting the HLA-G/ILT2 interaction should restore the TILs cytotoxic function against HLA-G positive ccRCC tumors. As listed previously, HLA-G is an excellent TAA since HLA-G expression in healthy tissues is highly restrained, but strongly neo-expressed on tumors. As HLA-G expression level is correlated with an advanced stage of the disease, implying a decrease of the number of cytotoxic effector cells and their function, blocking antibodies

would be insufficient in advanced stages. In this scenario, the cell therapies would be more adequate. Indeed, monoclonal anti-HLA-G antibodies could be used to develop anti-HLA-G CAR-T cells. These anti-HLA-G CAR-T cells would target directly and specifically the HLA-G expressing cells to eliminate the tumor.

Despite evidences that HLA-G expression is spread among hematopoietic and solid tumors, HLA-G expression is still largely underestimated. Insufficiency of biologic tools, in particular a wider specificity variety of anti-HLA-G monoclonal antibodies, make it difficult to determine and characterize HLA-G isoforms expressed, *de facto* limiting anti-HLA-G immunotherapies development.

AUTHOR CONTRIBUTIONS

MLo, JC, FA, MLe, and PL-D provided guidance and expertise in their respective areas of study. MLo, JC, and RD wrote the manuscript. All authors provided input, edited, and approved the final version of the manuscript.

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Soluble HLA-G and HLA-G Bearing Extracellular Vesicles Affect ILT-2 Positive and ILT-2 Negative CD8 T Cells Complementary

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Tumor immune escape is associated with both, the expression of immune checkpoint molecules on peripheral immune cells and soluble forms of the human leukocyte antigen-G (HLA-G) in the blood, which are consequently discussed as clinical biomarker for disease status and outcome of cancer patients. HLA-G preferentially interacts with the inhibitory receptor immunoglobulin-like transcript (ILT) receptor-2 in the blood and can be secreted as free soluble molecules (sHLA-G) or via extracellular vesicles (EV). To investigate the contribution of these two forms to the expression of checkpoint molecules in peripheral blood, we primed peripheral blood mononuclear cells with purified soluble sHLA-G1 protein, or EV preparations derived from SUM149 cells transfected with membrane-bound HLA-G1 or control vector prior to anti-CD3/CD28 T cell activation. Our study demonstrated that priming of PBMC with sHLA-G1 protein prior to 48 h activation resulted in enhanced frequencies of ILT-2 expressing $CD8^+$ T cells, and in an upregulation of immune checkpoint molecules CTLA-4, PD-1, TIM-3, and CD95 exclusively on ILT-2 positive $CD8^+$ T cells. In contrast, when PBMC were primed with EV (containing HLA-G1 or not) upregulation of CTLA-4, PD-1, TIM-3, and CD95 occurred exclusively on ILT-2 negative $CD8^+$ T cells. Taken together, our data suggest that priming with sHLA-G forms induces a pronounced immunosuppressive/exhausted phenotype and that priming with sHLA-G1 protein or EV derived from HLA-G1 positive or negative SUM149 cells affects $CD8^+$ T cells complementary by targeting either the ILT-2 positive or negative subpopulation, respectively, after T cell activation.

Keywords: HLA-G, ILT-2, immune checkpoint, extracellular vesicles, exosomes, breast cancer

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INTRODUCTION

The human leukocyte antigen-G (HLA-G) belongs to the non-classical class I HLA molecules and can exist in different isoforms expressed either as membrane-anchored structures or as secreted molecules (1–4). Additionally, HLA-G can be released as membrane-anchored molecules from various cell types via extracellular vesicles (EV) (5). EV are phospholipid bilayer-enclosed vesicles

that are present in biofluids and cell culture media (6). Assembly of EV depends on their cell of origin and differs remarkably encompassing a broad spectrum of antigens, cell surface-expressed receptors and/or ligands, metabolites, and nucleic acids (7). Generally, the unique molecular signature of EV guides their biodistribution, uptake and internalization (7). As multifactorial vehicles, EV orchestrate various systemic processes, triggering changes of the state of the recipient cell (8). In malignancies, EV play a critical role in the establishment and maintenance of the tumor microenvironment (TME) (6), which enables tumor development by continuous crosstalk between tumor cells and their microenvironment and by providing the tumor with cellular and soluble components including nutrients, oxygen, metabolites, and several other soluble factors (9). EV can either directly fuse with a target cell enabling the transfer of bioactive molecules to both, adjacent and distant sites, or be internalized via phagocytosis, endocytosis or micropinocytosis, thereby contributing to an intracellular signaling mechanism (10). Of note, fusion depends on an acidic micro-environment which naturally occurs inside tumors (11–14), while uptake and internalization of EV are primarily receptor-mediated via adhesion molecules (15). Thereby, tumor-derived EV (TEV) may represent an alternative mechanism of immunosurveillance deficiency impairing diverse immune cell lineages (6).

HLA-G preferentially serves as a ligand for inhibitory receptors present on different immune cells including the immunoglobulin-like transcript (ILT) receptor-2 (LILRB1/CD85j), ILT-4 (LILRB2/CD85d) and the killer immunoglobulin-like receptor 2DL4 (KIR2DL4/CD158d). ILT-2 is broadly expressed on monocytes, B cells, dendritic cells, and a subset of natural killer (NK) and T cells, whereas ILT-4 expression is myeloid-specific (16). Thus, HLA-G is able to impair functions of effector cells of both, the adaptive and the innate immune system. The ILT-2 receptor interacts with HLA-G molecules associated to β2-microglobulin and HLA-G dimers bind to ILT-2 with a higher affinity and avidity than monomers (17). Of note, similar to classical soluble HLA class I, soluble HLA-G (sHLA-G) can interact with the CD8 T cell co-receptor, which increases surface expression and secretion of FasL – the ligand of the Fas (CD95) receptor – inducing cell apoptosis (18).

Physiologically, HLA-G has a restricted tissue expression, whereas neo-expression of HLA-G and its diverse structures is induced in various pathological situations (2). Due to the role of HLA-G in tumor immune escape, it is proposed to be an immune checkpoint (IC) molecule (19). Indeed, expression of HLA-G or sHLA-G has been associated with poor survival, prognosis, therapy response, clinical status, and outcome in various malignancies [reviewed in Carosella et al. (19)]. Lately, HLA-G bearing EV (HLA-G_{EV}) originated from liquid biopsies of blood samples derived from breast and ovarian cancer patients have been introduced as novel cancer biomarker (20, 21). Strikingly, in these studies exclusively HLA-G_{EV}, but not sHLA-G, were of prognostic relevance suggesting self-contained effects of both structures. However, the structural diversity concerning monomers, dimers, and HLA-G expressing EV in liquid biopsies such as peripheral blood samples makes it difficult to implement HLA-G as a meaningful clinical biomarker with its functional consequences for peripheral immune effector cells (22). In this context, it is of note that the ILT-2 receptor is the sole inhibitory HLA-G receptor being expressed on peripheral blood cells, albeit only a minority of blood effector cells express ILT-2 (23). Thus, it has been proposed that the functional consequences of HLA-G and its soluble forms for immune cells in the blood should be focused on HLA-G sensitive effectors, namely the ILT-2 positive ones (23).

Besides HLA-G and ILT-2, additional IC molecules such as programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte-associated protein (CTLA-4), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and CD95 are associated in tumor-driven immune escape mechanisms acting locally at the tumor site or systemically in the peripheral blood (24–26). The continuous up-regulation and co-expression of multiple IC, being often observed in cancer and chronic infections, are indicative for an immunosuppressive/exhausted phenotype of T cells and are associated with loss of effector functions and immunosurveillance (27, 28). Hitherto, no data exists on the relation between sHLA-G or HLA-G_{EV} and the expression of ICs on peripheral blood cells. Hence, the aim of this study was to analyze the contribution of soluble forms of HLA-G to the surface expression of IC molecules. Purified sHLA-G1 molecules (29) and EV preparations derived from the human breast cancer (BC) cell line SUM149 either stable transfected with HLA-G1 or with a control vector served as antigen sources in functional assays. To model whether presence of sHLA-G1 or HLA-G_{EV} in the peripheral blood modulates immune effector cells regarding their expression of ICs, peripheral blood mononuclear cells (PBMC) were primed with sHLA-G1 or with HLA-G1 positive or negative EV preparations overnight prior to T cell activation with anti-CD3/CD28. As EV harbor multiple types of molecules, structures, and genetic information, we placed emphasis on both, the ILT-2 positive and ILT-2 negative T cell population.

MATERIALS AND METHODS

Cell Culture

Human BC cell line SUM149 was stable transfected with a GFP construct targeting HLA-G G1 (SUM149 LV2 G1-GFP) or with a control vector encoding GFP only (SUM149 LV2 N3-GFP). Cells were cultured in RPMI-1640 supplemented with 1% Penicillin/Streptomycin (both Thermo Fisher Scientific, Darmstadt, Germany) and 10% FBS Good Forte (PAN-Biotech GmbH, Aidenbach, Germany) at 37°C and 5% CO_2. Conditioned media (CM) were collected for EV enrichment and frozen at −20°C.

Isolation and Characterization of Extracellular Vesicles Derived From Conditioned Media

To isolate EV derived from CM of HLA-G1 transfected SUM149 cells (G1 EV) and the respective control cells (N3 EV), CM were thawed and centrifuged at 2,800 × *g* for 30 min at 4°C

and concentrated by tangential flow filtration (Repligen, Breda, Netherlands) with a 750 kDa/115 cm^2 mPES filter (D02-E500-05-N). The concentrate was subjected to ultra-centrifugation at 100,000 × *g* for 2 h at 4°C in a swinging bucket SW40 Ti rotor (Beckman Coulter, Krefeld, Germany). The pelletized EV were resuspended in 0.9% NaCl supplemented with 1% Penicillin/Streptomycin (Thermo Fisher Scientific).

EV fractions were analyzed as previously recommended as a minimal requirement for the definition of EV (30, 31). Nanoparticle tracking analysis (NTA) on the ZetaView Laser Scattering Video Microscope (Particle Metrix, Meerbusch, Germany) and its corresponding software (version 8.03.08.02) revealed a size distribution (mean ± SD nm) of 136.7 ± 3.3 and 133.4 ± 3.3 for the G1 EV or N3 EV preparation (**Supplementary Table S1**), which corresponds to the known size of EV, ranging between 30 and 150 nm (32). Particle concentration was determined by NTA and protein concentration was defined by protein assay (Thermo Fisher Scientific) (**Supplementary Table S1**). Expression of components associated with EVs and classical HLA class I was verified by SDS PAGE and western blot (**Supplementary Figure S1A**). 15 μg of EV suspensions were used for immunoblotting and 10 μg cell lysate derived from the respective cells served as control. Both preparations showed the typical EV marker profile including presence of TSG101 (clone: T5701; Sigma-Aldrich, St. Louis, MO, United States), classical HLA class I [α-heavy chain HLA class I; (33)], Syntenin (clone EPR8102; Abcam), and CD81 (clone: 5A6; BioLegend, Koblenz, Germany) and absence of Calnexin (Abcam) excluding cellular protein contamination. Additionally, western blot analysis revealed that HLA-G (clone 4H84; Exbio, Praha, Czechia) present in both, cell lysate and EV fraction of SUM149 LV2 G1-GFP cells and absent in cell lysate and EV fraction of the control SUM149 LV2 N3-GFP cells (**Supplementary Figure S1B**). Both, the NTA results and the EV marker profile fulfill the minimal requirement for the definition of EV (30, 31).

Stimulation of Peripheral Blood Mononuclear Cells

Frozen PBMC of healthy donors [for isolation and storage of PBMC see Kordelas et al. (34)] were thawed in complete medium consisting of RPMI-1640, 1% Penicillin/Streptomycin, 10% human AB serum (Transfusion Medicine, University Hospital Essen, Germany), and 0.556 μg DNAse (Roche, Mannheim, Germany). In a 96-U-bottom plate 6×10^5 PBMC/well were cultured in 200 μL of DNAse-free complete medium at 37°C and 5% CO_2 alone (control), in the presence of 1.2 ng purified HLA-G1 (sHLA-G1) protein (29) or in presence of 40 μg EV either derived from HLA-G1 transfected SUM149 cells (G1 EV) or from the respective control cells (N3 EV). 40 μg G1 EV corresponds to a mount of 1.2 ng HLA-G1 defined by HLA-G ELISA as previously described (20, 21, 35, 36). After 24 h, primed and unprimed PBMC were stimulated with beads coated with CD3/CD28 (Thermo Fisher Scientific) in a bead to cell ratio of 1:3 for 48 h. Influence of stimulation on the expression of IC molecules on T cells was assessed (**Supplementary Figure S2**). Additionally, viability of T cells upon stimulation and priming was analyzed (**Supplementary Figure S3**).

Flow Cytometric Analysis

LIVE/DEAD Violet™ Dead Cell Stain Kit was used according to manufacturer's instructions (Thermo Fisher Scientific) to analyze cell viability. Surface expression was analyzed by staining with fluorchromes-conjugated mononuclear antibodies targeting CD3 (BV510 clone OKT3), CD8 (PerCP-Cy5.5 clone SK1), PD-1 (AF488 clone EH12.2H7), CD95 (BV510 clone DX2), TIM-3 (PerCP/Cy5.5 clone F38-2E2), or CTLA-4 (BV605 clone BNI3). All antibodies were provided by BioLegend (Koblenz, Germany) with the exception of CD3 (Beckman Coulter). Isotype matched antibodies served as negative controls (BD Bioscience, Heidelberg, Germany). Samples were subjected to multicolor flow cytometry using a CytoFlexS cytometer (Beckman Coulter). Data acquisition of at least 200.000 events was performed with CytExpert Version 2.1 software (Beckman Coulter) and analyzed with Kaluza Analysis 2.1 software. General gating strategy for flow cytometric analysis is visualized in **Supplementary Figure S4**. Analysis strategy for multiple-positive T cells is given in **Supplementary Figure S5**.

Statistical Analysis

Data is presented as median with the 10th and 90th percentile. Frequencies of a certain cell population are either expressed as% or as fold change (FC). For FC, frequencies of sHLA-G1- or EV-primed cells were normalized to the corresponding stimulations obtained without priming. After testing for Gaussian distribution, statistical significance was determined by paired *t*-tests or Wilcoxon test for testing of two groups or by two-way ANOVA for comparison of multiple groups. Statistical analysis was performed by using GraphPad Prism V8.3 software (GraphPad Software, San Diego, CA, United States). *p*-values <0.05 were considered to be statistically significant.

RESULTS

Priming With sHLA-G1 Modulates the ILT-2 Expression of $CD8^+$ T Cells

To mimic whether the expression of ICs on T cells can be modulated by the presence of sHLA-G1 in the peripheral blood, PBMC ($n = 6$) of healthy individuals were primed with sHLA-G1 overnight prior to stimulation with anti-CD3/CD28. Flow cytometric analysis (**Supplementary Figure S2**) revealed similar frequencies of ILT-2 positive $CD4^+$ and $CD8^+$ T cells [median (range) in%: 19.6 (14.8–24.5) and 22.7 (11.5–39.6), respectively] in unprimed PBMC upon stimulation with CD3/CD28. However, priming with sHLA-G1 resulted in a significant increase of ILT-2 on the $CD8^+$ T cell subpopulation [53.8 (22.2–64.9)], while ILT-2 on $CD4^+$ T cells was only marginally increased [23.8 (14.4–41.8); **Supplementary Figure S6A**]. In contrast to ILT-2, pre-incubation with sHLA-G1 did not influence the frequency of the IC molecules CTLA-4, PD-1, TIM-3, or CD95, neither in $CD4^+$ nor $CD8^+$ T cell subpopulations (**Supplementary Figures S6B–E**).

Priming With sHLA-G1 Modulates the Expression of Immune Checkpoint Molecules Exclusively on ILT-2 Positive CD8$^+$ T Cells

As the immunomodulatory effect of sHLA-G1 is preferentially mediated via its interaction with ILT-2, CD4$^+$ and CD8$^+$ T cells were stratified according to their ILT-2 expression. Focusing on the ILT-2 positive CD4$^+$ subpopulation, frequencies of IC molecules were not significantly altered by priming with sHLA-G1. However, among the ILT-2 positive CD8$^+$ T cells, priming with sHLA-G1 resulted in a significant increase of CTLA-4, PD-1, TIM-3, and CD95 (**Figures 1A–D**) frequencies. For the comparison of ILT-2 positive and negative CD8 subpopulations,

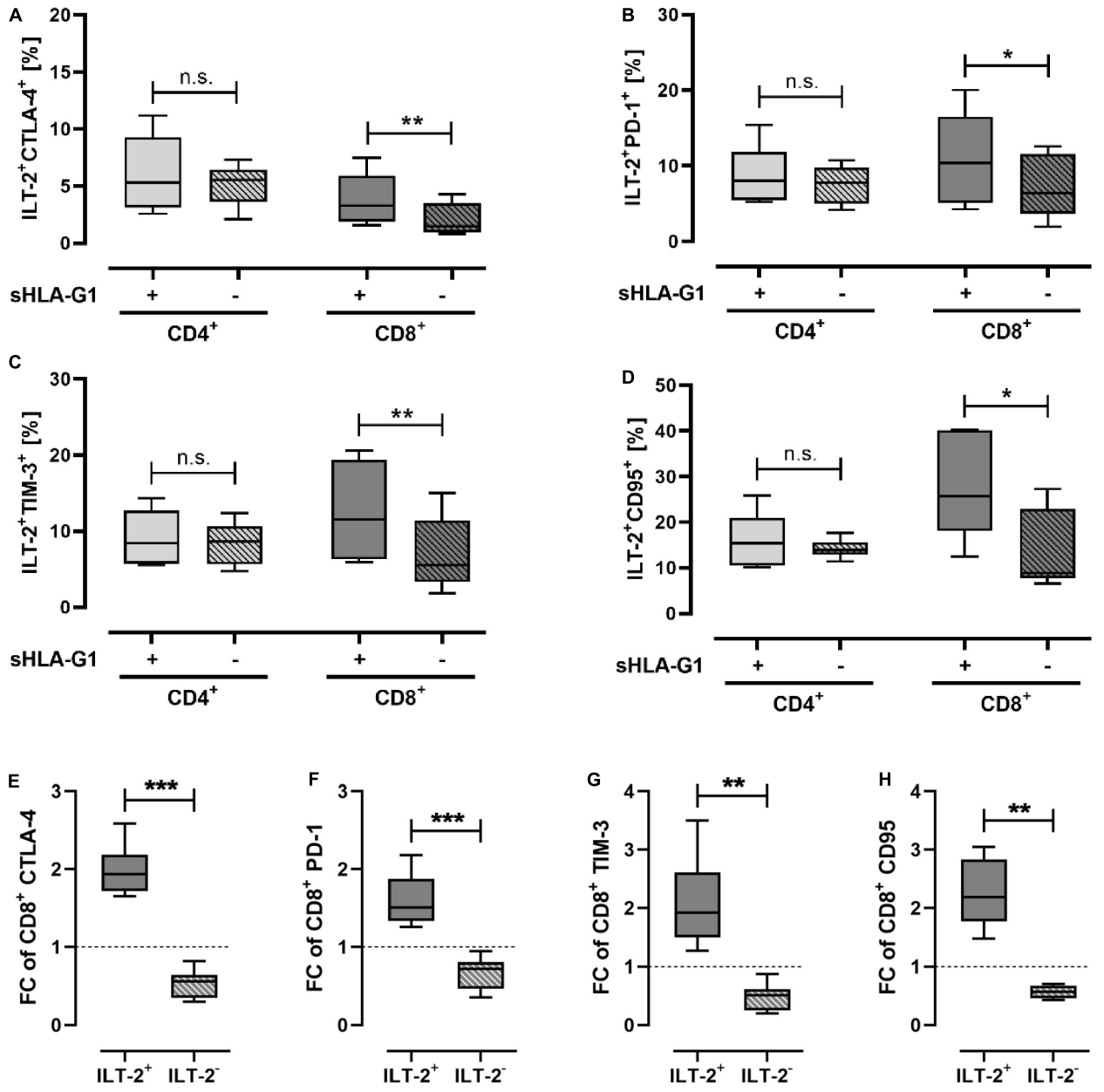

FIGURE 1 | Priming with sHLA-G1 significantly increases surface frequency of immune-modulatory molecules of ILT-2 positive CD8$^+$ T cells, but not that of CD4$^+$ T cells. Flow cytometric analysis of **(A–D)** ILT-2 positive CD4$^+$ and CD8$^+$ T cell populations and comparison of **(E–H)** ILT-2 positive and negative CD8$^+$ T cells regarding the immune checkpoint molecules CTLA-4, PD-1, TIM-3, and CD95. PBMC of six healthy donors were primed with (+) or without (-) sHLA-G1 overnight followed by stimulation with anti-CD3/CD28 beads for 48 h. **(A–D)** Population frequencies of the CD4$^+$ or CD8$^+$ ILT-2 positive parent population are given. **(E–H)** For comparison of ILT2 positive and negative CD8$^+$ subpopulation, frequencies obtained after stimulation of sHLA-G-primed cells were normalized to the corresponding stimulation obtained without priming and expressed as fold change (FC). Data is presented as median with the 10th and 90th percentile. Statistical significance was determined by two-tailed paired *t*-test. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

frequencies of a certain cell population obtained after stimulation of sHLA-G1-primed cells were normalized to the corresponding ones obtained without priming. Strikingly, analysis revealed that the FC of CTLA-4, PD-1, TIM-3, and CD95 (**Figures 1E–H**) was significantly elevated on ILT-2 positive $CD8^+$ T cells compared to ILT-2 negative ones. Comparison of ILT-2 negative and positive $CD4^+$ T cells showed no statistically different FC of any IC molecules. Combined these data evidence that sHLA-G -priming mediates an increase in IC molecules specifically on ILT-2 positive $CD8^+$ T cells, but not on ILT-2 negative ones.

Priming With EV Preparations Modulates Immune Checkpoint Molecules Exclusively on ILT-2 Negative $CD8^+$ T Cells

To elucidate the immune-modulatory effect of the different EV preparations compared to sHLA-G1, PBMC were primed either with sHLA-G1 protein or with 40 μg of G1 EV, which corresponded to a mount of 1.2 ng HLA-G1 or with 40 μg N3 EV prior to CD3/CD28 stimulation. For comparison, frequencies of a certain cell population obtained after stimulation of sHLA-G1- or EV-primed cells were normalized to the corresponding ones obtained without priming. Priming with sHLA-G1 or EV did not significantly result in an altered FC of ILT-2 positive or negative $CD4^+$ T cells (**Figure 2A**). However, compared to sHLA-G1-treated cells, EV-priming lead to a significantly reduced FC of ILT-2 positive $CD8^+$ T cells, while the FC of its negative counterpart increased significantly (**Figure 2B**). Concerning the IC molecules CTLA-4, PD-1, and CD95, sHLA-G1- and EV-priming showed opposing effects: among the ILT-2 positive cells, EV-treatment mediated a decline of the FC of $CTLA\text{-}4^+$, $PD\text{-}1^+$ and $CD95^+$ $CD8^+$ T cells compared to sHLA-G1, while among the ILT-2 negative cells, priming with EV resulted in an enhanced FC of $CTLA\text{-}4^+$, $PD\text{-}1^+$ and $CD95^+$ $CD8^+$ T cells compared to sHLA-G1 (**Figures 2C,D,F**). Further, although not reaching significance, priming with G1 EV induced a substantially elevated ($p = 0.07$) FC of CTLA-4 in ILT-2 negative $CD8^+$ T cells compared to N3 EV-primed cells. Considering TIM-3, FC was significantly increased among the ILT-2 positive $CD8^+$ T cells upon priming with sHLA-G1 compared to EV-treatment, while among the ILT-2 negative $CD8^+$ T cells FC of TIM-3 was not differentially altered by priming with sHLA-G1 or EV preparations (**Figure 2E**).

Priming With sHLA-G1 or EV Preparations Drives ILT-2 Positive or Negative $CD8^+$ T Cells, Respectively, Toward an Immunosuppressive/Exhausted Phenotype

As co-expression of multiple IC molecules is a feature of an immunosuppressive/exhausted phenotype, we analyzed the influence of sHLA-G- or EV-priming on the co-expression of CTLA-4, PD-1, TIM-3, and CD95 on ILT-2 positive and negative $CD8^+$ T cells (**Figure 3**). Strikingly, FC of at least two co-expressed IC was significantly increased upon sHLA-G1-priming compared to EV treatment in ILT-2 positive $CD8^+$ T cells, while among the ILT-2 negative $CD8^+$ T cells EV-priming led to significantly elevated FC of at least two co-expressed ICs compared to sHLA-G1-priming. Thus, sHLA-G1-priming and priming with EV originated from HLA-G1 positive or negative SUM149 cells appear to act complementary toward an immunosuppressive/exhausted phenotype by targeting either ILT-2 positive or ILT-negative $CD8^+$ T cell subpopulations, respectively.

