# THE BIOLOGICAL AND CLINICAL ASPECTS OF HLA-G, VOLUME II

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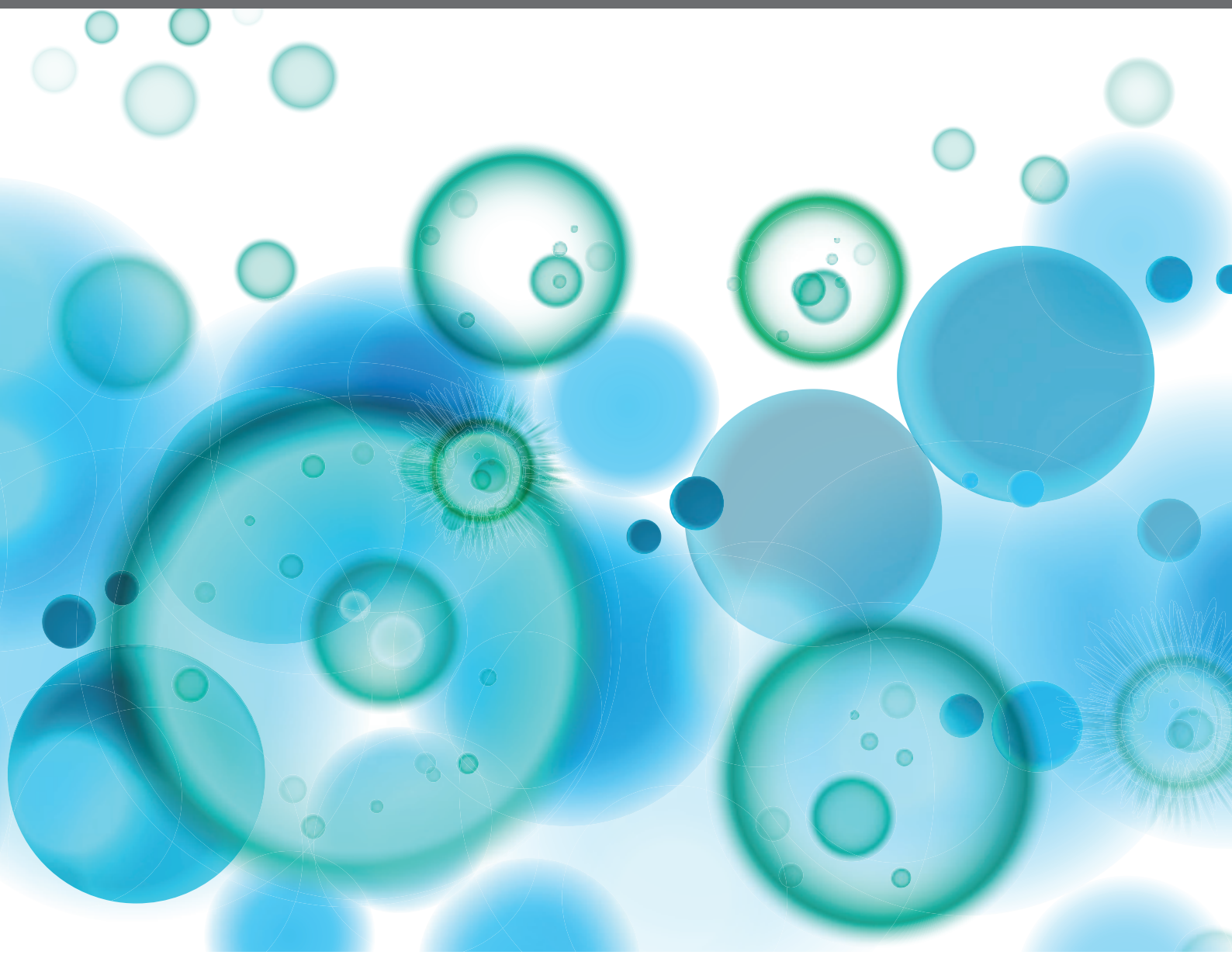

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# THE BIOLOGICAL AND CLINICAL ASPECTS OF HLA-G, VOLUME II

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EDITORIAL
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# Editorial: The Biological and Clinical Aspects of HLA-G, Volume II

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**Keywords: HLA-G, receptor, immune regulation, cancer, autoimmune, infection, reproduction**

**Editorial on the Research Topic:**

**The Biological and Clinical Aspects of HLA-G Volume II**

Human leukocyte antigen G (HLA-G), a non-classical class HLA-I antigen, is an important immune regulator in homeostasis and disease progression. Biological and clinical significance of HLA-G expression and genetic variation of *HLA-G* in different setting have been extensively investigated since its discovery.

In this Research Topic "The Biological and Clinical Aspects of HLA-G volume II", fifteen in-depth reviews, perspectives and original research articles on different aspects of HLA-G were published, which focused on HLA-G and its receptors in cancer, autoimmune and infectious diseases, skin, allergy and reproduction immunity.

Multiple HLA-G isoforms generated by primary transcript alternative splicing were identified, such as α1 domain-containing (HLA-G1~HLA-G7) and α1 domain-deleted isoforms. It is expected that all HLA-G spliceforms are immune inhibitory, but given the extent of their structural diversity, it is also expected that differences in their biological functions exist. The immune checkpoint functions of HLA-G were shown to be either beneficial or deleterious, depending on the biological context. Indeed, HLA-G was shown to promote fetal-maternal immune tolerance, limit inflammation, and prolong transplanted grafts acceptance. Inversely, it was also shown to impair host immune responses against virus-infected cells and malignant cells, and enhance the capability of these abnormal cells to escape immune clearance. The HLA-G receptors ILT2/LILRB1, ILT4/LILRB2, and KIR2DL4 were shown to bind α1 domain-containing isoforms. KIR2DL4 recognizes HLA-G through its α1-α2 domains, while ILT2 and ILT4 primarily recognize HLA-G α3 domain of B2M-associated isoforms (ILT2) or B2M-free isoforms (ILT4). Functionally, such interactions lead to immune inhibition. In this regard, an increasing number of clinical trials on HLA-G and/or ILTs targeted immunotherapy for solid cancers have been started. Binding of ILT2 and ILT4 receptors to α1-deleted isoforms is unknown. However, α1 domain-deleted isoform has been recently found to be with stimulatory property, raising questions regarding the receptors involved and warranting more work to explore HLA-G functional complexity.

Fetal trophoblasts are key players in fetal-maternal interaction during pregnancy. They constitutively express HLA-G, especially extravillous trophoblast cells, and this expression is required for maternal-fetal tolerance and successful implantation. Eikmans et al. present a method to culture trophoblasts from first-term placentas, and differentiate them into extravillous

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trophoblasts expressing HLA-G. This is a unique model to study naturally expressed HLA-G and conduct research on fetal-maternal immune cross-talk.

Neo-expression of HLA-G is commonly observed among almost all types of cancers. Through engagement with its receptors, HLA-G expression by cancer cells exerts an immune checkpoint function and induces immune suppression by inhibiting the functions of immune effectors, and by induction the proliferation of immune regulatory cells. Thus, there is a growing interest in blocking HLA-G:HLA-G-Receptor signaling blockade in cancer immunotherapy. Martı´n-Villa et al. provide an exhaustive review on the genetic variation of the *HLA-G* gene, on the mechanisms of HLA-G-mediated immune suppression and its relevance in cancer and autoimmune diseases. Lin and Yan review the possible HLA-G:ILTs-targeting immunotherapies for solid cancers, while also highlighting the major challenges that remain to be addressed in this context, including the diversity of HLA-G isoforms, theand intra- and inter-tumor heterogeneity of HLA-G expression. This should be taken into consideration when developping clinical applications. In this regard, HLA-G/KIR2DL4 signaling can impair ADCC of NK cells with the treatment of Her-2 antibody trastuzumab. Zheng et al. discuss the HLA-G/KIR2DL4 signaling in breast cancer microenvironment and its mechanisms and effects in breast cancer immunotherapy, providing a new light on significance of HLA-G/KIR2DL4 in cancer cell immune evasion.

Genetic variation of *HLA-G* has been shown to be related to HLA-G expression levels and predisposition to certain types of diseases. In this context, Scavuzzi et al. present the "MHC Cusp Theory" which postulates that in addition to its main role in antigen presentation, the MHC codes for allele-specific molecules that act as ligands in a conformationally-conserved cusp-like fold, which upon interaction with cognate receptors can trigger MHC-associated diseases. Based on the structural and functional features shares among HLA-G and other HLA antigens, and on how specific HLA-G allelic molecules differently affect NK functions, the authors postulate that HLA-G isoforms and allelic products might also contain cusp domain-binding sites. Piekarska et al. investigate association of haplotypes and diplotypes containing single nucleotide polymorphisms rs1632947: c.-964G>A, rs1233334: c.-725G>C/T in *HLA-G* promoter region, and rs371194629: c.*65_*66insATTTGTTCATGCCT in *HLA-G* 3' untranslated region (UTR) with levels of soluble HLA-G in semen and with male fertility status. They show that the G-C-ins haplotype is the most unfavorable one for male fertility, which is related to the lowest soluble HLA-G in semen. Rebmann et al. evaluate the relationships between variants in *HLA-G* UTR and disease outcome of patients with locally advanced, non-metastastic breast cancer. Their findings revealed that the UTR-1 or UTR-2 haplotypes are indicators for a better prognosis in term of a complete response to neoadjuvant chemotherapy or progression-free survival, while UTR-4 is a predictor for an inferior overall survival. Jasinski-Bergner et al. review the expression and clinical relevance of HLA-G expression in urological tumors, HLA-G use for diagnostic purposes, as prognostic biomarker, as a monitoring tool for immunotherapy, and as a therapeutic target in urological tumors. Li et al. highlight the significance of the detection of HLA-G molecule derived from different sources such as malignant lesions, peripheral circulating and body fluids, and extracellular vesicles for diagnosis and prognosis among cancer patients. Also, the authors review the limitations of the current HLA-G detection methods and offer opinions on how to standardize HLA-G detection methods.

In the context of inflammatory diseases, Negrini et al. show that increased peripheral soluble HLA-G levels and/or immune cell surface membrane-bound HLA-G molecule can be commonly observed in allergic diseases. In atopic dermatitis, HLA-G expression is found in papillary dermis and infiltrating immune cells such as T cells or Langerhans cells. In skin immunity and tolerance, Mestrallet et al. review the roles of epidermal keratinocytes in skin homeostasis and regeneration. They also highlight the therapeutic potential HLA-G expression modulation in keratinocytes to generate bioengineered universal donor cell sources for skin replacement. Mestrallet et al. further report that HLA-G is overexpressed in CD49f immature keratinocyte precursors with immunosuppressive properties, which can inhibit CD4+ T cell proliferation. DC-10 cells are a subset of DCs involved in IL-10-mediated tolerance. DC-10 are also characterized by a natural HLA-G expression. In patients with Type 1 diabetes (T1D), Amodio et al. reveal that low peripheral blood frequency of tolerogenic DC-10 during disease development is paralleled with the increased proportion of pro-inflammatory cDC2 cells, and that DC-10 impaired CD83 expression is associated with risk of developing T1D.

Induction of HLA-G expression by infected cells is known to be an immune eszcape tratégy for viruses. Jasinski-Bergner et al. elaborate mechanisms involved in the induction of HLA-G expression upon various virus infection; and Lin and Yan, review the immunopathological aspects of HLA-G:HLA-G-receptor signalling in SARS-CoV-2 infection, which could provide a better understanding of COVID-19 disease progression and identify potential immunointerventions to counteract SARS-CoV-2 infection.

The field of HLA-G research is fast expanding. With multiple application fields, multiple pathological contexts, biological aspects, and a growing number of new unanswered questions to be investigated, it may be difficult for newcomers to find their mark. We hope the readers of this second Research Topic on HLA-G that focuses on the current advances in the biological and clinical aspects of HLA-G, will find useful information, opinions and perspectives that will help them advance the field. We deeply appreciate all contributions from authors, reviewers and editors alike.

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REVIEW
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# HLA-G/ILTs Targeted Solid Cancer Immunotherapy: Opportunities and Challenges

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Immune checkpoint inhibitors (ICIs) have become a promising immunotherapy for cancers. Human leukocyte antigen-G (HLA-G), a neoantigen, its biological functions and clinical relevance have been extensively investigated in malignancies, and early clinical trials with "anti-HLA-G strategy" are being launched for advance solid cancer immunotherapy. The mechanism of HLA-G as a new ICI is that HLA-G can bind immune cell bearing inhibitory receptors, the immunoglobulin-like transcript (ILT)-2 and ILT-4. HLA-G/ILT-2/-4 (HLA-G/ILTs) signaling can drive comprehensive immune suppression, promote tumor growth and disease progression. Though clinical benefits could be expected with application of HLA-G antibodies to blockade the HLA-G/ILTs signaling in solid cancer immunotherapy, major challenges with the diversity of HLA-G isoforms, HLA-G/ILTs binding specificity, intra- and inter-tumor heterogeneity of HLA-G, lack of isoform-specific antibodies and validated assay protocols, which could dramatically affect the clinical efficacy. Clinical benefits of HLA-G-targeted solid cancer immunotherapy may be fluctuated or even premature unless major challenges are addressed.

**Keywords: HLA-G, immune checkpoint, immune checkpoint inhibitor, immunoglobulin-like transcript, cancer immunotherapy**

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## INTRODUCTION

Immune checkpoint inhibitors have become a promising immunotherapy for cancers, but durable clinical benefits are limited for existing agents (1). It's an exciting news released in Cancer Discovery "Gilead Buys into Tizona's Anti-HLA-G Strategy" that an early clinical trial with human leukocyte antigen-G (HLA-G) inhibitor TTX-080 is being launched for advance solid cancer patients (NCT04485013) (2).

HLA-G, firstly observed on extravillous cytotrophoblast, has been considered to play critical roles in maintaining maternal immune tolerance for the semi-allograft fetus during pregnancy (3, 4). In the context of malignancies, aberrant HLA-G expression in melanoma lesions but not adjacent normal tissues was reported by Paul and co-workers in 1998 for the first time (5). This pioneering investigation has been testified with thousands of samples in more than 30 types of

cancers. Ever increasing studies on HLA-G expression by solid tumor lesions have revealed that high levels of HLA-G expression was associated with advanced disease stage, tumor metastasis, poor prognosis, or shorter disease-free survival. However, either among patients with different types of cancers, or among patients with the same type of cancer, intertumor and intratumor heterogeneity of HLA-G expression is evident, such as among patients with breast cancer (6–15), colorectal cancer (16–24), cervical cancer (25–27), endometrial cancer (28–31), esophageal squamous cell carcinoma (32–34), Ewing sarcoma (35), gastric cancer (36–38), glioblastoma (39), hepatocellular carcinoma (40–42), lung cancer (43–45), classical Hodgkin lymphoma (46, 47), diffuse large B-cell lymphoma (48), cutaneous T- and B-cell lymphoma (49), nasopharyngeal carcinoma (50), oral squamous cell carcinoma (51), ovarian cancer (52–55), pancreatic adenocarcinoma (56–59), and thyroid cancer (60, 61) (**Table 1**). HLA-G expression in solid cancers is now well acknowledged in promoting cancer cell immune escaping and tumor development, and associated with disease progression and poor survival either among cancer patients or pre-clinical murine models (62).

Engagement of HLA-G/ILTs can induce either unresponsive or tolerogenic state of a wide range of immune effector cells. The underlying mechanisms for HLA-G as an immune checkpoint is that HLA-G can either directly bind tyrosine-based inhibitory motifs (ITIMs) containing immune inhibitory receptors, the immunoglobulin-like transcript (ILT)-2/CD85j/LILRB1 and ILT-4/CD85d/LILRB2, which is expressed on various immune competent cells such as T lymphocytes, natural killer cells (NK), dendritic cells (DC) (62), or indirectly by intercellular transfer by the process of trogocytosis and exosomes to drive a comprehensive immune suppression (63). Immune suppression induced by the HLA-G/ILTs signaling pathway includes inhibition of cytotoxicity (64), inflammatory cytokine production (65), chemotaxis and proliferation of T cells and NK cells (66, 67), inhibition antibody production of B cells and maturation of antigen presenting cells (68, 69), and dampen the anti-tumor functions of invariant natural killer T (iNKT) cells and tumor-infiltrating $CD8^+PD\text{-}1^-ILT\text{-}2^+$ T cells (70, 71). Also, HLA-G/ILTs engagement can also induce expansion of myeloid derived suppressive cells (MDSCs) and generation of regulatory T cells (72, 73). In addition to immune suppressive functions, HLA-G/ILTs can promote intratumor vascular remodeling by enhancing vascular endothelial growth factor-C (VEGF-C) expression, and increase tumor metastasis by inducing cancer promoting factor matrix metalloproteinases (MMPs) expression (74, 75) (**Figure 1**). Consequently, both innate and adaptive antitumor immune responses are impaired, thus favoring tumor cell immune evasion and disease progression. In this scenario, restoring antitumor functions of ILT-bearing immune cells with HLA-G inhibitors such as TTX-080 sounds reasonable.

As HLA-G expression is specifically induced in most types of solid cancer cells, clinical benefits of HLA-G inhibitors could be expected for cancer immunotherapy. However, challenges such as multiple HLA-G isoforms with distinct extracellular domains, different binding sites between HLA-G and ILT-2/ILT-4 interaction, intratumor or intertumor heterogeneity of HLA-G expression, and isoform-specific antibody and validated assay protocol lacking, remain tremendous hurdle in terms of HLA-G/ILTs antibody-based solid cancer immunotherapy.

## HLA-G ISOFORMS MOLECULAR STRUCTURE

The *HLA-G* gene contains eight exons and seven introns. However, most full-length transcripts carry only seven exons because exon seven is usually spliced out. Due to a premature stop codon in E6, the HLA-G full-length protein has 338-amino acids, which is relatively shorter compared with classical HLA class I molecules. Among these exons, E1 generates the signal peptide, E2-E4 generate extracellular α1, α2, and α3 domains, respectively. E5 generates the transmembrane domain, and E6 generates the intracellular cytoplasmic tail of HLA-G (76).

Due to its primary transcript alternative splicing, diverse molecular structures of HLA-G have been observed. Seven HLA-G isoforms including four membrane-bound (HLA-G1–HLA-G4) and three soluble (HLA-G5–HLA-G7) monomers have been identified. With a premature stop codon in E6, membrane-bound (HLA-G1–HLA-G4) isoforms have a unique truncated cytoplasmic tail comparing to other classic HLA class I molecules. Soluble HLA-G5 and HLA-G6 isoforms are resulted from a stop codon in intron 4, and HLA-G7 are generated from a stop codon in intron 2, which prevents the translation of their transmembrane domain (77, 78) (**Figure 2**).

Each HLA-G isoform has its unique extracellular structure. HLA-G1 is the only full-length isoform with extracellular α1, α2, and α3 domains; HLA-G2 has α1 and α3 domains; HLA-G3 has the only α1 domains; HLA-G4 has α1 and α2 domains. Similarly, HLA-G5 has the extracellular α1, α2, and α3 domains; HLA-G6 has α1 and α3 domains, and HLA-G7 has the only α1 domains. α1 and α2 domains form the peptide binding cleft, and α3 domain non-covalently bind to the light chain $\beta_2$-microglobulin ($\beta_2$m). Novel HLA-G isoforms such as lacking a transmembrane region and α1 domain have been predicted with RNAseq technology (79) (**Figure 3**). Moreover, higher molecular weight of HLA-G has been associated with post-translational modifications. Homo- and hetero-HLA-G dimers can be formed through intermolecular disulfide bonds with $Cys^{42}$ or $Cys^{147}$ in the extracellular α1 or α2 domain; others such as glycosylated, nitrated, and ubiquitinated HLA-G molecules have also been confirmed (80–83).