DISCUSSION

Immune effector cell dysfunction in the periphery of cancer patients can tremendously shape the evolution of tumors by mediating a suppressive/tolerogenic immune microenvironment impeding successful tumor elimination. Both, the expression of IC molecules on peripheral immune cells and soluble forms of HLA-G in the blood are associated with tumor immune escape and consequently discussed as clinical biomarker for disease status and outcome of cancer patients (19). Considering that HLA-G can be secreted as free sHLA-G molecules or via EV, we investigated the contribution of these two forms to the expression of the checkpoint molecules PD-1, CTLA-4, TIM-3, and CD95. In our experimental design we primed PBMC with purified sHLA-G1 protein or with EV preparations derived from the BC cell line SUM149 either HLA-G1 transfected or not prior to T cell stimulation with anti-CD3/CD28 to mimic the situation in peripheral blood. The results of our study demonstrate that priming with purified sHLA-G1 protein before T cell activation resulted (i) in enhanced frequencies of ILT-2 positive $CD8^+$ T cells, and (ii) in enhanced frequencies of the IC molecules CTLA-4, PD-1, TIM-3, and CD95 exclusively on ILT-2 positive $CD8^+$ T cells. (iii) Priming with HLA-G1 positive or negative EV preparations prior to T cell activation lead to enhanced frequencies of CTLA-4, PD-1, and CD95 exclusively on ILT-2 negative $CD8^+$ T cells. (iv) Accordingly, the co-expression of at least two IC, being indicative for a pronounced immunosuppressive or exhausted phenotype, was enhanced on ILT-2 positive $CD8^+$ T cells upon sHLA-G1 priming and on ILT-2 negative $CD8^+$ T cells upon EV priming. (v) Combined, priming with sHLA-G1 and EV derived from HLA-G1 positive or negative transfected SUM149 BC cells seem to affect $CD8^+$ T cells complementary by targeting either the ILT-2 positive or ILT-2 negative subpopulation, respectively.

We demonstrated that priming with sHLA-G1 significantly increased the frequency of ILT-2 on $CD8^+$ T cells, while frequencies of classical immune-modulatory molecules such as CTLA-4, PD-1, TIM-3, and CD95 were not altered. In fact, it has already been demonstrated that HLA-G1 is capable of signaling transcriptional and phenotypical changes in immune cells as described by the upregulation of ILT-2, ILT-3, ILT-4, and KIR2DL4 in antigen presenting cells, NK cells, and T cells (37). ILT-2 expression is considered to be more prominent on $CD8^+$ T cells compared to $CD4^+$ T cells with almost exclusive presence on previously activated cells (38–40). Our data, however,

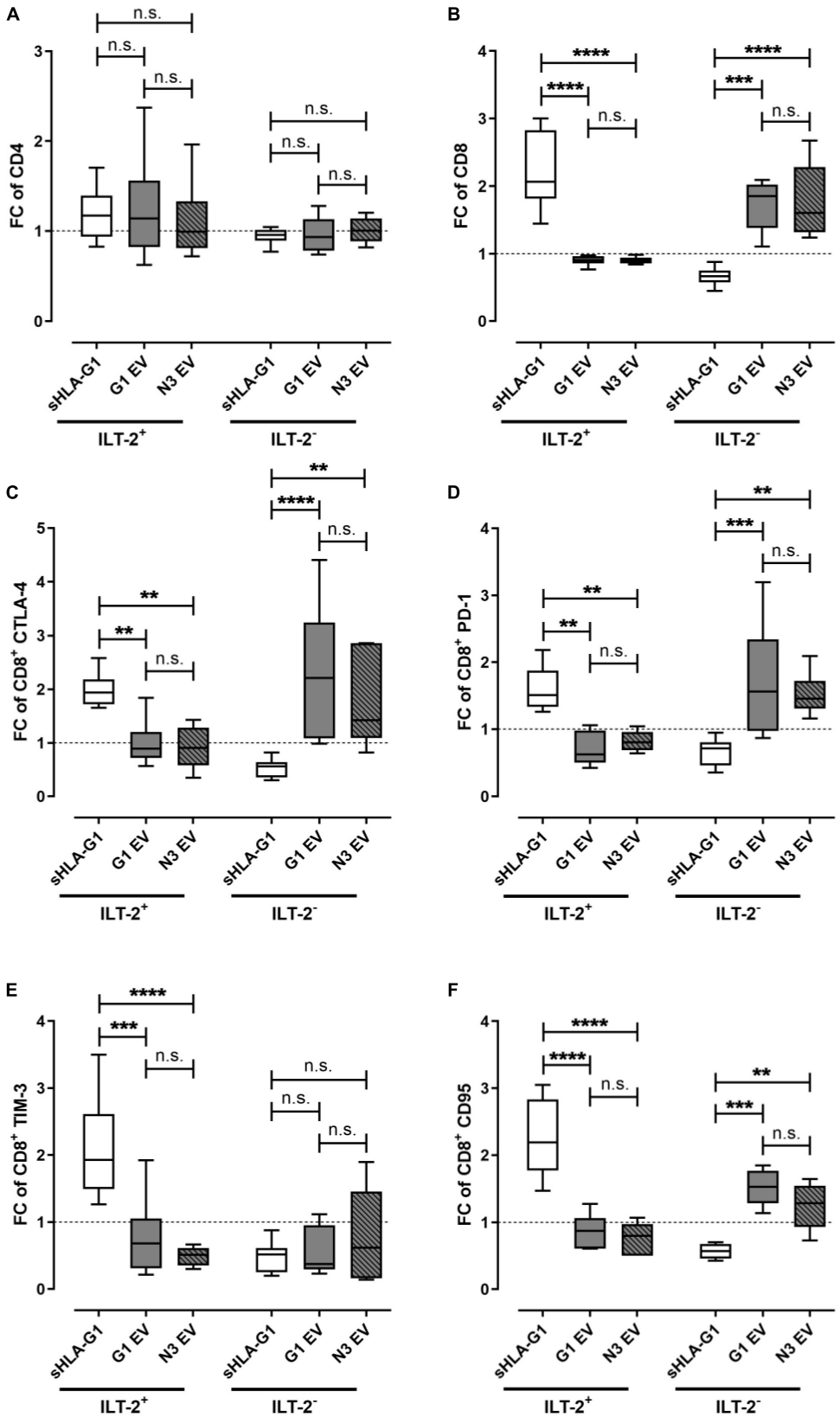

FIGURE 2 | Continued

FIGURE 2 | Priming with EV preparations derived from SUM149 cells significantly increase immune-modulatory molecules on ILT-2 negative CD8$^+$ T cells compared to sHLA-G-priming. Flow cytometric analysis of **(A)** CD4$^+$ and **(B–F)** CD8$^+$ T cell populations regarding **(A,B)** ILT-2 and the immune checkpoint molecules **(C)** CTLA-4, **(D)** PD-1, **(E)** TIM-3, and **(F)** CD95. PBMC of six healthy donors were primed with either sHLA-G1, or EV derived from SUM149 LV2 G1-GFP cells (G1 EV) or SUM149 LV2 N3-GFP (N3 EV) overnight followed by stimulation with anti-CD3/CD28 beads for 48 h. For comparison of ILT2 positive and negative CD8$^+$ subpopulation, frequencies obtained after stimulation of sHLA-G-primed cells were normalized to the corresponding stimulation obtained without priming and expressed as fold change (FC). Data is presented as median with the 10th and 90th percentile. Statistical significance was determined by two-way ANOVA. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p < 0.0001$.

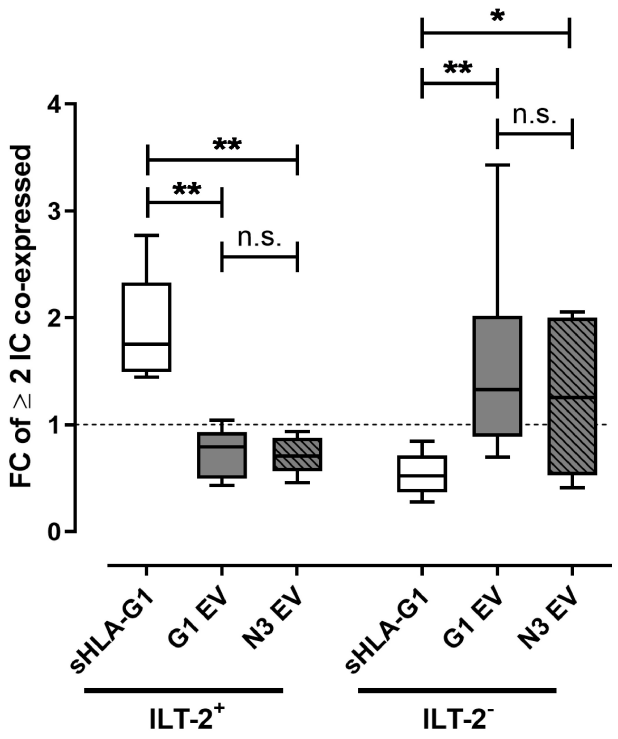

FIGURE 3 | Priming with sHLA-G1 or EV preparations drives ILT-2 positive or negative CD8$^+$ T cells, respectively, toward an immunosuppressive/exhausted phenotype. Flow cytometric analysis of the ILT-2 positive and negative CD8$^+$ T cell populations regarding the multi-positivity of immune checkpoint molecules (IC) including CTLA-4, PD-1, TIM-3, and CD95. IC greater or equal two was considered as multiple-positivity. PBMC of six healthy donors were primed with or without sHLA-G1 or EV derived from SUM149 LV2 G1-GFP cells (G1 EV) or SUM149 LV2 N3-GFP (N3 EV) overnight followed by stimulation with anti-CD3/CD28 beads for 48 h. For comparison of ILT-2 positive and negative CD8$^+$ subpopulation, frequencies obtained after stimulation of sHLA-G-primed cells were normalized to the corresponding stimulation obtained without priming and expressed as fold change (FC). Data is presented as median with the 10th and 90th percentile. Statistical significance was determined by two-tailed paired *t*-test. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

showed similar frequencies of ILT-2 in CD3/CD28 stimulated unprimed CD4$^+$ and CD8$^+$ T cells, whereas ILT-2 frequencies were more pronounced within the CD8$^+$ subpopulation upon sHLA-G1-priming.

Although generic analysis of CD4$^+$ and CD8$^+$ T cells did not reveal any sHLA-G-mediated alteration of tolerogenic molecules despite ILT-2, stratification of CD4$^+$ and CD8$^+$ T cells into ILT-2 positive and negative subpopulations revealed that sHLA-G1 predominantly and preferentially influences the IC molecule profile on ILT-2 positive CD8$^+$ T cells as compared to their ILT-2 negative counterpart. This is in line with Jacquier et al. (23) who requested that functional analyses of the immunosuppressive potential of HLA-G on PBMC should be refined toward ILT-2 positive, and thus, HLA-G-sensitive, cells.

A major mechanism by which tumor cells can impair immune effector function is hijacking of ICs as that mediated by the PD-1/PD-L1 pathway (41). Probably, different ICs (e.g., PD-1, CTLA-4) and HLA-G influence each other as it has been reported, for instance, for TIM-3 expression on CD8$^+$ tumor infiltrating lymphocytes that is closely associated with PD-1 expression (42). Indeed, this is the first study describing a synergy of sHLA-G1 and the frequency of IC molecules on certain T cell subsets. In this context, Contini et al. (18) have already reported that sHLA-G can bind CD8 without T cell receptor interaction inducing apoptosis in activated CD8$^+$ T cells through upregulation of FasL expression. Further, up-regulation of the expression of cell surface molecules such as FasL in cancer cells may mediate the dampening of cytotoxic T cell attacks (41). Thus, upregulation of the corresponding receptor CD95 (Fas) on sHLA-G1-primed CD8$^+$ T cells – as observed in our study – may increase the probability of apoptotic T cell death as both, the ligand and the corresponding receptor are upregulated. Similarly, cancer cells express high levels of inhibitory ligands such as PD-L1 and PD-L2, which, upon binding to PD-1 on T cells inhibits response of T cells toward cancer cells (41). Again, sHLA-G1 priming resulted in elevated frequency of PD-1 on CD8$^+$ T cells, potentially contributing to the establishment of immune escape via the PD-1/PD-L1 axis. Hence, sHLA-G1-priming reinforces an immunosuppressive TME rendering ILT-2 positive cytotoxic T cells unresponsive to cancer cells. Similarly, Dumont et al. (43) have demonstrated that CD8$^+$ tumor infiltrating lymphocytes (TIL) expressing ILT-2 showed a higher cytotoxicity and IFNγ production compared to their ILT-2 negative or PD-1 expressing counterparts and that cytotoxicity of ILT-2 positive TIL, but not that of ILT-2 negative or PD-1 positive TIL could be inhibited by HLA-G. Combined, Dumonts study and our study suggests that various IC pathway act concomitantly in the TME.

Hitherto, the majority of clinical studies analyzed sHLA-G molecules as a prognostic marker in various malignancies (19). Previously, we established that discrimination of sHLA-G forms represents diametric prognostic impacts on the clinical outcome of BC patients (21) and that only HLA-G_{EV}, but not the total amount of sHLA-G is an independent predictor for progression in ovarian cancer patients (20). Thus, we compared the effect of sHLA-G1 with that of EV derived from CM of SUM149 cells, transfected with (G1 EV) or without (N3 EV) HLA-G1. In our study we demonstrated that (iv) sHLA-G1 and EV impact CD8$^+$

T cells complementary: while sHLA-G predominantly influenced ILT-2 positive cells, ILT-2 negative cells were highly affected by EV. Thereby, the surface expression pattern of immune-modulatory molecules on $CD8^+$ T cells was substantially influenced toward an immunosuppressive/exhausted phenotype by both, sHLA-G and HLA-G_{EV} in an ILT-2-dependent or -independent manner, respectively. Of note, ILT-2 positive cells – and thus, per definition HLA-G-sensitive cells – represent only a minority of immune subsets (23). Hence, it is tempting to speculate that HLA-G_{EV} have a larger pool of cells to interact with, potentially explaining the prognostic relevance of HLA-G_{EV}, but not of total sHLA-G, in breast and ovarian cancer patients (20, 21). However, the prognostic potential of sHLA-G or HLA-G_{EV} might be changed in situations with increased frequencies of ILT-2 positive $CD8^+$ T cells such as during aging or chronic viral infections. Additionally, we demonstrated that our EV preparations carry classical HLA class I molecules. As classical HLA class I molecules are generally not expressed as a dimer, it is unlikely that they interact with the ILT-2 receptor which preferentially binds HLA-G dimers (44) rationalizing sHLA-G's preference to bind to the ILT-2 receptor on T cells. On the other hand, CD8 is the cognate receptor of classical HLA class I molecules explaining the preference of EV to interact with ILT-2 negative $CD8^+$ T cells.

Moreover, these results raise questions concerning the relation between these HLA-G structures in physiological and pathological situations. How does priming with a combination of sHLA-G1/HLA-G_{EV} affect the phenotype of T cells? Is the effect of sHLA-G1 and HLA-G_{EV} in the periphery additive, synergistic or competitive? Does one of the structures dominate? What is the ratio of sHLA-G1 to HLA-G_{EV} structures in the periphery of cancer patients in comparison to healthy individuals?

Despite these open questions, our data underline that EV are soluble carriers enhancing the immunosuppressive properties of the TME. As EV represent multifactorial vehicles, it should be acknowledged that the composition of the applied EV preparations is not restricted to HLA-G. In fact, TEV may expose ligands or antigens on their membrane that interact with cellular HLA receptors, thereby altering immune function (45). Moreover, TEV can carry immunosuppressive molecules such as FasL, TGF-β1, TRAIL, PD-L1, and NKG2D ligands, which are involved in immunosuppression (46, 47). Of note, TEV can affect the behavior of immune cells through receptor-ligand binding interaction or by internalization (10). Recently, it has been reported that the modulation of T cell function by TEV is not exerted via internalization by T cells, but rather via signaling molecules that they carry and deliver to the cell surface (46). Accordingly, we demonstrated that EV – irrespective of their composition – modify ILT-2 negative cells, while ILT-2 positive cells are unaffected. Notably, EV preparations are a heterogeneous group of diverse EV subsets. Comprehensive analysis of the EV preparations, especially considering classical IC molecules, might shed further light on the functionality of the EV-driven immunological modifications. In this context, elucidating the structural diversity of HLA-G on EV with regards to the monomeric vs. dimeric conformation, may explain the affinity toward ILT-2 negative $CD8^+$ T cells observed under our experimental conditions. Of note, another open, but highly interesting question is the sensitivity of $CD8^+$ T cells toward priming followed by anti-CD3/CD28 stimulation. Two major mechanisms by which TEV can contribute to tumor evasion are the initiation of apoptosis in cytotoxic $CD8^+$ T cells and the conversion of conventional $CD4^+$ T cells into regulatory T cells (48). Thus, the sensitivity of $CD8^+$ T cells to priming with sHLA-G forms observed in our study might be explained by our lack of emphasis on the regulatory phenotype of $CD4^+$ T cells biasing the analyses toward the $CD8^+$ T cell subpopulation.

A limitation of our study is the lack of blocking experiments demonstrating HLA-G specificity of the G1 EV preparation and the lack of functional assays demonstrating the functionality of T cells with an immunosuppressive phenotype. Generally, the capability of G1 EV and N3 EV to modify the surface expression of immune-modulatory molecules was similar. Nevertheless, our data clearly show an EV-driven effect compared to sHLA-G-priming or compared to T cell stimulation without priming. Moreover, FC of $CD95^+$ $CD8^+$ T cells was tentatively increased upon priming with G1 EV compared to N3 EV. Here, homogeneous HLA-G_{EV} preparations might enhance the effects observed in our study; however, due to the current technical limitations in the EV field, purification of homogeneous EV fractions is impossible.

Concluding, our data elucidate that priming of immune effector cells by discrete sHLA-G forms, including purified sHLA-G1 protein as well as HLA-G1 positive and negative EV, differentially modifies the phenotype of these cells. Here, we report that sHLA-G1 preferentially influences ILT-2 positive $CD8^+$ T cells, while HLA-G_{EV} mediate phenotypic alterations in ILT-2 negative $CD8^+$ T cells. Thus, it seems that discrete soluble HLA-G structures affect ILT-2 positive and ILT-2 negative $CD8^+$ T cells complementary suggesting that HLA-G-mediated inhibition of effector immune cells is not restricted to cells expressing the corresponding receptor ILT-2. Further, we provide first evidence that immune-modulation by soluble HLA-G might involve other IC molecules toward an immunosuppressive or exhausted phenotype. Combined, our data highlight that analyses of HLA-G functionality should be extended to discrete structures reinforcing its complexity in the periphery of cancer patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

ES: study design, data acquisition, statistical analysis, and manuscript writing. JL: provision of transfected SUM149 cells and manuscript editing. G-GH and CB-D: characterization and provision of sHLA-G1 protein and manuscript editing. EC and PH: manuscript editing. VR: study design, statistical analysis, and manuscript writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02046/full#supplementary-material

FIGURE S1 | EV characterization by western blotting. Marker expression analysis for **(A)** Calnexin, Tsg101, classical HLA class I, Syntenin, and CD81, and **(B)** HLA-G in EV fractions derived from SUM149 cell lines either transfected with a control vector (N3) or with HLA-G (G1) and their respective cell lysates. Cell lysates were used as positive control for Calnexin. Cell culture supernatants were collected and EV were purified by Tangential Flow Filtration and Ultra-centrifugation.

FIGURE S2 | Stimulation of PBMC with anti-CD3/CD28 beads increases frequency of surface expression of several markers on $CD4^+$ and $CD8^+$ T cells. Flow cytometric analysis of $CD4^+$ and $CD8^+$ T cell populations regarding **(A)** the HLA-G receptor ILT-2, and the immune checkpoint molecules **(B)** CTLA-4, **(C)** PD-1, **(D)** TIM-3, and **(E)** CD95. PBMC of six healthy donors were stimulated with (+) or without (-) anti-CD3/CD28 beads for 48 h. Population frequencies of the $CD4^+$ or $CD8^+$ parent population are given. Data is presented as median with the 10th and 90th percentile. Statistical significance was determined by two-tailed paired *t*-test. $*p \leq 0.05$, $**p \leq 0.01$.

FIGURE S3 | Effects of pre-incubation of PBMC on the viability of $CD4^+$ and $CD8^+$ T cells. Flow cytometric analysis of T cell populations regarding **(A)** the viability of $CD4^+$ and $CD8^+$ T cells and **(B)** the expression of CD95 on dead $CD4^+$ and $CD8^+$ T cells and **(C)** the expression of CD95 on dead ILT-2 positive and negative $CD8^+$ T cells. PBMC of six healthy donors were pre-incubated **(A,B)** with (+) or without (-) sHLA-G1, or **(C)** with sHLA-G1, G1 EV or N3 EV prior to stimulation with anti-CD3/CD28 beads for 48 h. **(A,B)** Population frequencies of the $CD4^+$ or $CD8^+$ parent population are given. **(C)** Data was normalized to stimulation without pre-incubation and is given as fold change. Data is presented as median with the 10th and 90th percentile. Statistical significance was determined by **(A,B)** two-tailed paired *t*-test ($*p \leq 0.05$) or **(C)** two-way ANOVA ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

FIGURE S4 | General gating strategy of flow cytometric analysis to characterize T cell subpopulations in PBMC. Total lymphocytes were first gated on forward scatter (FSC)/side scatter (SSC) plot. After gating on single cells, dead cells were dismissed via the fluorescent dye Live/Dead^{TM}. T cells were identified by the expression of the T cell receptor CD3. T cells were classified as $CD8^+$ ($CD3^+CD8^+$) or $CD4^+$ ($CD3^+CD8^-$) T cells. **(A)** Within the $CD4^+$ and $CD8^+$ population expression frequencies of ILT-2, CTLA-4, PD-1, TIM-3, and CD95 were determined. **(B)** $CD4^+$ and $CD8^+$ population were distinguished by ILT-2. Within the ILT-2^+ and ILT-2^- $CD4^+$ or $CD8^+$ T cell populations expression frequencies of CTLA-4, PD-1, TIM-3, and CD95 were assessed. Data were analyzed using the Kaluza software and population frequencies expressed as percent of the $CD4^+$ and $CD8^+$ parent population or the $CD4^+$ or $CD8^+$ and ILT-2^+ or ILT-2^- parent population.

FIGURE S5 | General analysis strategy of multi-positive T cells. A tree analysis including gates of ILT-2, PD-1, CTLA-4, TIM-3, and CD95 was performed based on the $CD4^+$ or $CD8^+$ T cell population divided into ILT-2 positive and negative subpopulation resulting in 32 receptor combinations (16 for ILT-2 positive and ILT-2 negative $CD4^+$/$CD8^+$ T cells, respectively). Due to low numbers of recorded frequencies for multi-positive cells, frequencies of cells with more than 1 receptor were added up for further analysis. A representative analysis of the $CD8^+$ population is shown.

FIGURE S6 | Priming with sHLA-G1 significantly increases frequency of ILT-2 on $CD8^+$ T cells, while frequency of immune checkpoint molecule is not altered by priming with sHLA-G1. Flow cytometric analysis of $CD4^+$ and $CD8^+$ T cell populations regarding **(A)** the HLA-G receptor ILT-2, and the immune checkpoint molecules **(B)** CTLA-4, **(C)** PD-1, **(D)** TIM-3, and **(E)** CD95. PBMC of six healthy donors were primed with (+) or without (-) sHLA-G1 overnight followed by stimulation with anti-CD3/CD28 beads for 48 h. Population frequencies of the $CD4^+$ or $CD8^+$ parent population are given. Data is presented as median with the 10th and 90th percentile. Statistical significance was determined by two-tailed paired *t*-test. $*p \leq 0.05$, $**p \leq 0.01$.

TABLE S1 | EV characterization by Nanoparticle Tracking Analysis and protein assay. Particle concentration and particle size of EV fractions derived from SUM149 cell lines either transfected with a control vector (N3) or with HLA-G (G1) was determined by Nanoparticle Tracking Analysis, while total protein concentration was assessed by MacroBCA. Cell culture supernatants were collected and EV were enriched by Tangential Flow Filtration and Ultra-centrifugation.

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HLA-G Expressing Immune Cells in Immune Mediated Diseases

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HLA-G is a HLA class Ib antigen that possesses immunomodulatory properties. HLA-G-expressing CD4+ and CD8+ T lymphocytes, NK cells, monocytes, and dendritic cells with immunoregulatory functions are present in small percentages of patients with physiologic conditions. Quantitative and qualitative derangements of HLA-G+ immune cells have been detected in several conditions in which the immune system plays an important role, such as infectious, neoplastic, and autoimmune diseases as well as in complications from transplants and pregnancy. These observations strongly support the hypothesis that HLA-G+ immune cells may be implicated in the complex mechanisms underlying the pathogenesis of these disorders.

Keywords: HLA-G, immune mediated diseases, lymphocytes, dendritic cells, monocytes, NK cells, regulatory cells

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INTRODUCTION

HLA-G is a HLA class Ib antigen characterized by a restricted tissue expression, low polymorphism and seven isoforms (HLA-G1 to HLA-G7) (1, 2). In both membrane-bound and soluble form, HLA-G exerts several immune-modulatory effects. It inhibits allogeneic proliferation of CD4+ T cells (3), natural killer (NK) and CD8+ T cells cytotoxicity (4), maturation of dendritic cells (DC) (5), and activation of B cells (6). In addition, soluble HLA-G molecules (sHLA-G) are able to trigger apoptosis in antigen specific CD8+ T lymphocytes (4, 7, 8).

HLA-G also seems to be involved in the tuning of immune responses. The incubation of peripheral blood mononuclear cells (PBMC) with HLA-G-expressing cells, favors a shift toward a Th-2 cytokine profile; whereas incubation with sHLA-G may have a counterbalancing effect, creating an anti-inflammatory environment due to the release of interleukin (IL)-10 (9, 10). Based on these findings, it has been recently proposed that HLA-G should be categorized as an "immune checkpoint" molecule (2).

HLA-G+ IMMUNE CELLS IN PHYSIOLOGIC CONDITIONS

T and NK Cells

Immune tolerance is based on a complex series of mechanisms that ultimately facilitate the elimination of foreign antigens, preventing collateral damage to host tissues. Immune tolerance is broadly classified into central and peripheral tolerance. Central tolerance occurs during lymphocyte development in the primary lymphoid organs, namely thymus (T cells) and bone marrow (B cells). Peripheral tolerance takes place in the peripheral tissues and lymph nodes, and consists of different immunologic mechanisms capable of controlling self-reactive lymphocytes that have escaped from central deletion (11). Immune regulation is crucial in the maintenance of peripheral tolerance

and is mediated by the action of T regulatory (Treg) lymphocytes (12). Several subsets of Treg lymphocytes with distinct phenotypes and mechanisms of action have been described within both CD4+ and CD8+ T lymphocytes, and it has been clearly demonstrated that these cells play an important role in physiological and pathological conditions such as autoimmune, infectious, or neoplastic diseases (12, 13).

In 2007 Feger et al. described novel subsets of T cells that express the immunomodulatory molecule HLA-G, identifying them as distinct subpopulations of Treg lymphocytes (14). In recent years, many other studies have confirmed the importance of HLA-G+ Treg lymphocytes in physiology and disease. Similar to the classical CD4+CD25+FoxP3+Tregs, human HLA-G+ Treg cells originate from thymus and are present in variable percentages in the peripheral blood of healthy subjects (0.1–8.3%). However, HLA-G+ Treg cells can be differentiated from classical CD4+ Tregs on the basis of their distinctive phenotype, lacking Forkhead Box P3 (FoxP3), CD39, and CD25 expression (14). CD4+HLA-G+T cells have a low proliferative capacity that, differently from classic Tregs, cannot be overcome by the addition of exogenous IL-2 (14). *In vitro*, CD4+HLA-G+ Tregs inhibit T-cell responses mainly through cell-to-cell contact and independent mechanisms (15); whereas classical CD4+CD25+FoxP3+ Tregs exert their suppressive function mainly through cell-to-cell dependent mechanisms (13, 16). In both Tregs subpopulations suppressive activity depends on an optimal TCR stimulation. CD4+HLA-G+ Tregs and classical CD4+CD25+FoxP3+ Tregs share common intracellular down-stream signaling events, following T cell receptor (TCR) ligation (17, 18). They show altered activation of the linker in activation of T cells (LAT) molecules involved in proximal TCR signaling, leading to reduced intracellular calcium influx when compared to non-regulatory T-cells (16). CD4+HLA-G+ Treg cells seem to exert their suppressive function via the secretion of various tolerogenic molecules such as sHLA-G5, IL-10, IL-35, and transforming growth factor (TGF)-β (15, 16). In this context, IL-10 and sHLA-G5 are the most important molecules responsible for the immunoregulatory activity of the CD4+HLA-G+ Treg (15, 16). Transforming growth factor-β and IL-35 do not seem to have a direct role in the immunomodulation exerted by CD4+HLA-G+ Treg, nevertheless, these cytokines may indirectly promote an immunosuppressive milieu, influencing the local differentiation of peripherally induced Tregs and/or supporting the survival of thymus-derived natural Tregs (19–21).

In vitro, CD4+HLA-G+ Tregs display a less efficient suppressive activity than classical $CD4^+CD25^+FoxP3^+$ Tregs; whereas *in vivo* the immunosuppressive capacity of the two Treg subsets is comparable (16). This notion suggests that CD4+HLA-G+ Tregs may modulate tissue inflammation within the target organs, in close proximity to effector T cells (16, 22).

Besides thymus-derived HLA-G+ Tregs, some normal resting and activated $CD4^+$ and $CD8^+$ T cells may acquire through trogocytosis the HLA-G1 molecule from antigen presenting cells (APCs), thus changing their function from effectors to regulatory cells capable of inhibiting alloproliferative responses (23). Interestingly, the acquisition of HLA-G via trogocytosis has also been described for monocytes and NK cells (24, 25). A non-cytolytic subset of HLA-G+ NK cells (NK-ireg) can be generated *in vitro* from peripheral blood CD34+ hematopoietic progenitors expressing membrane-bound IL-15. NK-ireg cells display a mature NK cell phenotype, release suppressive molecules (HLA-G, IL-10, and IL-21), and through these factors are capable of suppressing the cytotoxicity of DC and NK cells (26).

It has been recently reported that neutrophil gelatinase-associated lipocalin seems to be capable of upregulating HLA-G expression and expansion of Tregs cells in healthy donors (27). This observation is consistent with the knowledge that lipocalin family members act as modulators of many different physiological and pathologic processes, including cell differentiation, proliferation and apoptosis (28). Moreover, HLA-G expression is strongly regulated by methylation, and it has been recently observed that hypomethylating agents such as azacytidine and decitabine, can induce *de novo* expression of HLA-G on conventional T cells thus converting the latter into HLA-G+ Tregs (29). This data suggest the possibility of modulating the expansion of HLA-G-expressing T cells *in vivo* or generating them *in vitro* for adoptive immunotherapy in transplant patients or for other immunological disorders.

Monocytes

The expression of HLA-G in human mononuclear phagocytes and APC has been known for many years (30, 31). HLA-G cell surface expression has been detected at variable percentages in peripheral blood CD14+ monocytes from healthy individuals (32–36). HLA-G mRNA and intracellular HLA-G levels as well as surface HLA-G expression are selectively increased after *in vitro* treatment of monocytes with interferon (IFN)-β, IFN-γ, and IL-10 (30, 32).

As far as the functional role of CD14+HLA-G+ cells is concerned, it has been reported that they have limited *in vitro* immunostimulatory function and are able to inhibit T-cell alloproliferation when added in mixed lymphocyte cultures. The suppressive function of CD14+HLA-G+ cells is related to the expression of the HLA-G molecule, which can be antagonized by blocking HLA-G with specific monoclonal antibodies, and may also be mediated through sHLA-G, as suggested by transwell experiments. Further *in vitro* experiments have shown that co-incubation of CD4+ and CD8+ T cells with CD14+HLA-G+ cells decreases the surface expression of CD4 and CD8 molecules and inhibits both Th1 and Th2 cytokine production by antigen-stimulated autologous $CD4^+$ T cells (37, 38).

Monocytes can differentiate into a range of functional subsets including pro-inflammatory (M1) and anti-inflammatory (M2) cells. Recently published data indicates that M2 cells obtained from peripheral blood monocytes after *in vitro* activation with IL-4, express high amounts of HLA-G and drive upregulation of the HLA-G ligand immunoglobulin-like transcript (ILT)-2 on NK cells. This leads to the generation of hyporesponsive $CD56^{dim}$ NK cells with limited degranulation and cytotoxic activity (39).

Dendritic Cells

Peripheral blood DCs are APCs that regulate innate and adaptive immune responses. Different DC subsets have been

identified that can drive immune responses toward immunity or tolerance, including conventional monocytoid DCs that maintain immunological homeostasis and can induce tolerance, plasmacytoid DCs that present foreign antigens, activate Tregs, and tolerogenic DCs which promote tolerance.

The expression of HLA-G on DC may be regulated by cytokines. *In vitro* experiments have shown that TGF-β increases HLA-G expression by DC and that HLA-G+ DC down-regulate activation of CD4+ T cells and production of IL-6 and IL-17, suggesting the possibility that HLA-G+ DC plays a role in immunoregulatory *in vivo* (40).

Recently, a subset of human DC has been characterized. Termed DC-10, these human DC have the ability to secrete IL-10. DC-10 are found in peripheral blood and the spleen of healthy individuals. They can be generated *in vitro* by culturing peripheral monocytes in the presence of IL-10. Furthermore, DC-10 are highly represented in the decidua of pregnant women when compared to peripheral blood, suggesting that these cells may accumulate at the fetal maternal interface to promote tolerance to the semi-allogeneic fetus (41). DC-10 have a mature phenotype and express CD11c, CD14, CD16, CD141, and CD163. DC-10 also express HLA-G and ILT-4 and are able to induce T regulatory type 1 (Tr1) cells. The amount of HLA-G expression on DC-10 is genetically driven and is associated with specific variations in the 3' untranslated region of the HLA-G gene. Of particular interest are findings on the capacity of DC-10 to induce Tr1 cells, which correlates with the level of HLA-G expression. These data indicate that HLA-G expression plays a fundamental role in the tolerogenic activity of DC-10 and suggest a potential clinical use of DC-10 as an immunomodulatory treatment (42–44).

Collectively, results of *in vitro* and *in vivo* experiments indicate that HLA-G positive DC can affect the activity of NK cells, modulate the response of effector CD4+ and CD8+ T cells, and induce Tregs. These findings strongly support the notion that HLA-G expression by DC plays a central role modulating innate and adaptive immune responses in a healthy state and in pathological conditions.

HLA-G-EXPRESSING IMMUNE CELLS IN NON-AUTOIMMUNE DISEASES

T and NK Cells

Lymphocytes expressing HLA-G have been reported in several diseases in which the immune system plays a pivotal role, such as neoplastic, infectious, and autoimmune/inflammatory disorders.

An increase in the percentage of CD8+HLA-G+ T cells and the presence of HIV-1-specific CD8+HLA-G+ T lymphocytes have been described in HIV-1 patients, although their exact pathophysiologic role in the disease is still elusive (36, 45, 46). Other authors observed that HLA-G+ Treg may reduce harmful bystander immune activation, while minimally inhibiting antiviral T cell-mediated responses, thus suggesting the positive role of these cells in the natural history of HIV infection (47).

Reduced percentages of HLA-G expressing T cells and monocytes have been observed in pre-eclamptic patients, compared with women with a healthy pregnancy or healthy control subjects (48). By analogy with these results, Hsu and colleagues reported that CD4+HLA-G+ T cells are significantly expanded in the peripheral blood of pregnant women compared with non-pregnant controls and pre-eclamptic women (49). In addition, CD4+HLA-G+ T cells tend to accumulate in the decidua of healthy pregnant women; whereas this phenomenon is impaired in pre-eclamptic patients (41). A small subset of NKp46+HLA-G+IL-10+ NK cells has been described *in vivo* among the decidual NK cells of pregnant women, but the exact role of this cell subset requires further investigation (26).

Recently, a novel population of $CD4^{low}$HLA-G+ T cells, identified as IL-4-expressing Th17 cells, has been described in prostate cancer and their expansion seems to correlate with the increase of tumor aggressiveness (50). Increased percentages of HLA-G+CD3+ cells have been observed in the peripheral blood of breast cancer patients, suggesting that these cells may contribute to tumor development by down-modulating antitumor immunity (51). Moreover, it has also been reported that a subset of HLA-G+ NK cells possessing suppressive activity are considerably increased in the peripheral blood of breast cancer patients (52).

It is well-known that, in order to escape immune-surveillance, various malignant cells can aberrantly express HLA-G and/or secrete sHLA-G (53, 54). In addition, cancer cells can induce HLA-G-expressing immune cells (e.g., transferring HLA-G to T cells through trogocytosis) within the tumor microenvironment. This mechanism may increase the number of local immunosuppressive cells, thus facilitating tumor immune-escape (23, 55, 56).

A similar mechanism of immune-evasion has also been described for microbial infections, in fact *Pseudomonas aeruginosa* seems to be capable, at least *in vitro*, of inducing HLA-G expression in immune cells, creating a protected niche and facilitating bacterial survival (57).

In the context of kidney transplants, it has been reported that HLA-G expression on T cells increases after the transplant, but significantly decreases in subjects experiencing an acute rejection. This data suggests that HLA-G might be involved in the protection of transplants against rejection and the levels of HLA-G on CD4+ may represent a potential marker in predicting episodes of renal rejection after kidney transplantation (58, 59).

In patients that experience an allergic reaction, HLA-G expression as well as sHLA-G secretion are increased in CD4+ cells and monocytes after *in vitro* stimulation by the causal allergen, but not by non-specific stimuli and non-causal allergens (60). This data suggest that HLA-G may be involved into the pathogenetic mechanisms underlying allergic inflammation and allergen specific immunotherapy (60, 61).

Monocytes and Dendritic Cells

Several *in vivo* data-sets support the immunomodulatory properties of HLA-G+ monocytes. In fact, a high frequency of CD14+HLA-G+ cells have been detected in patients undergoing allogeneic hematopoietic cell transplantation. These HLA-G+

monocytes appear early post-transplant and remain at high levels for up to one year after the transplant. It is of interest that HLA-G+ monocytes have also been detected in skin biopsies of transplanted patients who developed graft-*versus*-host disease. It may be hypothesized that the increase of HLA-G+ monocytes could be related to an alloreaction occurring after transplant (37).

Elevated numbers of HLA-G+ monocytes have been found in the peripheral blood of HIV-1-infected individuals. The expression of HLA-G might either be directly caused by the HIV-1 virus infection or indirectly related to increased levels of IL-10, which is known to induce HLA-G expression in monocytes. By decreasing the antigen-presenting capacity of monocyte, the upregulated expression of HLA-G could be one of the strategies used by the HIV-1 virus to evade immune surveillance (36).

Collectively, *in vitro* and *in vivo* data suggests that monocyte activation by cytokines, infectious agents, and allogeneic stimuli induces HLA-G expression. Taking this into account, HLA-G+ monocytes may exert immunosuppressive effects on CD4+, CD8+, and NK cells, playing a role in down-regulation of the immune response.

Concerning DCs, it has been reported that monocytoid DC expressing high HLA-G levels can be found in the peripheral blood of stable and tolerant liver transplant recipients. The number of HLA-G+ DC correlates with the percentage of CD4+CD25^{high}CD127- Tregs and with the intensity of Foxp3 expression, thus supporting the hypothesis that HLA-G+ DC may play a tolerogenic role in alloimmune reactivity (62).

Mast Cells

Mast cells are bone marrow derived cells that circulate in an immature form and become mature after migration in a tissue site. Mast cells have been mostly viewed as effectors of IgE-mediated allergic diseases and host defense against parasites. The role of mast cells in both innate and adaptive immunity has been recognized recently. In addition, mast cells are involved in tissue repair through the secretion of several cytokines and growth factors that enhance fibroblast proliferation and collagen deposition, and inhibit degradation of the extra cellular matrix (63).

To our present knowledge it is not known whether mast cells express HLA-G in physiological conditions, and emerging research on the role for HLA-G+ mast cells in liver diseases is of interest (64, 65). It has been reported that mast cells infiltrating the livers of patients infected with hepatitis C virus (HCV) express HLA-G and secrete HLA-G in soluble form. The number of HLA-G+ mast cells is significantly associated with the areas of connective tissue and liver fibrosis located close to the hepatic arteries, veins and bile ducts of the portal tracts (66, 67). The presence of mast cells in the liver can be related to the production of TGF-β, a potent mast cell chemoattractant, by hepatic stellate cells (HSC)(68, 69). Then, HLA-G+ infiltrating mast cells promote HSC proliferation that, in turn, induces liver fibrosis (70). The expression and secretion of HLA-G by mast cells in HCV infected patients can be explained by the elevated amounts of IFN-α and IL-10 produced during HCV infection (71, 72). Accordingly, *in vitro* and *in vivo* data indicate that these cytokines strongly modulate HLA-G up-regulation in monocytes and other cells including trophoblasts, fibroblasts, and neoplastic cells (32, 73–78). The function of HLA-G+ mast cells during HCV infection remains to be clarified. It may be suggested that HLA-G expression may promote viral escape from the immune system by inhibiting both adaptive and innate immunity, thus protecting HCV-infected cells and favoring viral progression.

In summary, this data supports the assumption that HLA-G+ immune cells are implicated in the pathogenesis of a wide array of disorders. The role of HLA-G+ immune cells in the context of autoimmune diseases will be discussed in the following paragraphs.

HLA-G-EXPRESSING IMMUNE CELLS IN AUTOIMMUNE DISEASES

Multiple Sclerosis

Multiple sclerosis (MS) is an immune-mediated disorder of the central nervous system (CNS) leading to demyelination as well as axonal and neuronal damage, with progressive neurological impairment (79). The course of MS can follow four clinical patterns that include relapsing remitting MS (RRMS, which accounts for 80–90% of MS cases at onset), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive relapsing MS (PRMS) (80). Although the pathogenesis of MS is still not completely understood, it is known that central tolerance may be defective leading to the development of self-reactive T cells that transmigrate into the CNS where they can be activated by APCs and determine brain damage (79). The brain has long been considered an immunologically privileged site. This idea is based on the observation that tissue transplants in the CNS are not commonly rejected by the immune system. Commonly accepted explanations for the lack of an effective immune response to antigens in the brain are an anti-inflammatory and, with regard to invading immune cells, pro-apoptotic environment in the brain, the limited access of brain-derived antigens to the lymphoid organs, the presence of the blood–brain barrier, low major histocompatibility complex (MHC) expression in the brain parenchyma, and the absence of DCs (81, 82). However, numerous studies in infectious, autoimmune and tumor models have challenged this view by showing that potent immune reactions can and do occur in the CNS (83).

The main aspect favoring the autoimmune etiology of MS consists of the presence of activated IFN-producing T helper 1 (Th1) cells, that recognize peptides of the myelin sheath, including myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (80). HLA-G immunoreactivity was detected in the transition zone between the plaque center and the perilesional areas as well as in both acute and chronic active plaques. In proximity of MS lesions the adjacent normal appearing gray matter remained predominantly negative for HLA-G, whereas HLA-G expression in adjacent normal appearing white matter was similar to the expression levels of the lesion borders (83). Notably, in early and highly inflammatory MS lesions, HLA-G expression was abundant and detected on macrophages/activated

microglia cells. Similarly, perilesional activated microglia cells were immunoreactive for HLA-G. Furthermore, endothelial cells and meningeal vessels as well as arachnoidal cap cells show HLA-G immunoreactivity (83). However, expression of the inhibitory receptors for HLA-G, belonging to immunoglobulin-like transcript family ILT2 and ILT4, have been described in chronic active MS plaques. ILT2 immunoreactivity could be observed in the plaque center and the plaque border and paralleled HLA-G immunoreactivity. The main cellular sources for both molecules were macrophages and microglia (83). The cerebrospinal fluid (CSF) compartment has been proposed to partially constitute a functional equivalent of the lymphatic system for the CNS. Interestingly, the levels of HLA-G on $CD14^+$ monocytes were significantly elevated in the CSF of patients with MS compared with peripheral blood. Of note is the fact that HLA-G expressed by monocytes was identified as an important negative immune-regulatory factor, down-regulating the production of Th1 as well as Th2 cytokines, inhibiting antigen-specific and autologous $CD4^+$ T-cell activation, and inducing anergic T cells (37, 38). Furthermore, a small number of both $CD4^+$ and $CD8^+$ T cells, including $CD4^+$ Tregs, expressed HLA-G in the CSF of MS patients (83). Interestingly, CSF-derived HLA-G+CD4+ Tregs show high expression of the C-C chemokine receptor 5 that might favor their selective migration into the nervous system of MS patients, counteracting the activity of autoreactive T cells. The frequency of CSF-derived HLA-G+CD4+ Tregs correlates positively with the disease status in MS patients with active disease (22). Increased levels of HLA-G+CD4+ Tregs have been detected in MS patients responses to IFN or natalizumab treatment (80).

Taken together, these findings, seem to confirm that HLA-G expression on immune cells infiltrating CNS and detectable in CSF, may contribute to immune-regulation in MS.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease that can affect virtually any organ system, including skin, joints, kidneys, brain, and blood vessels (84). The development of SLE is dependent on a complex interplay between genetic, environmental, and immunological factors (85, 86). Among these, defective function of regulatory T cells and polyclonal activation of B lymphocytes leading to the production of auto-antibodies seems to play a major role (87–89).

Limited literature data are available on the expression of HLA-G in immune cells from SLE patients. Monsivais-Urenda et al. reported that monocytes from SLE patients as well as mature $CD83^+$ DC showed a reduced expression of HLA-G compared with healthy controls (35). In addition, monocytes from SLE patients showed a diminished induction of HLA-G expression in response to stimulation with IL-10, and when pre-treated with IFN-γ they exhibited an impaired capability to inhibit the proliferation of autologous lymphocytes. Interestingly, lymphocytes from SLE patients seem to display a lower capability to acquire HLA-G molecules by trogocytosis from autologous monocytes as compared to lymphocytes from normal subjects (35). By contrast, our and other groups reported that the percentage of HLA-G expressing cells among PBMC is significantly higher in SLE patients than in healthy controls (33, 90). In particular, the percentages of HLA-G-positive monocytes and HLA-G-expressing CD4+, CD8+, and CD4+/CD8+ double positive (DP) cells are significantly higher in SLE patients than in controls. Moreover, within the population of DP cells a subpopulation of $CD4^{dull}CD8^{high}$ cells displayed a high proportion of $HLA\text{-}G^+$ cells, while HLA-G was virtually absent in the same subpopulation of healthy subjects (33). The function of circulating HLA-G+ DP cells is not known, however, it is worth noting that DP cells seem to exert a suppressive role in the production of autoantibodies in SLE patients (91). In summary, it may be proposed that the up-regulation of HLA-G membrane expression by PBMC could reflect an effort to regulate the hyperactive immune status occurring in SLE.

Systemic Sclerosis

Systemic sclerosis (SSc) is a chronic connective tissue disease of unknown origin, more frequently affecting women. It is characterized by diffuse fibrosis, vasculopathy and immune dysregulation. In addition to skin involvement, SSc can affect multiple organ systems, including the musculo-skeletal, pulmonary, cardiac, gastrointestinal, and urinary systems (92, 93). Complex alterations of the normal functional balance within immune cells sub-populations, in particular Th17 lymphocytes and Tregs, including both CD4+ and CD8+ Tregs subsets, have been demonstrated in patients affected by SSc (94–97).

Our research group analyzed the role of both membrane HLA-G and sHLA-G in SSc patients. In particular, we recently reported that the percentage of HLA-G-positive monocytes, CD4+ T cells, CD8+ T cells and DP cells are significantly higher in SSc patients as compared to healthy subjects (34). Among DP cells a subpopulation of $CD4^{dull}CD8^{high}$ lymphocytes highly expressing HLA-G was detected. The function of circulating DP cells in SSc is under investigation, however, it is worth noting that these cells, which may exert potent suppressive effects, are present in the inflamed tissues of patients affected by immune mediated disorders and in the skin of patients with early active SSc. This may contribute, through IL-4 secretion, to the enhanced extracellular matrix deposition by fibroblasts (98). Plasma sHLA-G levels were higher in SSc patients when compared to healthy controls. Notably, plasma levels of sHLA-G1 and sHLA-G5 isoforms were comparable and no significant differences were detected in total sHLA-G, sHLA-G1 and sHLA-G5 levels between limited and diffuse SSc forms. The total sHLA-G plasma levels correlated with the elevated TGF-β levels circulating in SSc patients (34). This finding is in agreement with *in vitro* data demonstrating that the production of TGF-β by myelomonocytic cells is strongly increased after incubation with recombinant sHLA-G (99). In summary, it may be proposed that there is a possible involvement of HLA−G in SSc pathogenesis, as the elevated HLA−G membrane expression by PBMC and the increased sHLA−G plasma levels may reflect an attempt to control the immune derangement occurring in this disease and concur, through TGF-β up-regulation, with fibroblast activation and fibrosis development (34).

Skin Diseases

Psoriasis (Ps) is a common inflammatory, chronic, and disabling skin disease that affects 1–3% of the population (100). Distinct clinical phenotypes may be observed in this disease, including chronic plaque (Ps vulgaris), guttate, and pustular variants. At least 10% of patients can develop arthritis (101). In many cases, a marked infiltration of mononuclear leucocytes (T lymphocytes and DC) into the dermis and elongated/hyperplastic blood vessels in the papillary dermal region can be observed (102). Because Ps is considered to be an organ-specific autoimmune disease, Cardili et al. analyzed HLA-G expression in skin specimens obtained from patients with Ps and observed the presence of HLA-G molecules on lymphomononuclear cells within the dermis and to a higher extent, in the epidermis. The intensity of HLA-G expression was not correlated with Ps variants or severity. By contrast, skin specimens obtained from healthy individuals were negative for HLA-G expression (103). Other authors have reported HLA-G expression in $CD68^+$ $CD11c^+$ macrophages lining the dermo-epidermal junction in patients with Ps vulgaris (104). In addition, NK cells and $CD4^+$ T cells expressing the IL2 inhibitory receptor have been described in Ps skin infiltrates, suggesting that HLA-G may act as an inhibitory molecule to down-regulate the activation of effector cells (104). These findings lead to the assumption that HLA-G^+ macrophages could represent an internal control system that counteracts auto-reactive expression of T-cell cognate receptors for HLA-G.