## HLA-G/ILTs BINDING

ILT-2 and ILT-4 belong to the type I transmembrane glycoproteins, which have four extracellular immunoglobulin-like domains (D1-D4), a transmembrane region, and an intracellular tail with four or three immunoreceptor tyrosine-based inhibitory motifs (ITIMs). ILT-2 can be found on a variety

**TABLE 1** | HLA-G expression in solid cancers.

| Cancers | Lesions | Method (Ab) | HLA-G (%) | Immuno-staining evaluation | Main findings | Ref. |
|---|---|---|---|---|---|---|
| Breast cancer | 39 | IHC (4H84) | 41% | 0, negative; 1–5% (1); 6–25% (2); 26–75% (3); 76–100% (4). | Associated with shorter disease-free survival. | (6) |
| | 58 | IHC (4H84) | 70.7% | Negative (0); 1–25% (1); 26–50% (2); 51–75% (3); >75% (4). | Associated with advanced disease stage. | (7) |
| | 235 | IHC (HGY) | 66% | Negative (–); <25% (+) and/or weakly; 25–50% and/or moderately (++); >50% and/or strongly stained (+++). | An independent prognosis factor. | (8) |
| | 501 | IHC (4H84) | 60% | Positive, any staining of tumor cells; Negative, no staining. | A prognostic factor among classical HLA class I negative patients. | (9) |
| | 52 | IHC (5A6G7) | 59.6% | Negative, <25% positivity; positive (>25% positivity. | Associated with aggressiveness. | (10) |
| | 45 | IHC(MEM-G/2) | 62.2% | Positive, >15% of staining. | Associated with shorter survival. | (11) |
| | 102 | IHC (4H84) | 94.1% | Negative (–); weak staining (+); moderate staining (++) and strong staining (+++). | HLA-G$^{low}$ is associated with higher overall and relapse-free survival rates. | (12) |
| | 73 | HC (MEM-G/1) | 43.8% | Positive, >25% of staining, irrespective of staining intensity. | Not associated with clinical parameters. | (13) |
| | 2,042 | IHC (4H84) | 24% | Positive, any staining of tumor cells; Negative, no staining. | Not associated with clinical outcome. | (14) |
| | HER2+ (n = 28) | WB (4H84) (5A6G7) | HLA-G/GAPDH ratio | High and low levels of protein expression were determined by median. | Among HER2+ tumors, patients with HLA-G6 low had a higher pathological complete response. | (15) |
| Colorectal cancer | 81 | IHC (4H84) (MEMG/1) (MEM-G/2) | 29% 35% 19% | Based on presence or absence of positive stained cells. | HLA-G expressed in majority primary tumors but not in associated liver metastasis. | (16) |
| | 201 | IHC (HGY) | 64.6% | Without staining (–); < 25% and/or weakly (+); 25–50% and/or moderately (++); > 50% and/or strongly stained (+++). | An independent prognosis factor. | (17) |
| | 102 | IHC (MEM-G/2) | 70.6% | Based on presence or absence of positive stained cells. | Associated with worse survival. | (18) |
| | 457 | IHC (4H84) | 70.7% | HLA-G positive when >5%, irrespective of staining intensity. | HLA-G expression >55% associated with worse prognosis. | (19) |
| | 285 | IHC (4H84) | 22.1% | Intensity of staining (absent, weak, moderate, or strong). | Associated with worse survival and disease-free survival. | (20) |
| | 484 | IHC (4H84) | 27.7% | Intensity of staining (absent or faint in <20%), weak (faint to weak in >20% but ≤70%), moderate (weak to moderate in >70%), or strong (intense in 20~70%). | Associated with presence of the Foxp3+ cells. | (21) |
| | 88 | IHC (4H84) | 59.1% | Total score of the proportion and intensity scores for negative and positive tumor cell (ranges = 0–9). Cut point scores for positive and negative tumor cells are ≥4. | Increased expression of HLA-G correlated with tumor node metastasis staging. | (22) |
| | 379 | IHC (4H84) (5A6G7) | 70.7% 60.4% | The percentage of HLA-G positive tumor cells based on presence of HLA-G staining while irrespective the staining intensity. HLA-G >5% in a section was considered as positive. Difference of the percentage of HLA-G positive tumor cells (ΔHLA-G) in the case-matched CRC samples was calculated by the percentage of HLA-G detected with mAb 4H84 subtracted that with mAb 5A6G7. According to value of ΔHLA-G, three groups were obtained: ΔHLA-Gneg (ΔHLA-G> –5.0%), ΔHLA-Gcom (–5.0%≤ΔHLA-G ≤ 5.0%), and ΔHLA-Gpos (ΔHLA-G>5.0%). | HLA-Gneg in 64(16.9%), ΔHLA-Gcom in 159 (42.0%), and ΔHLA-Gpos in 156 (41.2%), mAbs 4H84neg5A6G7pos in 44 (11.6) CRC cases was observed. Both ΔHLA-G and its subgroups mAbs 4H84$^{neg}$5A6G7$^{pos}$ and 4H84 pos5A6G7 neg status were significantly related to survival. | (23) |
| | 157 | Flow cytometry (MEM-G/09) | Median of HLA-G:14.9% (range: 1.8–80.0%) | Among EpCMA+ colorectal tumor cells. | Higher HLA-G percentage associated with patient poor survival. | (24) |

*(Continued)*

**TABLE 1** | Continued

| Cancers | Lesions | Method (Ab) | HLA-G (%) | Immuno-staining evaluation | Main findings | Ref. |
|---|---|---|---|---|---|---|
| Cervical cancer | 58 | IHC (5A6G7) | 75.86% | No expression (0); 1–30% (1); 31–70% (2); 71–100% positive cells (3). | An early marker for progression. | (25) |
| | 143 | IHC (4H84) | 60% | Membrane or combined membrane and cytoplasmic expression of HLA-G were interpreted as positive. | Associated with disease progression. | (26) |
| | 79 | IHC (5A6G7) | 31.6% | Low expression when no signal or discrete staining; high expression when moderate or intense staining. | HLA-G detected in 17 (32.7%) without and 8 (29.6%) with metastasis. | (27) |
| Endometrial carcinoma | 44 | IHC(4H84) | 55% | Negative (0); 1–5% (1); 6–10% (2); 11–25% (3); 26–50% (4); >50% (5). | Associated with disease stage. | (28) |
| | 525 | IHC (4H84) | 39.8% | Negative (0); 1–5% (1); 5–25% (2); 25–50% (3); 50–75% (4), and >75% (5). The intensity scored 0: absent, 1: weak, 2: moderate, 3: strong. The sum of both scores. A score of ≥2.5 considered as up-regulation of HLA-G. | Not associated with survival. | (29) |
| | 40 | IHC (4H84) | 40% | Both membrane-bound and cytoplasmic HLA-G expression were considered as positive. | Not associated with survival. | (30) |
| | 113 | WB (MEMG/1) | HLA-G/ GAPDH ratio | High and low levels of protein expression were determined by median. | Higher levels of HLA-G 56 kDa isoforms were observed in patients with metastases to lymph nodes | (31) |
| Esophageal cancer | 121 | IHC (HGY) | 90.9% | Without staining(–); <25% and weakly (+); 25~50% and moderately (++); >50% and strongly stained (+++). | An independent prognosis factor. | (32) |
| | 79 | IHC (4H84) | 65.8% | HLA-G expression was graded as: negative, 1~25% (1+), 26~50% (2+), 51~75% (3+), and >75% (4+), irrespective of staining intensity. | HLA-G is an independent prognosis factor. | (33) |
| | 60 | IHC (MEM-G/ 1) | 70% | Without staining (0); <25% (1+); 25~50% (2+); and >50% (3+). Negative and 1+ as HLA-G negative, 2+ and 3+ as HLA-G positive. | Associated with cancer cell differentiation, lymph node metastasis. | (34) |
| Ewing sarcomas | 47 (primary) | IHC (4H84) | 30% | Graded by low, intermediate or strong densities. | Associated with tumor infiltrating T cells. | (35) |
| | 12 (relapse) | | 33% | | | |
| Gastric cancer | 160 | IHC (HGY) | 71% | Without staining (–); <25% and/or weakly (+); with 25~50% and/or moderately (++); >50% of the cancer tissues and/or strongly stained (+++). | An independent prognosis factor. | (36) |
| | 52 | IHC (5A6G7) | 31.% | HLA-G positivity when >10%. | An independent prognosis factor. | (37) |
| | 179 | IHC (4H84) | 49.7% | Negative; 1~25% (+); 25~50% (++); >50% (+++). | An independent prognosis factor. | (38) |
| Glioblastoma | 108 | IHC (MEM-G/ 2) | 60.2% | No details described. | HLA-G-negative patients were alive longer than HLA-G positive patients. | (39) |
| Hepatocellular carcinoma | 173 | IHC (MEM-G/ 1) | low (43%) high (57%) | The density of HLA-G staining evaluated with computerized image system. | Associated with poor survival and increased recurrence; | (40) |
| | 36 | WB (MEM-G/ 1) | 66.7% | No details described. | An independent prognosis factor. | (41) |
| | 219 | IHC (4H84) | 50.2% | Negative, and positive grouped as 1~25%, 26~50%, 51~75%, and >75%. | Associated with advanced disease stage. | (42) |
| Lung cancer | 106 | IHC (HGY) | 75% | Without staining (–); <25% and weakly (+); 25~50% and moderately (++); >50% and strongly stained (+++). | An independent prognosis factor. | (43) |
| | 101 | IHC (4H84) | 41.6% | Negative (0), 1~25% (1), 26~50% (2), and >50% (3), irrespective of staining intensity. | Associated with advanced disease stage. | (44) |
| | 131 | IHC (5A6G7) | 34% | Negative ≤ 5% and positive >5%. | Predominately expressed in adenocarcinoma. | (45) |
| Lymphoma (classical Hodgkin) | 175 | IHC (MEM-G/ 1) | 54% | Positive when >50% of neoplastic cells showed stronger staining. | Associated with absence of MHC class I expression on HRS cells and EBV negative status. | (46) |
| | 20 | IHC (4H84) | 55% | Negative staining (0), <25% (1), 26~50% (2), 51~75% (3), 76~100% (4). | Different patterns of HLA-G expression associated with different outcomes. | (47) |
| Lymphomas (Diffuse Large B-Cell) | 148 | IHC (4H84) | 24% | Positive when >25% of lymphoma cells expressed intermediate/ strong staining. | Negative HLA-G expression associated with worse survival. | (48) |

*(Continued)*

**TABLE 1** | Continued

| Cancers | Lesions | Method (Ab) | HLA-G (%) | Immuno-staining evaluation | Main findings | Ref. |
|---|---|---|---|---|---|---|
| Lymphomas (cutaneous T- and B-cell) | 45 | IHC (4H84) | 51% | HLA-G positivity as a strong (numerous cells) or as a single-cell positivity (scant, scattered cells throughout the infiltrate). | Associated with high-grade histology and advanced stage in CTCL. | (49) |
| Nasopharyngeal carcinoma | 552 | IHC (4H84) | 79.2% | Intensity as (neg); weak (1); moderate (2); strong (3). Percentage <5% (0); 5–25% (1); 26–50% (2); 51–75% (3); 76–100% (4). A score by adding intensity and positive cells. | Associated with poor prognosis, disease recurrence or metastasis. | (50) |
| Oral squamous cell carcinoma | 60 | IHC (MEM-G/2) | 50% | An immunoreactive score (IRS) calculated by multiplying the percentage and staining intensity. IRS = 0 (negative); <2 (low); >2 (high). | Lower HLA-G expression associated with longer survival. | (51) |
| Ovarian cancer | 40 | IHC (4H84) | Low (55%) moderate (20%) strong (25%) | 0–25% stained tumors and mild staining (1+); 25–50% and moderately staining (2+); >50% and strongly staining (3+). | HLA-G expression >17% associated with poor survival. | (52) |
| | 34 | IHC (MEM-G/2) | 35% | No details described. | Associated with high-grade histology. | (53) |
| | 118 | IHC (5A6G7) | 79.7% | Percentage of stained cells >5% (+); <5% (–). | Not associated with clinical parameters. | (54) |
| | 62 | IHC (4H84) | 72.4% | The scores correspond to the percentage of positive tumor cells of <1% (score 0); 1–5% (score 1), 6–25% (score 2), 26–50% (score 3), and >50% (score 4). Score 1 and score 2 were considered as "low positive percentage cells" scores. Whereas, score 3 and score 4 were considered as "high positive percentage cells" scores. | Positive HLA-G expression was highly represented in patients with ovarian carcinoma recurrence. | (55) |
| Pancreatic adenocarcinoma | 122 | IHC (Rabbit polyclonal) | low (36.1%) high (63.9%) | 0, none; 1, ≤25%; 2, 26~50%; 3, >50%). Intensity (0, none; 1, weak; 2, moderate; 3, strong). | An independent prognosis factor. | (56) |
| | 42 | IHC (4H84) | 66% | 1–25% (negative), 26–50%, 51~75%, and >75%, irrespective of staining intensity. | Associated with advanced stages | (57) |
| | 158 | IHC (not described) | 39.2% | Negative: <5%; local: 5–75%; diffuse: >75%, irrespective of staining intensity. | Associated with worse survival. | (58) |
| | 243 | IHC (4H84) | 36.7% | Strongly (+++), with almost all cancer cells (≥90%) staining strongly; moderately (++), with <90 and ≥50% of cancer cells staining strongly; weakly positive (+), with <50 and >10% of cancer cells staining strongly or >5% of cancer cells staining weakly; negative (–), with ≤5% of cancer cells staining. | High HLA-G associated with both shorter overall survival and disease-free survival. | (59) |
| Thyroid carcinoma | 138 | IHC (5A6G7) | 90.6% | Without staining (–); <25% (+); 25~50% (++); >50% of cell staining (+++). | Associated with poor prognosis | (60) |
| | 70 | IHC (MEM-G/2) | 44.3% | No details described. | Associated with lymph node metastasis. | (61) |

of immune cells, such as subpopulations of T cells, B cells, natural killer (NK) cells, myeloid-derived suppressive cells (MDSCs), dendritic cells (DCs), and monocytes/macrophages. ILT-4 is not expressed on lymphocytes, but on monocytes/macrophages, neutrophils, basophils, DCs, and MDSCs (84, 85).

A recent study revealed that ILT-2/-4 extracellular D1D2 are responsible for the interaction with HLA-G binding, while D3D4 act as a scaffold (86). Also, ILT-2/-4 are more accessible to the HLA-G dimer binding than that of HLA-G monomer, leading to much stronger inhibitory signals. ILT-2 and ILT-4 extracellular D1D2 binds to the extracellular α3 domain of HLA-G, but structurally dependents. ILT-2 only binds to the HLA-G heavy chain associated with $\beta_2$m, while ILT-4 can bind to both $\beta_2$m free HLA-G heavy chain and HLA-G heavy chain with $\beta_2$m. Moreover, residues $Tyr^{38}$ and $Tyr^{76}$ in ILT-2 are responsible for binding to HLA-G $Phe^{195}$ in the α3 domain, whereas residues $Tyr^{36}$ and $Arg^{38}$ in ILT-4 interact with the $Phe^{195}$–$Tyr^{197}$ loop in the HLA-G α3 domain (87). The different binding sites between ILT-2/-4 and HLA-G could be an explanation for the higher affinity of ILT-4 than the affinity of ILT-2 when they interact with HLA-G (88). Based on the different extracellular structure of HLA-G isoforms and ILT binding characteristics, ILT2 can bind to the $\beta_2$m associated HLA-G1 and HLA-G5 isoforms. However, ILT-4 can bind to both $\beta_2$m associated or $\beta_2$m free HLA-G isoforms, which include HLA-G1, -G2, -G4, -G5 and HLA-G6 isoforms (**Figure 4**).

Taking advantage of the early studies on fetal-maternal immune tolerance, HLA-G-mediated immune inhibition has been well-acknowledged in the broader spectrum of health and disease situations. HLA-G expression favors the acceptance of

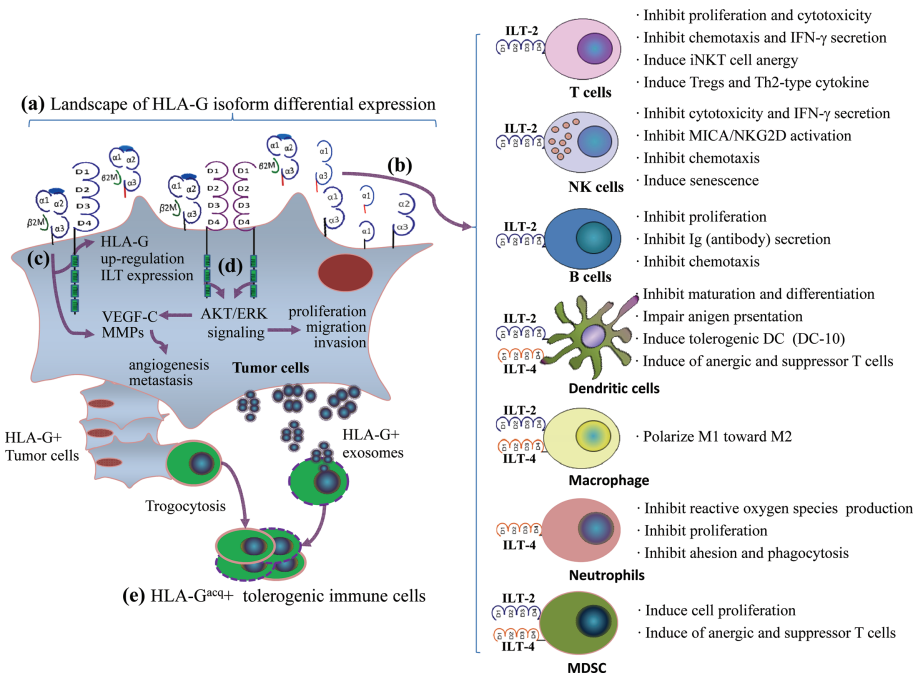


**FIGURE 1** | A comprehensive immune suppression mediated by HLA-G/ILTs engagement in cancer development. **(A)** Distinct profiles of HLA-G isoform expression in an individual cancer patient. The heterogeneous landscape of HLA-G isoform differential expression among cancer patients can be temporal and spatial dependent. **(B)** Immune inhibitory receptors ILT-2 and ILT-4 are expressed on different immune cell. ILT-2 recognizes HLA-G1 and HLA-G5 while ILT-4 recognizes HLA-G1, -G2, -G5, and HLA-G6 isoforms. HLA-G isoform-dependent ILT-2 and ILT-4 engagement induces a wide spectrum of immune suppression which benefits cancer cell escaping from host immune surveillance and anti-tumor immunity. **(C)** HLA-G expression up-regulates intratumor ILTs and MMPs expression. **(D)** ILTs induce VEGF-C expression and enhance cancer cell proliferation, migration and invasion through AKT/ERK signaling, which favors cancer cell angiogenesis and metastasis. **(E)** In addition to direct binding between HLA-G and ILTs, immune cells acquire HLA-G from neighboring HLA-G+ cancer cells through contact-dependent trogocytosis and from cancer cell derived HLA-G-bearing exosomes in a long-distance. HLA-G acquired immune cells became tolerogenic phenotype and immune functions are impaired.

allograft organ transplantation, whereas it provides an additional strategy for cancer cells to escape from immune surveillance and clearance (89, 90). HLA-G/ILTs engagement activates the phosphorylation of the ITIMs contained in the tyrosine residues, which present docking sites for Src homology 2 (SH2) protein tyrosine phosphates SHP-1 and SHP-2, thus initiating an inhibitory signaling cascade. Meanwhile, ITIM-dependent recruitment of SHP1/SHP2 can markedly suppress the ITAM activated Syk/Src signal cascades in immune cell activation (91).

## CHALLENGES IN HLA-G/ILTs TARGETED SOLID CANCER IMMUNOTHERAPY

One strategy successfully deployed by cancer cells for immune evasion is the impairment of the classical HLA class I and II antigens to hide infected cells from T cell recognition, while aberrant induction of HLA-G expression by cancer cells makes host anti-tumor immune system rather vulnerable (92). Though development of HLA-G/ILTs interaction targeted ICIs is promising for cancer immunotherapy, much real-world information on HLA-G/ILTs status is extremely necessary for both future basic and clinical investigations.