Atopic dermatitis (AD) is another chronic T-cell mediated skin disorder which, in contrast to Ps, exhibits a Th2 type cytokine profile including over-production of IL-10, which is known to up-regulate HLA-G (105). Khosrotehrani et al. investigated the role of HLA-G in patients with AD. They found that HLA-G was mainly expressed by infiltrating T cells and to a lesser extent, by macrophages and even DC (106). The epidermis was consistently negative for HLA-G expression, suggesting that, analogously to Ps, HLA-G up-regulation may either be the consequence of the permissive cytokine environment in AD or it may be a part of an internal regulatory system to control excessive inflammation.

DISCUSSION

Data in recent literature indicates that small percentages of HLA-G positive immune cells can be detected in the peripheral blood of patients with physiological conditions. In these conditions HLA-G positive immune cells seem to play an emerging role in maintaining immune homeostasis. However, increased percentages of circulating and tissue infiltrating HLA-G positive immune cells occur in various pathological conditions like infections, cancers, transplants, and immune-mediated diseases. Taking into account the immunoregulatory role of HLA-G, it may be suggested that T lymphocytes, NK cells and APCs that express HLA-G molecules are potentially involved in the pathogenesis of immune mediated diseases.

HLA-G positive cells can modulate both the priming and the effector phases of the immune response, thus contributing to peripheral immune tolerance. It may be proposed that HLA-G expressing immune cells represent an attempt to create an immune-suppressive milieu, as a way of controlling immune derangement in systemic autoimmune disorders. However, several important issues still need to be clarified in this context. A better understanding of the HLA-G gene regulation will greatly improve the possibility of manipulating this emerging immune check-point, which could alter the course of immunological diseases. Moreover, the role played by different molecular HLA-G isoforms and the contribution of specific HLA-G expressing subpopulations in each clinical situation needs to be better defined. Therefore further pre-clinical and clinical investigations are required in order to provide more detailed information on the role played by HLA-G expressing cells in the mechanisms underlying the onset and progression of immune-mediated diseases. These future studies are crucial for the development of potential HLA-G strategies of therapy.

AUTHOR CONTRIBUTIONS

All authors equally contributed to the conception of ideas and design of this manuscript.

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REVIEW
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Roles of HLA-G in the Maternal-Fetal Immune Microenvironment

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During pregnancy, the maternal uterus and fetus form a special microenvironment at the maternal-fetal interface to support fetal development. Extravillous trophoblasts (EVTs), differentiated from the fetus, invade into the decidua and interact with maternal cells. Human leukocyte antigen (HLA)-G is a non-classical MHC-I molecule that is expressed abundantly and specifically on EVTs in physiological conditions. Soluble HLA-G (sHLA-G) is also found in maternal blood, amniotic fluid, and cord blood. The abnormal expression and polymorphisms of HLA-G are related to adverse pregnancy outcomes such as preeclampsia (PE) and recurrent spontaneous abortion (RSA). Here we summarize current findings about three main roles of HLA-G during pregnancy, namely its promotion of spiral artery remodeling, immune tolerance, and fetal growth, all resulting from its interaction with immune cells. These findings are not only of great significance for the treatment of pregnancy-related diseases but also provide clues to tumor immunology research since HLA-G functions as a checkpoint in tumors.

Keywords: human leukocyte antigen G, pregnancy, extravillous trophoblasts, immunology, natural killer cells, spiral artery remodeling, fetal development

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INTRODUCTION

The HLA-G gene was first discovered in 1982 (1) and was denominated HLA-G in 1990 (2). HLA-G is a class I histocompatibility antigen, but unlike the classical class I major histocompatibility complex (MHC-I) HLA-A, HLA-B, and HLA-C genes, HLA-G displays limited polymorphism. Seven different HLA-G mRNA transcripts have been identified, with this variety attributed to alternative splicing of its seven exons. HLA-G2, G3, and G4 transcripts are translated into membrane-binding isoforms; HLA-G5, G6, G7 into soluble HLA-G (sHLA-G) isoforms; and HLA-G1 into both types of isoforms (3–6).

HLA-G appears to be especially relevant to pregnancy. After ovulation, the uterine stromal fibroblasts of the endometrium differentiate into decidual cells. In addition, uterine spiral arteries are formed. In early pregnancy, steroid hormones, progesterone, and β-estradiol act on maternal vascular endothelial cells and increase vascular permeability, promoting angiogenesis (7–9). Immune cells, especially natural killer (NK) cells, are recruited though maternal vessels. Extravillous trophoblasts (EVTs) of the embryo invade into the decidua and replace the endothelial cells (10). Together, these processes remodel spiral arteries and form the maternal-fetal interface to support the provision of oxygen and nutrients for fetal development.

HLA-G has been reported to be expressed on the surfaces of preimplantation embryos (11–13) and EVTs (14–16), while sHLA-G has been detected in culture medium of *in vitro*-fertilized (IVF) embryos (17, 18), maternal blood (19–21), amniotic fluid (22, 23), and cord blood (24, 25). HLA-G plays critical roles in the remodeling of spiral arteries, fetal development, and immune tolerance (10).

Considering these findings involving HLA-G, it is not surprising to also find relationships between HLA-G and complications associated with pregnancy. Specifically, HLA-G gene polymorphisms and decreased levels of sHLA-G have been found to be related to embryo implantation failure (18, 26–30), recurrent spontaneous abortion (31–36), placental abruption (37) and pre-eclampsia (38–48). For example, a multicenter study showed that the presence of soluble HLA-G in the culture medium of the embryo is significantly associated with increased pregnancy rates after assisted reproduction technique (ART) (26). Furthermore, Nowak et al. observed that soluble HLA-G level in the serum of patients is correlated with pregnancy outcome after ART. In addition, they revealed significantly association of G-C-ins (-725G>C SNP in the promoter region of HLA-G) haplotypes with infertility (27). HLA-G plasma level in women with placental abruption was significantly decreased (37). It is therefore critical to achieve a comprehensive understanding of the roles of HLA-G in pregnancy in general, and in maternal-fetal interactions specifically. Herein, we review three such major identified roles.

HLA-G PROMOTES THE REMODELING OF SPIRAL ARTERY

Fetal development in the uterus requires nutrients and oxygen provided by the maternal blood. Spiral artery remodeling is essential for accelerating and stabilizing placental blood flow during pregnancy. This remodeling begins with decidua-associated remodeling and involves the initial swelling and disorganization of the vascular smooth muscle of spiral artery. Next the vascular endothelium becomes liquefied and the elastic membrane disintegrates. These changes are mainly induced by angiogenic growth factors, which have been found to be produced by decidual NK cells and macrophages (49–53).

NK cells constitute less than 20% of human peripheral lymphocytes, but account for about 70% of lymphocytes in the first-trimester decidua (54), and are hence the most abundant lymphocytes in the early maternal-fetal interface. NK cells recognize MHC-I proteins on target cells by expressing receptors such as killer cell immunoglobulin-like receptors (KIRs). And then NK cells decide whether to kill target cells upon signals from inhibitory or activating receptors.

KIR2DL4 (also called CD158d), a member of the KIR family, has a structure, localization, and function that differs from that of other KIRs (55–57). KIR2DL4 is expressed by NK cells and activates the production of IFN-γ but does not promote cytotoxicity of resting peripheral NK cells (58, 59). One variant of KIR2DL4, 9A is unstable on the surface because of its truncated cytoplasmic tail (60). In addition, KIR2DL4 has been reported to be mainly localized in intracellular endosomes containing Rab5 (61) and this localization requires the Ig domain of KIR2DL4. The localization and function in endosomes is specific to KIR2DL4. Because the expression of other members of the KIR family on the cell surface is very high.

The ligand of KIR2DL4 is HLA-G. Rajagopalan et al. (57) reported that soluble KIR2DL4, a fusion protein composed of the extracellular region of KIR2DL4 and the IgG Fc region, binds to LCL 721.221 cells that express HLA-G but not HLA-Cw3 or HLA-B7. Furthermore, soluble HLA-G or HLA-G on the surface of target cells was observed to be endocytosed into vesicles of NK cells after interacting with KIR2DL4. Soluble HLA-G has been shown to activate the secretion of proangiogenic/proinflammatory cytokines and chemokines (i.e., IL-6, IL-1β, IL-8, IL-23, MIP-1-α, and MIP-3-α) by NK cells. In addition, the secretion of IL-8 was shown to require the cytoplasmic tail of KIR2DL4 (61). Rajagopalan et al. (62) later described the molecular mechanism involved in this process. In NK cells, KIR2DL4 was found to interact with DNA damage signaling kinase DNA-PKcs and trigger phosphorylation of Akt at position Ser^{473} following stimulation with soluble HLA-G. Phosphorylated Akt activates the NF-κB pathway and hence results in the production of proinflammatory and proangiogenic cytokines (62). In addition, Rajagopalan et al. (63) identified a mutation of the TRAF6-binding motif in the KIR2DL4 cytoplasmic tail that caused decreased IL-8 secretion in transfected 293T cells. A co-immunoprecipitation assay using NK cells demonstrated an association between TRAF6 and KIR2DL4. And the TRAF6 binding site was shown to be required for the NF-κB signaling pathway. Specifically, KIR2DL4 recruits TRAF6, which in turn phosphorylates TAK1 at position T187. TAK1 participates in the production of IL-6, IFN-γ, CXCL1, and P2RX5 in NK cells (63).

When peripheral NK cells were stimulated with soluble HLA-G (sHLA-G) or KIR2DL4 agonist antibody, DNA-PKcs was activated and induced the expression of cyclin-dependent kinase inhibitor p21. At the same time, heterochromatin protein 1-γ (HP1-γ) was phosphorylated at position Ser-83. These events are related to cell senescence. NK cells activated by KIR2DL4 and sHLA-G acquired senescence features, in particular they became enlarged and showed increased β–galactosidase (SA-β-gal) activity. However, proliferation and apoptosis were not induced. Supernatants from peripheral NK cells stimulated with agonist antibody of KIR2DL4 could enhance HUVEC vascular permeability and tube formation. This observation is consistent with the stimulation by TNF-α and IL-1β (64). These data acquired by Long et al. showed that sHLA-G can stimulate the production of senescence-associated secretory phenotype (SASP) in NK cells by binding to KIR2DL4, and hence promote vascular permeability and angiogenesis (65). Thus, in this way, HLA-G can facilitate spiral artery remodeling (**Figure 1**).

KIR2DL4 mRNA has been detected in peripheral NK cells of every donor tested (66). Nonetheless, protein levels of KIR2DL4 were reported to be very low on the surface of resting peripheral

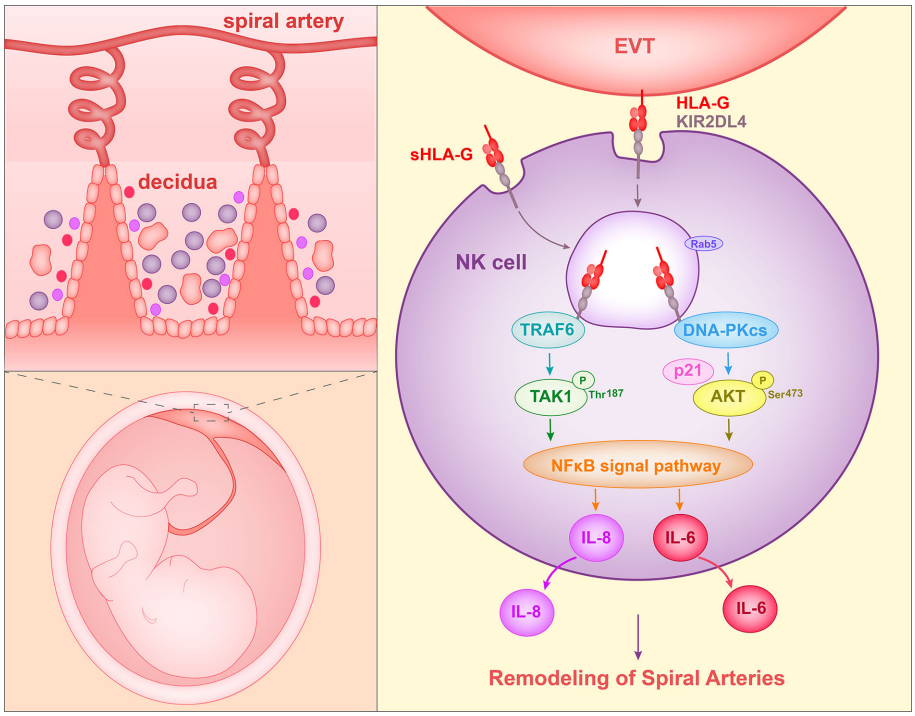

FIGURE 1 | Proposed interaction of HLA-G with decidual NK cells to promote spiral artery remodeling. HLA-G on EVTs or sHLA-G secreted by EVTs binds to KIR2DL4 on NK cells, and HLA-G and KIR2DL4 are then endocytosed into Rab5$^+$ endosomes of NK cells. The endocytosed KIR2DL4 binds to TRAF6, induces phosphorylation of TAK1 at Thr187 and activates the NF-κB pathway. In addition, KIR2DL4 interacts with DNA-PKcs, triggering phosphorylation of Akt at Ser473 and upregulating p21. Phosphorylated Akt then activates the NF-κB pathway and results in the expression of the senescence-associated secretory phenotype (SASP) and production of, for example, IL-6 and IL-8. The SASP promotes vascular permeability, angiogenesis and invasion of EVT.

NK cells and decidual NK cells (67). In addition, the investigations, which sHLA-G and KIR2DL4 are endocytosed into endosomes and induce secretion of proangiogenic cytokines that promote vascular permeability, were verified in peripheral NK cells. Peripheral NK cells are quite different from decidual NK cells, and hence whether this process and mechanism occur in decidual NK cells remains to be determined. Furthermore, Fu et al. (68) observed the expression of intracellular KIR2DL4 in first-trimester decidual NK cells. This observation is consistent with the findings that KIR2DL4 resides in endosomes of decidual NK cells.

HLA-G has been reported to be selectively expressed on the cell surface of extravillous trophoblasts (14, 15). In addition, Apps et al. (69) found a significant percentage of HLA-G expressed as a homodimer on the surfaces of trophoblast cells in first-trimester placenta. This HLA-G homodimer is disulphide-linked and β2m-associated (69, 70). LCL 721.221 cells that express homodimeric HLA-G could stimulate CD14$^+$ decidual macrophages or decidual NK cells to secrete cytokines such as IL-6, IL-8, and TNF-α (53). This stimulation is activated when homodimeric HLA-G binds to immunoglobulin-like transcript 2 (ILT2, also called LILRB1) on macrophages or to KIR2DL4 on NK cells. Macrophages differentiated from peripheral monocytes could exhibit the phenotype of decidual macrophages when stimulated by soluble HLA-G5. These macrophages were observed to secrete more IL-6 and CXCL-1 and to induce trophoblast invasion (71). In addition, soluble HLA-G5 could also form a homodimer (69). Although, it remains to be determined whether sHLA-G in decidua and maternal serum exists as a homodimer or monomer.

sHLA-G induces the secretion of IL-8 in NK cells and homodimeric HLA-G on EVTs can stimulate decidual NK cells and macrophages to produce IL-8. Hanna et al. (72) found that decidual NK cells highly express IL-8, which can bind to CXCR1 and CXCR3. In addition, a transwell assay showed that IL-8 neutralizing antibody could reduce invasiveness of trophoblasts induced by decidual NK cells. Furthermore, neutralizing antibody to IL-8 could inhibit trophoblast invasion induced by decidual NK cells in Matrigel on nude mice. Therefore, IL-8 secreted by decidual NK cells was determined to participate in trophoblast invasiveness. In addition, a population of ILT2^{hi} pregnancy-trained decidual NK cells has been identified in repeated pregnancies. HLA-G-expressing LCL721.221 cells could enhance the secretion of more VEGFα in decidual NK cells of women with multigravid pregnancies. VEGFα secreted by decidual NK cells promotes vascularization (73).

In addition, HLA-G1$^+$ APCs have been shown induce the differentiation of CD4$^+$ peripheral T cells into suppressive CD4$^+$

T cells (74). It has been reported that decidual Treg cells can increase trophoblast invasiveness. This effect is inhibited by anti-IL-10 neutralizing antibody and is increased by anti-TGF-β antibody (75).

The remodeling of spiral artery in women with pre-eclampsia is impaired (76). In patients with pre-eclampsia, the level of soluble HLA-G in serum was detected to be decreased whether in the first, second or third trimester of pregnancy (41, 50). In addition, HLA-G3 transcript was significantly reduced in the placenta of mild pre-eclampsia (42). Furthermore, the frequency of +14 bp/+14 bp genotype (14 bp insert in exon 8 of the HLA-G gene) in pre-eclampsia offspring was higher than in control offspring. In addition, fetal HLA-G*0106 in combination with maternal KIR2DL4*006 allele was reported to be associated with pre-eclampsia risk among multigravid pregnancies (51). Therefore, it will accelerate the treatment and prevention of preeclampsia to investigate the role of HLA-G in spiral artery remodeling.

HLA-G PARTICIPATES IN THE FORMATION OF MATERNAL-FETAL IMMUNE TOLERANCE

In the uterus, the fetus expresses paternal histocompatibility antigens, which are foreign antigens for the mother, yet the fetus is neither rejected nor attacked by the maternal immune system. This phenomenon is related to the specific immune tolerance microenvironment at the maternal-fetal interface. In the first trimester, immune cells account for up to 40% of the decidua. NK cells, macrophages, and T cells make up, respectively, about 70%, 15%–20%, and 5%–15% of decidual leukocytes (77). In addition, myeloid dendritic cells (DCs) have also been detected. Fetal-derived EVTs invade into the decidua and come into direct contact with maternal leukocytes. EVTs express classical MHC I HLA-C molecules and non-classical MHC I HLA-G and HLA-E molecules, and interact with leukocytes expressing receptors (such as KIR) to provide immune tolerance conditions (78).

NK cells are the most abundant leukocytes in the decidua during the first trimester. Expression of HLA-G has been suggested to protect NK-cell-sensitive cells from lysis by NK cell lines or decidual NK cells (79, 80). Moreover, NK cells in the maternal uterine blood were observed to not kill cytotrophoblast cells, whether the cytotrophoblast cells were isolated from the same mothers from whom the NK cells were derived or from other mothers. Cytotoxicity of NK cells was restored if an antibody that blocks both HLA-G and HLA-C was added into the co-culture medium. But an anti-HLA-C antibody did not reverse the protection against NK lysis. Therefore, HLA-G protected embryo-derived cytotrophoblast cells from being lysed by NK cells from the maternal uterine blood (81). However, cells overexpressing HLA-G have been reported to upregulate the surface expression of HLA-E, another nonclassical MHC molecule (82) since HLA-E can be loaded with HLA-G-derived peptide, the HLA-G leader sequence (83). Therefore, it is the HLA-E-NKG2A/CD94 interaction that inhibits cytotoxicity of NK cells to cell lines expressing HLA-G.

NK cells have been reported to express two HLA-G receptors: KIR2DL4 (57) and ILT2 (69). Co-culture of NK cell lines with HLA-G-expressing melanoma M8 cells restored the expression of KIR2DL4 or ILT2 on NK cells (84), and NK cells could use the trogocytosis to acquire HLA-G from tumor cells (85). Following co-culture with HLA-G1-expressing M8 cells or LCL 721.221 cells, IL-2-activated NK cells or IL-2-activated peripheral NK cells could acquire HLA-G1 on their cell surfaces and lose their cytotoxicity. Similarly, trogocytosis could transfer HLA-G to decidual NK cells from EVTs (86). While HLA-G mRNA was not detected, cell surface and intracellular HLA-G molecules were found in decidual NK cells after NK cells were co-cultured with EVTs. A transwell separation demonstrated NK cells obtaining HLA-G through direct cell contact. Therefore, decidual NK cells were shown to obtain HLA-G and then endocytose the HLA-G when in contact with EVTs. As mentioned above, HLA-G binds KIR2DL4 and is endocytosed by NK cells. Trogocytosis may play a role in NK cell tolerance.

Apps et al. (87) found that decidual NK cell degranulation was not affected when co-culturing with LCL 721.221 cells expressing HLA-G. The CD107a level in decidual NK cells was unchanged as a result of the stimulation of LCL 721.221 cells that express HLA-G. In addition, van der Meer et al. (88) reported that soluble HLA-G did not affect cytotoxicity of uterine mononuclear cells towards K562 cells and lytic activity of peripheral NK cells towards K562 cells was not affected by soluble HLA-G. However, soluble HLA-G could stimulate peripheral NK cells to produce interferon (IFN)-γ. Further, Poehlmann et al. (89) found that soluble HLA-G1 could inhibit cytotoxicity of term placentae NK cells towards K562 cells, NK cell sensitive cell lines, even when the NK cells were pre-stimulated with IL-2. And soluble HLA-G1 induced reduction of perforin in term placentae NK cells. Therefore, it is necessary to use a system of decidual NK cells and EVT to study the effects and underlying mechanism of HLA-G on decidual NK cells tolerance to EVTs. Du et al. (75) reported that lytic activity of decidual NK cells towards K562 cells was reduced when decidual NK cells were pre-cocultured with trophoblasts. Trophoblasts pre-incubated with Treg cells showed a greater downregulation effect, attributed to decidual $CD4^+CD25^+$ Treg cells upregulated HLA-G expression in the trophoblasts. Furthermore, a neutralizing antibody to HLA-G could rescue the cytotoxicity of decidual NK cells. Thus, Treg cells could promote the expression of HLA-G in trophoblasts and inhibit cytotoxicity of decidual NK cells, and hence facilitate the production of IL-4 and IL-10 by decidual NK cells. In addition, de Mendonça Vieira found that HLA-G expression on term placenta EVTs is higher than that on first trimester EVTs. Increased HLA-G could provide increased interaction with term pregnancy dNK cells through KIR2DL4 and ILT2. However, term pregnancy dNK cells showed increased degranulation capacity in response to PMA/ionomycin and K562 cells (90). Therefore, HLA-G may have different effects on dNK cells in different stages of pregnancy.

In first-trimester decidua, 10%–15% of leukocytes are T cells. This proportion has been shown to rise up to 70% at the end of pregnancy (77). At the same time, HLA-G expression has been observed to be higher in term pregnancy EVT cells than in first-trimester EVTs (91). Decidual T cells have been reported to express the HLA-G receptor ILT2. In 1999, Le Gal et al. (92) found that HLA-G specifically inhibited cytolytic T cell function. M8 cells were sensitized with influenza virus peptide and the cytotoxicity of peripheral antigen-specific $CD8^+$ CTL towards M8 cells was specifically decreased when M8 cells expressed HLA-G1. This inhibition could be rescued by anti-HLA-G1 mAb. In addition, Bainbridge et al. (93) reported that expression of HLA-G on C1R B-lymphocyte cells inhibited $CD4^+$ T cell proliferation when peripheral blood mononuclear cells were stimulated by C1R. Furthermore, HLA-G1 was reported to induce upregulation of ILT2 and KIR2DL4 mRNA in peripheral $CD4^+$ T cells when peripheral blood mononuclear cells (PBMCs) were co-cultured with HLA-G1-expressing LCL 721.221 cells (84). A proportion of peripheral $CD4^+$ T cells and $CD8^+$ T cells express ILT2 on their surfaces. Further, addition of anti-ILT2 mAb was observed to enhance cytotoxicity of $CD8^+$ CTL towards target cells (94). Soluble ILT2 and ILT4 could competitively inhibit binding of soluble recombinant CD8αα to soluble MHC-I molecules including HLA-G1 (95). LeMaoult et al. (96) found that peripheral $CD4^+$ T cells and $CD8^+$ T cells were able to acquire HLA-G1 by trogocytosis from LCL 721.221 cells, which express HLA-G1. This process did not require any interaction between HLA-G1 and receptors since addition of anti-HLA-G1 mAb or anti-ILT2 mAb did not affect the trogocytosis capability. Acquired HLA-G1 inhibited proliferation of peripheral $CD4^+$ T cells stimulated by IL-2 or allogeneic PBMCs. In addition, $CD4^+$ T cells that acquired HLA-G1 turned into regulatory cells and inhibited activation and proliferation of autologous T cells. Furthermore, peripheral T cells could also use trogocytosis to acquire ILT2 (97). Given that EVTs express high levels of HLA-G, and that Treg cells account for a high proportion of T cells in decidua, decidual T cells may also obtain HLA-G from EVTs and transform into Treg cells. Interestingly, Tilburgs et al. (98) reported that the co-culture of T cells with EVTs could increase the percentage of $CD4^+CD25^{hi}FOXP3^+CD45RA^+$ Treg cells in a population of decidual T cells or peripheral T cells. Recently, Salvany-Celades et al. (99) identified three types of Treg cells in decidua: $CD25^{hi}Foxp3^+$, $PD1^{hi}$, and $TIGIT^+$ Treg cells. In their experiments, all these Treg cells suppressed proliferation of decidual $CD4^+$ or $CD8^+$ T cells stimulated with anti-CD3 and anti-CD28 beads. $CD25^{hi}Foxp3^+$, $PD1^{hi}$ Treg cells could inhibit IFN-γ, TNF-α production of decidual $CD4^+$ or $CD8^+$ T cells. In addition, co-culture with EVTs was observed to increase the proportion of $CD25^{hi}Foxp3^+$ and $PD1^{hi}$ Treg cells but not of $TIGIT^+$ Treg cells that make up the population of $CD4^+$ peripheral T cells. This process would be expected to require cell-cell contact. However, addition of blocking antibody against HLA-G had no effect on the increase of the proportion of Treg cells in the population of peripheral T cells. Whether HLA-G mediates an increase in the quantity of Treg cells in decidua remains to be investigated.

$CD14^+$ macrophages constitute the second largest population of leukocytes in the decidua and express HLA-G receptors ILT2 and ILT4. Decidual macrophages in the first trimester consist of two distinct populations: $CD11c^{hi}$ and $CD11c^{lo}CD209^{hi}CD206^{hi}$. These two subsets have been shown to constitutively produce IL-6, TNF-α, and TGF-β, whereas IL-10 and MIP-1β have been shown to be mainly secreted by $CD11c^{hi}$ decidual macrophages (100). Expression of ILT2 and ILT4 were increased in peripheral monocytes stimulated with M8 cells expressing HLA-G1 or HLA-G5 (95). In addition, HLA-G1-transfected antigen presenting cells (APC) lines could inhibit proliferation of $CD4^+$ T cells, which was not mediated by the release of HLA-G1 in the medium. Further, APCs expressing HLA-G1 induced the differentiation of $CD4^+$ T cells into suppressive T cells (74). The inclusion of decidual macrophages was observed to increase the percentage of Treg cells in the population of peripheral $CD4^+$ T cells (99). However, it is still unclear whether HLA-G participates in immune tolerance mediated by macrophages.

$DC\text{-}SIGN^+$ ($CD11c^+CD1c^+$) cells make up about 3% of decidual leukocytes (101). IL-10-producing DCs have been reported to be present in PBMCs, and are termed DC-10. DC-10 has been indicated to be able to induce a differentiation of naïve $CD4^+$ T cells into IL-10-producing Tr1 cells when subjected to an ILT4/HLA-G signal. And this interaction induced the expression of HLA-G on $CD4^+$ T cells (102). Guo et al. (103) found that trophoblasts express thymic stromal lymphopoietin (TSLP) and secret soluble TSLP. Decidual $CD1c^+$ DCs were observed to secrete a lot of IL-10 and CCL-17 when stimulated with soluble TSLP, thereby promoting the differentiation of decidual $CD4^+$ T cells into Th2 cells. In addition, decidual DCs stimulated by TSLP could induce decidual $CD4^+CD25^-$ T cells to differentiate into $CD4^+CD25^+FOXP3^+$ Treg cells through TGF-β1 (75).

In addition, granulocytic myeloid-derived suppressor cells (GR-MDSCs) were found to accumulate in the term placenta (104). GR-MDSCs in the peripheral blood of pregnant woman express ILT2 and ILT4. Soluble HLA-G can increase the suppressive activity of placental GR-MDSCs on T cell proliferation (105). Therefore, GR-MDSCs contribute to the formation of immune tolerance in placenta.

HLA-G FACILITATES FETAL GROWTH

In addition to promoting remodeling of spiral arteries and immune tolerance, HLA-G has been found to facilitate fetal growth by stimulating secretion of growth promoting factors (GPFs) in NK cells, according to recent studies (68, 106, 107).

Fu et al. (68) found that decidual NK cells in the first trimester expressed high quantities GPFs such as pleiotrophin (PTN), osteoglycin (OGN), and osteopontin (OPN) at the mRNA and protein levels. Most GPF-positive NK cells were $CD49a^+Eomes^+$ tissue resident NK (trNK) cells. However, a smaller percentage of the decidual NK cells in the first trimester from recurrent spontaneous abortion (RSA) patients were trNK cells, and these first-trimester decidual NK cells from RSA patients

showed decreased secretion of GPFs, while expression of GPFs in trNK cells from patient decidua was decreased. In order to explore whether the defect of trNK cells and GPFs could affect fetal development, Fu et al. constructed a pregnancy model of NK cell knockout mice, pregnant *Nfil3*$^{-/-}$ mice. In the uteruses of pregnant *Nfil3*$^{-/-}$ mice, the number of trNK cells and the GPF levels were decreased. In addition, the average weight of fetuses from pregnant *Nfil3*$^{-/-}$ mice was decreased and the development of the embryonic skeletal system was defective. Fetal growth restriction (FGR) in pregnant *Nfil3*$^{-/-}$ mice could be rescued by transferring induced CD49a^{+} uterus-like trNK cells (108). Furthermore, pregnant GPF knockout mice showed the same fetal growth defect as did *Nfil3*$^{-/-}$ mice. CD49a^{+} uterus-like trNK cells differentiated from GPF knockout mice could reach the uterus but could not rescue the fetal development defect in pregnant *Nfil3*$^{-/-}$ mice. Moreover, injection of anti-PTN or anti-OGN antibody caused significantly reduced fetal weight. And injection of PTN could restore the fetal weight defect in pregnant *Nfil3*$^{-/-}$ mice. These findings revealed that decidual trNK cells promote fetal development by secreting growth-promoting factors.

When co-cultured with EVTs *in vitro*, decidual NK cells expressed higher levels of GPFs. Since HLA molecules on EVTs and their receptors on NK cells are important for the maternal-fetal interface, Fu et al. co-cultured decidual NK cells with LCL 721.221 cells, which expressed HLA-G or HLA-C. The co-culture assay revealed that HLA-G promote expression of PTN, OGN, and OPN in dNK cells. TrNK cells expressed HLA-G receptors ILT2 and intracellular KIR2DL4. In the co-culture assay, HLA-G antibody and ILT2 antibody reduced GPF secretion significantly in decidual NK cells stimulated by EVTs. At the same time, GPF expression levels in decidual NK cells transfected with KIR2DL4 siRNA also decreased when the NK cells were co-cultured with EVTs. Therefore, HLA-G in EVTs could promote GPF secretion in trNK cells by acting on the receptors ILT2 and KIR2DL4 (68). Zhou et al. (109) analyzed the changes of signaling pathways in decidual NK cells stimulated with EVT and found that the PI3K-Akt signaling pathway was significantly altered. Phosphorylation of AKT1 at Ser-473 and the expression of PDK2 were increased in decidual NK cells co-cultured with EVTs. In addition, transcription factor PBX1, which is already expressed in high quantities in decidual NK cells, showed even higher levels of expression in EVT-stimulated first-trimester decidual NK cells. SiRNA and phosphorylation inhibitor of AKT1 or PDK2 could reduce the levels of expression of PBX1 in decidual NK cells stimulated with EVTs. HLA-G and ILT2 blocking antibody were each shown to reduce the levels of phosphorylation of AKT1 at Ser-473 and reduce the levels of PBX1 expression in decidual NK cells co-cultured with EVTs. Therefore, HLA-G in EVTs could stimulate the PDK2-AKT1 signaling pathway and increase PBX1 expression by interacting with ILT2 in decidual NK cells. Zhou et al. also demonstrated that PBX1 could enhance the expression of growth-promoting factors PTN and OGN by directly binding to their promoters. PBX1 gene was mutant and protein level of PBX1 was reduced in decidual NK cells of RSA patients. In addition, trNK cell number and PTN and OGN levels were decreased and fetal development was impaired in mice having PBX1 knocked out specifically in NK cells. Therefore, these results indicated that HLA-G in EVTs interacts with ILT2 in decidual NK cells and activates the PDK2-AKT1 signaling pathway in NK cells, and in turn PBX1 promotes fetal growth by upregulating PTN and OGN (**Figure 2**).

Imbalance of maternal-fetal immune tolerance and fetal growth embryo development can lead to miscarriage. Plasmatic levels of soluble HLA-G, both sHLA-G1 and HLA-G5, in women with abortion was much lower than those in pregnant women. In addition, sHLA-G1 was absent in the serum of women with RSA (31). Furthermore, Nowak et al. found that women who were heterozygous in −716 HLA-G (−716 T>G SNP in the promoter region of HLA-G) had a lower possibility of spontaneous miscarriage (110). In the promoter region of HLA-G, -1573 T>C SNP and -1746 C>A SNP were also reported to be associated with RSA (33). Therefore, studying the role of HLA-G during pregnancy could be beneficial to the understanding of RSA.

CONCLUSIONS

To summarize the above reports, three roles of HLA-G have been found during pregnancy. HLA-G interacts with ILT2 and KIR2DL4 on macrophages and NK cells to enhance the production of proangiogenic cytokines and to enhance the EVT invasion of decidua, thereby promoting spiral artery remodeling. In addition, HLA-G binds to ILT2, ILT4, and KIR2DL4 on NK cells, T cells and macrophages, inhibits the cytotoxicity of NK cells and CD8^{+} T cells, and causes an increase in the percentage of Treg cells in the population, and thereby contributes to immune tolerance. Furthermore, HLA-G on EVTs could induce the production of growth-promoting factors by decidual NK cells, thereby regulating fetal growth (**Figure 3**).

Since mice do not express HLA-G, samples are difficult to obtain and *in vitro* culture cannot be maintained for a long time, thus, it is difficult to study the function and mechanism of HLA-G without functional experimental models. Interestingly, Turco et al. (111) constructed, from first-trimester villi, long-lasting genetically stable trophoblast organoids that could differentiate into EVTs. Experiments deploying this system are expected to be used to further investigate the role and mechanism of HLA-G in pregnancy and to test the current models.

HLA-G has been reported to be abnormally expressed in many kinds of tumor tissues and has been detected in the plasma of cancer patients. In addition, the expression of HLA-G was found related to the outcome in various tumors (112). Since HLA-G is abnormally and specifically expressed in tumor tissues, it may represent a checkpoint in tumor immunology (113). At the maternal-fetal interface, HLA-G has been found to inhibit the cytotoxicity of T cells and NK cells and increase the proportion of Treg cells. It may also perform similar functions in tumor microenvironment. Therefore, the roles of HLA-G in pregnancy may provide clues for further understanding of tumor immunology.

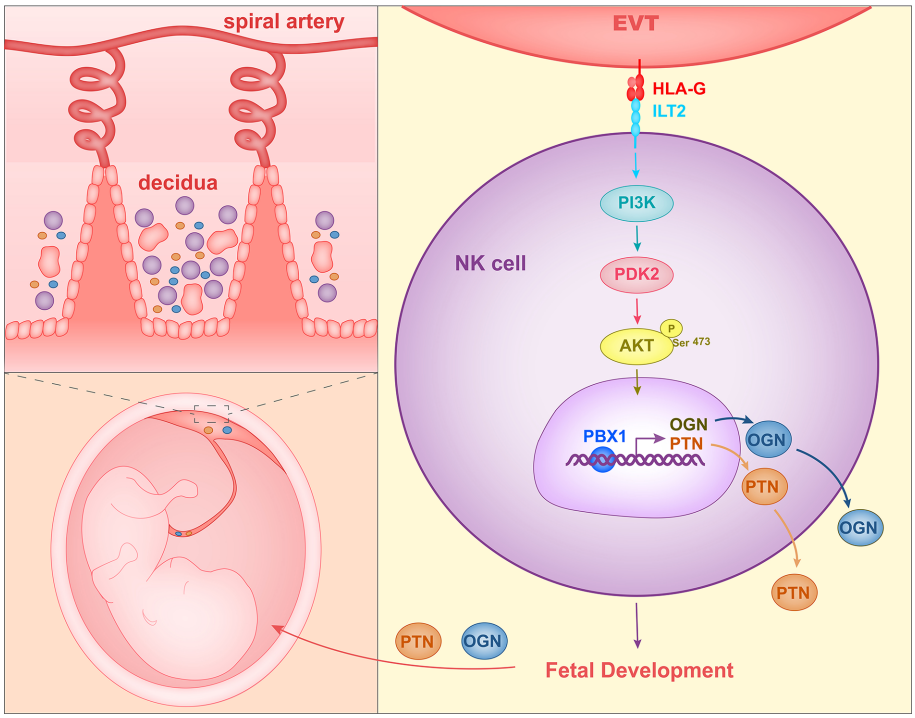

FIGURE 2 | Mechanism of HLA-G interacting with decidual NK cells to promote fetal growth. HLA-G on EVTs binds to ILT2 on NK cells, activates the PI3K-AKT signal pathway and induces expression of transcription factor PBX1. PBX1 upregulates the secretion of growth-promoting factor PTN and OGN, facilitating early fetal growth.

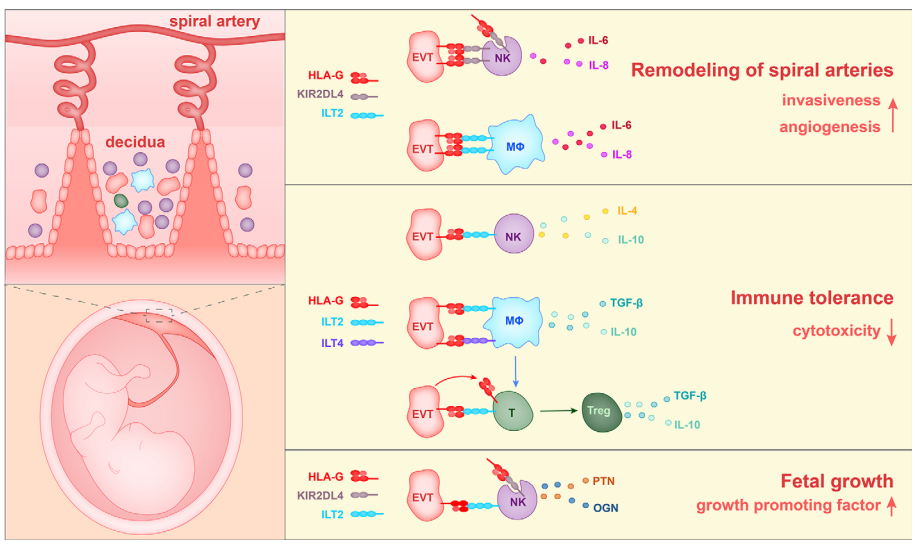

FIGURE 3 | The currently identified roles of HLA-G in the pregnancy microenvironment. 1) HLA-G on extravillous trophoblasts (EVTs) binds to KIR2DL4 on NK cells or ILT2 on macrophages, and in this way stimulates the production of IL-6, IL-8, and VEGFα. Soluble HLA-G binds to KIR2DL4 on NK cells and induces the production of IL-6 and IL-8. Thus HLA-G promotes vascular permeability, angiogenesis and EVT invasiveness, and thereby participates in the remodeling of spiral arteries. 2) HLA-G on EVTs binds to ILT2 on NK cells, ILT2 and ILT4 on macrophages, and ILT2 on Treg cells, and reduces levels of cytotoxicity towards fetal tissues. 3) HLA-G on EVTs binds to ILT2 and KIR2DL4 on NK cells and promotes the secretion of growth-promoting factors PTN and OGN, thereby facilitating fetal growth.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. XX drafted the manuscript and figures. HW and YZ edited/reviewed the article.

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Intratumor Heterogeneity of HLA-G Expression in Cancer Lesions

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Signaling pathway between human leukocyte antigen (HLA)-G and immune inhibitory receptors immunoglobulin-like transcript (ILT)-2/4 has been acknowledged as one of immune checkpoints, and as a potential target for cancer immunotherapy. Like other immune checkpoints, inter- and even intratumor heterogeneity of HLA-G could render a rather complexity for HLA-G-target immunotherapy. However, little information for intratumor heterogeneity of HLA-G is available. In this study, HLA-G expression in a serial section of colorectal cancer (CRC) lesions from three CRC patients (each sample with serial section of 50 slides, 10 randomized slides for each antibody), three different locations within a same sample (five CRC), and three case-matched blocks that each includes 36 esophageal cancer samples, were evaluated with immunohistochemistry using anti-HLA-G antibodies (mAbs 4H84, MEM-G/1 and MEM-G/2 probing for all denatured HLA-G isoforms, 5A6G7, and 2A12 probing for denatured HLA-G5 and HLA-G6 isoforms). Our results revealed that, in addition to the frequently observed inter-tumor heterogeneity, intratumor heterogeneous expression of HLA-G is common in different areas within a tumor in CRC and esophageal cancer samples included in this study. Moreover, percentage of HLA-G expression probed with different anti-HLA-G antibodies also varies dramatically within a tumor. Given HLA-G has been considered as an important immune checkpoint, intratumor heterogeneity of HLA-G expression, and different specificity of anti-HLA-G antibodies being used among studies, interpretation and clinical significance of HLA-G expression in cancers should be with caution.

Keywords: HLA-G, tumor, heterogeneity, isoform, antibody, colorectal cancer, esophageal cancer

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INTRODUCTION

Immune suppressive functions induced by the interaction between human leukocyte antigen-G (HLA-G) and its immune inhibitory receptors, the immunoglobulin-like transcripts (ILTs), have been widely acknowledged (1). Receptors ILT-2 and ILT-4 express on various immune cells, the immune tolerogenic effects induced by HLA-G are comprehensive (2). Due to alternative splicing of its primary transcripts, seven confirmed HLA-G isoforms (HLA-G1~HLA-G7), and recently predicted novel isoforms such as lacking a transmembrane region and α1 domain have been reported (3).

In the context of cancers, different degree of inter-tumor HLA-G expression has been observed in most histological types of cancers studied, and the significance of HLA-G/ILTs signaling pathway as an immune checkpoint in cancer biology has been highlighted (4). Look back to its expression firstly observed in cancer, the melanoma lesions in 1998 (5), immune tolerance induced by HLA-G has been solidified by large numbers of studies both *in vitro* and *in vivo* preclinical experimental animal models (6–8).

HLA-G/ILTs binding can inhibit the proliferation of natural killer cells (NK), T and B lymphocytes and maturation and antigen presentation of dendritic cells (DC), suppress NK and T cell's cytotoxic function, B cell's immunoglobulin production and neutrophils' reactive oxygen species production and phagocytosis capability (9–11). To the contrary, HLA-G/ILTs binding can promote myeloid-derived suppressor cells (MDSC) proliferation and polarize M1 macrophages towards to M2 type (12, 13). Moreover, immune tolerance can be induced by HLA-G-bearing exosomes between cells at long-distance, and by cellular membrane fragments containing HLA-G through trogocytosis in a close cell-to-cell contact manner (14, 15). In preclinical murine models, HLA-G could promote tumor immune escape and growth through murine MDSC proliferation and Th2 cytokine production, or reduce T and B cell tumor infiltrate, impair B cell immune responses in immunocompetent mice (8, 16). Findings also revealed that HLA-G expression in ovarian cancer cells could enhance the tumor cell migration and metastasis in tumor-bearing immunodeficient nude mice through induction of matrix metalloproteinase-15 (MMP-15) expression (7, 17). Moreover, a recent study showed that depletion of $CD4^{low}HLA\text{-}G^{+}$ T cells could favor the castration-resistance prostate cancer therapy (18). Echoing the above mentioned *in vitro* and *in vivo* preclinical experimental observations, lesion HLA-G expression was observed to be closely associated with tumor metastasis, poor tumor cell differentiation, advanced disease stage and worse survival in a variety of cancers in clinical settings (14).

Inter- and intratumor heterogeneity of immune checkpoints is the main obstacle for immune checkpoint inhibitor (ICI) immunotherapy. Consequently, the benefits of the ICI therapy varies dramatically among patients (19). As a new immune checkpoint, the inter-tumor heterogeneous pattern of HLA-G expression is well evidenced; however, information for the intratumor heterogeneity of HLA-G is very limited. Previous studies revealed that the degree of HLA-G or its receptors ILT2/4 expression varies markedly among different locations in a primary renal cell cancer tumor lesion, indicating the complexity of intratumor heterogeneity of HLA-G and its receptor expression (3, 20).

In this study, inter- and intratumor heterogeneity of HLA-G expression was evaluated with immunohistochemistry using a panel of anti-HLA-G antibodies (mAbs 4H84, MEM-G/1 and MEM-G/2 probing for all denatured HLA-G isoforms, 5A6G7 and 2A12 probing for denatured HLA-G5 and HLA-G6 isoforms) in a serial section of colorectal cancer lesions from three CRC patients, three different locations within a same sample from five CRC patients, and three case-match blocks that each includes 36 esophageal cancer samples, and our findings solidify the heterogeneity of HLA-G in cancers.

MATERIALS AND METHODS

Tumor Lesion Specimen

Tumor lesion specimen and clinical records were retrospectively reviewed. In this study, three CRC lesions #598937 (Female, 65 years, AJCC stage IIIA), #624267 (Female, 72 years, AJCC stage I A) and #681878 (Female, 80 years, AJCC stage I A; **Table 1**), and each sample was serially sectioned for 50 slides. Slides from three

TABLE 1 | Percentage of HLA-G expression in serial section of colorectal cancer lesions.

Samples	Antibodies	Percentage of HLA-G positive tumor cells (%)											*p*
		#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	Mean	
CRC #598937													
Female, 65 years, AJCC stage IIIA													
Group 1	mAb 4H84	88.8	86.9	81.9	88.1	85.0	86.3	91.9	91.3	85.6	91.3	87.71	<0.001
(All isoforms)	mAb MEM-G/1	65.0	55.0	65.0	61.3	62.9	62.5	67.5	72.5	76.3	62.5	65.05	
	mAb MEM-G/2	57.5	65.0	68.8	57.5	77.5	77.5	70.0	67.5	75.0	77.5	69.38	
Group 2	mAb 5A6G7	55.0	45.0	60.0	45.0	41.3	53.8	58.3	50.0	60.0	76.3	54.47	0.108
(HLA-G5/6)	mAb 2A12	47.5	48.8	50.0	41.3	53.8	46.3	43.8	50.0	57.5	35.0	47.40	
CRC #624267													
Female, 72 years, AJCC stage IA													
Group 1	mAb 4H84	94.5	94.1	95.0	94.1	94.1	92.7	94.1	94.1	94.5	92.3	93.95	0.453
(All isoforms)	mAb MEM-G/1	94.5	89.1	94.1	93.6	94.5	94.1	94.5	93.6	91.8	94.1	93.39	
	mAb MEM-G/2	93.6	92.7	93.2	94.1	95.0	93.6	93.6	92.7	92.7	94.5	93.57	
Group 2	mAb 5A6G7	92.7	90.2	90.9	86.8	78.2	92.7	90.0	88.6	93.2	88.6	89.19	0.190
(HLA-G5/6)	mAb 2A12	91.1	93.2	80.0	89.8	89.9	85.9	89.5	82.3	77.3	84.1	86.31	
CRC #681878													
Female, 80 years, AJCC stage IA													
Group 1	mAb 4H84	81.9	80.0	80.6	82.5	84.4	80.6	80.6	84.4	85.0	91.3	83.13	<0.001
(All isoforms)	mAb MEM-G/1	43.8	45.0	27.5	46.3	50.1	32.5	43.8	60.0	26.3	33.8	40.91	
	mAb MEM-G/2	0.00	36.3	21.3	18.8	14.4	22.5	6.30	5.00	10.6	26.3	16.15	
Group 2	mAb 5A6G7	68.3	85.6	76.3	68.8	61.9	61.9	60.6	52.5	39.4	63.8	63.91	0.105
(HLA-G5/6)	mAb 2A12	69.4	62.5	61.3	68.1	70.6	71.9	72.5	83.8	76.9	72.5	70.95	

different locations within a same sample from another five CRC samples were obtained #1022488 (Male, 49 years, AJCC stage III B), #1022363 (Male, 70 years, AJCC stage I A), #1020932 (Male, 75 years, AJCC stage IV A), #1023081 (Male, 75 years, AJCC stage II A) and #444345 (Male, 86 years, AJCC stage II A; **Table 2**). Furthermore, slides from three case-matched blocks that each includes 36 esophageal squamous cell carcinoma (ESCC) samples were included in the study. Among 36 ESCC patients (27 male and nine female; median age: 58 years; range from 47 to 79 years), there were one patient with stage I B, six patients with II A, 14 patients with II B, seven patients with III A, seven patients with III B, and one patient with III C. The detailed clinical information was shown in **Table 3**. The clinicopathological findings were determined according to 7th American Joint Committee on Cancer (AJCC) Tumor-Node-Metastasis (TNM) staging system (21). None of them received radiotherapy, chemotherapy, or other medical interventions before the study. All these patients were diagnosed and treated at Taizhou Hospital of Zhejiang Province, China, and samples were retrieved by Biological Resource Center, Taizhou Hospital of Zhejiang Province (National Human Genetic Resources Platform of China YCZYPT [2017]02). Written informed consent was obtained from each participant before the surgical operation, and this study was approved by Medical Ethics Review Board of Taizhou Hospital of Zhejiang Province.

HLA-G Antibodies and Immunohistochemistry

Five anti-HLA-G murine antibodies were used in this study. mAbs 4H84 (dilution 1:200), MEM-G/1 (dilution 1:100) and MEM-G/2 (dilution 1:100), IgG1 antibodies detect denatured heavy chain of all HLA-G isoforms (Exbio, Prague, Czech Republic); mAbs 5A6G7 and 2A12, IgG1 antibodies probe denaturized heavy chain of HLA-G5/HLA-G6 isoforms (dilution 1:100; Exbio, Prague, Czech Republic). Immunohistochemistry assay was performed on 4-μm-thick, formalin-fixed and paraffin-embedded tumor lesion sections. Details of the protocols was according to our previous study (22). Immunohistochemistry staining was visualized with a Dako EnVison kit (Dako, Glostrup, Denmark). The percentage of HLA-G positive tumor cells was determined by presence of HLA-G staining while irrespective of staining intensity. HLA-G staining was evaluated by two reviewers who were blind to the patient clinicopathological information. Membrane or/and cytoplasmic expression of HLA-G were interpreted as positive. Percentage of HLA-G-positive tumor cells was determined by each observer, and the average of scores was calculated.