### Lack of HLA-G Isoform-Specific Monoclonal Antibodies

The most fundamental task is to develop HLA-G molecule universal or distinct HLA-G isoform-specific mAbs. As high as 80% amino acid sequence identity sharing in the extracellular domain of all HLA class I antigens, only distinct feature of HLA-G is its molecular weight of 39 kDa (HLA-G1 isoform) which is less than 45 kDa of the other classical HLA class I antigens (93), and this is rather similar to other non-classical HLA class I antigens including HLA-E and HLA-F (https://www.uniprot.org/uniprot/P17693; https://www.uniprot.org/uniprot/P13747; https://www.uniprot.org/uniprot/P30511). CLUSTALO sequence alignment results showed that, among HLA-G, HLA-F, and HLA-E molecules, the full-length amino acid sequence are identity round 62%, 75.9% between HLA-G and HLA-F, 71.2% between HLA-G and HLA-E, and 40.1% between HLA-E and HLA-F (**Supplementary Figure 1**). Thus, the cross-reactivity of anti-HLA-G mAbs to other classical and non-classical HLA class I antigens remains a huge task to be improved

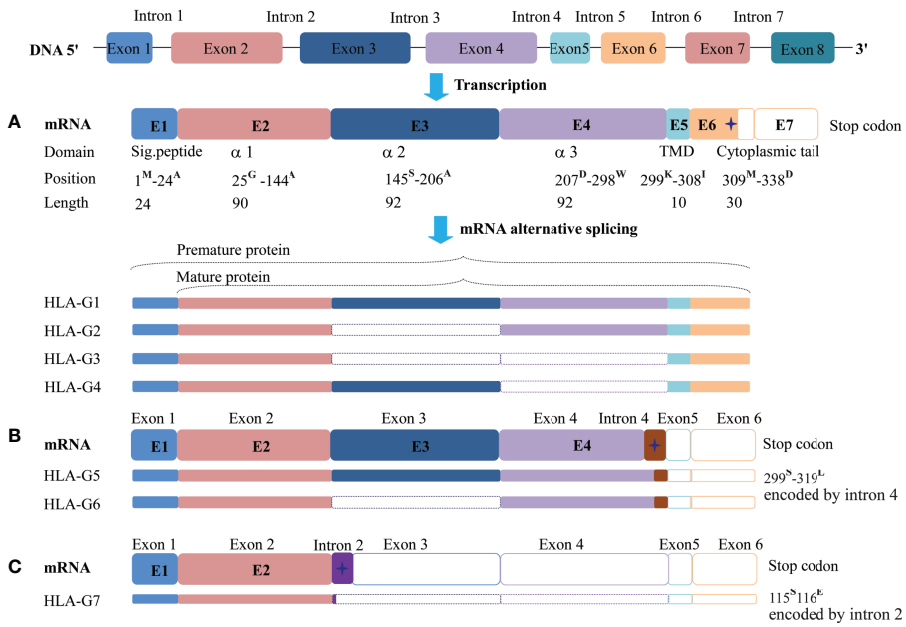


**FIGURE 2** | Seven identified HLA-G isoforms generated from its primary transcript alternative splicing. **(A)** The heavy chain of membrane-bound isoforms HLA-G1, -G2, -G3, -G4 generated by an mRNA containing a stop codon in exon 6. **(B)** Soluble isoforms HLA-G5 and HLA-G6 generated by an mRNA with a pre-stop codon in intron 4, which terminates transmembrane and cytoplasmic tail transcription. **(C)** Soluble isoforms HLA-G7 generated by an mRNA with a pre-stop codon in intron 2, which terminates the following domain transcription. Sig.peptide, Signal peptide; TMD, transmembrane domain; ✦, stop codon. The superscript capital letter represents amino acid at the position.

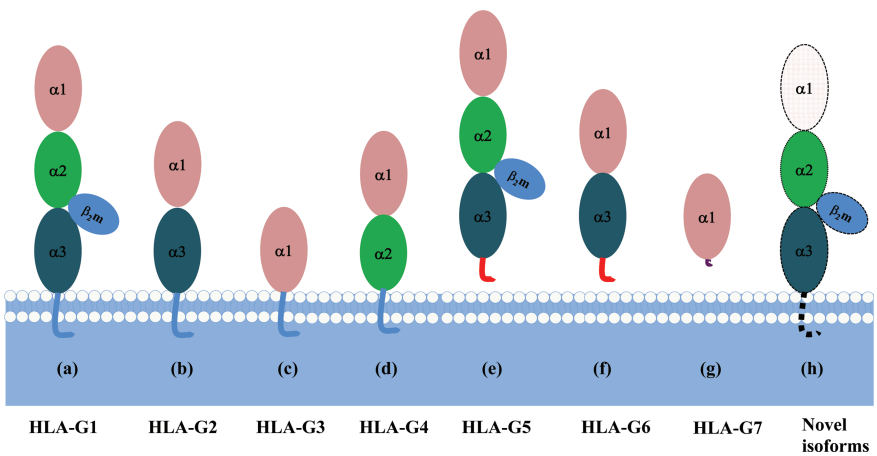


**FIGURE 3** | A schematic structure of HLA-G isoforms. **(A)** HLA-G1 have α1, α2, and α3 extracellular domains; **(B)** HLA-G2 have α1, and α3 extracellular domains; **(C)** HLA-G3 have α1 extracellular domains; **(D)** HLA-G4 have α1, and α2 extracellular domains; **(E)** HLA-G5 have α1, α2, and α3 extracellular domains; **(F)** HLA-G6 have α1 and α3 extracellular domains; **(G)** HLA-G7 has α1 extracellular domain followed by two C- terminal amino-acids encoded by intron 2; **(H)** Novel HLA-G isoforms such as lacking a transmembrane region and α1 domain have been predicted, but their structure remains confirmed.

(94). Indeed, cross-reactivity of the most widely used such as mAb 4H84 for denatured HLA-G form has been observed to react with other HLA class I antigens (95, 96). Moreover, due to *HLA-G* primary transcript alternative splicing, in addition to seven ever-identified HLA-G isoforms and more novel isoforms can be expected, lack of HLA-G isoform-specific mAb prevents advances in characterizing their biological functions and clinical significance (97). To be noted, a panel of novel HLA-G isoforms predicted by deep transcriptome analysis have been reported. Among these isoforms, HLA-G1L have extra five amino acids (NKTPR) ahead

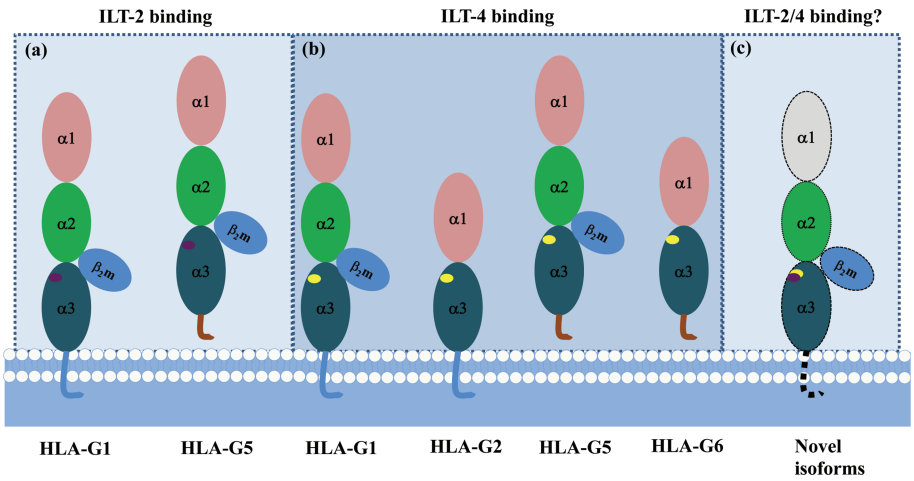


**FIGURE 4** | Different binding sites between HLA-G/ILT-2 and HLA-G/ILT-4. ILT-2 residues Tyr38 and Tyr76 bind Phe195 in HLA-G α3 domain, ILT-4 residues Tyr36 and Arg38 bind Phe195–Tyr197 loop in HLA-G α3 domain. **(A)** ILT-2 binds HLA-G heavy chain associated with $\beta_2$m (HLA-G1 and HLA-G5). **(B)** ILT-4 binds both $\beta_2$m-free (HLA-G2 and HLA-G6) and $\beta_2$m-associated (HLA-G1 and HLA-G5) HLA-G heavy chain. **(C)** A panel of novel HLA-G isoforms including isoforms without α1 domain and transmembrane region, or with an extended 5'-region generated by HLA-G mRNA alternative splicing were predicted. However, molecular structure of these novel isoforms and remain to be identified, and interaction with ILTs is unknown yet. and represent ILT-2 and ILT-4 binding site in HLA-G isoforms.

the beginning residue Methionine at the N-terminal ends, others such as isoforms lack α1 or both α1 and α2 extracellular domains, and novel soluble HLA-G isoforms with distinct C-terminal ends generated by skipping exons 5 and 6.

Among anti-HLA-G antibodies, mAbs 4H84 and 5A6G7 are with known recognizing epitope in HLA-G heavy chain. mAb 4H84 generated by amino acid residues from 61 to 83 (in α1 domain HLA-G, which probes all denatured HLA-G isoforms (98). mAb 5A6G7 generated by a 22-mer C-terminal amino acid sequence in HLA-G5 and HLA-G6, which probes both native and denatured HLA-G5 and HLA-G6 isoforms (99, 100). mAbs 4H84 and 5A6G7 are the most widely used antibodies for evaluating total HLA-G (mAbs 4H84 and MEM-G/1) or HLA-G5/6 isoforms (mAb 5A6G7) respectively in malignant lesions with immunohistochemistry. As a result, novel isoforms without α1 domain can't be detected by the mAb 4H84 (23, 79). In this scenario, the interpretation of clinical significance of HLA-G in cancers seems rather premature unless more reliable specific anti-HLA-G mAbs are used (**Figure 5**).

Furthermore, evaluation criteria for lesion HLA-G expression including staining protocols, cut-off levels, and cross-assay concordance are far from standardized, which could dramatically affect the definition of HLA-G expression and interpretation of its clinical significance, even with a same mAb to detect HLA-G within a certain type of cancer (62, 96). In this context, a reference criterion should be recommended by international community for HLA-G staining to minimize discrepancies across studies is urgently warranted.

Also, given different binding specificity of HLA-G between ILT-2 and ILT-4 resulted from its extracellular structure, that ILT-2 binds HLA-G1 and HLA-G5 while ILT-4 binds HLA-G1, -G2, -G4, -G5, and HLA-G6 isoforms, the landscape of HLA-G isoforms and their degree of expression in cancers can tremendously affect the benefits of HLA-G/ILTs based cancer immunotherapy.

## Heterogeneity of HLA-G Expression in Malignancies

Accumulating evidence solidify the concept that tumor heterogeneity, including inter-patient, inter-tumor, and intra-tumor heterogeneity, is the main cause for variable responses and clinical outcomes to anti-cancer therapy (101). Since the first report HLA-G expressed in tumors, the degree or proportion of HLA-G expression in thousands of malignant lesions among over thirty different types of cancers have been explored. Data revealed that HLA-G expression is restricted to malignant lesions, but not in adjacent non-tumorous tissues, and that neo-HLA-G expression is strongly related to metastasis, advanced disease stage, poor prognosis, and clinical outcome (89). However, inter-patient, inter-tumor, and intra-tumor heterogeneity of the HLA-G expression in each histopathological type of malignancies is also evident (102, 103). In addition to the HLA-G heterogeneity caused by tumor cell itself, such as clonal growth with genetic alterations, epigenetic and post-translational modifications, explanation of HLA-G expression could be biased due to usage of different current available anti-HLA-G monoclonal antibodies, and different assay protocols (62, 96). Very recently, using the method of flow cytometry, our data showed that HLA-G expression in 157 epithelial cell adhesion molecule (EpCAM) positive-gated colorectal tumor lesions is with a median of 14.90% (range: 1.81~79.90%) (24).

For an example, the inter-patient proportion of HLA-G expression in cancers has been observed from 24 to 94.1% in breast cancers (12, 14), and from 22.1 to 70.7% in colorectal cancers (19, 20). Noteworthy, only few studies on the

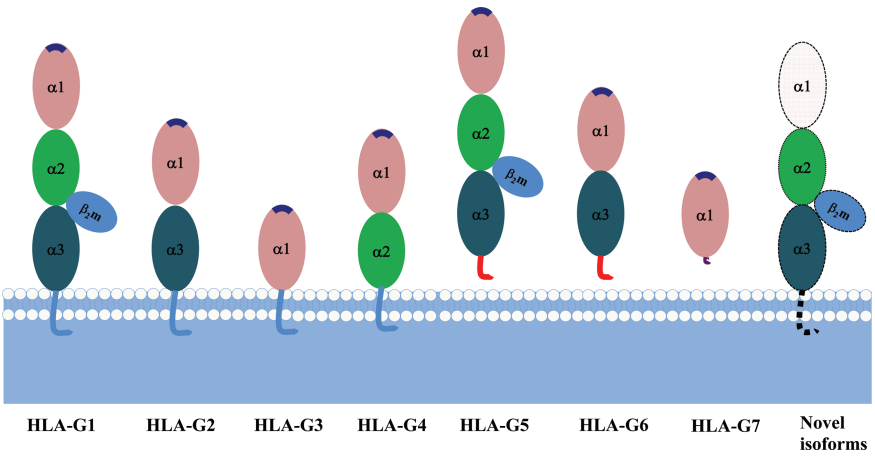


**FIGURE 5** | Epitopes in HLA-G recognized by mAbs 4H84 and 5A6G7. Among HLA-G antibodies, only mAbs 4H84 and 5A6G7 were generated with definite immunogen epitopes. mAb 4H84 generated by 61st~83rd amino acids (EEETRNTKAHAQTDRMNLQTLRG) in HLA-G α1 domain which recognizes denatured heavy chain of all seven identified HLA-G isoforms containing α1 domain. mAb 5A6G7 generated by 21-mer C-terminal amino acid (SKEGDGGIMSVRESRSLSEDL) in HLA-G5 and -G6 isoforms, which recognizes both native and denatured heavy chain of HLA-G5 and HLA-G6 isoforms. Novel HLA-G isoforms such as isoforms without α1 domain and transmembrane region, or with an extended 5'-region were predicted. However, no current antibody is available to detect. and represent mAb 4H84 and 5A6G7 recognizing site in HLA-G isoforms.

intrapatient inter-tumor and intra-tumor heterogeneity of the HLA-G expression were available. In a cohort of 136 primary cervical cancers, inter-tumor heterogeneity of the HLA-G expression was detected in 25% of these lesions and 11% of case-matched lymph node (LN) metastases. Among pathological subtypes, HLA-G was positive in 22% in squamous cell carcinoma lesions and 20% in LN metastatic tissues, 31% in adenocarcinoma lesions, and 28.0% in their LN metastatic samples. In patients with invasive cervical cancer, positive proportion of HLA-G expression was found in 31.6 and 29.6% of the primary and LN metastatic lesions, respectively (104). Similarly, a study by Swets et al. (16) showed that HLA-G expression was found in 29% of the primary colorectal cancer and 30% of the corresponding liver metastases. Finally, regarding the intra-tumor heterogeneity of the HLA-G, Rouas-Freiss et al. (103) reported that, among 19 clear cell renal-cell carcinoma lesions, the proportion of HLA-G expression varies dramatically on CA9+ clear cell renal-cell carcinoma cells in different zones in each sample, which could be ranged from negative to almost totally positive for HLA-G expression. Also with clear cell renal-cell carcinoma lesions, a study by Tronik-Le Roux et al. (79) released that HLA-G isoform expression including HLA-G1, -G5, and HLA-G6 are extremely heterogeneous among distinct subcellular locations and zones within a same tumor. In a serial section study with colorectal and esophageal cancer lesions, our recent findings further revealed that intratumor heterogeneous expression of HLA-G is a very frequent phenomenon among different zones within a tumor (102).

## Native HLA-G Isoform Expression in Cancer Lesions Needs Evaluated

As aforementioned, most currently available information of HLA-G expression in cancer lesions were evaluated by immunohistochemistry with the mAbs 4H84 and/or 5A6G7, which represents all α1 domain containing HLA-G or HLA-G5/6 isoform expression. Whether these data are consistent with the levels of tumor cell surface HLA-G expression remain elusive. Previous evidence showed that no correlation has been established between the degree of cancer lesion HLA-G5/6 expression evaluated with immunohistochemistry and peripheral soluble HLA-G levels (42). Given the fact that HLA-G/ILTs interaction is conformation dependent, tumor cell surface native or conformational HLA-G expression be evaluated with assays such as flow cytometry is necessary (105). To address this issue, more reliable and specific anti-native or -conformational HLA-G mAbs are yet to be explored.

## OTHER HLA-G RECEPTORS

In addition to the ILT-2 and ILT4, receptors including killer inhibitory receptor (KIR) 2DL4/CD158d, CD8, CD160 could also bind HLA-G (106). To be noted, NKG2A/CD94 has been recently reported which could bind to allelic specific products of HLA-G (107).

KIR2DL4 is a member of the killer cell immunoglobulin (Ig)-like receptor (KIR) family, but with an atypical feature owing to a D0 and D2 hybrid extracellular domain, a positively charged arginine residue in the transmembrane region and one ITIM domain in its cytoplasmatic tail. The charged arginine residue enables KIR2DL4 to associate with the Fc fragment receptor γ (FcRγ), which contains two cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs). With this unique structure, KIR2DL4 is of both the activation (ITAM) and inhibitory (ITIM) signaling domains (108). The biological function of KIR2DL4 and HLA-G binding is to work as an

activating receptor, which induces strong pro-inflammatory cytokine and chemokine immune responses through the endosomal DNA-dependent protein kinase (DNA-PKcs) signaling pathway, but not the NK cell cytotoxicity (109). KIR2DL4 is predominately expressed in decidual NK cells and HLA-G/KIR2DL4 interaction plays critical roles in the regulation of maternal-fetal immune microenvironment, spiralartery remodeling and fetal growth (4).

The heterodimer NKG2A/CD94, a member of the C-type lectin-like receptor family, is a well-known immune inhibitory receptor for HLA-E binding. NKG2A/CD94 is mainly expressed on CD8+ T lymphocytes and subsets of NK cells (110). Additionally, NKG2A/CD94 has recently been found to be an HLA-G allelic product dependent receptor. Hò et al. (107) revealed that a remarkably higher binding affinity was observed for the HLA-G*01:04/NKG2A/CD94 interaction than those for the HLA-G*01:03/NKG2A/CD94 and HLA-G*01:01/NKG2A/CD94 interactions. Moreover, no engagement was observed between the activating receptor NKG2C/CD94 and HLA-G. HLA-G allelic product dependent binding of NKG2A/CD94 could result from the single amino acid residue in the HLA-G*01:01 heavy chain differing from HLA-G*01:04 (p.110L > I) and HLA-G*01:03 (p.31T > S), and these different residues may affect the peptide repertoire and receptor recognition (111). Consequently, different biological function modulation could be expected for the different allelic HLA-G molecules on the immune cells expressing NKG2A/CD94.

Other receptors are the glycosylphosphatidylinositol-anchored receptor CD160, which is expressed on activated endothelial cells, $CD16^{+}CD56^{dim}$ NK cells, and $CD8^{+}$ T cells (112, 113). CD160 signaling depends on adapter proteins, such as the phosphoinositide-3 kinase, to activate endothelial cell migration and angiogenesis, and immune cells, such as cytokine releasing and target cell lyses (112, 114). Moreover, the cytotoxic T cell surface marker CD8 is reported to interact with the HLA-G protein, which can induce apoptosis of the $CD8^{+}$ T cells and $CD56^{+}CD8^{+}$ NKT cells through the Fas/FasL pathway (115).

## CONCLUSIONS AND PERSPECTIVES

In most scenarios, HLA-G/ILTs interaction promotes cancer cells to escape immune surveillance and anti-tumor immunity (97, 116). Interference or blockade of HLA-G/ILTs interaction can restore host anti-tumor immune responses, which providing a strong rationale and opportunities to develop HLA-G/ILTs-targeted ICIs for solid cancer immunotherapy. Indeed, early clinical trials based on this immune checkpoint is being launched for different advanced solid cancer treatment[2]. However, many challenges remain to be addressed.

First, multiple identified HLA-G isoforms and more can be expected, which have distinct extracellular domain(s) for each of them. This information indicates that biological function of different isoforms is diverse. Being lack of isoform-specific antibodies, their tumor tissue expression characteristics and clinical significance is unclear. Currently available mAb 4H84, which probes all isoforms containing α1 domain, can't distinguish either a distinct isoform or combination of different isoforms expressed on tumor tissues. Novel isoforms without α1 domain can't be detected with any available mAbs which can be ignored in tumor tissues. These findings sharply compounded the clinical relevance of HLA-G in tumor patients (62). Second, being lack of international validated or recommended assay protocols, both performance and cut-off points are diverse and unconcordance in interpretation of HLA-G expression across studies is rather common (117). Third, being the landscape of HLA-G isoform expression in tumor tissues can't be specified, application of HLA-G/ILTs-targeted ICIs for cancer treatment can be aimless. Furthermore, HLA-G/ILTs engagement is isoform dependent, where HLA-G1 and HLA-G5 binds ILT-2, and HLA-G1, -G2, -G4, -G5, and HLA-G6 binds ILT-4 (118). Differential expression of HLA-G isoforms does exist in tumor cells whereas the underlying mechanisms are yet to be explored. In this scenario, patient individualized landscape and degree of HLA-G isoform expression should be defined before therapy due to which can tremendously affect clinical benefits of HLA-G-based solid cancer immunotherapy.

In summary, exploration of more reliable and HLA-G isoform-specific antibodies, implementation of international community validated HLA-G detection protocols, deeper insight of patient individualized landscape of HLA-G expression, and development of isoform matched both HLA-G and ILT-2/-4 blocking antibodies, are future directions warranted for the HLA-G/ILTs-targeted solid cancer immunotherapy. Despite these challenges, owing to HLA-G expression is restrict to malignant tissues, and HLA-G/ILTs signaling is involved in a broader spectrum of immune responses than CTLA-4/B7 and PD-1/PD-L1 does, the clinical effects of the immune checkpoint HLA-G/ILTs is optimistic.

## AUTHOR CONTRIBUTIONS

Conceptualization, data curation, funding acquisition, writing — original draft, review and editing: W-HY and AL. Both 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.2021.698677/full#supplementary-material

**Supplementary Figure 1 |** CLUSTALO sequence alignment either among or between full-length HLA-G, HLA-F, and HLA-E molecules. Results of sequence alignment **(A)** among full length of HLA-G, HLA-F, and HLA-E molecules. **(B)** Between full length of HLA-G and HLA-F molecules. **(C)** Between full length of HLA-E and HLA-F molecules. **(D)** Between full length of HLA-E and HLA-G molecules.

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# Altered Frequency and Phenotype of HLA-G-Expressing DC-10 in Type 1 Diabetes Patients at Onset and in Subjects at Risk to Develop the Disease

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Type 1 diabetes (T1D) is a chronic autoimmune disease resulting in progressive destruction of β-cells. Several factors affecting lymphocyte and antigen-presenting cells, including dendritic cells (DCs), contribute to defective maintenance of tolerance in T1D. DC-10 are a subset of human DCs involved in IL-10-mediated tolerance. A precise monitoring of DC-10 in the peripheral blood is possible thanks to the discovery of specific biomarkers. DC-10, being cells that naturally express HLA-G, may be used for the appropriate staging of the disease. By enumerating and phenotypically characterizing DC-10 in the peripheral blood of subjects at different stages of T1D development—first-degree relatives (FDRs) of T1D patients, without ($Ab^{neg}$) or with ($Ab^{pos}$) autoantibodies, T1D patients at onset, and age-matched healthy controls (HCs)—we showed that DC-10 contain a high proportion of HLA-G-expressing cells as compared with monocytes. We reported that a low frequency of DC-10 during disease development is paralleled with the increased proportion of pro-inflammatory cDC2 cells. Moreover, DC-10 number and phenotype differ from $Ab^{neg}$ FDRs, $Ab^{pos}$ FDRs, and T1D patients compared with HCs, and DC-10 from T1D patients express low levels of CD83. Finally, multiple regression analysis, considering DC-10 and HLA-G-related parameters, showed that $Ab^{neg}$ FDRs are more similar to subjects with autoimmunity than to HCs. This is the first demonstration that impairment in DC-10 number and phenotype, specifically CD83 expression, is associated

with risk of developing T1D, suggesting a possible use of CD83$^+$ DC-10 to stratify individuals at risk of T1D in conjunction with classical prognostic factors.

**Keywords: DC-10, type 1 diabetes (T1D), HLA-G, tolerogenic dendritic cells, pro-inflammatory myeloid cells, first-degree relatives (FDRs)**

## INTRODUCTION

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease characterized by progressive destruction of pancreatic β cells (1). Both genetic and environmental factors, by affecting lymphocyte activities (2) and antigen-presenting cells (APCs), contribute to defective induction/maintenance of tolerance in T1D. Dendritic cells (DCs) are professional APCs implicated in dictating the activation and differentiation of T cells. Conventional (c)DCs promote the initiation of pathogenic T-cell responses, whereas non-activated DCs, or specialized subsets of DCs, termed tolerogenic (tol)DCs, induce or activate regulatory T cells leading to tolerance (3). Aberrant activation of cDCs or defects in tolDC activities contribute to breaking self-tolerance in T1D (4). Activated cDCs, which stimulate auto-reactive T cells and secrete pro-inflammatory cytokines, and defective tolDCs have been reported in pancreatic islets and lymph nodes of T1D patients (5, 6). Moreover, cDC subsets have been described in the peripheral blood of T1D patients (4). The lack of specific biomarkers for identifying tolDCs has limited their identification and study in T1D patients.

DC-10 are a subset of HLA-G-expressing tolDCs, which induce adaptive T regulatory type 1 (Tr1) cells *in vitro via* the IL-10-dependent HLA-G/ILT4 pathway (7). HLA-G is a well-recognized immune-modulatory molecule that regulates innate and adaptive immune responses and promotes tolerance (8). Polymorphisms located at the 3′ un-translated region (UTR) of *HLA-G* locus, which can arrange in a limited number of haplotypes and finely tune the HLA-G expression (9), have been associated with T1D (10–12). Moreover, the tolerogenic potential of DC-10 is associated with genetic variations of the *HLA-G* locus encoding for elevated levels of protein expression (13). The discovery that DC-10 are present *in vivo* (7, 14), modulate T-cell responses, induce Tr1 cells *in vitro* in a HLA-G-dependent manner, and are associated with tolerance (15) prompted us to postulate that DC-10 represent an important subset of naturally occurring HLA-G-expressing DCs involved in promoting tolerance in T1D than can be used as biomarker for staging the disease in conjunction with classical prognostic factors. The discovery of CD141, CD163, CD14, and CD16 as specific DC-10 biomarkers (14) allows a precise monitoring of DC-10 in the peripheral blood and tissue.

In this study, we evaluated the presence and frequency of HLA-G-expressing DC-10 in the peripheral blood of first-degree relatives (FDRs) of T1D patients with negative (Ab$^{neg}$) or positive (Ab$^{pos}$) serology, T1D patients at onset, and age-matched healthy controls (HCs). We show that DC-10 number and phenotype differ from Ab$^{neg}$ FDRs, Ab$^{pos}$ FDRs, and T1D patients, compared with HCs. In addition, in T1D patients, the ratio between tolerogenic DC-10 and pro-inflammatory cDC2 is weighted toward the immunogenic compartment. Finally, comparison of six variables associated with DC-10 and HLA-G revealed that Ab$^{neg}$ FDRs are different from HCs and that a progressive decrease of CD83$^+$ DC-10 percentage is associated with disease development.

## MATERIALS AND METHODS

### Study Subjects

Adult HCs (>18 years) were admitted to the "Servizio Medicina Preventiva" at the San Raffaele University Hospital, Milan, Italy. Pediatric HCs (≤18 years) were children with no immune system-related diseases undergoing surgery only for congenital disease at the Orthopedic Pediatric Department at the San Raffaele Hospital. Both adult and pediatric HCs had no family members with T1D.

Adult T1D patients were recruited at the Endocrinology Department. For genetic analysis, both new-onset (blood withdrawal within 3 months from diagnosis) and long-standing adult T1D individuals were included in the study. Pediatric T1D subjects were enrolled at the Pediatric and Neonatology Department at the San Raffaele Hospital. Only children with a blood withdrawal within the first 10 days after diagnosis (new-onset T1D) were included in the study. The diagnosis of T1D was made based on sustained hyperglycemia (documented by repeated glucose measurements and HbA1c measurement), fasting C-peptide levels, 1.0 ng/ml, or the presence of at least one islet-specific autoantibody (16, 17).

FDRs of T1D patients were recruited under the umbrella of the Type 1 diabetes TrialNet Pathway to Prevention Trial (TN01) at the TrialNet Clinical Center of the San Raffaele Hospital. These subjects were divided in two groups based on the presence of circulating islet-specific autoantibodies (e.g., GAD65, IA2, mIAA, or ZnT8) at blood withdrawal: FDRs without (Ab$^{neg}$) or with at least one (Ab$^{pos}$) autoantibody.

Human peripheral blood was collected upon informed consent in accordance with the Declaration of Helsinki and with local ethical committee approvals: IRB#DRI-003 for HCs and T1D patients and IRB#NHPROT32803-TN01 for Ab$^{neg}$ and Ab$^{pos}$ subjects. For pediatric subjects, the informed consent has been provided by parents. All the individuals included in the study were European Caucasians, and samples were collected from 2016 to 2017. A full blood count laboratory test was performed for all the samples received. Subjects with ongoing infections were excluded from the analysis.

Characteristics of subjects enrolled in the study are listed in (**Table 1**) and in (**Table 2**).

### Flow Cytometry Analysis

The frequencies of DC-10 and other monocyte/myeloid populations, and the HLA-G, ILT4, CD83, and HLA-DR

**TABLE 1** | Characteristics of subjects enrolled in the study.

| Variables | HCs (n = 40) | $Ab^{neg}$ FDRs (n = 37) | $Ab^{pos}$ FDRs (n = 21) | T1D (n = 22) |
|---|---|---|---|---|
| Age (years) | 12 (5–17) | 11 (4–17) | 10 (5–17) | 12 (3–17) |
| Female sex, n (%) | 18 (45) | 18 (49) | 9 (43) | 8 (36) |
| # of auto Abs | N.A. | 0 | 3 (1–5) | 3 (0–4) [4 missing] |
| Days from diagnosis | N.A. | N.A. | N.A. | 5 (2–10) |
| HbA1c (%) | N.A. | N.A. | 5.3 (4.4–5.8) [2 missing] | 11.9 (9.4–15.4) |
| C-peptide (ng/ml) | N.A. | N.A. | N.A. | 0.32 (0.02–1.27) [1 missing] |

*The sample includes individuals ≤17 years old. Continuous variables are presented as median (min–max); categorical variables are presented as frequency (%). Clinical parameters that were not available for one or more $Ab^{pos}$ FDRs and T1D patients are indicated as missing.*
*N.A., not available; HCs, healthy controls; FDRs, first-degree relatives; T1D, type 1 diabetes.*

**TABLE 2** | Characteristics of subjects enrolled for genetic studies.

| Variables | HCs (n = 78) | $Ab^{neg}$ FDRs (n = 97) | $Ab^{pos}$ FDRs (n = 52) | T1D (n = 43) |
|---|---|---|---|---|
| Age (years) | 14 (5–43) | 13 (2–44) | 15 (4–49) | 13 (3–41) |
| Female sex, n (%) | 33 (42) | 53 (55) | 25 (48) | 20 (47) |
| # of auto Abs | N.A. | 0 | 2 (1–5) | 3 (0–4) [5 missing] |
| Days from diagnosis | N.A. | N.A. | N.A. | 6 (0–6668) |
| HbA1c (%) | N.A. | N.A. | 5.1 (4.4–6) [4 missing] | 10.5 (5.4–15.4) [1 missing] |
| C-peptide (ng/ml) | N.A. | N.A. | N.A. | 0.37 (0.00–1.30) [4 missing] |

*Continuous variables are presented as median (min–max); categorical variables are presented as frequency (%). Clinical parameters that were not available for one or more $Ab^{pos}$ FDRs and T1D patients are indicated as missing.*
*N.A., not available; HCs, healthy controls; FDRs, first-degree relatives; T1D, type 1 diabetes.*

expression levels were assessed on EDTA peripheral blood within 6 h after blood withdrawal. In brief, 50 µl of antibody (Ab) mix was directly added to 180 µl of whole blood and incubated for 15 min at room temperature in the dark. The samples were then incubated for additional 13 min at room temperature in the dark with lysis buffer (EDTA 1 mM, $KHCO_3$ 10 mM, and $NH_4Cl$ 150 mM) to perform red blood cell lysis, washed twice, and re-suspended in phosphate-buffered saline (PBS) (Sigma, CA, USA) with 2% fetal bovine serum (FBS) (Lonza, Italy). Cells were identified using a multiparametric approach based on the combination of monoclonal Abs (**Supplementary Table S1**). Samples were acquired within 12 h after the staining using a FACSCanto II flow cytometer (Becton Dickinson, Mountain View, CA). Samples were processed and stained in batches to reduce technical variability. Moreover, to ensure a standardized sample analysis and a reliable mean fluorescence intensity (MFI) evaluation among samples, flow cytometer was calibrated daily using 8-peak rainbow calibration particles (Spherotech, IL, USA) before sample acquisition. MFI was evaluated on the whole gated populations. Data were analyzed with FCS express v6 (*De Novo* Software, Glendale, CA), and quadrant markers were set accordingly to the relative fluorescence minus one (FMO) staining performed for all the donors analyzed.

## Statistical Analysis

Statistical methods applied for each set of data in **Figures 1**, **2** are reported in the related figure legend. For **Figures 3**, **4A, B**, and **Supplementary Figures S3, S4C** and **S5**, comparisons among groups were performed with linear mixed-effects (LME) models to account for the presence of several subjects within the same family. In each analysis, to meet the assumption of normality of the residuals of the models, an appropriate transformation, indicated in the figure legend, was eventually applied to the response variable; and, when necessary, few outliers identified by the specific model were not included in that analysis. For the sake of completeness, these outliers have been included in the figures as black dots (**Figures 3B, C**, **Supplementary Figures S3A** and **S5**)

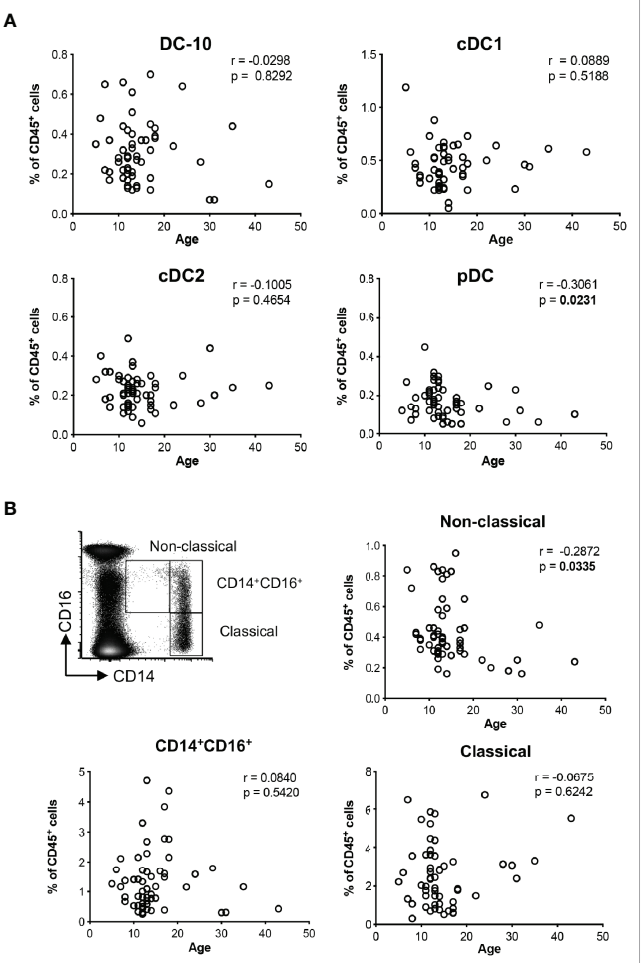


**FIGURE 1** | Distributions of the major dendritic cell and monocyte subsets with age. The percentage of DC-10 ($CD11c^+CD14^+CD16^+CD141^+CD163^+$), cDC1 ($CD11c^+CD14^-CD141^+$), cDC2 ($CD11c^+CD1c^+$), and pDC ($CD11c^-CD303^+$), and $CD14^+CD16^+$, and non-classical ($CD14^{low}CD16^+$, and classical ($CD14^{high}CD16^-$) monocytes were evaluated by multiparametric flow cytometry in the peripheral blood of healthy donors (HCs) at different ages. Scatterplot and Spearman's correlation analyses between age and the indicated DC populations **(A)** and monocyte subsets **(B)** are shown. Each data point represents a donor (n = 55). Data distribution, Spearman's rank correlation coefficient (r), and p-values are shown for each analysis. Statistically significant p-values are indicated in bold.

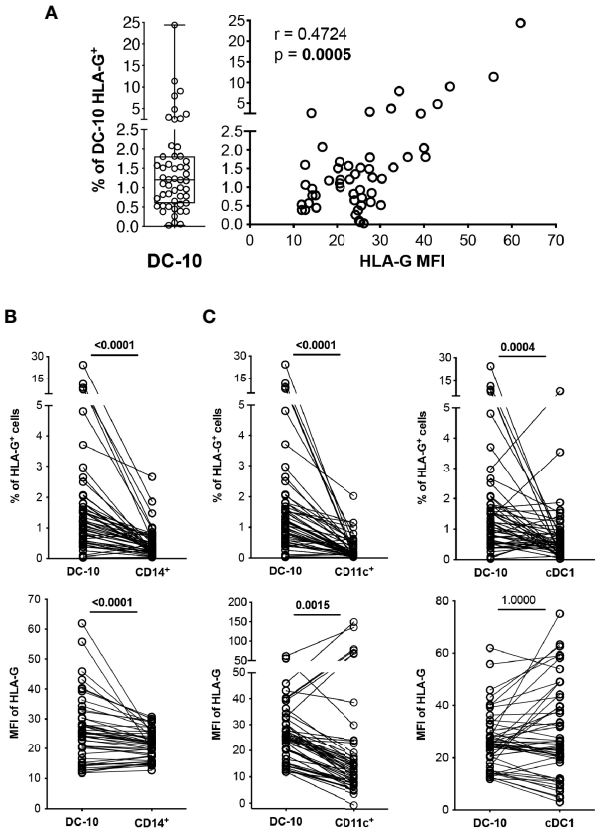


**FIGURE 2** | DC-10 contain a high proportion of HLA-G-expressing cells. DC-10 ($CD11c^+CD14^+CD16^+CD141^+CD163^+$), $CD14^+$, $CD11c^+$, and cDC1 ($CD11c^+CD14^-CD141^+$) cells were identified, and expression levels of HLA-G were evaluated by multiparametric flow cytometry in the peripheral blood of 51 healthy donors at different age. **(A)** Percentages of HLA-G-expressing DC-10. Each dot represents a single donor, line indicates median, and whiskers are minimum and maximum levels (left panel). Scatterplot and Spearman's correlation analyses between the percentage of DC-10 expressing HLA-G and HLA-G mean fluorescence intensity (MFI) are shown. MFI was evaluated on the whole gated populations. Each data point represents a donor. Data distribution, Spearman's rank correlation coefficient (r), and p-value are shown (right panel). **(B)** Percentages of HLA-G-expressing cells (top panel) and HLA-G MFI (bottom panel) on DC-10 and $CD14^+$ cells. **(C)** Percentages of HLA-G-expressing cells (top panels) and HLA-G MFI (bottom panels) on DC-10, $CD11c^+$, and cDC1 cells. For **(B, C)**, each dot represents a single donor; lines connect the data of the same donor. Numbers indicate p-values of paired Wilcoxon's test after Bonferroni's correction. The correction was performed for all comparisons in **(B, C)**, separately for the type of data (either the percentage of HLA-G-expressing cells or the HLA-G MFI). Statistically significant p-values are indicated in bold.

and considered in the computation of the reported descriptive statistics. For testing difference between all pairs of groups, a post-hoc analysis was performed by using the R package phia and by applying Bonferroni's correction to account for multiple comparisons. For (**Figure 4C**), the linear model was employed to evaluate the dependency between % of $CD83^+$ DC-10 and % of $HLA\text{-}G^+$ DC-10, within each group. The standard linear regression was estimated for the groups HC, $Ab^{pos}$, and T1D, since all subjects were independent, while the LME model was used in case of $Ab^{neg}$ in order to account for the presence of several subjects within the same family. In each analysis, in order to meet the assumption of normality of the residuals of the model, an appropriate transformation was applied to the response variable (% of $HLA\text{-}G^+$ DC-10).

For the genetic analyses of HLA-G 3′UTR and 5′URR (PROMO) regions reported in (**Figure 5**, **Supplementary Figure S6, Supplementary Tables S5** and **S7**), logistic mixed-effects models were employed for comparing the frequency of the single haplotypes or genotypes between each group HC, $Ab^{pos}$, and T1D *versus* $Ab^{neg}$. The models account for the presence of several subjects within the same family and, in case of the haplotypes, also for the presence of two alleles per subject. Comparisons have been performed only for haplotypes or genotypes with at least two groups with absolute frequency of at least 10. P-Values were adjusted by applying Bonferroni's correction to account for multiple testing.