Statistical Analysis

Statistical analysis was performed with the SPSS 13.0 statistical software package (SPSS, Inc., Chicago, IL, USA). Comparison between groups was analyzed with non-parametric Mann-Whitney U or Kruskal-Wallis H test. $p<0.05$ (two-tailed) was considered statistically significant.

RESULTS

To evaluate the heterogeneity of HLA-G expression in cancers, three different types of tumor tissue samples were prepared. a) For three CRC tissue samples (#598937, #624267 and #681878), 50 slides was serially sectioned for each sample. Among 50 slides, 10 randomized slides for each antibody probing. b) Slides from three different zones within a same sample from another five CRC samples (#1022488, #1022363, #1020932, #1023081, and #444345), and c) slides from three case-matched blocks that each includes 36 esophageal cancer samples. These slides were probed with five different anti-HLA-G antibodies. Anti-HLA-G antibodies were divided into two

TABLE 2 | Percentage of HLA-G expression in different zones of colorectal cancer lesions.

Sample	Sex	Age	AJCCStage	Percentage of HLA-G positive tumor cells (%)				
				4H84	MEM-G/1	MEM-G/2	5A6G7	2A12
CRC #1022488	Male	49	III B					
Zone 1				0.57	2.43	0.27	9.16	2.84
Zone 2				0.71	1.67	0.21	20.29	9.69
Zone 3				2.00	2.22	0.00	7.18	4.42
CRC #1022363	Male	70	I A					
Zone 1				14.88	9.25	2.60	22.05	11.90
Zone 2				50.00	0.00	0.00	4.12	24.80
Zone 3				22.00	0.44	0.67	9.42	12.89
CRC #1020932	Male	75	IV A					
Zone 1				45.00	8.93	8.74	32.61	24.53
Zone 2				15.58	26.38	7.13	16.13	19.50
Zone 3				13.19	7.00	0.50	24.19	18.13
CRC #1023081	Male	75	II A					
Zone 1				59.49	34.14	56.57	4.33	16.43
Zone 2				15.19	55.20	59.23	32.3	19.70
Zone 3				36.32	24.48	14.10	2.74	7.58
CRC #0444345	Male	86	II A					
Zone 1				45.30	25.40	34.80	16.23	0.00
Zone 2				13.00	58.82	32.35	41.14	30.59
Zone 3				32.74	33.23	37.42	31.42	27.10

TABLE 3 | Percentage of HLA-G expression in different blocks of esophageal squamous cell carcinoma.

No.	Sex	Age	AJCC stage	Percentage of HLA-G positive tumor cells (%)					
				Blocks	4H84	MEM-G/1	MEM-G/2	5A6G7	2A12
1	Male	61	III B	1#	40	10	0	0	0
				2#	58	10	0	0	0
				3#	0	0	5	0	0
2	Male	62	II B	1#	30	30	10	0	0
				2#	65	60	40	0	0
				3#	80	75	45	0	0
3	Female	54	III A	1#	65	80	45	1	0
				2#	98	30	0	0	40
				3#	80	80	20	0	0
4	Female	47	II B	1#	60	10	40	0	0
				2#	80	30	0	0	0
				3#	98	90	90	80	85
5	Male	60	II A	1#	95	90	90	0	80
				2#	70	80	30	0	0
				3#	95	85	80	0	85
6	Male	53	II B	1#	80	90	70	3	20
				2#	80	60	3	0	0
				3#	55	70	5	0	3
7	Male	56	II B	1#	60	60	0	0	0
				2#	60	55	0	0	0
				3#	95	80	85	10	0
8	Female	72	II A	1#	80	85	65	0	0
				2#	95	85	15	20	45
				3#	90	90	80	5	1
9	Male	72	III A	1#	90	90	90	70	30
				2#	70	90	60	0	0
				3#	95	85	40	0	1
10	Male	65	I B	1#	0	0	0	0	0
				2#	0	0	0	0	0
				3#	40	10	0	0	0
11	Male	51	II B	1#	0	0	0	0	0
				2#	0	0	0	0	0
				3#	20	0	0	0	0
12	Male	56	II B	1#	0	0	0	0	0
				2#	30	20	0	0	0
				3#	75	20	0	0	0
13	Male	58	III A	1#	0	0	0	0	0
				2#	20	0	0	0	0
				3#	40	0	80	0	0
14	Male	59	II B	1#	70	55	55	0	0
				2#	40	10	0	0	0
				3#	98	5	70	0	0
15	Male	79	II A	1#	35	15	30	10	10
				2#	35	30	20	0	0
				3#	85	5	55	0	10
16	Male	57	II B	1#	70	5	80	60	10
				2#	30	65	40	0	0
				3#	98	60	30	0	0
17	Female	58	III A	1#	70	80	10	0	0
				2#	80	60	90	0	0
				3#	40	0	80	0	0
18	Male	59	III A	1#	95	90	80	0	60
				2#	95	95	90	2	40
				3#	95	85	70	65	45
19	Male	48	II A	1#	20	0	10	0	10
				2#	10	80	10	0	0
				3#	80	0	0	0	0
20	Male	59	III B	1#	80	80	0	0	0
				2#	60	0	0	0	0
				3#	40	2	0	0	0
21	Female	58	II B	1#	60	60	0	0	0
				2#	40	0	0	0	0
				3#	30	0	0	0	0

(Continued)

TABLE 3 | Continued

No.	Sex	Age	AJCC stage	Percentage of HLA-G positive tumor cells (%)					
				Blocks	4H84	MEM-G/1	MEM-G/2	5A6G7	2A12
22	Female	73	II B	1#	60	0	0	0	0
				2#	65	0	0	0	0
				3#	70	0	0	0	0
23	Male	50	III A	1#	3	0	0	0	0
				2#	0	0	0	0	0
				3#	15	0	0	0	0
24	Female	58	III B	1#	80	60	30	0	0
				2#	85	0	20	0	0
				3#	85	70	60	0	0
25	Male	50	III B	1#	60	10	0	0	0
				2#	70	0	0	0	0
				3#	75	30	30	0	0
26	Female	50	III C	1#	80	55	0	0	0
				2#	70	0	55	0	0
				3#	85	80	0	0	0
27	Male	70	II B	1#	60	40	0	0	0
				2#	10	0	0	0	0
				3#	20	10	0	0	0
28	Male	49	II A	1#	85	80	80	80	0
				2#	70	0	80	0	3
				3#	95	90	85	80	70
29	Male	55	II B	1#	90	40	30	0	0
				2#	10	0	0	0	0
				3#	85	20	40	0	0
30	Male	53	II A	1#	95	80	80	0	2
				2#	60	2	15	2	2
				3#	90	70	80	30	10
31	Male	59	III B	1#	65	2	10	0	0
				2#	80	0	55	0	0
				3#	85	80	70	0	0
32	Male	59	III B	1#	90	90	90	60	0
				2#	85	0	40	0	0
				3#	95	90	90	30	0
33	Male	51	III A	1#	95	80	70	0	2
				2#	90	0	80	3	0
				3#	95	30	20	0	2
34	Female	53	II B	1#	90	3	80	0	0
				2#	80	0	0	0	0
				3#	90	85	60	0	0
35	Male	69	II B	1#	0	0	0	0	0
				2#	0	0	0	0	0
				3#	80	20	0	0	0
36	Male	54	III B	1#	70	30	20	0	0
				2#	90	0	0	0	0
				3#	90	60	0	0	0

groups according to the specificity of these antibodies. Group 1: mAbs 4H84, MEM-G/1 and MEM-G/2, which detect denatured heavy chain of all HLA-G isoforms; Group 2: mAbs 5A6G7 and 2A12, which detect denaturized heavy chain of HLA-G5/HLA-G6 isoforms. The representative immunohistochemistry HLA-G staining patterns of CRC and ESCC were shown in **Figure 1**.

Intratumor Heterogeneity of HLA-G

Intratumor heterogeneous expression of HLA-G was observed among different sections and antibodies used in three CRC tissue samples (#598937, #624267, and #681878).

For the Group 1 antibodies (mAbs 4H84, MEM-G/1, and MEM-G/2), HLA-G expression was dramatically different in samples CRC#598937 ($p<0.001$) and CRC#681878 ($p<0.001$), while comparable degree of HLA-G expression was observed in sample CRC#624267 ($p=0.453$). Among these samples, no significant variation of HLA-G expression was found for the Group 2 antibodies (mAbs 5A6G7 and 2A12; **Table 1**).

Moreover, HLA-G expression in samples from different zones of a same tumor also varied significantly when detected with a distinct anti-HLA-G antibody. CRC#1022488 for an example, the percentage of HLA-G expression detected with mAbs 5A6G7 and 2A12 are much higher than that probed with mAbs 4H84, MEM-G/1 and MEM-G/2. Zone 2 particularly, percentage of HLA-G expression detected by mAb 5A6G7 is 20.29% while HLA-G is nearly negative detected by mAb 4H84 (0.71%). In CRC#1022363,

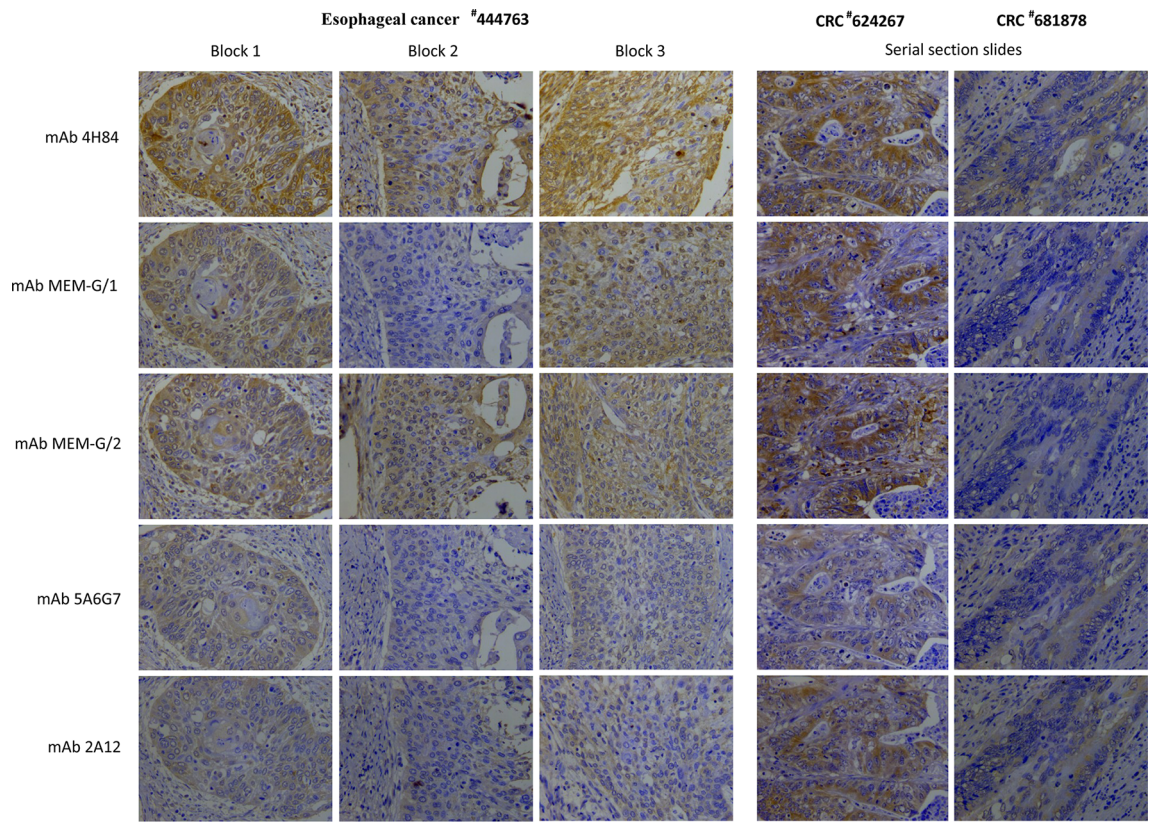

FIGURE 1 | Representatives of intratumor heterogeneous staining of HLA-G expression with different anti-HLA-G antibodies in three blocks of a esophageal squamous cell carcinoma sample (#444763), and serial sections of two colorectal cancer samples (#624267 and #681878) (400×).

the degree of HLA-G detected by mAb 4H84 was 14.88%, 50.0% and 22.0% in zone 1, zone 2, and zone 3, respectively. HLA-G expression in zone 2 and 3 was almost undetectable, while HLA-G was positive in zone 1 when detected by mAbs MEM-G/1 and MEM-G/2. Moreover, HLA-G expression was observed in all three zones when detected with mAb 5A6G7 and mAb 2A12, respectively (**Table 2**). Similarly, intratumor heterogeneity of HLA-G expression was also found in case-matched esophageal cancer blocks (**Table 3**).

Intratumor Heterogeneity of HLA-G Isoforms

Distinct pattern and variation of HLA-G expression was also observed for each antibody for HLA-G detection among 10 randomized slides from a same tumor sample. No significant variation of HLA-G expression was observed when detected by mAb 2A12 in CRC#598937 (p=0.1151), mAb 4H84 in CRC#681878 (p=0.154), and mAbs MEM-G/1 (p=0.203) and MEM-G/2 (p=0.386) in CRC#624267. HLA-G expression was found varied dramatically among 10 slides when probed with a distinct anti-HLA-G antibody (**Figure 2A**). To be noted, previously considered as unexpected immunohistochemistry staining patters such as mAb $4H84^{neg}$ mAb $5A6G7^{pos}$ was observed in this study (**Table 2**). In CRC#1022488, HLA-G expression is low/negative stained with mAbs 4H84, MEM-G/1, and MEM-G/2, while HLA-G is positive when stained with mAbs 5A6G7 and 2A12. This staining pattern now could be explained by the findings that novel HLA-G isoforms such as lacking the α1 domain was depicted by Tronik-Le Roux et al. (3) in a renal cancer study. Similar data were also observed in slides from three different zones within a same sample from another five CRC samples (#1022488, #1022363, #1020932, #1023081, and #444345; **Figure 2B**).

Among 36 ESCC samples, HLA-G expression could be detected by mAbs 4H84, MEM-G/1 and MEM-G/2, while HLA-G expression is negative detected by mAbs 5A6G7 and 2A12 in most cases. Moreover, the staining pattern for mAbs 4H84 and 5A6G7 seems more consistent according to their recognizing epitope in the HLA-G heavy chain, that no mAbs $4H84^{neg}5A6G7^{pos}$ was observed (**Table 3**).

DISCUSSION

Inter-tumor HLA-G expression in various types of tumor tissues has been widely investigated and its clinical significance has been well acknowledged. A large body of studies have evidenced that

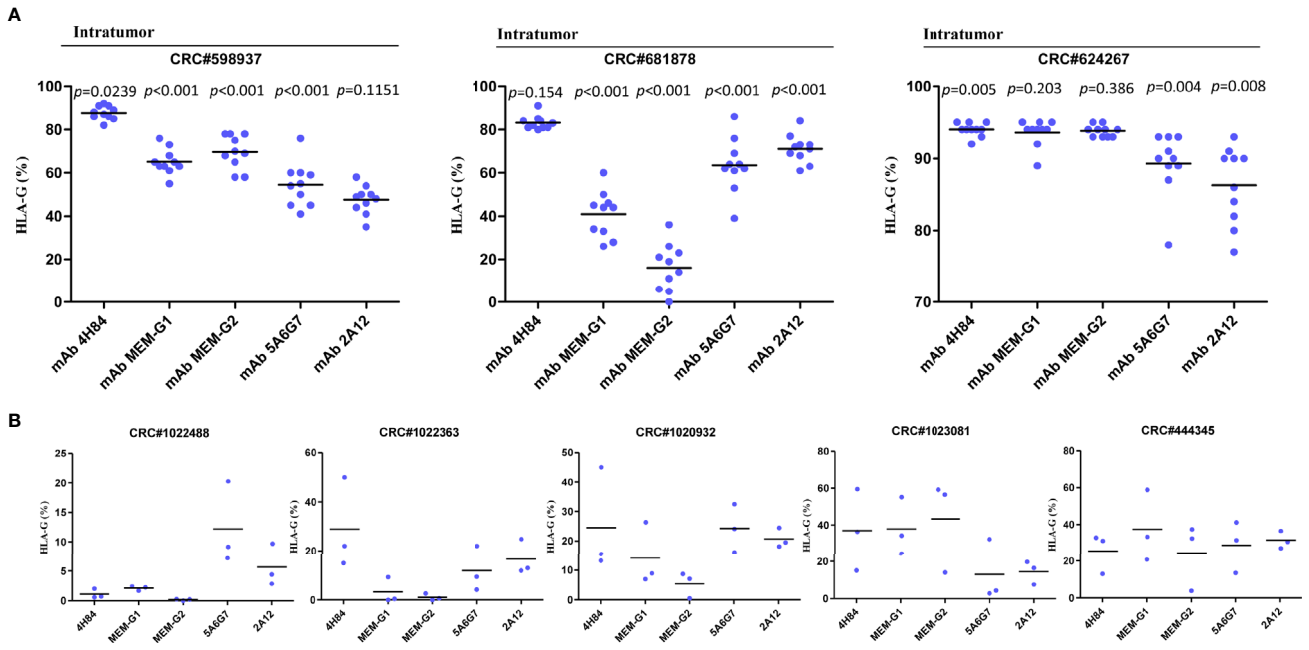

FIGURE 2 | Intratumor heterogeneous staining of HLA-G expression with different anti-HLA-G antibodies in **(A)** serial sections of three colorectal cancer samples (each sample with 50 serial sections, 10 randomized slides for each antibody). Dot represents each section. Black line represents median. Comparison among the sections was analyzed with analyzed with Kruskal-Wallis H test. **(B)** three different zones within a same sample (five CRC samples). Dot represents each zone. Black line represents median.

higher degree of HLA-G expression in cancers is related to disease progression and worse clinical outcome (14). Based on the signaling pathway of HLA-G/ILTs and its clinical relevance, HLA-G as a potential immune checkpoints is expected (1). Though ICIs such as targeting the PD-L/PD-L1 is certainly an effective and promising strategic regime for cancer immunotherapy, limited effects of the ICIs therapy resulted from inter- and intratumor heterogeneous expression of immune checkpoints is gaining concern (19).

Indeed, the degree and percentage of HLA-G in cancers varies significantly among different types of cancers which have been observed to be negative in uveal melanoma to totally positive in hydatidiform moles (23, 24), and inconsistent HLA-G findings among different cohorts or laboratories existed in most cases even on a same type of cancer such as breast cancer (25–27) and CRC (22, 28–30). These controversies might be raised by the different specificities of HLA-G monoclonal antibodies, varied laboratory technical procedures, or different composition and HLA-G genetic backgrounds of the included cohorts (14, 31). In line with this, our data showed that different staining pattern of HLA-G expression has been observed between the CRC and ESCC, where HLA-G is almost negative in ESCC but positive in CRC samples when detected by mAbs 5A6G7 and 2A12. This finding indicated that HLA-G isoforms could be differentially regulated among different types of cancers. Moreover, mechanisms involved in regulation of HLA-G expression are complex. In addition to the HLA-G genetic variations both in 5′-upstream regulatory region and in 3′-untranslated region which comprise binding sites for transcription factor and microRNAs and epigenetic modifications (32), other environmental factor such as hypoxia, cytokines, hormones, and even immunotherapy chemicals and radiation have been acknowledged to be related to the regulation of HLA-G expression (33–35).

Intratumor heterogeneity of HLA-G expression has been firstly detailed in 19 primary renal cell cancer (RCC) tumor tissues. HLA-G expression was sharply differed either between samples or inside a tumor tissue (20). In that study, with mAb 4H84, authors revealed that various degree of HLA-G expression exists among different areas (zones) as they illustrated in sample RCC#2 (70% in area T1, 37% in T2, 58% in T3 and T4, respectively), while no HLA-G expression was observed in the T1 or T2 areas in sample RCC#10. In line with their findings, as our data in this study revealed that intratumor heterogeneous expression of HLA-G is a common phenomenon among different zones within a sample in CRC and ESCCs. According to these results, similar findings that intratumor HLA-G heterogeneity could be expected in other malignancies. Shortly afterwards, with transcriptome analysis in RCC samples, they further depicted that, besides the already identified HLA-G1~HLA-G7 isoforms, novel HLA-G isoforms without an α1 domain and transmembrane region could be existed (3). This important finding do explain previously unexpected immunohistochemistry staining patters such as mAb 4H84^{neg} mAb 5A6G7^{pos}, which was observed in our study such as the CRC#1022488 and other samples. In this context, in an our previous study, we found 44 out of 379

(11.6%) CRC patients were with the staining pattern of mAbs $4H84^{neg}$ $5A6G7^{pos}$, and CRC patients with the patterns of mAbs $4H84^{neg}$ $5A6G7^{pos}$ had a longer survival time than those with the pattern of mAbs $4H84^{pos}5A6G7^{neg}$ (36). However, future investigations for the biological functions and clinical significance of novel HLA-G isoforms with mAbs $4H84^{neg}$ $5A6G7^{pos}$ are extremely necessary.

However, our study have notable limitations. First, this study is based on a very limited size of patients and types of cancers, the real-world of the heterogeneity of HLA-G expression in more different types of cancers and in larger cohorts of cancer patients remain to be explored. Second, being the very limited size of the patients included, clinical significance of the heterogeneity of HLA-G and HLA-G isoform expression in cancers is still unknown. Third, potential mechanisms underlying the heterogeneity of HLA-G in cancers remain to be uncovered. Finally, more specific antibodies for HLA-G isoforms are needed to define the clinical significance of a particular HLA-G isoforms.

In summary, our study revealed a rather high degree of intratumor heterogeneity of HLA-G expression in cancers, and degree of HLA-G expression is also varied among anti-HLA-G antibodies with different specificities. Therefore, to evaluate the clinical significance of HLA-G expression in cancers, important issues including location of the tumor tissues isolated, HLA-G isoforms and specificity of the anti-HLA-G antibodies should be concerned.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Review Board of Taizhou Hospital of Zhejiang Province. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

W-HY: study design. XZ, JG, and AL: performed experiments. J-GZ, Q-YH, Q-YC, Y-HY, W-JZ, and H-HX: material support and data acquisition. W-HY: performed statistical analysis and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Inhibition of iNKT Cells by the HLA-G-ILT2 Checkpoint and Poor Stimulation by HLA-G-Expressing Tolerogenic DC

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Invariant Natural Killer T (iNKT) cells are a small and distinct population of T cells crucial in immunomodulation. After activation by alpha-GalactosylCeramide (αGC), an exogenic glycolipid antigen, iNKT cells can rapidly release cytokines to enhance specific anti-tumor activity. Several human clinical trials on iNKT cell-based anti-cancer are ongoing, however results are not as striking as in murine models. Given that iNKT-based immunotherapies are dependent mainly on antigen-presenting cells (APC), a human tolerogenic molecule with no murine homolog, such as Human Leucocyte Antigen G (HLA-G), could contribute to this discrepancy. HLA-G is a well-known immune checkpoint molecule involved in fetal-maternal tolerance and in tumor immune escape. HLA-G exerts its immunomodulatory functions through the interaction with immune inhibitory receptors such as ILT2, differentially expressed on immune cell subsets. We hypothesized that HLA-G might inhibit iNKT function directly or by inducing tolerogenic APC leading to iNKT cell anergy, which could impact the results of current clinical trials. Using an ILT2-transduced murine iNKT cell line and human iNKT cells, we demonstrate that iNKT cells are sensitive to HLA-G, which inhibits their cytokine secretion. Furthermore, human HLA-G$^+$ dendritic cells, called DC-10, failed at inducing iNKT cell activation compared to their autologous HLA-G$^-$ DCs counterparts. Our data show for the first time that the HLA-G/ILT2 ICP is involved in iNKT cell function modulation.