Multiple mixed-effects regression analyses were performed to identify potential predictive variables of T1D development, since these models can account for the presence of several individuals within the same family. The logistic mixed-effects model was employed for predicting the probability to be $Ab^{neg}$ *versus* HCs. The cumulative link mixed model was used for predicting the probability to be: a subject at risk without ($Ab^{neg}$) or with autoimmunity ($Ab^{pos}$) or to be a person with T1D (considering the groups as ordered). For both analyses, the variables considered in the starting model were Age, % of DC-10, % of cDC2, % of $HLA\text{-}G^+$ DC-10, % of $CD83^+$ DC-10, and 3′UTR genotype (considering the comparisons of all genotypes with respect to DelC/DelC genotype). The final models were obtained with a backward procedure of variable selection.

In all the analyses, p-values less than 0.05 were considered significant. All p-values of the analyses were reported in the figures and/or (**Supplementary Tables S2, S5** and **S7**). Statistical analysis was performed using R 3.5.0 (http://www.R-project.org/).

# RESULTS

## The Frequency of Circulating DC-10 Is Stable in Children and Adults

Using the newly identified set of markers CD14, CD16, CD141, and CD163 (14), and the gating strategy shown in (**Supplementary Figure S1A**), we evaluated the frequency of circulating DC-10 in a cohort of 55 healthy donors (HCs). The distribution of classical (c) DC1 ($CD11c^+CD14^-BDCA\text{-}3^+$), cDC2 ($CD11c^+BDCA\text{-}1^+$), and plasmacytoid (p)DCs ($CD11c^-BDCA\text{-}2^+$) subsets (**Supplementary Figure S1B**) was analyzed in parallel. The frequency of DC-10 was constant with respect to the age among the donors analyzed and represented on average 0.31% ± 0.15% (range 0.07%–0.70%) of $CD45^+$ cells (**Figure 1A**). Similarly, the frequency of cDC1 and cDC2 subsets was stable in young and adult HCs being on average 0.45% ± 0.20% (range 0.05%–1.19%) and 0.23% ± 0.09% (range 0.06%–0.49%) of $CD45^+$ cells, respectively. In line with previous studies (18), the frequency of pDC decreased with age (r = −0.3061, p = 0.0231) and represented on average 0.17% ± 0.08% (range 0.05%–0.45%) of $CD45^+$ cells (**Figure 1A**).

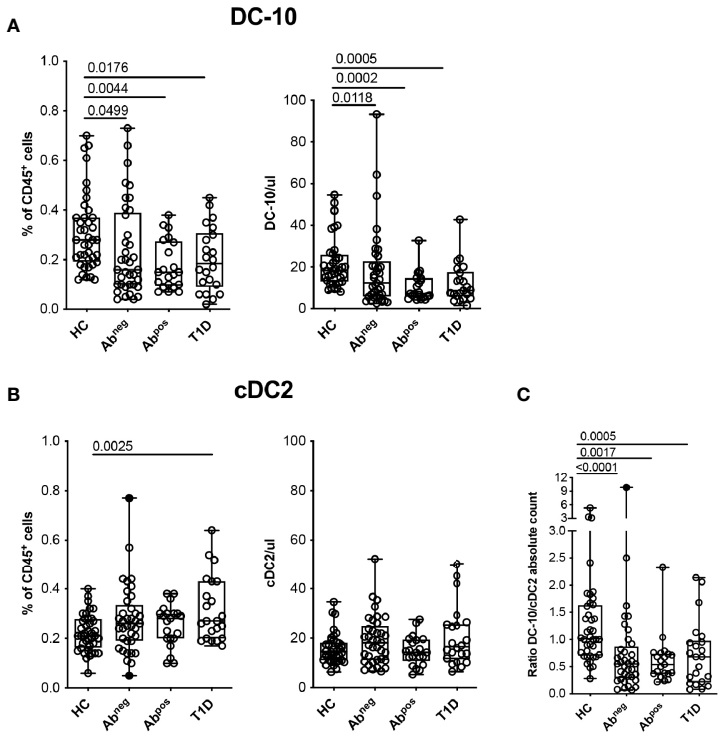


**FIGURE 3** | Tolerogenic DC-10 are reduced in type 1 diabetes (T1D) patients and in both $Ab^{neg}$ and $Ab^{pos}$ first-degree relative (FDR) subjects. DC-10 ($CD11c^+CD14^+CD16^+CD141^+CD163^+$) and cDC2 ($CD11c^+CD1c^+$) were identified by multiparametric flow cytometry in the peripheral blood of pediatric healthy donors (HCs; n = 40), of age-matched first-degree relatives of T1D patients without ($Ab^{neg}$, n = 37) or with ($Ab^{pos}$, n = 21) autoantibodies, and pediatric T1D patients at onset (T1D, n = 22). The frequency and absolute cell count of **(A)** DC-10 and of **(B)** cDC2 in the indicated cohort of donors are shown. **(C)** Ratio between DC-10 and cDC2 as absolute cell count was calculated for each donor. The absolute cell counts of DC-10 and cDC2 were obtained by normalization of the $CD45^+$ cells based on the full blood count laboratory test. Each dot represents a single donor, and black dots in the $Ab^{neg}$ group indicate donors identified as outliers by the corresponding linear mixed-effects (LME) model used for the comparing groups; lines indicate medians, and whiskers are minimum and maximum levels. For each set of data, *post-hoc* analysis of the LME model with the R package phia was performed for testing the difference between all groups. Numbers indicate statistically significant Bonferroni's adjusted p-values. The p-values of all comparisons are reported in **Supplementary Tables S2, S4**. In the LME analysis, data on the percentage of DC-10 and cDC2 cells were used in square root scale, while data on absolute cell counts (even the ratio DC-10/cDC2) were used in natural logarithmic scale in order to meet the assumptions of the model.

DC-10 are a subset of $CD14^+CD16^+$ cells (14); thus, we analyzed the frequency of circulating monocyte subsets according to the standard classification (19): classical ($CD14^{high}CD16^-$), $CD14^+CD16^+$, and non-classical ($CD14^{low}CD16^+$) monocytes. The proportion of non-classical monocytes decreased with age ($r = -0.2872$, $p = 0.0335$) with an average of 0.44% ± 0.21% (range 0.16%–0.95%) in $CD45^+$ cells, while the frequencies of classical and $CD14^+CD16^+$ monocytes were constant in young and adult HCs, with an average of 2.55% ± 1.65% (range 0.31%–6.74%) and of 1.36% ± 1.02% (range 0.25%–4.72%) in $CD45^+$ cells (**Figure 1B**).

Overall, in healthy conditions, the distribution of DC-10 in the peripheral blood is constant in young and adults.

## DC-10 Contain a Higher Proportion of HLA-G-Expressing Cells Than Do Total $CD14^+$ Cells, $CD11c^+$ Cells, and cDC1

*In vitro* differentiated DC-10 express HLA-G (7, 13); thus, we investigated whether this feature is shared by their *in vivo* counterpart. Circulating DC-10 from HCs expressed variable levels of HLA-G ranging from 0.03% to 24.37%, with the percentage of HLA-G-positive DC-10 correlating with HLA-G expression levels, indicated by the MFI ($r = 0.4724$, $p = 0.0005$) (**Figure 2A**).

$CD14^+$ monocytes constitute the main subset of immune cells expressing HLA-G, although at low and variable levels [e.g., 0.12% ± 0.07% of $HLA\text{-}G^+CD14^+$ cells (20–24)]. In our cohort of HCs, circulating DC-10 expressed significantly higher HLA-G levels compared to those expressed by total $CD14^+$ cells in terms of both percentage of $HLA\text{-}G^+$ cells (2.19% ± 3.84% and 0.66% ± 0.93%, respectively, $p < 0.0001$) and levels of protein expression (MFI of HLA-G, 25.95% ± 10.82% and 26.23% ± 18.96%, respectively, $p < 0.0001$) (**Figure 2B** and **Supplementary Figure S2A**). The frequency of $HLA\text{-}G^+$ DC-10 was also significantly higher compared with the percentage of $HLA\text{-}G^+CD11c^+$ (0.30% ± 0.37%, $p < 0.0001$) and of $HLA\text{-}G^+$cDC1 cells (0.78% ± 1.17%, $p = 0.0004$) (**Figure 2C** and **Supplementary Figure S2B**). Moreover, HLA-G MFI on DC-10 was significantly higher compared with that on $CD11c^+$ cells (32.55% ± 47.25%,

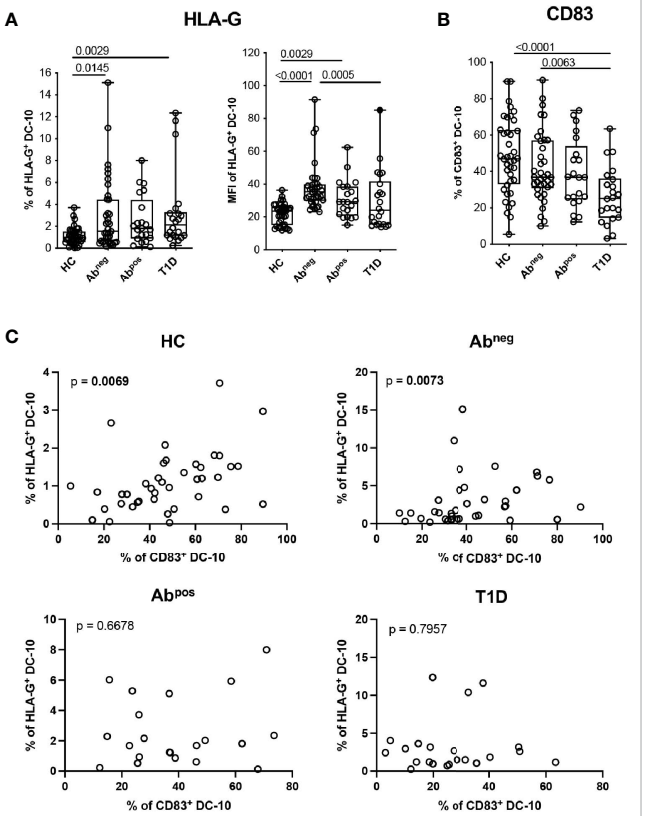


**FIGURE 4** | DC-10 from type 1 diabetes (T1D) patients and first-degree relative (FDR) subjects show an altered phenotype. DC-10 were identified as $CD11c^+$ $CD14^+CD16^+CD141^+CD163^+$, cells and the expression levels of the indicated markers were measured by multiparametric flow cytometry in the peripheral blood of pediatric healthy donors (HCs; n = 40), age-matched first-degree relatives of T1D patients without ($Ab^{neg}$, n = 37) or with ($Ab^{pos}$, n = 21) autoantibodies, and pediatric T1D patients (T1D, n = 22). **(A)** Percentages of HLA-G-expressing cells (left panel) and HLA-G MFI (right panel) on DC-10. **(B)** Percentages of $CD83^+$ DC-10. Each dot represents a single donor, and the black dot in the T1D group indicates a donor identified as outlier by corresponding linear mixed-effects (LME) model used for the comparing groups; lines indicate medians, and whiskers are minimum and maximum levels. For each set of data, *post-hoc* analysis of the LME model with the R package phia was performed for testing the difference between groups. Numbers indicate statistically significant Bonferroni's adjusted p-values. The p-values of all comparisons are reported in **Supplementary Table S2**. In the LME analysis, the % of HLA-$G^+$ DC-10 and MFI of HLA-$G^+$ DC-10 were used in natural logarithmic scale, while the % of $CD83^+$ DC-10 were used in square root scale in order to meet the assumptions of the model. **(C)** Analysis of dependency between the expression of HLA-G and CD83 in DC-10 in the indicated cohorts of donors are shown. Data distribution and p-values are shown for each analysis. Statistically significant p-values are indicated in bold. In the analysis, the response variable (% of HLA-$G^+$ DC-10) was used in square root scale for the HC and $Ab^{pos}$ groups, and in natural logarithmic scale, for the $Ab^{neg}$ and T1D groups in order to meet the assumptions of the model.

p = 0.0015), but similar to that detected on cDC1 (31.84% ± 19.08%, p = 1.0000) (**Figure 2C** and **Supplementary Figure S2B**).

Altogether, these results indicate that DC-10 contain a higher percentage of HLA-G-expressing cells among the myeloid cell subsets analyzed.

## Tolerogenic DC-10 Are Reduced in Type 1 Diabetes patients at Onset and in Both $Ab^{neg}$ and $Ab^{pos}$ First-Degree Relative Subjects

Hypothesizing that DC-10 frequency could be altered in diseases characterized by breakage of tolerance, we investigated possible variations of DC-10 frequency in subjects with different stages of T1D. Glucose variability in long-standing T1D patients can affect myeloid and stem and progenitor cell compartments (25, 26); thus, we performed the analysis in pediatric new-onset T1D patients, in comparison with age-matched FDRs of T1D patients without or with autoantibodies ($Ab^{neg}$ and $Ab^{pos}$, respectively), and age-matched HCs (**Table 1**). Circulating DC-10 were significantly lower as frequency and as absolute cell count in T1D patients (0.20% ± 0.13%, p = 0.0176; and 12.20% ± 9.44 cells/ml, p = 0.0005, respectively), $Ab^{pos}$ FDRs (0.18% ± 0.10%, p = 0.0044; and 10.53 ± 6.96 cells/ml, p = 0.0002, respectively), and $Ab^{neg}$ FDRs (0.24% ± 0.19%, p = 0.0499; and 18.25 ± 18.87 cells/ml, p = 0.0118, respectively) compared with HCs (0.31% ± 0.15%, and 22.02 ± 12.22 cells/ml) (**Figure 3A** and **Supplementary Table S2**). The proportion, but not the total cell number, of classical cDC2 was significantly higher in T1D patients compared with HCs (0.32% ± 0.13% and 0.22% ± 0.07%, respectively; p = 0.0025) (**Figure 3B** and **Supplementary Table S2**). Notably, the DC-10/cDC2 absolute cell number ratio was significantly higher in HCs (1.31 ± 0.92) compared with $Ab^{neg}$ FDRs (0.89 ± 1.59, p < 0.0001), $Ab^{pos}$ FDRs (0.62 ± 0.45, p = 0.0017), and T1D patients (0.73 ± 0.60, p = 0.0005) (**Figure 3C** and **Supplementary Table S2**). No differences in the frequency of classical cDC1 and pDC were observed among the different cohorts (**Supplementary Figure S3A** and **Supplementary Table S2**). According to previous data (27, 28), the frequency of $CD11c^+HLA\text{-}DR^+$ myeloid DCs was reduced in T1D patients (36.60% ± 10.15%) compared with that present in HCs (47.06% ± 13.45%, p = 0.0107) and in $Ab^{neg}$ FDRs (46.86% ± 14.00%, p = 0.0424) but was similar to that observed in $Ab^{pos}$ FDRs (38.13% ± 14%) (**Supplementary Figure S3B** and **Supplementary Table S2**). Finally, in T1D patients, and in $Ab^{neg}$ FDRs and $Ab^{pos}$ FDR subjects, the frequency of the different monocyte subsets was comparable with that of HCs, except for classical monocytes being significantly higher in $Ab^{neg}$ FDRs compared with HCs (3.57% ± 1.45% and 2.42% ± 1.65%, respectively; p = 0.0008) (**Supplementary Figure S3C** and **Supplementary Table S2**).

Overall, these findings indicate a specific reduction in the tolerogenic DC-10 compartment that is parallel to the increase of the pro-inflammatory cDC2 in T1D patients, $Ab^{neg}$ FDRs, and $Ab^{pos}$ FDRs.

## DC-10 From Type 1 Diabetes Patients and First-Degree Relatives Show an Altered Phenotype

We further characterized circulating DC-10 focusing on the expression of HLA-G and of ILT4, tolerogenic molecules associated with DC-10 regulatory activity (13, 14), and the activation marker CD83. A higher percentage of HLA-$G^+$

DC-10 was identified in $Ab^{neg}$ FDRs (2.96% ± 3.28%, p = 0.0145) and in T1D patients (3.24% ± 3.51%, p = 0.0029) and, as a tendency, in $Ab^{pos}$ FDRs (2.56% ± 2.23%, p = 0.0593) compared with HCs (1.11% ± 0.78%) (**Figure 4A** and **Supplementary Table S2**). Notably, in $Ab^{neg}$ FDRs, DC-10 expressed significantly higher levels of HLA-G (MFI of HLA-G 37.74% ± 14.16) than did HCs (MFI of HLA-G, 21.89 ± 6.41, p < 0.0001) and T1D patients (MFI of HLA-G 30.07 ± 17.90, p = 0.0005) (**Figure 4A** and **Supplementary Table S2**). Moreover, despite the high variability, $Ab^{neg}$ FDRs and HCs showed on average the highest percentage of $CD83^+$ DC-10, which was significantly higher compared with that of T1D patients (42.17% ± 19.16% and 48.19% ± 20.59% *vs.* 26.61% ± 15.43%, p = 0.0063 and p < 0.0001, respectively) (**Figure 4B**, **Supplementary Figure S4A** and **Supplementary Table S2**). The percentage of $CD83^+CD11c^+$ and of $CD83^+$ cDC1 cells was comparable among the groups analyzed (**Supplementary Figure S5** and **Supplementary Table S2**). Interestingly, the percentage of HLA-$G^+$ DC-10 correlated with the percentage of $CD83^+$ DC-10 in $Ab^{neg}$ FDRs (p = 0.0073). The dependency between the percentage of HLA-$G^+$ DC-10 and the percentage of $CD83^+$ DC-10 was also detected in HCs (p = 0.0069) but not in $Ab^{pos}$ FDRs or T1D patients (subjects with autoimmunity) (**Figure 4C**). No differences in the proportion of $ILT4^+$ DC-10 were detected among the different cohorts (**Supplementary Figures S4B, C** and **Supplementary Table S2**).

These findings indicate that in T1D patients and $Ab^{neg}$ and $Ab^{pos}$ FDRs, despite the lower proportion, DC-10 expressed HLA-G at higher levels compared with DC-10 from HCs. Moreover, in subjects with autoimmunity (e.g., T1D patients and $Ab^{pos}$ FDRs), DC-10, but not other myeloid cell subsets, are less activated compared with those present in subjects without autoimmunity (e.g., $Ab^{neg}$ FDRs and HCs).

## $Ab^{neg}$ First-Degree Relative Subjects Differ From Age-Matched Healthy Controls

Our data indicate that in T1D patients at onset and in subjects at risk to develop the disease (FDRs), DC-10 number and phenotype are different from those in HCs. Moreover, in $Ab^{neg}$ FDRs, despite that DC-10 are present at lower frequency compared with those in HCs, these cells expressed high levels of HLA-G (**Figure 4A**). An association between specific HLA-G genotypes/haplotypes and T1D has been previously demonstrated (10–12); thus, we analyzed nine specific variations of the 3′UTR HLA-G locus and inferred haplotypes and genotypes in the different cohorts of subjects (**Supplementary Tables S3** and **S4**).

To further define differences between $Ab^{neg}$ FDRs and HCs, we performed multiple regression analysis considering the following variables: age of the persons, % of DC-10, % of cDC2, % of HLA-$G^+$DC-10, % of $CD83^+$DC-10, and 3′UTR HLA-G genotype (considering the comparisons InsG/InsG, DelC/InsG, and UTR-3/X genotypes with respect to the DelC/DelC genotype) (**Table 3**). Multiple regression analysis identified all variables investigated to significantly predict the probability of being $Ab^{neg}$ FDRs, with the only exception of the DelC/InsG *vs.* DelC/DelC comparison (**Table 3**), indicating that overall $Ab^{neg}$ FDR subjects differ from HCs with respect to these parameters. These findings confirm and further support previous evidence that $Ab^{neg}$ FDRs cannot be considered healthy, as they already have signs of metabolic and immunological alterations (29–31).

**TABLE 3** | Multiple regression analysis to compare $Ab^{neg}$ FDRs with healthy controls.

| Variables | Coefficient | Standard error | p-Value |
|---|---|---|---|
| Intercept | 39.379 | 18.919 | **0.0374** |
| Age | −4.113 | 1.624 | **0.0113** |
| % of DC-10 | −131.960 | 48.966 | **0.0070** |
| % of cDC2 | 86.396 | 42.981 | **0.0444** |
| % of HLA-$G^+$ DC-10 | 24.331 | 8.567 | **0.0045** |
| % of $CD83^+$ DC-10 | −1.246 | 0.403 | **0.0020** |
| 3′UTR genotype | | | |
| InsG/InsG *vs.* DelC/DelC | 59.319 | 22.684 | **0.0089** |
| DelC/InsG *vs.* DelC/DelC | 20.380 | 10.875 | 0.0609 |
| UTR-3/X *vs.* DelC/DelC | 23.947 | 11.107 | **0.0311** |

*Final logistic mixed-effects model for predicting the type of group (individual at risk of develop T1D without autoimmunity, n = 37 $Ab^{neg}$ FDRs vs. n = 40 healthy controls), by accounting for the presence of several subjects within the same family. The final model was obtained with a backward procedure of variable selection as described in the Material and Methods. Statistically significant p-values are indicated in bold.*
*FDRs, first-degree relatives; T1D, type 1 diabetes.*

## The Frequency of $CD83^+$ DC-10 Predicts the Probability to Be a Type 1 Diabetes Patient

To define whether the same set of variables that distinguish $Ab^{neg}$ FDRs from HCs can predict the probability to be an individual at risk without ($Ab^{neg}$) or with autoimmunity ($Ab^{pos}$) or a T1D patient, considering the groups as ordered, we employed a cumulative link mixed model. The analysis showed that only the percentage of $CD83^+$ DC-10 can be considered a significant risk factor to develop the disease (coefficient of the model = −0.0881, p = 0.0373). Indeed, a progressive decrease of $CD83^+$ DC-10 percentage is associated with disease progression (e.g., from $Ab^{neg}$ FDRs, to $Ab^{pos}$ FDRs, to overt disease). The observation that none of the other variables, including the % of DC-10 or the % of HLA-$G^+$ DC-10, were retained in the final cumulative link mixed model while they were retained in the previous final model predicting $Ab^{neg}$ FDRs *vs.* HCs indicates that $Ab^{neg}$ FDRs share several similarities with $Ab^{pos}$ FDRs and with T1D patients, while they differ from HCs with respect to these variables.

## HLA-G UTR-3 Is More Represented in $Ab^{neg}$ First-Degree Relative Subjects

The observations that specific 3′UTR HLA-G genotypes can predict the probability of being $Ab^{neg}$ FDRs, and that $Ab^{neg}$ FDRs are more similar to subjects with autoimmunity, prompted us to perform an additional HLA-G genetic analysis to explore if HLA-G genetic variations represent risk factors for developing T1D. To this aim, we enlarged the analysis by including adult subjects (**Table 2**), and by genotyping both the 3′UTR and promoter (PROMO) regions of HLA-G. The frequency of UTR-3 allele was significantly higher in $Ab^{neg}$ FDRs (20.1%)

compared with HCs (8.3%, p = 0.0283; **Figures 5A, B**, **Supplementary Table S5**) and comparable with the frequency assessed in $Ab^{pos}$ FDRs (10.6%, p = 0.2603) and in T1D patients (11.6%, p = 0.5331). We also analyzed 28 different single-nucleotide polymorphisms in the PROMO region of HLA-G gene (**Supplementary Table S6**). We inferred almost a hundred different haplotypes, of which 16 was already described, six with a frequency >0.5%, and 78 with a frequency <0.5%, which clustered in four major PROMO families (data not shown). Among them, we observed a significantly higher frequency of the PROMO-0104 allele in $Ab^{neg}$ FDRs compared with HCs (17.5% *vs.* 7.1%, p = 0.0390) (**Supplementary Figure S6** and **Supplementary Table S7**). These results, in conjunction with the high frequency of UTR-3 in $Ab^{neg}$ FDRs, confirm previous data on the linkage disequilibrium between the 3′UTR-3 and the PROMO-0104 alleles (9). However, probably the smaller sample size of $Ab^{pos}$ FDRs and T1D groups did not allow to define the protective role of PROMO-0104 alleles *versus* disease progression, although it was present with a lower percentage in these two groups with respect to $Ab^{neg}$ FDRs (for $Ab^{pos}$ FDRs: 6.7%, p = 0.0664; for T1D: 8.1%, p = 0.2073).

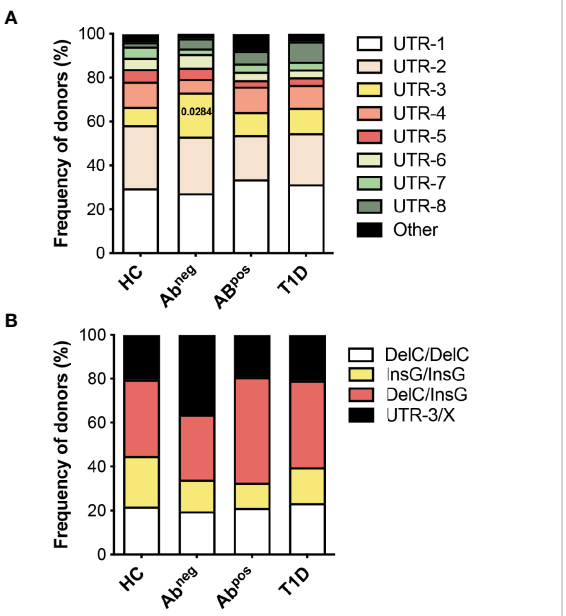


**FIGURE 5** | HLA-G UTR-3 is more represented in $Ab^{neg}$ first-degree relatives (FDRs). **(A)** Allele relative frequencies based on the analysis of nine polymorphic sites present in the 3′UTR of HLA-G locus in healthy donors (HC; n = 78), first-degree relatives of type 1 diabetes (T1D) patients without ($Ab^{neg}$, n = 97) or with ($Ab^{pos}$, n = 52) autoantibodies, and T1D patients (T1D, n = 43) are shown. **(B)** Genotype relative frequencies for 14-bp Ins/Del and +3142 G/C polymorphisms present in the 3′UTR of HLA-G locus in the indicated cohorts of donors are shown. The logistic mixed-effects models were employed for comparing the relative frequency of the single haplotypes or genotypes of all groups *vs*. HCs, as described in the *Materials and Methods*. Numbers indicate statistically significant Bonferroni's adjusted p-values. The p-values of all comparisons are reported in **Supplementary Table S5**.

## DISCUSSION

In this study, we report that naturally occurring DC-10 contain a higher frequency of HLA-G-expressing cells compared with $CD14^+$, $CD11c^+$, and cDC1 cells. Moreover, we show, for the first time, that the frequency and number of circulating DC-10 are reduced in pediatric T1D patients at onset and in individuals at risk of developing the disease, while the proportion of pro-inflammatory cDC2 cells increases in these groups. In addition, the frequency of $CD83^+$DC-10 progressively decreases from $Ab^{neg}$ FDRs to $Ab^{pos}$ FDRs, to T1D patients, and a low proportion of $CD83^+$DC-10 is associated with disease progression. Multiple regression analysis of variables related to DC-10 and HLA-G confirms that $Ab^{neg}$ FDRs differ from healthy subjects and are more similar to T1D patients and to $Ab^{pos}$ FDRs.

Thus far, the presence and the frequency of DC-10 have been described in the peripheral blood of adults (7, 13, 14, 32). The present study reveals that, similar to other myeloid DC subsets, the frequency of circulating DC-10 is stable throughout life and represents on average the 0.3% of total leucocytes, while the proportion of plasmacytoid DCs decreased in adults (18). The expression of HLA-G is a specific feature of *in vitro* differentiated DC-10 (7), and high HLA-G expression is a key determinant for their tolerogenic activity (13). Here, we show that, although the frequency of $HLA\text{-}G^+$ DC-10 is variable among the donors analyzed, DC-10 contain a higher proportion of HLA-G-expressing cells compared with $CD14^+$ monocytes, previously described to be the major myeloid population expressing/secreting HLA-G (21, 22, 33, 34), and to other DC subsets, such as $CD11c^+$ and cDC1 cells.

Studies on the frequency of myeloid cells in T1D patients have been limited to pro-inflammatory cells, including DCs (27, 28, 35). We scrutinized for the first time the presence and the frequency of pro-inflammatory and anti-inflammatory APCs (e.g., DC-10) in pediatric $Ab^{neg}$ FDRs and $Ab^{pos}$ FDRs and in T1D patients at onset. We showed a reduced frequency and number of DC-10 in T1D patients and in subjects at risk to develop the disease compared with HCs. DC-10 reduction is paralleled with the increased frequency of pro-inflammatory cDC2, and the ratio DC-10/cDC2 progressively decreases from HCs to $Ab^{neg}$ FDRs, $Ab^{pos}$ FDRs, and T1D patients. The DC-10 contraction is specific since $CD14^+CD16^+$ monocytes, containing DC-10, are present at comparable frequency in the different cohorts analyzed independently from the stage of the disease.

The increased cDC2 frequency in T1D patients seems to be in contrast with two recent publications showing a reduction in the proportion of myeloid DCs in T1D patients compared with HCs (27, 28). However, the latter analysis was performed using different gating strategies and combinations of markers: myeloid DCs were defined as $lin^-HLA\text{-}DR^+CD123^-CD11c^+$ cells. Using the same strategy, we confirmed the reduced frequency of $HLA\text{-}DR^+CD11c^+$ cells in T1D patients compared with HCs. Notably, with our detailed analysis, which allows the segregation of myeloid $HLA\text{-}DR^+CD11c^+$ cells in CD1c/BDCA-1- and CD141/BDCA-3-expressing DCs, we showed the specific expansion of the former compartment, known to be the main

producers of IL-12, tumor necrosis factor-α (TNF-α), IL-8, and IL-10, and inducers of cytotoxic T cells (36–38). This result supports the hypothesis that the enriched frequency of pro-inflammatory myeloid DCs is associated with the activation and differentiation of auto-reactive T cells sustaining T1D development. Moreover, our results suggest that the decrease of DC-10 and the DC-10/cDC2 ratio weighted toward the immunogenic compartment could be indicators of break of tolerance.

DC-10 from $Ab^{pos}$ FDRs and T1D patients express higher levels of HLA-G, in terms of percentage both of positive cells and of protein expression, compared with HCs. Notably, the percentage of DC-10 expressing CD83 decreases significantly in T1D patients and, as a tendency, in $Ab^{pos}$ FDRs when compared with both healthy subjects and $Ab^{neg}$ FDRs. Correlation analysis between the percentage of HLA-$G^+$ DC-10 and $CD83^+$ DC-10 revealed a dependency between these two parameters in $Ab^{neg}$ FDRs and in HCs but not in $Ab^{pos}$ FDRs and in T1D patients. This result suggests a possible correlation between DC-10 phenotype and function that it is lost during disease progression. Multiple regression analyses supported this hypothesis, since it revealed that the percentage of $CD83^+$ DC-10 not only discriminates between $Ab^{neg}$ FDRs and HCs but also represents a risk factor for developing T1D. Indeed, a reduction of $CD83^+$ DC-10 percentage is associated with disease progression. The downregulation of CD83 on DCs resulted in a lower stimulatory capacity (39); therefore, we can speculate that, despite the high expression of HLA-G, DC-10 in $Ab^{pos}$ FDRs and in T1D patients are poorly activated and might have an impaired ability to activate and/or induce regulatory T cells. The decrease in CD83 expression is specific for DC-10, since other myeloid cells (e.g., $CD11c^+$ and cDC1) expressed CD83 independently from the stage of the disease, suggesting that the latter pro-inflammatory cells are not impaired in their activation and possibly functions. Future dedicated functional studies using *ex vivo* isolated DC-10 from subjects at different stages of the disease are warranted to verify our hypothesis.

Single parameter and multiple regression analyses further support previous evidences that $Ab^{neg}$ FDRs differ from HCs and cannot be considered healthy, as they already have signs of metabolic and immunological alteration such as altered neutrophil-specific and type I IFN signatures (29–31). Results from the cumulative link mixed analysis, demonstrating that $Ab^{neg}$ FDRs are more similar to $Ab^{pos}$ FDRs and to T1D patients, further sustain this conclusion. Indeed, several DC-10-related parameters, including the % of DC-10 and the % of HLA-$G^+$ DC-10, can discriminate between $Ab^{neg}$ FDRs and HCs, but they seem not to distinguish $Ab^{neg}$ FDRs from $Ab^{pos}$ FDRs and from T1D patients. Based on these findings, we can speculate that the proportion of DC-10 and of HLA-$G^+$DC-10 can discern also $Ab^{pos}$ FDR from HCs and T1D patients from HCs and could be considered risk factors to develop the disease. Further studies including larger cohorts of patients are warranted to confirm this hypothesis.

Genetic variants of the *HLA-G* locus have been associated with the risk to develop T1D (40), with contrasting results. Silva and colleagues reported an increased frequency of the 14 base pair deletion (14DEL) polymorphism in T1D patients compared with HCs (12). Conversely, a study performed considering HLA-G 3′UTR extended haplotypes described a lower frequency of the UTR-3 haplotype, containing the 14DEL polymorphism, in T1D patients compared with HCs (10). In the present study, we found a similar frequency of UTR-3 in T1D patients and HCs. However, the different genetic backgrounds derived from the geographical origin of our cohorts of patients compared with the study from Brazilian groups can influence the frequency of genetic variants and should be considered.

In conclusion, for the first time, we show an increase of the pro-inflammatory arm of the myeloid immunity (cDC2) and a reduction of the tolerogenic compartment (DC-10) in individuals at risk of developing T1D and in T1D patients at onset. Interestingly, the monitoring of the myeloid compartment, and, in particular, of $CD83^+$ DC-10, might be used to follow changes in the immunological status from the absence of signs of autoimmunity to the overt disease. Moreover, the high frequency of HLA-G UTR-3, previously shown to be associated with HLA-G expression levels, in individuals at risk to develop T1D without signs of autoimmunity ($Ab^{neg}$ FDRs) could represent a protective factor toward the disease development. These results pave the way to additional longitudinal studies, based on longer follow-up and including larger cohorts of subjects, to investigate the functional role of DC-10 in controlling/preventing T1D development and to further define if HLA-G genetics, in addition to the HLA-DR/DQ screening, can be used as an additional risk/protection factor for disease development.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ospedale San Raffaele ethics committee: approval IRB#DRI-003 and IRB#NHPROT32803-TN01. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

GA designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. AM contributed to the analysis of the data. RC performed some of the experiments. AS performed sample processing and

biobanking. PMVR performed the statistical analysis and CDS supervised them. RB, MP, and EB provided the samples. MB conceived the scientific idea and helped in the data interpretation. SG conceived the scientific idea, supervised the experimental design and data interpretation, and wrote the manuscript. SG is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. 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.2021.750162/full#supplementary-material

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# HLA-G/sHLA-G and HLA-G-Bearing Extracellular Vesicles in Cancers: Potential Role as Biomarkers

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As a non-classic major histocompatibility complex (MHC) class I molecule, human leukocyte antigen G (HLA-G) is expressed in fetal-maternal interface and immunoprivileged site only in healthy condition, and in pathological conditions such as cancer, it can be *de novo* expressed. It is now widely accepted that HLA-G is a key molecule in the process of immune escape of cancer cells, which is ubiquitously expressed in the tumor environment. This raises the possibility that it may play an adverse role in tumor immunity. The expression level of HLA-G has been demonstrated to be highly correlated with clinical parameters in many tumors, and its potential significance in the diagnosis and prognosis of cancer has been postulated. However, because HLA-G itself has up to seven different subtypes, and for some subtypes, detected antibodies are few or absent, it is hard to evaluate the actual expression of HLA-G in tumors. In the present work, we described (a) the structure and three main forms of HLA-G, (b) summarized the mechanism of HLA-G in the immune escape of tumor cells, (c) discussed the potential role of HLA-G as a tumor marker, and reviewed (d) the methods for detecting and quantifying HLA-G.

**Keywords: HLA-G, tumor, immune escape, extracellular vesicles, biomarker**

## INTRODUCTION

As early as 1983, human leukocyte antigen G (HLA-G) is first observed on the cytotrophoblast at the fetal-maternal interface (1). As a class of major histocompatibility complex (MHC) I molecules, HLA-G showed low polymorphic in the coding region, while several polymoprphism have been describe in non-coding region of the locus (3'UTR, and 5'URR regions) (2). The exons and introns of the HLA-G gene are the same as those of classic MHC class molecules, consisting of eight exons and seven introns (3, 4). However, HLA-G shows only limited genetic polymorphism. The main reason can be attributed to that the terminator of HLA-G is located in the second codon of exon 6, and thus most of exon 6 and all of exons 7 and 8 cannot be translated into protein (5, 6). HLA-G can exist in a variety of structures, which can not only be expressed on the cell surface but also exist in

the form of secretion (7, 8). There are seven isoforms, which encode four membrane-bound (HLA-G1, -G2, -G3, and -G4) and three soluble (HLA-G5, -G6, and -G7) protein isoforms (9). Each HLA-G subtype contains one to three spherical domains (α1, α2, and α3) encoded by exon 2, exon 3, and exon 4 (**Figure 1**).

HLA-G is initially considered to be useful for fetal establishment and maintenance of maternal-fetal immune tolerance (10, 11). Therefore, initial studies have mainly focused on its role in regulating maternal immune cell responses and protecting the fetus from natural killer (NK) cell-mediated lysis (12–14). Fetal and tumor development are similar and characterized by rapid tissue proliferation, which is associated with the expressions of anti-apoptotic factors and telomerase (14–17). It is worth noting that since immunoregulatory sites can be shared between tumor development and placenta formation, both placenta and tumor are protected by the immune system and resistant to induction by the immune microenvironment. Therefore, the research focus of HLA-G has gradually shifted to tumors (18).

In the malignant environment where tumors occur, the expression of HLA-G in melanoma is first reported in 1998 (19), and then the abnormal expression of HLA-G is observed in a variety of malignant tumors, such as lung cancer (20–28), gastric cancer (29–37), ovarian cancer (OC) (38–47), breast cancer (48–54), and hematopoietic tumor (55–58). With the deepening of research, the expression of HLA-G in solid malignant tumors and its potential clinical relevance have attracted increasing attention (59). Abnormal expression of HLA-G plays a variety of roles in the progression of malignant tumors, such as inducing apoptosis, inhibiting immune cytolysis and cytotoxicity, and chemotaxis of regulatory cells and damage of different immune effector cells through receptor binding and/or trogocytosis (60).

In addition, HLA-G is secreted in a variety of body fluids, as either free soluble HLA-G (sHLA-G) or part of extracellular vesicles (EVs), and it has been extensively studied as tumor markers. Among them, sHLA-G is derived from the secretion of sHLA-G homotypes, such as HLA-G5, HLA-G6, and HLA-G7, and the shedding of membrane-bound HLA-G homotypes, such as HLA-G1, cleaved by proteolytic hydrolysis (61). Soluble isoforms can be detected in saliva (62, 63), ascitic fluid (47), plasma (26, 33, 48, 49, 56, 64–69), thymus (70), seminal plasma (71), cerebrospinal fluid (CSF) (72, 73), human first trimester and term placentas *in situ* and *in vitro* (74), and cell culture supernatant (75, 76). High levels of sHLA-G are correlated with tumor histological type, lymph node metastasis, and patient survival, which can be used as a tumor marker to provide a basis for early diagnosis, differentiation, and prognosis (21). However, it remains largely unclear whether HLA-G-bearing EVs are produced by tumor cells, while there is a functional association between the HLA-G-bearing EVs and various tumors, such as melanoma, breast cancer, and kidney cancer (77). In summary, sHLA-G and HLA-G-bearing EVs may provide unpredictable diagnostic opportunities to monitor tumor status and progression.