Keywords: Human Leucocyte Antigen G, Natural Killer T cells, immune regulation, tolerogenic dendritic cells, ILT2/CD85j/LILRB1

INTRODUCTION

Natural Killer T (NKT) cells are a subset of T cells expressing distinct αβ T cell receptor (αβTCR). Initially, co-expression of T cell and Natural Killer (NK) cell markers (CD56 or CD161) were used to identify this population, therefore named NKT cells. NKT cells are now better characterized as CD1d-dependent T cells with potent cytokine production capacity (1–3). CD1d is a MHC-class-I-

like molecule that mediates the presentation of lipid or glycolipid antigens to T cells. Invariant NKT (iNKT) cells, are a small subtype of the NKT population. They recognize lipids presented by of CD1d, in particular the marine-sponge-derived alpha-galactosylceramide (αGC), and express a canonical invariant TCR α chain (Vα24Jα18 in humans and Vα14Jα18 in mice) and TCR β chains that use limited Vβ segments (Vβ11 in humans and Vβ8.2 in mice) (4).

iNKT cells play an important role in anti-tumor immunity by linking the innate and adaptive immune responses. Stimulation of iNKT cells by the CD1d-αGC complex leads to a rapid production of Th1 and Th2 cytokines (e.g. IFN-γ and IL-4) and recruitment of dendritic cells (DC), NK cells, B cells, helper T cells, and cytotoxic T cells. In mice, this capability of αGC-stimulated iNKT cells to boost cellular immune responses was strong enough to generate specific responses against tumor cells, such as the B16F10 melanoma cell line, leading to long-term tumor rejection (5–7). Besides this adjuvant effect, iNKT cells can also directly control tumor growth by cytotoxicity (8). Clinical data show that iNKT cell numbers correlate with better survival of cancer patients (9) and on the contrary, that abnormal numbers and functions of iNKT cells are associated with poor clinical outcome (10, 11). Thus, there is an increasing interest in iNKT cell-based immunotherapy strategies to treat cancer.

iNKT-based anti-tumor strategies rely, so far, on harnessing iNKT cells to optimize anti-tumor vaccination through (i) intravenous injection of αGC (12) (ii) adoptive transfer of αGC pulsed APC (13) and (iii) adoptive transfer of *ex vivo* activated iNKT cells (14, 15). Clinical trials were mainly based on infusions of either αGC-loaded APC preparations or αGC-expanded enriched iNKT, which gave promising results in mouse models (16, 17). However, unlike in murine studies, results obtained with human iNKT cells are not yet convincing (18). Given that iNKT-based immunotherapies are dependent on APC, human-specific immune checkpoint-expressing or tolerogenic APCs could dampen their activation. It was shown that intravenous injection of αGC leads to iNKT cell anergy in a PD-1/PDL-1 dependent manner. Indeed, iNKT cells functions were decreased by PD-L1/PD-L2 expressed by APCs (19).

Thus, it is possible that the striking differences observed after iNKT-based anti-tumor immunizations in mice and humans could be due to differential expression of regulatory molecules in humans and mice, including species-specific murine-only and/or human-only molecules. In this work, we investigated the possible impact of the HLA-G/Immunoglobulin-like Transcript 2 (ILT2) interaction on the function of iNKT cells.

HLA-G is a molecule involved in fetal-maternal tolerance and in tumor immune escape. This non-classical HLA class I molecule has low polymorphism, unlike classical HLA class I molecules, and presents four membrane-bound (HLA-G1 to G4) and three soluble isoforms (HLA-G5 to G7). The most common and best-characterized isoforms, HLA-G1 and HLA-G5, are non-covalently associated with β-2-microglobulin (B2M) (20, 21). HLA-G physiological expression is tissue-restricted, mainly to trophoblast, thymus, cornea, and mesenchymal stem cells in physiological conditions. However, HLA-G can be induced under pathological conditions such as viral diseases, inflammatory disorders, transplantation and cancer (22).

HLA-G immuno-modulatory functions on all immune cell subsets are exerted through specific binding to inhibitory receptors. ILT2/CD85j/LILRB1 is one of the known HLA-G receptors, which is expressed on various proportions of monocytes, DC, B, NK, and T cells (23). ILT2 has four tandem Ig-like extracellular domains and four immunoreceptor tyrosine-based inhibitory receptor motifs (ITIM) in its cytoplasmic tail. In the case of T and NK cells, HLA-G:ILT2 interaction was reported to inhibit alloproliferation (24–28), alter cytokine secretion (25, 29–32), and inhibit the antigen-specific cytolytic functions of cytotoxic T lymphocytes (CTLs) (33, 34), uterine NK cells and peripheral blood NK cells (35, 36).

HLA-G-expressing tumor cells or high levels of HLA-G in plasma have been reported in numerous types of cancers and associated with higher grade and worse prognosis (22, 37–41). Indeed, HLA-G plays the role of an immune escape mechanism through inhibition of anti-tumor effectors, alteration of cytokine expression patterns (14, 37, 38), and generation of regulatory cells (39, 40). Furthermore, tumors can induce HLA-G expression by other cells such as tolerogenic APCs (e.g. DC-10 cells), leading to T cell anergy and induction of regulatory T cells (42, 43). Interestingly, ILT2 expression has also been associated with tumor immune escape (44). Thus, HLA-G:ILT2 is a potent immune checkpoint and constitutes a potential new target in anti-tumor therapies.

iNKT cells are related to both NK and T cells since they are T cells expressing markers mostly associated with NK cells, in particular inhibitory receptors (45). Since human NK cells and classical T cells were shown to be inhibited by HLA-G through ILT2 receptor expression, we reasoned that iNKT cells could be sensitive to HLA-G that would be expressed by the tumor cells themselves or by antigen-presenting cells such as the recently discovered HLA-G-positive DC-10 tolerogenic DC subset. Our results show that this is indeed the case. As HLA-G is known to be present in the tumor microenvironment, it could inhibit iNKT cell reactivity to αGC and impair the effectiveness of the iNKT cell-based immune therapy.

MATERIALS AND METHODS

Human PBMC Isolation

Blood in ETDA tubes or from plateletpheresis residues was collected at the French Blood Center (EFS, Saint Louis Hospital, France) from healthy donors with informed consent. Human PBMC from healthy donors were used for ILT2 analysis, and for $CD14^+$ monocyte and iNKT cells isolation. PBMC were isolated by density gradient separation using either Ficoll (Sigma-Aldrich) or Leucosep™ tube (Grenier Bio-One), washed twice in 0.9% NaCl (Versylène® Fresenius), and counted using trypan blue dye in KOVA counting slides (Fisher Scientific). Viability was always >90%. Processing was completed within less than 10 h for all sample specimens.

Flow Cytometry Analysis

Surface markers and intracellular cytokines were analyzed by flow cytometry. Labeling steps were performed by using between 0.2–3×10^6 cells per test according to the experimental requirements in either FACS tubes or 96-well U-bottom plates. Washing steps were performed with PBS followed by centrifugation at 800 g for 1 min. Surface labeling was carried out by blocking Fc receptors, either with anti-mouse CD16/32 (eBioscience) for murine cells or human immunoglobulin G (Sigma) for human samples, for 5 min at room temperature, followed by antibody incubation for 30 min at 4°C in the dark using concentrations according to the manufacturer's instructions. Cells were fixed in 200 µl PBS containing 1% formaldehyde and acquired within 24 h after two washing steps. To detect intracellular cytokines, surface-labeled cells were fixed with 4% PFA for 10 min at 4°C, and then permeabilized for the intracellular staining with Perm/Wash solution (BD Bioscience) following manufacturer's instruction prior to incubation with cytokine specific antibodies.

Antibodies used in flow cytometry analysis were: i) Anti-murine: CD1d-PE (clone 1B1, BD Phamingen), CD3-APC (clone REA606, Miltenyi Biotech), IL-2-FITC (clone JES6-5H4, eBioscience), and PIR-A/B-PE (clone 10-1-PIR, BD Phamingen); ii) Anti-human: CD1d-PE (clone 51.1, eBioscience), CD1d tetramer-APC (pre-loaded with and without αGC, ProImmune), CD3-eFluor450 (clone OKT3, eBioscience), CD11c-APC (clone 3.9, eBioscience), CD14-PerCP-Cy5.5 (clone 61D3, eBioscience), CD56-PE-Cy7 (clone CMSSB, eBioscience), CD86-PE-Cy7 (clone IT2.2, eBioscience), HLA-DR-FITC (clone MEM-12, Exbio), HLA-G (clone MEM-G/09, Exbio), IFNg-PerCP-Cy5.5 (clone 4S.B3, eBioscience), IL-4-APC (clone 8D4-8, eBioscience), ILT2-PE (clone HP-F1, eBioscience), CD161-APC (clone HP-3G10, eBioscience), and Va24Ja18 TCR-FITC (clone 6B11, eBioscience). The matched isotype controls were systematically used.

Acquisition was performed on either a BD FACSCanto™ II equipped with BD FACSDiva™ software (version 6.0, BD Bioscience) or a MACSQuant® Analyser 10 equipped with MACSQuantify™ Analysis Software (version 2.8, Miltenyi Biotech). The PMT voltages were adjusted for each fluorescence channel using unstained cells and compensations were set using a mixture of unstained and single color stained cells with antibodies. Analyses were performed by FlowJo software (version 10, FlowJo LLC).

ILT2 Expression on Lymphocytes

Three million freshly isolated PBMC from 14 healthy donors were used to perform ILT2 expression analysis by flow cytometry. The CD3-eFluor450, CD56-PE-Cy7, Vα24Jα18 TCR-FITC, CD1d tetramer-APC, and ILT2-PE and their matched isotype controls were used as a five-color staining. The ILT2 expression was analyzed on $CD4^+$ T cells ($CD3^+CD56^-CD4^+$ lymphocytes), $CD8^+$ T cells ($CD3^+CD56^-CD8^+$ lymphocytes), NK cells ($CD3^-CD56^+$ lymphocytes), $CD3^+CD56^+$ NKT cells ($CD3^+CD56^+$ lymphocytes) and iNKT cells ($CD3^+6B11^+$ lymphocytes). Cytometry analysis was set according to the isotype controls and all results were expressed as % of $ILT2^+$ among studying subsets.

Murine iNKT and APCs

The murine NKT1.2 [Vα14i NKT cell hybridoma 1.2 in (46)], C57BL/6) and A20CD1d (CD1d-transduced A20 B cells line, ATCC, Balb/c) lines were used as iNKT and APCs, respectively. They were kindly provided by Pr. Mitchell Kronenberg (La Jolla Institute, CA, USA) and cultured in X-VIVO-10 medium (Lonza) at 37°C with 5% CO_2.

HLA-G-expressing and ILT2–expressing stable cell lines were generated by transduction and the lentiviral particles were generated as follows: specific sequences corresponding to native ILT2 cDNA (NM_006669.6), HLA-G1 cDNA (NM_002127.5) modified K334A and K335A according to Zhao et al. (47), and human beta-2-microglobulin (hB2M) cDNA (NM_004048.2) were cloned separately into a pTrip plasmid vector by digestion/ligation after extraction by PCR with specific primers, under CMV immediate early promoter. HIV-1-derived vector particles were produced by calcium phosphate co-transfection of HEK-293T cells (ATCC) with the recombinant plasmid pTRIP, an envelope expression plasmid encoding the glycoprotein from VSV, serotype Indiana glycoprotein, and the p8.74 encapsidation plasmid. Viral stocks were titrated by real-time PCR on cell lysates from transduced HEK-293T cells and expressed as transduction unit (TU) per ml.

To generate A20CD1d-HLA-G/hB2M and NKT1.2-ILT2 cell lines, 1×10^5 A20CD1d or NKT1.2 cells were seeded in 12-well plate with in 500 µl of X-VIVO-10 medium and 10^6 TU (293T) of Trip CMV-HLAG plus Trip CMV-hB2M or Trip CMV-ILT2 vectors. Cells were incubated for 1 h at 37°C and then centrifuged 1 h at 37°C 1,200 g. Afterwards, 1 ml of X-VIVO-10 medium was added and incubated at 37°C. Two weeks later, positive cells were sorted by flow cytometry using anti-HLA-G or anti-ILT2 antibodies. The expression of, HLA-G, ILT2, murine CD1d, and PIR-B were evaluated by flow cytometry before the iNKT activation assay.

Human mDC and DC-10 Differentiation

Human $CD14^+$ monocytes were isolated from fresh PBMC by positive selection using CD14 MicroBeads (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Cells were cultured in RPMI 1640 (Lonza, Italy) supplemented with 10% Fetal Calf Serum (Lonza, Italy), 2 mM L-glutamine (Lonza, Italy), and 100 U/ml penicillin/streptomycin (Lonza, Italy) at 37°C. To induce mature DC (mDC), $CD14^+$ monocytes were kept in culture with 10 ng/ml rhIL-4 (R&D Systems, Minneapolis MN, USA) and 100 ng/ml rhGM-CSF (Genzyme, Seattle WA, USA) for 5 days and maturation was induced by the addition of 1 µg/ml of LPS (Sigma, CA, USA) for additional 2 days. To differentiate DC-10, $CD14^+$ monocytes were kept in culture with 10 ng/ml rhIL-4, 100 ng/ml rhGM-CSF, and10 ng/ml of rhIL-10 (BD, Pharmigen, CA, USA) for 7 days. DCs were harvested and analyzed for lineage maturation makers (CD14, CD1a, CD11c, HLA-DR, and CD86), CD1d and HLA-G by flow cytometry before the iNKT activation assay.

Human iNKT Isolation and Expansion

Human $CD14^+$ monocytes and iNKT cells were isolated from fresh PBMC by positive selection using CD14 MicroBeads and anti-iNKT MicroBeads (Miltenyi Biotech, Germany), respectively, according to manufacturer's instructions. iNKT expansion was performed as described (48) with modifications. Briefly, iNKT were co-cultured with $CD14^+$ monocytes at 1:1 ratio in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Gibco), 10 µg/ml gentamicin (Gibco), and 0.25 µg/ml fungizone (Gibco) in the presence of 20 ng/ml GM-CSF (Peprotech), and 20 ng/ml IL-4 (R&D Systems), and 100 ng/ml αGC (Cayman Chemical). Half of the medium was replaced and 20 U/ml IL-2 (Chiron, Emeryville, USA) was added every day from day 2 to day 21 in order to reach around 1×10^6 cells. ILT2 expression was evaluated and iNKT cells were phenotypically validated by flow cytometry before the iNKT activation assay. Purity of the iNKT cell population was systematically higher than 90%. The iNKT cells used in the activation assay with DC-10 cells were isolated and maintained in culture with the presence of IL-2 and αGC till the autologous mDC and DC-10 cells were differentiated.

iNKT Activation Assays

The APC (A20CD1d, mDC, or DC-10) and NKT cells (NKT1.2, or human iNKT cells) were co-incubated at 1:1 ratio (1×10^6 cells/ml) in 96-well U-bottom plates for 24 and 4 h respectively for the assay of NKT1.2 cells and human iNKT cells. αGC-loaded APCs were prepared by incubating APC with 100 ng/ml of αGC in DMSO during 1 h at 37°C. Control APCs were prepared concomitantly by adding the same volume of DMSO without αGC. For blocking experiments, both APC and NKT cells were pretreated with either anti-HLA-G (functional grade, clone 87G; Exbio) or anti-ILT2 (functional grade, clone GHI/75; BioLegend) at 37°C for 1 h prior to the co-incubation. The protein transport inhibitor (eBioscience) was used at 1× to stop the cytokine release. NKT cell phenotypes and intracellular cytokines labeling was performed as described above. The expression of murine IL-2 and human IFN-γ and IL-4 were analyzed in NKT1.2 cells ($CD3^+$ cells) and human iNKT cells (singlet $CD3^+6B11^+$ lymphocytes or $CD3^+CD161^+$ lymphocytes), respectively. The gates for cytokine expression were set according to the non-activated controls in each independent experiment.

Statistics

Shapiro-Wilk normality test, one-way ANOVA, Bonferroni's Multiple Comparison Test, and statistical plots were performed in Prism 5 software (GraphPad). P-value ≤ 0.05 was considered statistically significant.

RESULTS

Human iNKT Cells Express Cell-Surface ILT2 Upon Activation

To determine whether NKT cells can be inhibited by HLA-G, we first evaluated their ILT2 expression in comparison to autologous $CD4^+$ T cells ($CD3^+CD56^-CD4^+$ lymphocytes), $CD8^+$ T cells ($CD3^+CD56^-CD8^+$ lymphocytes), and NK cells ($CD3^-CD56^+$ lymphocytes). As shown in **Figure 1A**, $CD3^+CD56^+$ NKT cells (i.e. $CD3^+CD56^+$ lymphocytes) generally represented almost 10% of peripheral lymphocytes. On the other hand, iNKT (i.e. $CD3^+6B11^+$ lymphocytes), showing double positivity for CD3 and Vα24Jα18 expression ($6B11^+$) represented only 0.06% of peripheral lymphocytes. Subgating of this small subset showed them to be CD1d-αGC-reactive, but only 30% of them expressed CD56 (**Figure 1B**).

ILT2 expression by $CD3^+CD56^+$ NKT and iNKT cell subsets was then investigated in peripheral blood monocuclear cells (PBMC) of 14 healthy donors. As shown on **Figure 1C**, only a minority of iNKT expressed ILT2 at their surface. We compared ILT2 expression by $CD3^+CD56^+$ NKT and iNKT cells to those of other lymphocyte cell subsets (**Figure 1D**). Approximately 1% of $CD4^+$ T cells, 20% of $CD8^+$ T cells, and 35% of NK cells expressed ILT-2 receptors at their surface, which is in accordance with what has been reported (22). The $CD3^+CD56^+$ NKT cell population expressed ILT2 the most (39.82%), with values similar to those of NK cells (~35%) (**Figure 1D**). This was in sharp contrast to iNKT cells, which barely expressed ILT2 (~5%).

It is known that cell-surface ILT2 expression can be induced on T cells following activation (49). Thus, we stimulated $6B11^+CD3^+$ iNKT cells with autologous αGC-loaded $CD14^+CD1d^+$ APCs purified from healthy donors, as previously reported (48). We showed (**Figure 1E**) that ILT2 expression is indeed induced upon iNKT cell activation, resulting in a 10-fold increase compared to unstimulated iNKT cells.

HLA-G:ILT2 Pathway Inhibits iNKT Cell Activation in a Murine *In Vitro* Model

Human iNKT cells represent only 0.01% to 0.1% of peripheral lymphocytes and their ILT2 expression is dependent on their activation state, reducing even further the numbers of human iNKT cells available for setting up the conditions of our functional assays. Given this and the fact that no human iNKT cell lines exist, we first developed an *in vitro* assay using murine cell lines. The murine line A20CD1d, transduced with HLA-G1 and hB2M, was used as APCs presenting αGC to the ILT2-transduced NKT1.2 effector cell line, as described in Materials and Methods. HLA-G1 expression on A20CD1d-HLA-G1-hB2M cells and ILT2 expression on NKT1.2-ILT2 cells are shown in **Figure 2**. Of note, no association between HLA-G1 heavy chain and murine B2M was observed, and HLA-G1 was expressed at the cell surface only in A20CD1d cells transduced with both HLA-G1 and human B2M (**Supplemental Figure 1**). Although HLA-G does not have a murine homolog, it can interact with PIR-B, the functional murine homolog of human ILT receptors (50). As can be seen in **Figure 2**, PIR-B was not expressed by either A20CD1d or NKT1.2 cell lines, ruling out any effect of HLA-G binding endogenous murine receptors, leaving transduced ILT2 as sole HLA-G receptor on NKT1.2-ILT2 cells.

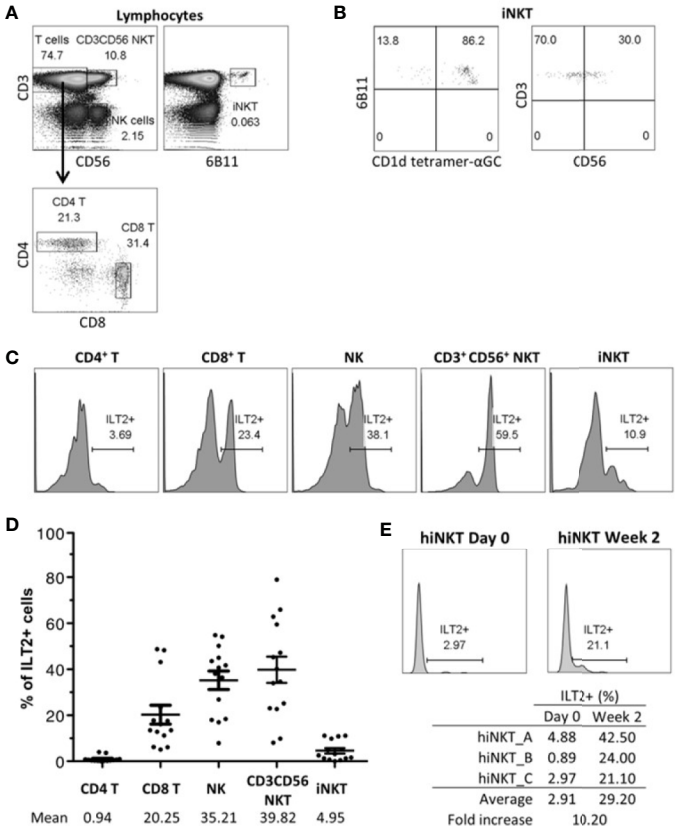

FIGURE 1 | Human Invariant Natural Killer T (iNKT) cells express cell-surface Immunoglobulin-like Transcript 2 (ILT2). ILT2 expression on $CD3^+CD56^+$ NKT cells and $CD3^+6B11^+$ iNKT cells was evaluated with fresh PBMC by flow cytometry and was compared to that of $CD4^+$ T, $CD8^+$ T, and NK cells. **(A)** Gating strategy to identify $CD4^+$ T, $CD8^+$ T, NK, $CD3^+$ $CD56^+$ NKT, and iNKT cells from lymphocytes gated according to the FSC-SSC profile and to the CD3, CD4, CD8, CD56, and 6B11 expression. Percentage of parent population is indicated. **(B)** CD1d tetramer-αGC reactivity and CD56 expression of iNKT cells. Percentage of parent population is indicated. **(C)** ILT2 expression on different subsets of one representative blood donor. Percentage of $ILT2^+$ cells is indicated. **(D)** Percentage of $ILT2^+$ cells of each subpopulation from 14 healthy donors. Bars indicate mean ± SEM. **(E)** ILT2 expression on iNKT cells during expansion. iNKT cells at day 0 and 2 weeks after stimulation by CD1d-αGC and cytokines are shown. Percentage of $ILT2^+$ cells is indicated.

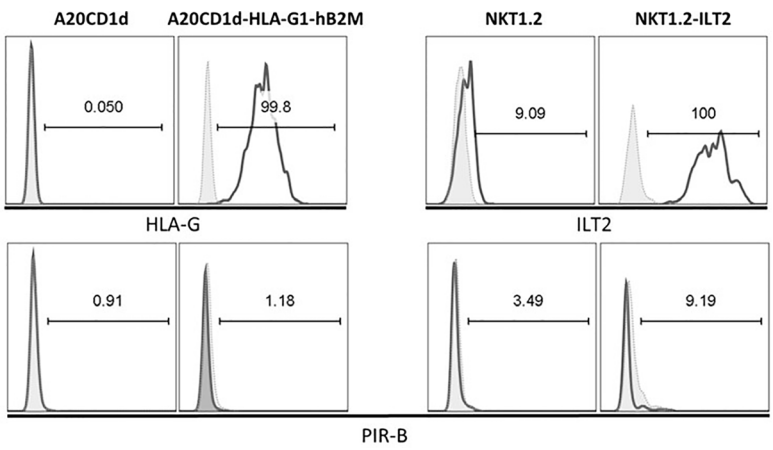

FIGURE 2 | A20CD1d and NKT1.2 cells lines generated to evaluate Human Leucocyte Antigen G (HLA-G):Immunoglobulin-like Transcript 2 (ILT2) inhibitory effect. A20CD1d and NKT1.2 cells were transduced to express HLA-G and ILT2, respectively. Flow cytometry was performed to measure the expression of HLA-G, ILT2, and PIR-B. Open histograms: staining for the indicated antibodies. Shaded histograms: isotype controls. Percentage of the parent population is indicated.

To evaluate the capability of HLA-G to inhibit iNKT cells through ILT2, 24-h co-incubations between A20CD1d ± HLA-G/hB2M, and effector NKT1.2 cells ± ILT2 in the presence or absence of αGC were performed, and intracellular IL-2 expression by NKT1.2 cells was evaluated by flow cytometry (51).

Figure 3 summarizes the results obtained from six independent experiments and demonstrates that intracellular IL-2 expression was increased in NKT1.2 cells after αGC stimulation in the absence of HLA-G ($p \leq 0.001$), and was significantly decreased for NKT1.2-ILT-2 cells only in the presence of HLA-G ($p \leq 0.001$). Addition of a blocking anti-HLA-G antibody restored IL-2 expression upregulation ($p \leq 0.05$), demonstrating that the inhibition observed was indeed due to the HLA-G:ILT2 interaction.