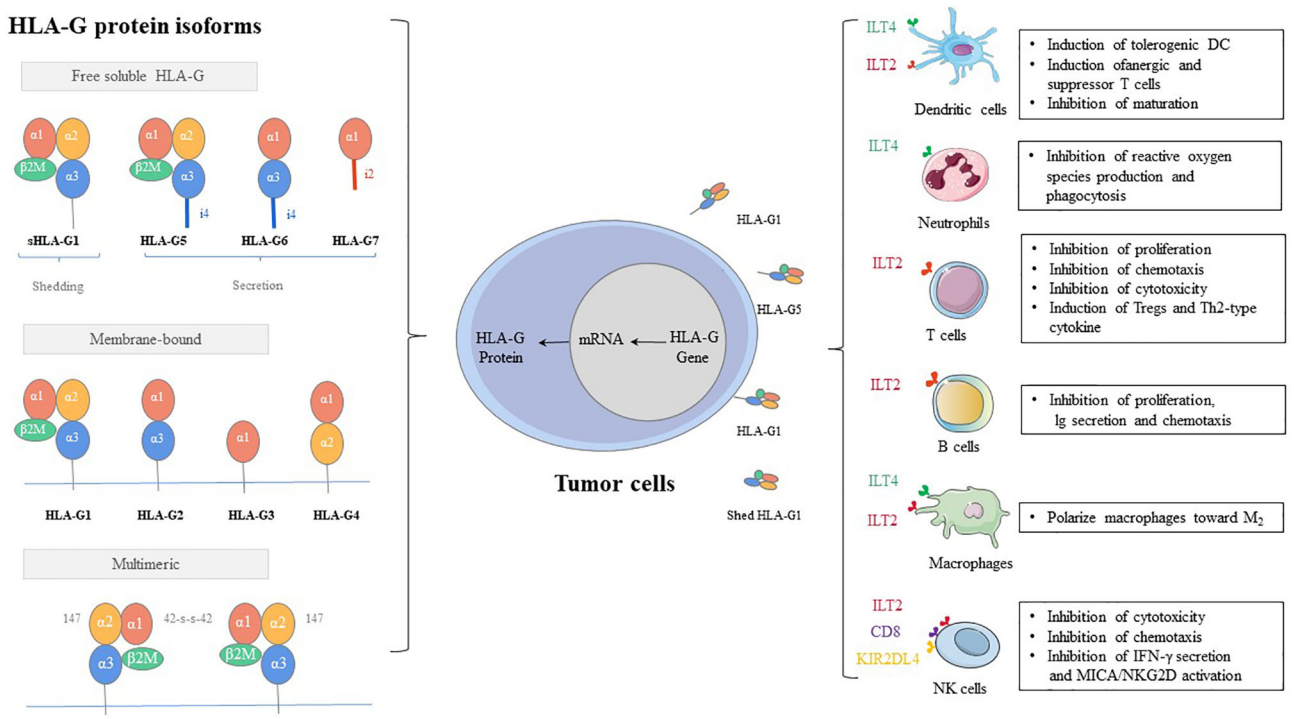


**FIGURE 1** | HLA-G protein isoforms and immune-inhibitory function.

## HLA-G: A KEY IMMUNE EVASION MOLECULE IN TUMORS

In cancer, abnormal expression of HLA-G is considered a key strategy of tumor cells to evade immune surveillance, which is strongly supported by the high incidence of tumors in patients treated with immunosuppressive agents after organ and stem cell transplantation (78). The continued construction of tumor phenotypes is thought to be a result of immune-mediated tumor recognition, a phenomenon known as immune editing of cancer. Three stages define the process of immune editing: elimination (immune surveillance), balance (duration/dormancy), and escape (progression) (79, 80). These three stages integrate the immune system's ability to protect the host from cancer and promote cancer development (81). HLA-G is involved in all three stages, and it is highly necessary to understand the role of HLA-G in tumor immune escape to better develop effective anti-tumor strategies. In the present work, we summarized the main mechanisms of HLA-G-mediated immunosuppression in three aspects as follows:

1. Inhibitory receptors of HLA-G, such as KIR2DL4/CD158d, ILT-2/CD85j, ILT-4/CD85d, CD8, and CD160, can directly exert the immunosuppressive effect by HLA-G. These inhibitory receptors can express in all monocytes, as well as B cells, T cells, and NK cells. Especially, ILT2 receptors are present in subgroups of dendritic cells (DCs) and myeloid-derived suppressive cells (MDSCs) (82–85). ILT4 receptors are mainly expressed in DCs, neutrophils, monocytes, and MDSCs (86–88). KIR2DL4 receptors are mainly expressed in decidual NK cells (89). The mechanisms by which these receptors participate in immunosuppressive effects induced by HLA-G include inhibition of differentiation, cytokine secretion and chemotaxis, immune cell proliferation, cytotoxicity, and induction of MDSCs or M2-type macrophages and regulatory cells (90, 91) (**Figure 1**).
2. MDSCs, regulatory T cells (Tregs), and tolerogenic DCs can participate in immune escape regulated by HLA-G *via* an indirect immunosuppressive way. The sub-population of tolerogenic DCs, DC-10s, expresses a high level of HLA-G and induces adaptive type 1 Tregs (Tr1) through the HLA-G/ILT4 signaling pathway (92). On the other hand, tolerogenic DCs produce CD8+CD28+ and CD4+CD25+CTLA-4+ Tregs under the induction of HLA-G, which further strengthens the ability of immune escape of tumor cells (93).
3. HLA-G uses dynamic transfer mechanisms between cells, such as trogocytosis (membrane-bound HLA-G) and EVs (membrane-bound and sHLA-G) (77, 94–96) (**Figure 2**). Trogocytosis is the process of transporting secretory vesicles or other membrane vesicles from the cell through the cell membrane (97). Activated T cells and NK cells obtain membrane fragments containing functional HLA-G from HLA-G+ or tumor cells through the process of exocytosis. HLA-G-modified cells can immediately reverse immune

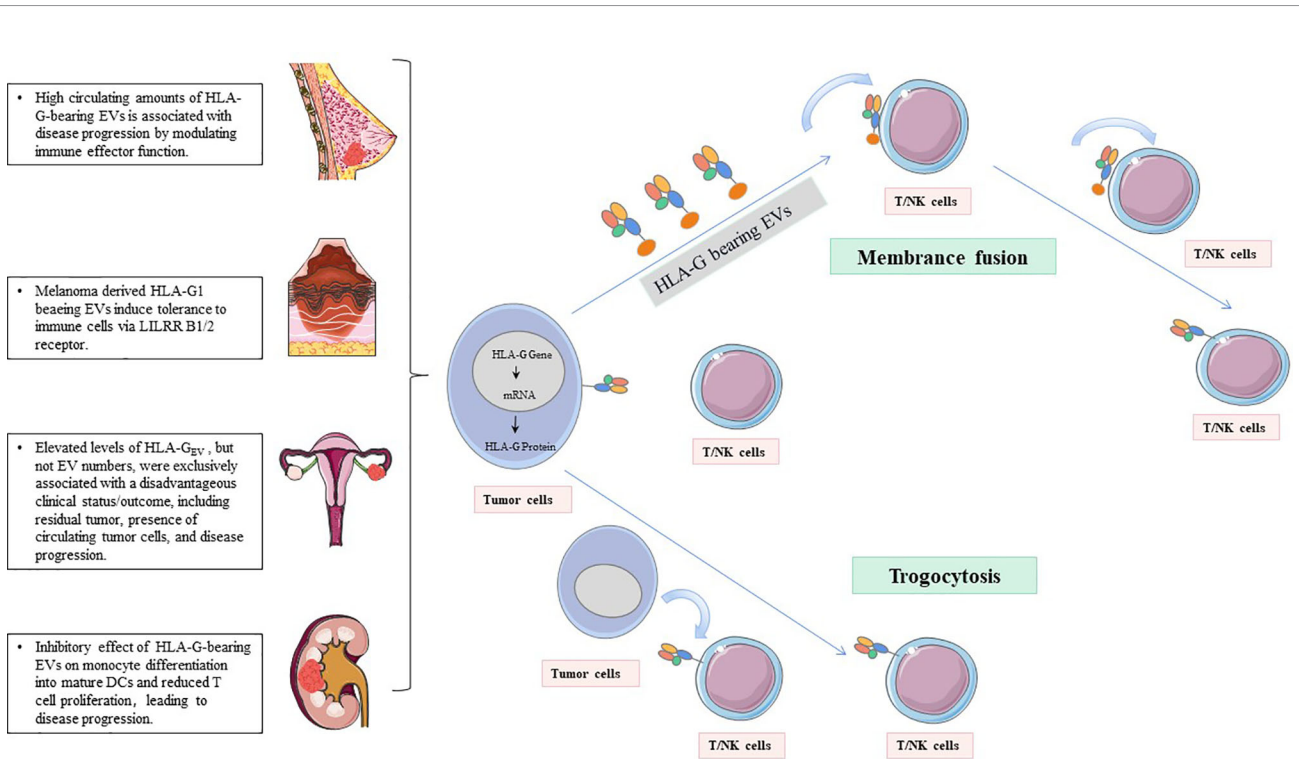


**FIGURE 2** | HLA-G-bearing EVs with potential immunological and clinical relevance. NK cells and T cells acquire HLA-G from HLA-G+tumor cells or HLA-G+immune cells *via* the process of trogocytosis and/or EVs.

effector functions to tolerogenic function (98, 99). The generic term EVs are phospholipid bilayer-enclosed vesicles, which are highly heterogeneous in size, molecular content, and membrane composition depending on the state, micro-environment, and the cell of origin (100, 101). According to biogenesis, EVs are characterized by apoptotic bodies (AB) (>500 nm), microvesicles (100-1,000 nm), and exosomes (70-150 nm) (102). In the tumor state, especially in the established acidic microenvironment, EVs can directly fuse with cancer cells, or carry out the transportation and exchange of biologically active substances through endocytosis, phagocytosis, and micropinocytosis, thereby contributing to the intercellular signaling mechanism, providing the tumor with oxygen, metabolites, nutrients and other soluble factors, and making tumor development possible (103).

## ELEVATED HLA-G IN TUMOR PATIENT TISSUES AS TUMOR INDICATORS

Over the years, many studies have reported that HLA-G is preferentially detected in the primary tumor site and metastatic tumor site, while it is rarely detected in the tumor spontaneous regression site, adjacent tissues, or healthy tissues (104–106). Immunohistochemical (IHC) staining is usually used to detect the expression frequency of HLA-G in tissues, which is combined with clinical results for analysis. In most cancers, the expression of HLA-G is related to the patient's poor clinical outcome (25, 107, 108).

In liver tissue, HLA-G is detected in the primary site of hepatocellular carcinoma, while its expression is low in benign lesions represented by liver cirrhosis (109). In thyroid tissue, the cell staining efficiency of HLA-G antigen in follicular adenocarcinoma and thyroid cancer is significantly higher compared with the normal thyroid and goiter (110). In ovarian tissue, HLA-G is always co-localized with CA125 protein, indicating that OC cells express HLA-G, while normal cells do not (39). In cervical cancer lesions, the expression of HLA G is an important predictor of CIN I and age, which is not affected by other variables. In addition, HLA-G interacts with immunosuppression induced by human papillomavirus infection, leading to more serious clinical outcomes observed in patients with CIN III and invasive cervical cancer (111, 112).

In oral squamous cell carcinoma (OSCC), according to IHC and reverse transcription-polymerase chain reaction (RT-PCR) results, the higher the TNM stage, the higher the protein expression level of HLA-G, and the histological grade and lymph node metastasis are positively correlated with the expression of HLA-G (107). These results indicate that HLA-G is related to the malignant transformation of tumors, supporting that HLA-G is an indicator of early diagnosis and dynamic monitor. The expression of HLA-G in tissues may also be an important indicator for the prognosis of cancer patients. In 201 colon cancer patients, IHC staining shows that the survival time of patients with HLA-G-positive tumors is significantly shorter compared with those carrying HLA-G-negative tumors (113). In multivariate analysis, HLA-G shows the potential as an independent prognostic factor.

In a joint analysis of HLA class I, HLA-E, and HLA-G to predict the prognosis of colorectal cancer (CRC), three tumor immune phenotypes are generated by comprehensively analyzing the expressions of all markers, resulting in strong immune system tumor recognition, intermediate immune system tumor recognition, and poor immune system tumor recognition. These immune phenotypes represent important and independent clinical prognostic characteristics of colon cancer (108). In nasopharyngeal carcinoma (NPC), HLA-G is positively correlated with CD68+ macrophages and IL-10 expression, indicating that HLA-G may regulate immune escape in NPC (114). Among 522 NPC specimens, the expression of HLA-G at the protein level is detected in 79.2% of cases. In addition, the high expression of HLA-G predicts the low survival rate of NPC patients (114). Moreover, HLA-G is suggested to be an independent predictor of cancers, such as esophageal squamous cell carcinoma (115), gastric cancer (37, 116), breast cancer (52, 117, 118), and OC (38, 44, 119). Finally, the differential expressions of HLA-G can also help predict and diagnose different subtypes. Significant differences between groups have been observed between low-grade glioma and high-grade glioma tissues (120). Besides, the expression of HLA-G in the non-luminal subtypes of invasive ductal carcinoma of the breast is significantly higher compared with the luminal subtypes (121).

## sHLA-G IN THE BLOOD OF TUMOR PATIENTS AS CIRCULATING TUMOR MARKERS

In the serum of healthy people, the content of HLAG is 20 ng/mL and significantly lower compared with cancer patients. sHLA-G is produced and secreted mainly by immune cells and tumors (122). For example, in acute leukemia, the level of sHLA-G in T cells and monocytes in the serum is detected by enzyme-linked immunosorbent assay (ELISA), which is averagely five times higher compared with healthy controls. Moreover, sHLA-G can be secreted *in vitro* by DCs, lymphocytes, plasma cells, and monocytes/macrophages (56), and these secreted sHLA-G molecules cause anti-tumor reactions locally in the tumor or along with the circulatory system to the whole body. Next, we described the current status of sHLA-G in the serum of tumor patients as tumor markers from the aspects of diagnosis, prognosis, and identification (**Table 1**).

In terms of diagnosis, sHLA-G is abnormally expressed in the plasma in breast ductal carcinoma (131), head and neck squamous cell carcinoma (129), gastric cancer (33), CRC (125), and papillary thyroid carcinoma (65), which is considered to be a preoperative diagnosis of cancer histopathology potential marker of aggressiveness. It is worth noting that in gastric cancer, researchers have found that sHLA-G in combination with common serum tumor markers, such as CA72-4, CA125,

**TABLE 1 |** Clinical research involving diagnosis and prognosis of sHLA-G in the blood.

| Cancer type | Sample source | Sample size | Methods (Ab) | Experimental result evaluation | Expression evaluation of HLA-G | Ref. |
|---|---|---|---|---|---|---|
| **Breast cancer** | plasma | 142 (procured before NACT) 154 (after) | ELISA | The total concentration of sHLA-G plasma levels (median [range] ng/mL) from BC patients (n = 102) before (41.3 [4.4–117.6]) and after (44.6 [3.1–117.6]) NACT was significantly increased compared with 16 healthy female controls (16.3 [4.0–37.8]). | The free soluble and vesicular HLA-G as prognostic markers, whereas the total sHLA-G levels without dividing into subcomponents were not related to clinical outcome. | (48) |
| | plasma | 92 (patients) 70 (controls) | ELISA | Concentration of the plasma sHLA-G was with the median of 82.19 U/mL (range 13.50 –191.37) for BC patients, and 9.65 U/mL (range 4.38 – 69.69) for normal controls, | Plasma sHLA-G levels might be a useful preoperative biomarker for diagnosis | (49) |
| **Ovarian cancer** | plasma | 79 (patients) 80 (controls) | ELISA (MEM-G9) | In OC patients, sHLA-G1 levels were more increased than HLA-G5 levels. | As a potential biomarker for advanced and complicated OC. | (66) |
| **Lung cancer** | plasma | 137 | ELISA | In lung cancer patients, the plasma levels (median[range]) of sHLA-G were significantly increased compared with healthy controls (34 ng/mL [3.6–160] vs. 14 ng/mL [0–98]). | Plasma levels of sHLA-G is potent predictors for overall survival (OS) in lung cancer patients. | (26) |
| | plasma | 91(patients) 150 (controls) | ELISA | The median plasma sHLA-G was 34.0 U/mL (range 3.13 – 275.5) in NSCLC patients and 20.4 U/mL (range 0.97 -270.6) in controls. | HLA-G may be a potential therapeutic target, and plasma sHLA-G of NSCLC patients can be used as a prognostic factor for NSCLC. | (23) |
| **Colorectal cancer** | plasma | 133 | ELISA (MEMG/9) | sHLA-G levels were higher in patients with mucinous carcinoma (MC). | A useful prognostic marker and predictive biomarker of therapeutic response in advanced CRC. | (64) |
| **Endometrial cancer** | plasma | 40 (patients) 45 (controls) | ELISA | The majority of EC patients expressed the sHLA-G1 subtype (75%), and only 25% expressed the HLA-G5 isoforms. | Related to clinical progress. | (123) |
| **Thyroid carcinoma** | plasma | 85 (patients) 77 (30 days after surgery) | ELISA (MEM-G/9) | sHLA-G was decreased in patients with invasion. | Associated with tumor invasion. | (65) |
| **Thyroid carcinoma** | plasma | 121 (patients) 183 (underwent PTC surgery) | ELISA (MEM-G9) | sHLA-G level was significantly higher in PTC patients than those without markers of aggressiveness | sHLA-G as a potential novel marker of PTC aggressiveness | (67) |
| **Esophageal squamous cell carcinoma** | plasma | 41 (patients) 153 (controls) | ELISA | The median plasma concentration of sHLA-g in ESCC patients was 152.4 U/mL (range 28.8-239.5) *versus* 8.9 U/mL (range 4.6-63.5) in normal controls. | May be a useful biomarker for preoperative diagnosis. | (115) |
| **Breast cancer** | serum | 80 (patients) 80 (controls) | ELISA | Levels of sHLA-G were higher in the breast cancer group (median117.2 U/mL) compared with the control group (median 10.1 U/mL, P, 0.001). | Measurement of sHLA-G concentrations has diagnostic value for detecting breast cancer and metastasis. | (124) |
| **Lung cancer** | serum | 191 (patients) 191 (controls) | ELISA | The mean serum level of sHLA-G in NSCLC patients (53.3 ± 4.6 U/mL) was significantly increased compared with controls (8.36 ± 0.4 U/mL). | Serum sHLA-G levels in NSCLC patients could be useful biomarkers for the diagnostic and prognosis of NSCLC. | (22) |
| **Colorectal cancer** | serum | 398 | ELISA (MEMG/9) | Median sHLA-G was significantly higher in cancer compared with normal CRC, hyperplastic polyps, inflammatory bowel disease, and adenomas (all $P < 0.001$). | May be a useful indicator in differentiating colorectal cancer from benign colorectal diseases. | (125) |
| **Thyroid carcinoma** | serum | 145 | ELISA | sHLA-g in serum was increased in patients with thyroid carcinoma compared with healthy controls ($P < 0.05$). | Affects the progression of thyroid cancer. | (126) |
| **Oral squamous cell carcinoma** | serum | 216 (patients) 193 (controls) | ELISA | sHLA-G levels were higher in the OSCC patients than healthy subjects (85.694 ± 69.966 U/mL *vs.* 11.404 ± 10.424 U/mL). | sHLA-G may act as a promising biomarker for non-invasive diagnosis of OSCC. | (127) |
| **Head and neck squamous** | venous blood | 383 (patients) | PCR | Individuals with Del/Ins and Ins/Ins genotypes were at greater risk of HNSCC disease than those with Del/Del genotypes. | The C/C, Del/Ins and Ins/Ins genotypes as well as C and Ins alleles may be the major | (128) |

*(Continued)*

**TABLE 1** | Continued

| Cancer type | Sample source | Sample size | Methods (Ab) | Experimental result evaluation | Expression evaluation of HLA-G | Ref. |
|---|---|---|---|---|---|---|
| **cell carcinoma** | | 383 (controls) | | | risk factors for the strong influence of tobacco on HNSCC in Indian. | |
| | serum | 120 (patients) 99 (controls) | ELISA | Compared with the control group (6.45 ± 1.31 ng/L), the levels of SHLA-G in patients were significantly higher (8.25 ± 1.74 ng/L). | Potential diagnostic serum protein markers. | (129) |
| **Esophageal cancer** | EDTA blood | 239 (patients) 467 (controls) | PCR | In the Kazakh region, individuals with the -14 bp/-14 bp and C/C genotypes had a 2.82 times higher risk of developing EC than those with the +14 bp/+14 bp and C/C genotypes. | The 14 bp deletion/insertion of HLA-G gene may play a role in EC susceptibility of Kazakh. | (130) |

and CA19-1, can improve the clinical screening of gastric cancer compared with sHLA-G alone (132). In patients with breast cancer, the expression of HLA-G is negatively correlated with proliferation factor (133). In addition, the concentration of sHLA-G in plasma helps predict and diagnose cancers of different subtypes. Compared with patients with simple lobular carcinoma and simple ductal carcinoma, patients with mixed lobular lesions and breast ducts have a more significant increase in sHLA-G (131). ELISA also shows that the plasma levels of sHLA-G in patients with liver cancer are higher compared with the control group (134). These results support that sHLA-G can be used for tumor early diagnosis.