HLA-G:ILT2 Pathway Inhibits Human iNKT Cell Activation

In order to evaluate whether the reactivity of human iNKT cells naturally expressing ILT2 could also be inhibited through the HLA-G:ILT2 interaction, we first stimulated human iNKT cells from healthy donors with αGC-loaded A20CD1d and A20CD1d-HLA-G1-hB2M cells. Indeed, it was reported that human iNKT cells can be stimulated by αGC presented in the context of murine CD1d (52). Because resting human iNKT cells barely express ILT2 as shown earlier in this work, our experiments required that ILT2 receptor expression be boosted by an *in vitro* expansion step prior to use, following published protocols (48) with modifications described in Materials and

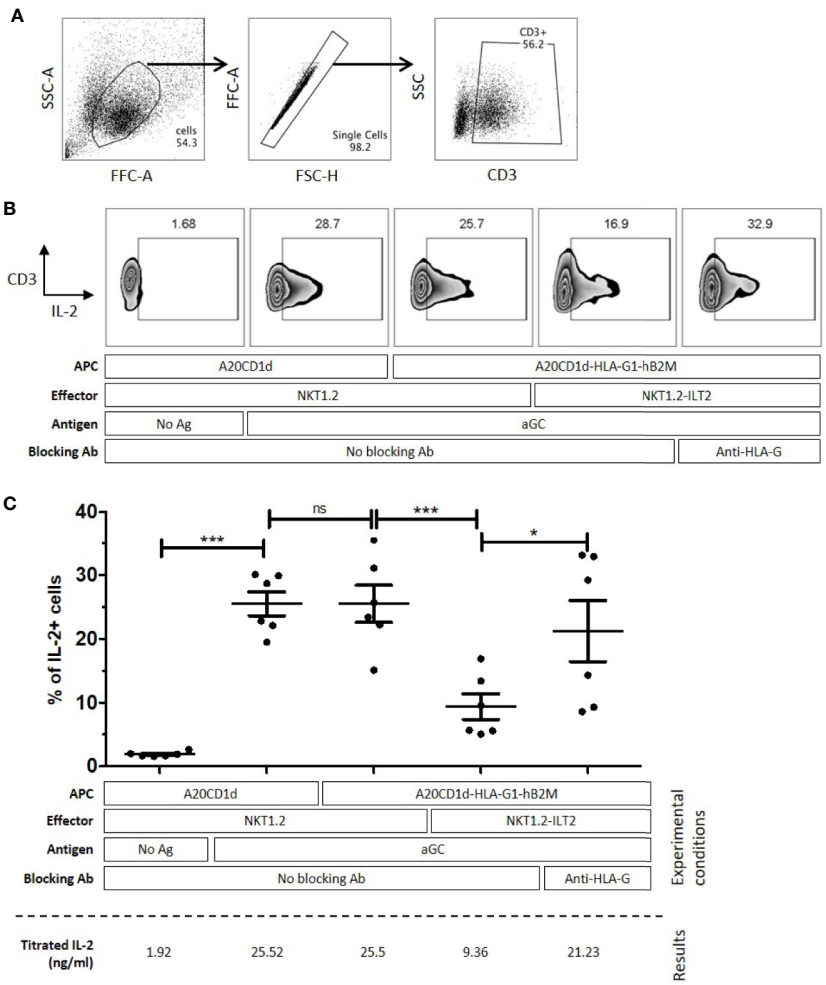

FIGURE 3 | NKT1.2-ILT2 cells are inhibited through the Human Leucocyte Antigen G (HLA-G)/Immunoglobulin-like Transcript 2 (ILT2) interaction. A20CD1d or A20CD1d-HLA-G1-hB2M cells were used as Antigen Presenting Cells (APC) and NKT1.2 or NKT1.2-ILT2 cells were used as effector cells. Intracellular IL-2 expression of effector NKT cells was evaluated by flow cytometry after 24 h of co-culture with APC loaded or not with αGC as indicated. Anti-HLA-G antibody (Ab), 87G, was used to block the HLA-G/ILT2 interaction when indicated. **(A)** Gating strategy to identify NKT1.2 cells after the coculture assay. FSC-SSC profile was used to identify living cells, then the NKT1.2 cells are identified as CD3+ singlet living cells. Percentage of lymphocytes is indicated. **(B)** Intracellular IL-2 expression in NKT1.2 cells of one representative experiment. The gate of IL-2 expression is based on the no activation control in each experiment. **(C)** Six independent experiments were performed. Each bar indicates mean ± SEM. Horizontal bars indicate statistical significance (One-way ANOVA and Bonferroni *post hoc*, *$p \leq 0.05$; ***$p \leq 0.001$).

(**Figure 1E**). Two donors were used for these experiments, and results did not reach significance but only show tendency. Following amplification, 25% of iNKT cells from both donors expressed cell-surface ILT2 (data not shown). Stimulation results show that in the absence of αGC, only 1%–4% of iNKT expressed IFN-γ and 5%–7% of iNKT expressed intracellular IL-4 (**Figure 4**). Intracellular IFN-γ and IL-4 expression increased to 17.6% and 18.2% respectively when stimulated by αGC-loaded A20CD1d for 4 h, whereas intracellular IFN-γ and IL-4 expression was respectively 6.72% and 8.73% after a 4-h co-incubation with αGC-loaded A20CD1d-HLA-G1-hB2M cells (**Figure 4**). The presence of blocking anti-HLA-G or anti-ILT2 antibodies restored intracellular IFN-γ and IL-4 expression by iNKT cells stimulated by αGC-loaded A20CD1d-HLA-G1-hB2M cells, demonstrating that human iNKT inhibition was dependent on HLA-G:ILT2 interaction.

These results demonstrate that human iNKT cells are sensitive to HLA-G inhibition through ILT2 receptors engagement.

αGC-Loaded HLA-G-Expressing Tolerogenic DC-10 Cells Do Not Activate Human iNKT Cells

DC-10 cells are human tolerogenic DC expressing high levels of HLA-G. They are regulatory cells capable of inhibiting allogeneic responses through HLA-G and IL-10, of inducing IL-10-producing T regulatory type 1 (Tr1) cells (42, 43), and they were shown to increase in cancer patients (53, 54). Accordingly, we investigated whether tolerogenic DC-10 cells could inhibit the functions of autologous human iNKT. Human iNKT cells were stimulated with αGC-loaded autologous mDC or autologous DC-10 cells, and IFN-γ and IL-4 expression was analyzed. Freshly isolated monocytes from two healthy donors were differentiated into mDC and DC-10 cells as previously described (42), and in parallel, iNKT cells were isolated from the same donors and amplified *in vitro* in the presence of IL-2 and αGC. As expected (42, 43), DC-10 *in vitro*-differentiated from peripheral monocytes expressed cell-surface HLA-G1, while mDC barely expressed HLA-G1 (**Figure 5**). Conversely, mDC and DC-10 expressed similar cell-surface expression levels of CD1d, indicating that both cell types were capable of presenting αGC to autologous human iNKT cells.

iNKT cells were then co-incubated for 72 h with either autologous mDC or DC-10 and their intracellular IFN-γ and IL-4 expression was evaluated by flow cytometry. It is unfortunate that we could obtained all required cell populations from two donors only, preventing the results from reaching statistical significance, although tendencies were clear: **Figure 6B** shows the results obtained for one donor, and **Figure 6C** presents data for both donors. As can be seen for both experiments combined, 11%–12% of iNKT cells expressed IFN-γ and 16%–17% expressed IL-4 when stimulated with unloaded mDC and DC-10. When iNKT cells were stimulated with αGC-loaded mDC, IFN-γ and IL-4 expression increased, to 26% and 31%, respectively (**Figure 6C**). Conversely, when iNKT cells were stimulated with αGC-loaded DC-10, no increase in IFN-γ and IL-4 expression was observed, and the proportion of iNKT cells positive for IFN-γ and IL-4 remained at or below the baseline obtained without αGC (7% and 10% respectively) (**Figure 6C**). These results indicated that HLA-G-positive αGC-loaded DC-10 cells do not seem to support human iNKT activation.

DISCUSSION

We sought to prove that the interaction of HLA-G with ILT2 could inhibit iNKT activation. Thus, we first evaluated the ILT2 cell-surface expression on iNKT cells. Surprisingly, although $CD3^+CD56^+$ NKT cells expressed high levels of cell-surface ILT2, the iNKT cell subset, specific for αGC presented in the context of CD1d, barely expressed ILT2 (**Figure 1D**). Indeed, ILT2 expression on resting human iNKT cells from peripheral blood was higher than that of $CD4^+$ T cells but lower than that of $CD8^+$ T cells or NK cells. Therefore, following these first results, it seemed unlikely that HLA-G could have any significant direct inhibitory effect on iNKT cells. However, it must be considered that even though $CD4^+$ T cells express ILT2, even less so than iNKT cells, they are well known to be sensitive to HLA-G inhibition anyway (27, 28, 55). This discrepancy between weak ILT2 expression and sensitivity to HLA-G can be explained by two hypotheses: first, T cell inhibition by HLA-G may not be direct; HLA-G could act on stimulating APC or through the generation of tolerogenic APC. This mechanism is very relevant to iNKT cells since monocytes/DC that are required for αGC-induced iNKT activation constitutively express the ILT2 and ILT4 HLA-G receptors (23, 56), and are known to be inhibited by HLA-G (57–59). Second, ILT2 might not be readily present on peripheral blood resting T cells, but be upregulated upon activation. In this case, HLA-G would not act directly on resting iNKT cells, but only on already activated ones. Several studies demonstrated that ILT2 is upregulated by $CD4^+$ and $CD8^+$ T cells upon activation (49). In agreement with these results, our experiments showed that ILT2 was upregulated on iNKT cells in response to stimulation, and that *in vitro* expanded iNKT cells expressed cell-surface ILT2 at a level comparable to that of polyclonal NK cells (**Figure 1E**). Thus, according to our results, it seems that HLA-G direct inhibition should only impact activated iNKT cells.

To demonstrate that activated iNKT cells could be inhibited by HLA-G, we developed an *in vitro* model using a murine NKT cell line. Indeed, iNKT cells, and even more so ILT2-positive iNKT cells, represent a very small subset of peripheral lymphocytes which is difficult to isolate. Thus, to overcome this limitation, we set up HLA-G/ILT2 ICP inhibition experiments in the already described NKT1.2 vs A20CD1d model and transduced them with ILT2 and HLA-G/hB2M respectively. We showed that NKT1.2 cells rapidly expressed IL-2 in response to stimulation by αGC-loaded A20CD1d cells expressing HLA-G or not (**Figure 3**). However, IL-2 upregulation was hampered when ILT2-expressing NKT1.2-ILT2 cells were stimulated by HLA-G-expressing αGC-loaded A20CD1d-HLA-G1-hB2M cells (**Figure 3**). IL-2 expression inhibition was due to the ILT2:HLA-G1 interaction given that

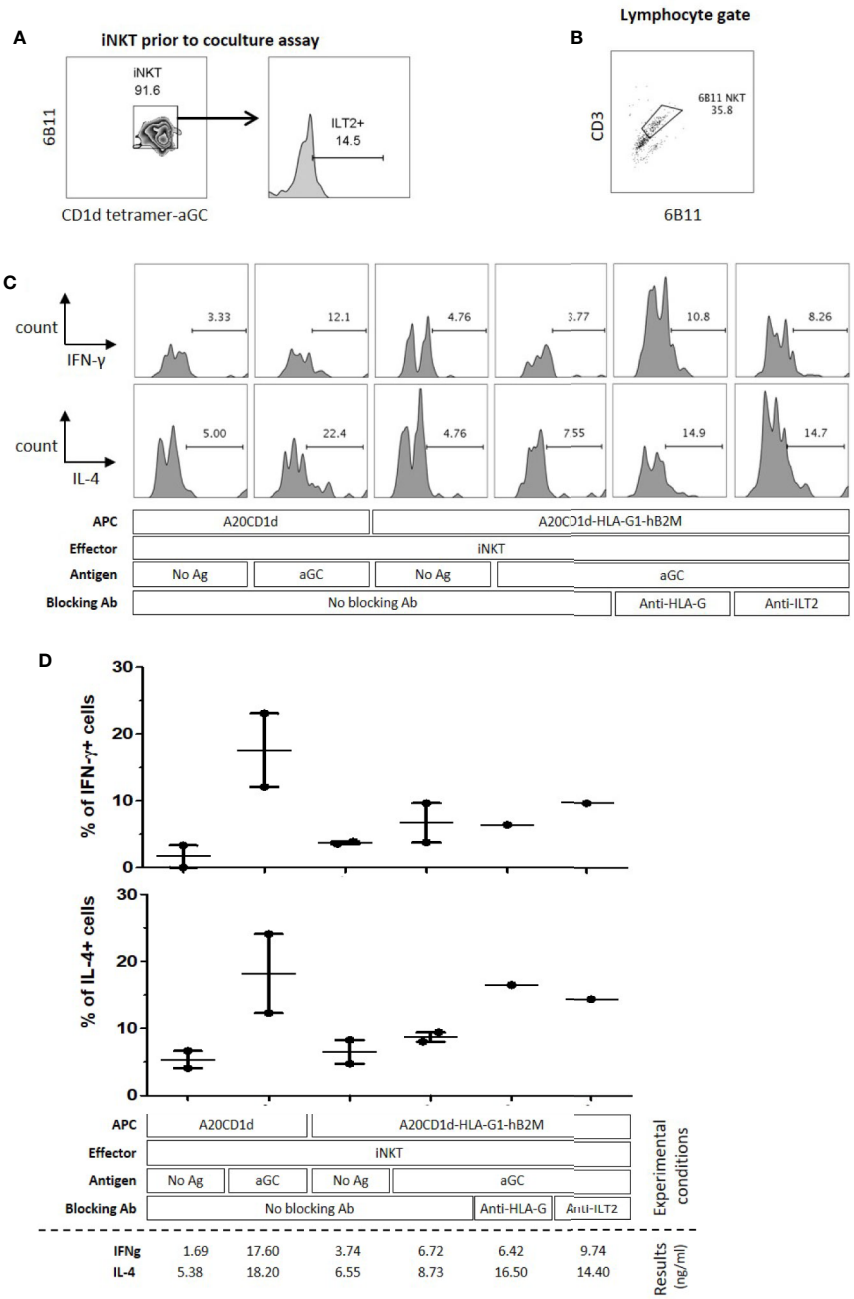

FIGURE 4 | Activation of human Invariant Natural Killer T (iNKT) cells is inhibited through the Human Leucocyte Antigen G (HLA-G):Immunoglobulin-like Transcript 2 (ILT2) interaction. A20CD1d or A20CD1d-HLA-G1-B2M cells were used as Antigen Presenting Cells (APC) and human iNKT cells were used as effector cells. Intracellular IFN-γ and IL-4 expression by effector NKT cells was evaluated by flow cytometry after a 4-h co-culture with APC loaded or not with αGC as indicated. Anti-HLA-G antibody 87G, or anti-ILT2 antibody GHI/75, were used to block the HLA-G/ILT2 interaction as indicated. **(A)** Before functional assay, expanded human iNKT cells were checked using 6B11 and CD1d-tetramer-αGC. Their surface ILT2 levels were measured. **(B)** Gating strategy to identify human iNKT cells after the coculture assay with stimulator cells, gated on lymphocytes according to the FSC-SSC profile and CD3+ and 6B11+ expression. Percentage of lymphocytes is indicated. **(C)** IFN-γ (upper panel) and IL-4 (lower panel) expression levels by effector cells of one representative experiment. The gate of cytokine expression is based on the no activation control in each experiment. **(D)** Two independent experiments were performed; bar indicates mean ± SEM.

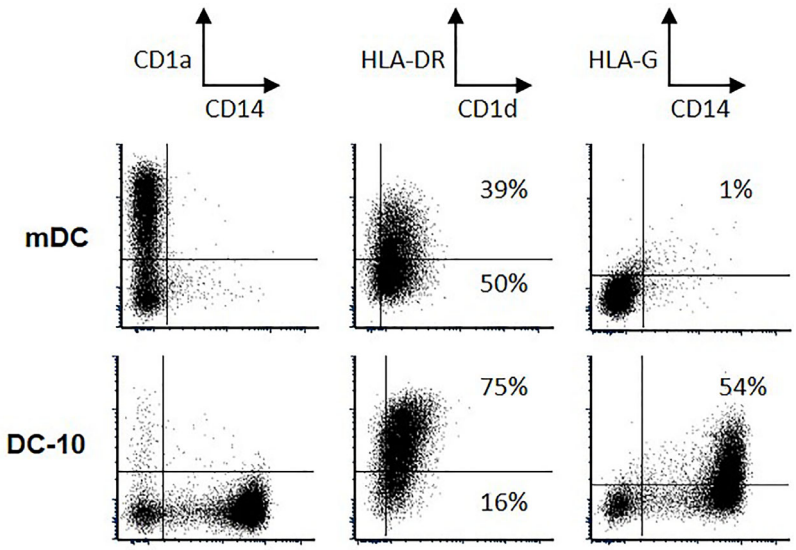

FIGURE 5 | Phenotypic characteristics of mature dendritic cell (mDC) and autologous DC-10 cells. Phenotype was validated based on the expression of CD14, CD1a, CD1d, HLA-DR, and Human Leucocyte Antigen G (HLA-G); numbers indicate the percentages of positive cells for CD1d and HLA-G.

anti-HLA-G blocking antibodies restored IL-2 expression (**Figure 3**). We demonstrated that HLA-G could directly inhibit ILT2-expressing iNKT cells. Transduced murine cell lines may not be representative of human iNKT cells, however the human iNKT cells interaction with αGC-loaded murine CD1d could be used to study human iNKT cells inhibition by HLA-G. αGC-loaded murine A20CD1d/A20CD1d-HLA-G1-hB2M cells were used to stimulate human *in vitro*-expanded iNKT cells (**Figure 4**). In these experiments, iNKT cells had been expanded *in vitro* in order to induce ILT2 expressions sufficiently so that the ILT2$^+$ population was analyzable. It is unclear whether naïve, resting iNKT could respond to HLA-G as activated iNKT did. One can hypothesize that only previously stimulated iNKT would respond to HLA-G. Those iNKT cells, recognizing specific CD1d-lipid complexes would then be akin to memory T cells and sensitive to HLA-G inhibition in recall immunization or in the context of chronic stimulation. One can also hypothesize that resting, naïve iNKT cells would be insensitive to HLA-G but only until they are fully activated and upregulate ILT2. In this case, HLA-G would not prevent the early functions of iNKT, but rather their late functions, and possibly shorten their activation.

When studying the possible effect of HLA-G:ILT2 ICP on iNKT cell activation and functions, it is necessary to consider the stimulatory cells. Indeed, in the context of cancer, HLA-G can be expressed by the tumor cells and/or by the infiltrating APCs (monocytes/macrophages/DC). Myeloid cells constitutively express both ILT2 and ILT4 HLA-G receptors, and are efficiently inhibited by this molecule (23). Following exposure to HLA-G, APC lose their capability to stimulate T cells (57) and therefore might lose their capability to stimulate iNKT cells as well. Furthermore, HLA-G induces the differentiation of regulatory cells, including regulatory myeloid cells (42, 60). These cells do not support regular T cell activation and might not stimulate iNKT cells either. In order to test this hypothesis, we investigated if HLA-G-expressing tolerogenic DC, called DC-10, were capable of stimulating iNKT cells. DC-10 cells are particularly interesting in our context because they express the same levels of CD1d as mature DC (mDC). Indeed, iNKT cells have been stimulated by artificial APC, expressing costimulatory molecules such as CD80 and CD86. Thus, the lack of stimulation of DC-10 cells compared to mDC could not be explained by lack of costimulation. Generating autologous mDC, DC-10, and ILT2-expressing iNKT to set up and then perform functional studies proved to be a very problematic and uncertain task, which limited the number of experiments we could perform, ultimately preventing our results from reaching statistical significance. However, the tendency was clear and indicates that whereas mDC efficiently activate iNKT cells, HLA-G-expressing tolerogenic DC-10 do not (**Figure 6**). We could not block and prove that HLA-G caused iNKT cell inhibition, for lack of sufficient autologous DC-10 and purified iNKT cells. However, in light of the other results presented here, an HLA-G-mediated inhibition of iNKT following stimulation by αGC-loaded DC-10 is a relevant hypothesis, even though inhibition by IL-10 cannot be excluded, since DC-10 modulatory activity relies not only on the expression of HLA-G but also on the secretion of IL-10 (42). These two mechanisms are actually not mutually exclusive and could act synergically as it has been shown in other contexts (42, 43, 61). It is well known that HLA-G, and especially HLA-G-expressing APCs such as DC-10, not only inhibit the classical function of T cells, but also induce their differentiation into regulatory cells (42). Yamaura et al. demonstrated that IL-10-secreting DCs can induce iNKT cells to produce more IL-10 which will further have an anergic phenotype, and potently inhibit allogeneic CD4$^+$ T cell proliferation *in vitro* (62). We do not know the function of

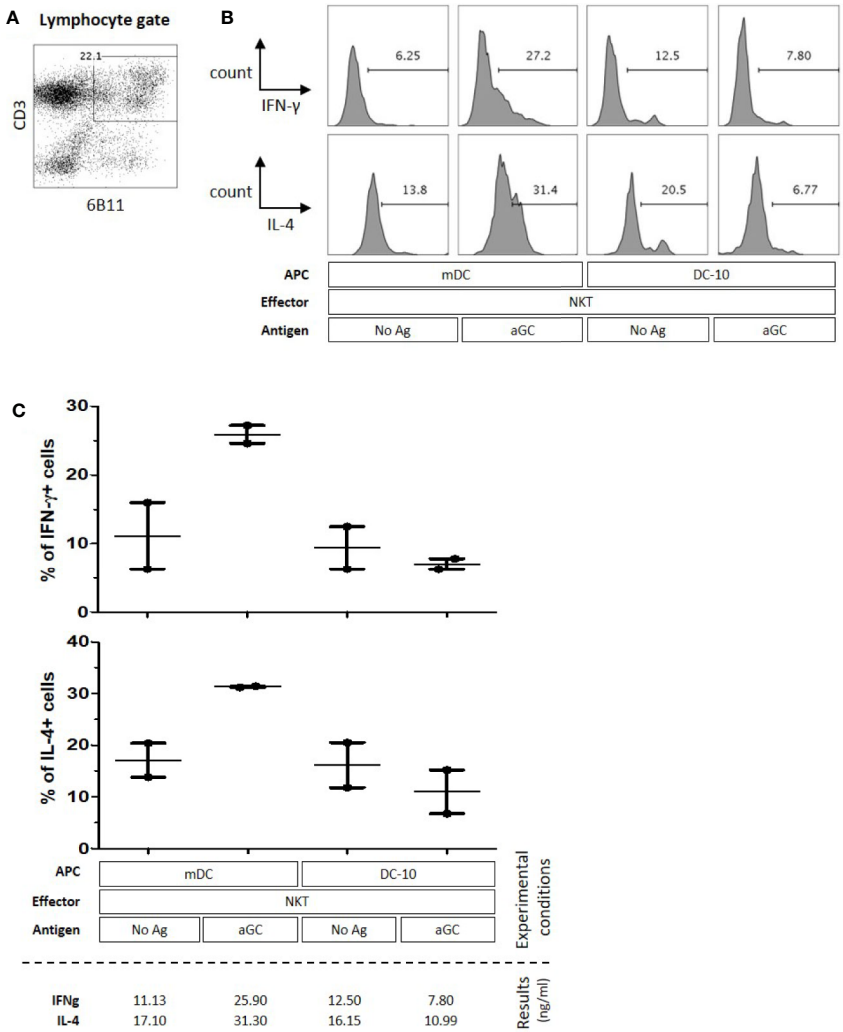

FIGURE 6 | Tolerogenic DC-10 cells do not support human Invariant Natural Killer T (iNKT) cell activation. Mature Dendritic Cells (mDC) or tolerogenic DC-10 cells were used as antigen-presenting cell (APC), and autologous human iNKT cells were used as effector cells. Intracellular IFN-γ and IL-4 expression of effector cells was evaluated by flow cytometry after 72 h of co-culture with APC loaded or not with αGC antigen as indicated. **(A)** Gating strategy to identify iNKT cells after the coculture assay with stimulator cells, gated on lymphocytes according to the FSC-SSC profile and $CD3^+$ and $CD161^+$ expression. Percentage of lymphocytes is indicated. **(B)** IFN-γ (upper panel) and IL-4 (lower panel) expression levels by effector cells of one representative experiment. The gate of cytokine expression is based on the no activation control in each experiment. **(C)** Two independent experiments were performed; bar indicates mean ± SEM.

DC-10-stimulated iNKT cells, but their differentiation toward a regulatory type is a possibility that should be investigated. Nevertheless, our data show for the first time that iNKT cells are not activated if tolerogenic stimulators are present. This is an important headway in the context of anti-tumor adjuvant therapy using iNKT cells, given that these therapeutic strategies rely on the proper activation of iNKT cells by autologous DC. Thus, because tolerogenic DC or myeloid suppressive cells have been reported in the pathological context of cancer, this might explain the lack of iNKT response in human trials, possibly amplified by iNKT cell differentiation in tolerogenic iNKT cells (62) which would achieve the opposite of the intended goal. Thus, without proper identification, DC-10 cells could very well be present within the autologous myeloid cell population used for iNKT cell stimulation in iNKT-based immunotherapies.

iNKT cells have the ability to rapidly release large amounts of cytokines to link both innate and adaptive immune responses. Hence, iNKT cells possess a potent adjuvant activity: IFN-γ secretion by activated iNKT cells can activate NK cells, mature DC, and prime Ag-specific T cell responses (63) IFN-γ and IL-4 secretions can also contribute to antibody secretion and memory B cell induction (64, 65). Our work demonstrates that HLA-G and myeloid regulatory cells such as HLA-G-expressing DC-10 cells prevent proper activation of iNKT cells by αGC, a mechanism that may very well occur in iNKT cell-based anti-tumor therapy trials and reduce therapy efficiency.

More generally, our results emphasize the need for factoring in the functions of ICPs and regulatory cells in iNKT cell-based anti-tumor therapies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

C-LW: performed experiments, collected and analyzed data, wrote the manuscript. JC: designed and performed experiments, analyzed data, wrote the manuscript. GA: performed and analyzed experiments. FA: performed experiments. ML: analyzed data, wrote the manuscript. SG: wrote the manuscript. PL-D: manuscript review. JL: designed study, analyzed data, wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.608614/full#supplementary-material

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