In terms of prognosis, the high expression of sHLA-G in patients with non-small cell lung cancer is significantly related to poor overall survival (OS), especially in patients with advanced cancer (135). An interesting phenomenon has been found in the Tunisian population that patients with HLA-G*01:04:01 alleles have elevated plasma levels of sHLA-G, while patients who do not carry HLA G*0105N alleles (cannot encode HLA-G1 protein) express a dramatically reduced level of sHLA-G in plasma (22). In CRC, the prognostic value of plasma sHLA-G is mainly reflected by predicting the risk of liver metastasis in patients with stage II and III CRC. Hauben and colleagues have found that the sHLA-G levels are associated with shorter liver metastasis-free survival (LMFS) in patients with stage II CRC and longer LMFS in patients with stage III CRC. The possible reason can be attributed to the fact that the stage III patients receive chemotherapy before the sample collection, and the tumor cells are damaged (64). Therefore, the differential level of plasma sHLA-G is a predictive biomarker of treatment response to advanced CRC, and it is also a potential prognostic marker.

## sHLA-G IN BODY FLUIDS OF TUMOR PATIENTS AS TUMOR MARKERS

The source of sHLA-G is related to the inflammatory factors present in the cancer microenvironment and the reduction of NK cells and memory T cells (136, 137). Researchers have revealed that the expression of HLA-G in ascites, saliva, and bronchial lavage fluid can be useful for tumor diagnosis and prognosis (**Table 2**).

## ASCITES

Results obtained from Ullah's group have shown that sHLA-G in ascites is mainly expressed by ascites cells, tumor cells, and stromal cells (39). Moreover, Sun et al. have found that the level of sHLA-G in malignant ascites induced by various solid tumors is significantly higher compared with benign ascites. Especially for ascites caused by gynecological tumors and gastrointestinal tumors, the levels of related tumor markers CEA and CA199 are also elevated in malignant ascites, while its specificity and sensitivity are significantly lower compared with sHLA-G (139). The level of sHLA-G in malignant ascites caused by OC and breast cancer is significantly higher compared with benign ascites. In addition, the specificity, sensitivity, and AUC are greatly improved when the critical value of 13 ng/mL is achieved. Besides, in OC, sHLA-G1/G5 is negatively correlated with CD3-/CD56+ subgroups and CD4+ CD45RO+ memory cells, suggesting that sHLA-G plays a role in the tumor microenvironment by up-regulating T-reg cells and down-regulating NK cells (39). In cytology-negative malignant ascites, sHLA-G also has outstanding diagnostic performance. In 32 cases of cytology-negative ascites, the positive rate of sHLA-G is 75%, which is significantly higher compared with CEA and CA199 (50). Collectively, sHLA-G can be used as an independent indicator for early diagnosis of malignant ascites, and it is helpful to screen for malignant ascites when cytology is negative.

## SALIVA

Saliva is a body fluid that can be collected easily, quickly, and non-invasively (140). Biomolecules in saliva have been used as promising markers for early diagnosis, detection, and treatment in OSCC (141, 142), breast cancer (143, 144), OC (145), and other tumors. In OSCC, saliva is the body fluid that has the closest contact with oral tumors. Researchers have collected the saliva samples from 22 OSCC patients. Compared with non-metastatic OSCC, the HLA-G level of metastatic OSCC is dramatically elevated and correlated with poor OS (63). The sHLA-G level in CRC patients is also significantly higher compared with healthy controls, especially in patients with stage III-IV tumor (62). In the bronchial lavage fluid of

**TABLE 2 |** Clinical research involving diagnosis and prognosis of HLA-G in the body fluid.

| Cancer type | Sample source | Sample size | HLA-G type | Methods (Ab) | Experimental results evaluation | Expression evaluation of HLA-G | Ref. |
|---|---|---|---|---|---|---|---|
| **Breast cancer Ovarian cancer** | ascites specimens | 24 (malignancy) 19 (negative) | sHLA-G | ELISA (W6/32) | The levels of sHLA-G were significantly higher in malignant compared with benign ascites | Measurement of sHLA-G is a useful molecular adjunct to cytology in the differential diagnosis of malignant *versus* benign ascite | (50) |
| **Ovarian cancer** | peritoneal fluid | 16 | sHLA-G1 HLA-G5 | ELISA (MEM-G/9、5A6G7) | The level of HLA-G5 isoform was specifically higher in seven samples. | sHLA-G, particularly HLA-G5, may affect antitumor immune response both *in situ* and in circulation. | (39) |
| **Breast cancer** | effusions | 46 | HLA-G | IHC (4H84) | IHC showed predominantly focal HLA-G expression in 12 of 46 (26%) breast carcinoma effusions and 16 of 39 (41%) solid lesions | Associated with shorter disease-free survival. | (51) |
| **Ovarian cancer** | effusions | 148 | HLA-G | IHC (4H84) | HLA-G was detected in cancer cells in 49/148 (33%) effusions, 33/66 (50%) primary tumors, and 59/122 (48%) solid metastases. | A new role for HLA-G as a prognostic indicator in advanced-stage OC in effusions. | (47) |
| **Colorectal cancer** | saliva | 20(patients) 10(controls) | sHLA-G1 HLA-G5 | ELISA | In patients diagnosed with CRC, salivary sHLA-G values were significantly higher compared with the control group of healthy patients | sHLA-G can be a good prognostic and diagnostic biomarker in CRC. | (62) |
| **Colorectal cancer** | single-cell suspensions | 157 | HLA-G | Flow Cytometry Analysis (MEM-G/09) | The median percentage of HLA-G expression was 14.90% (range:1.81% to 79.90%). | HlA-G is closely associated with the survival of CRC patients. | (138) |
| **Oral squamous cell cancer** | saliva | 22 (patients) 23 (controls) | sHLA-G | ELISA | There was no significant difference in shLA-G concentration between OSCC and control groups. | It helps tumor cells evade immune defense mechanisms. | (63) |
| **Lung cancer** | bronchoalveolar fluid | 31 | sHLA-G | ELISA | The mean value of soluble HLA-G was 49.04 ng/mL, and the level of HLA G varied greatly in metastatic tumors. | HLA-G soluble protein is significantly associated with patients with metastatic tumor and can be used as a prognostic marker of lung cancer. | (24) |

patients with different histological types of lung cancer, the level of sHLA-G is significantly correlated with lower Karnofsky index in metastatic tumors and can be used as a prognostic marker for lung cancer (24). The exudate of OC patients is collected before, during, or after chemotherapy. It is found that the expression of HLA-G is decreased after chemotherapy, and the decreased expression of HLA-G predicts the improvement of patient survival rate. This may be related to the preferential sensitivity of HLA-G-expressing cells (47). Therefore, researchers believe that saliva sHLA-G can be used as a diagnostic and prognostic biomarker for multiple cancers, and it is a less invasive alternative to venipuncture. However, more studies are needed to confirm the significance of saliva sHLA-G as a tumor indicator.

## HLA-G DERIVED FROM EVs AS A TUMOR MARKER

EVs are composed of growth factors, biologically active lipids, genetic information, and antibodies/ligands/receptors, and they are resistant to RNase, enduing their great potential as a tumor biomarker (146–148). Secreted HLA-G can exist in the form of free sHLA-G or be secreted by EVs and found in various body fluids, such as plasma, ascites, and pleural exudate (96) (**Figure 2**). Recent studies have found that tumor cells, cytotrophoblast cells, and mesenchymal stem cells can secrete HLA-G-carrying EVs, playing a role in regulating the tumor microenvironment and immunosuppressive function (149). Therefore, HLA-G can be shedding from the cell surface by metalloproteases or released from various cells, incorporated into EVs, and serve as a promising tumor indicator (77, 104, 150).

Riteau et al. have isolated an HLA-G-positive cell line from primary and metastatic lymph node melanomas and named it Fon. For the first time, this melanoma cell line is found to secrete exosomes containing HLA-G1. They speculate that the immune tolerance produced by melanoma-derived HLA-G exosomes may be a method for tumors to regulate host immunity. So far, the specific mechanism of this method is still unclear (150). In breast cancer patients receiving neoadjuvant chemotherapy (NACT), the relationship between exosomes carrying HLA-G and the prognosis of the disease has been evaluated. Before NACT, the sHLA-$G_{EV}$ levels are correlated with circulating stem cell-like tumor cells. The total amount of sHLA-$G_{EV}$ is

significantly increased after NACT, and it is related to the disease process, while the total sHLA-G level has nothing to do with the clinical prognosis (48). In addition, HLA-G-bearing EVs released by renal cancer cells damage the differentiation of monocytes into DCs and inhibit the maturation process of DCs. These findings suggest that HLA-G-mediated tumor immune escape mechanisms can spread to HLA-G-negative tumor cells through the EV pathway (151, 152). In epithelial ovarian cancer (EOC), the levels of HLA-$G_{EV}$ are increased by seven times compared with healthy controls, and the elevated HLA-$G_{EV}$ can serve as independent 3-year and 10-year progression-free survival (PFS) prognostic factors. It is worth noting that all patients with high levels of HLA-$G_{EV}$ experience disease progression within approximately 5 years after the initial diagnosis. Schwich's group has also shown that HLA-$G_{EV}$ serves as an independent risk assessment marker for disease progression of EOC (40).

## HLA-G DETECTION AND QUANTIFICATION

Based on the current understanding of HLA-G and its various forms that have multiple immune tolerance regulating functions in malignant tumors, HLA-G is generally recognized as a biomarker that can be used to monitor the disease state and progression in cancer patients (153). However, due to the diversity of HLA-G structures, the standardization of HLA-G detection methods has been a topic of discussion from the past to the present (154). HLA-G test results vary greatly in different locations of HLA-G acquisition, between different tumors, or the same tumor in different laboratory test results.

## DETECTION OF HLA-G IN TISSUES

The status of HLA-G in tumor tissues is usually detected by IHC. Although IHC is an experimental technique that has been widely used, there is controversy about the use of such a method to detect HLA-G. Because HLA-G has multiple subtypes, and each antibody recognizes only specific epitopes, leading to different staining results (52, 54, 124, 155). In addition, the experimental procedures of IHC also differ greatly, such as the type of antibody used and its dilution, incubation time, and staining evaluation criteria. The differences in treatment history, tumor subtypes, and individual tumor microenvironment between different patients will also affect the evaluation of HLA-G expression levels (156–158).

In 81 patients with colon cancer, Swets and colleagues have used three monoclonal antibodies (4H84, MEM-G/1, and MEM-G/2) to evaluate the expression of HLA-G. In primary tumors, the positive staining rates of HLA-G using monoclonal antibody 4H84, MEM-G/1, and MEM-G/2 are 29%, 6%, and 10%, respectively. They have found that different epitopes of HLA-G detected by different monoclonal antibodies are differentially expressed in CRC tissues (159–161). Although it has been confirmed that 4H84 and MEM-G/1 can recognize all subtypes of HLA-G, the reason for this difference in expression is generally believed to be a cross-reaction with HLA-I (160, 161). For example, 4H84 has been shown to cross-react with the existence of β2M free classic HLA class I molecules on activated leukocytes. This may lead to an overestimation of HLA-G expression in pathological tissues recognized by leukocyte infiltration (such as CRC), leading to differences among studies (162). Therefore, it is recommended to use a variety of different monoclonal antibodies to detect HLA-G.

In addition, whether these antibodies can block the binding of HLA-G to its receptors (ILT2, ILT4, and KIR2DL4) is also a challenge (163). Antibodies targeting the α3 domain and β2M of HLA-G can block the binding of HLA-G to ILT2 and ILT4. The antibody targets the α2 domain and can block the binding of HLA-G to KIR2DL4. It has been confirmed that MEM-G/1 blocks the binding site of HLA-G2 and ILT4, while MEM-G/9 and G233 can bind to HLA-G1, depriving the binding site of ILT2. Besides, antibody 87G can block the interaction between HLA-G1 and its receptor (164). Therefore, to block the interaction of HLA-G with all its receptors, it is necessary to develop an antibody mixture that can recognize all HLA-G subtypes and block the binding of HLA-G to ILT2, ILT4, and KIR2DL4, thereby reducing HLA-G cross-reaction with its receptors.

## DETECTION OF sHLA-G IN FLUIDS

sHLA-G is also expressed and released by cancer cells, which is a potential biomarker in the body fluids of cancer patients (62). In particular, sHLA-G in the supernatant of IVF embryos has been considered as an independent factor predicting pregnancy outcome (165–167). Therefore, as early as 2004, in the Wet Workshop for the quantification of sHLA-G, the standardization of sHLA-G detection and quantification methods has been discussed, including sensitivity, standard reagents, and antibody specificity (168). At present, ELISA is mainly employed to quantify the level of sHLA-G in body fluids. The monoclonal antibody MEM-G/9 is used to simultaneously detect the shedding of HLA-G1 and the secreted HLA-G5. MEM-G/9 specifically captures β2M-related HLA-G1 and HLA-G5 as well as polyclonal anti-human β2M antibodies (169). In this seminar, HLA-G-expressing EVs are also discovered. This structure cannot be detected by the combination of MEM-G/9 and anti-β2 but can be detected by an antibody combination of mAb 5A6G7 and W6/32. Given the often-antagonistic composition and complex structure of EVs, the various components carried by EVs may drive the functional activity of EVs expressing HLA-G and eliminate or enhance the immune tolerance function of HLA-G.

ExoQuick™ precipitation is now usually used to extract EVs from samples and to quantify the number of EV particles and the number of vesicular-bound HLA-G (HLA-$G_{EV}$) (48). The results show that in OC, poor clinical status and presence of CTC, and PFS are associated with elevated HLA-$G_{EV}$ levels (40). In breast cancer patients receiving NACT, high levels of vesicular sHLA-G are also associated with disease progression (48). Given these,

future work should focus on the standardization process. Before being applied to routine clinical practice, a larger research cohort, prospective research, and internationally recommended standardized testing methods are needed to verify the application of HLA-G in diseases. Importantly, it is necessary to develop more specific antibodies against HLA-G subtypes, explore new undiscovered HLA-G subtypes, and make full use of existing experimental techniques to evaluate the role of various subtypes in various tumors.

## CONCLUSIONS

After more than 30 years of research, it has been shown that compared with other tumor markers, HLA-G has unique characteristics as follows: 1) it participates in the immune tolerance network in healthy individuals and tumor patients; 2) it is barely expressed in normal tissues and frequently identified in tumor cells. Given these characteristics of HLA-G, one of the current research goals is to serve as a biomarker for diagnosis, prognosis, and clinical testing. However, many factors affect the interpretation of the clinical significance of HLA-G, such as the number of patients included in the study, different clinical parameters of patients (such as medical history, disease stage, and treatment plan), and differences in testing protocols. To better explain the structural diversity of HLA-G, sHLA-G, HLA-$G_{EV}$, and their expression and clinical significance in tumors, future efforts should be devoted to studies focusing on multi-center and large sample analysis, and the establishment of standardized and feasible HLA-G detection methods.

## AUTHOR CONTRIBUTIONS

PL, NW and YZ wrote and edited the manuscript. CW and LD edited the manuscript. All authors contributed to the article and approved the submitted version.

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# Perspective of HLA-G Induced Immunosuppression in SARS-CoV-2 Infection

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COVID-19, the disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has threatened public health worldwide. Host antiviral immune responses are essential for viral clearance and disease control, however, remarkably decreased immune cell numbers and exhaustion of host cellular immune responses are commonly observed in patients with COVID-19. This is of concern as it is closely associated with disease severity and poor outcomes. Human leukocyte antigen-G (HLA-G) is a ligand for multiple immune inhibitory receptors, whose expression can be upregulated by viral infections. HLA-G/receptor signalling, such as engagement with immunoglobulin-like transcript 2 (ILT-2) or ILT-4, not only inhibit T and natural killer (NK) cell immune responses, dendritic cell (DC) maturation, and B cell antibody production. It also induces regulatory cells such as myeloid-derived suppressive cells (MDSCs), or M2 type macrophages. Moreover, HLA-G interaction with CD8 and killer inhibitory receptor (KIR) 2DL4 can provoke T cell apoptosis and NK cell senescence. In this context, HLA-G can induce profound immune suppression, which favours the escape of SARS-CoV-2 from immune attack. Although detailed knowledge on the clinical relevance of HLA-G in SARS-CoV-2 infection is limited, we herein review the immunopathological aspects of HLA-G/receptor signalling in SARS-CoV-2 infection, which could provide a better understanding of COVID-19 disease progression and identify potential immunointerventions to counteract SARS-CoV-2 infection.

**Keywords: HLA-G, immune receptor, SARS-CoV-2, COVID-19, immune suppression**

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## INTRODUCTION

COVID-19, the disease caused by the highly contagious virus "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2), has become a serious global public health concern (1). Although unprecedented comprehensive virus transmission prevention measures have been strictly implemented, such as travel restrictions, public social distancing, personal hygiene, and patient quarantine requirements, the virus has spread widely and caused more than 5,054,267 deaths worldwide since its outbreak in December 2019 (2–4).

The clinical manifestation of COVID-19 can be asymptomatic, mild to moderate, or severe, or critical pneumonia with symptoms of acute respiratory distress syndrome, multi-organ failure, and/or shock (5). Among patients with COVID-19, risk factors such as advanced age and pre-existing conditions are associated with increased disease severity (6). From an immunological perspective, a host's innate and adaptive immune responses are indispensable in controlling viral infection and disease progression. However, abnormal host immune responses are common in patients with severe COVID-19, including cytokine storm resulting from hyper-inflammatory immunological humoral reactions, as well as impairment of cellular antiviral immune responses (7, 8). Our previous studies revealed that total lymphocytes, CD3+, CD4+, and CD8+ T cells were dramatically lower among patients with severe COVID-19 than among non-severe patients at admission. These cells returned to normal levels by the second week after discharge, however, lower CD8+ T cell count is an independent risk factor for longer viral positivity duration and is related to an increased risk for discharged patients with SARS-CoV-2 re-positivity (9–11). Pro-inflammatory cytokines and chemokines, such as interleukin (IL)-1β, IL-6, IL-8, tumour necrosis factor (TNF)-α, macrophage inflammatory protein (MIP) 1α/CCL3, interferon (IFN)-γ-induced protein 10 (IP10), monocyte chemoattractant protein 1 (MCP1), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), were substantially increased (12, 13). Moreover, antiviral cellular immune responses were compromised because of the following: (a) the dramatically decreased absolute number of CD3+ lymphocytes, subpopulations of CD4+ and CD8+ T cells, CD3+CD56+ NKT cells, B cells, and natural killer (NK) cells (14, 15); (b) various functions were impaired and/or exhausted, such as the cytotoxicity of these immune effectors (16–18); and (c) immune regulatory cells, including myeloid-derived suppressor cells, were notably expanded in severe cases (19).

Although an increasing number of clinical and immunological findings on the immunopathological features of COVID-19 are being reported, the molecular mechanisms involved in the dysregulation of cellular immune responses against SARS-CoV-2 infection are yet to be discovered (20). During viral infection, various strategies to escape the host antiviral immune attack and to favour replication and disease progression have been developed by the virus particles (21, 22). Alteration and intervention of human leukocyte antigen (HLA) and/or its receptor expression is one of the strategies applied by viruses (23). In patients with COVID-19, HLA-E receptor CD94/NK group 2 member A (NKG2A), a member of the immune inhibitory receptors, is remarkably increased in CD8+ T and NKT cells, resulting in their functional exhaustion. Notably, high levels of NKG2A expression are significantly reduced when patients recover from the disease (18).

HLA-G is a non-classical HLA class I antigen, which is a strong immune inhibitory mediator *via* receptor signalling. Because of the alternative splicing its primary transcript, at least seven HLA-G isoforms have been identified, which include four membrane-bound (HLA-G1, HLA-G2, HLA-G3, and HLA-G4) and three soluble (HLA-G5, HLA-G6, and HLA-G7) isoforms (24). HLA-G can be upregulated by various viral infections, including SARS-CoV-2, which can render comprehensive immunosuppressive roles in favouring virus immune evasion and subsequent disease progression (25, 26). Several immune cell surface-expressed receptors have been identified that bind to HLA-G, including immunoglobulin-like transcripts-2 (ILT-2)/CD85j/LIR1, ILT-4/CD85d/LIR2, killer inhibitory receptor (KIR) 2DL4/CD158d, CD8, and CD160 (24). The glycosylphosphatidylinositol-anchored transmembrane glycoprotein receptor CD160 is closely related to the KIR2DL4, although their homology is rather weak only with 29% identity and 44% similarity (27). In this scenario, HLA-G/receptor signalling among various immune cells is important in COVID-19 pathogenesis and progression (**Figure 1**).

Herein, we focus on the implication of HLA-G/receptor signalling on immune response impairment during SARS-CoV-2 infection.

## IMMUNE MODULATION OF HLA-G MOLECULE

The *HLA-G* gene was identified by Geraghty et al. in 1987, and HLA-G protein expression was first found in extravillous cytotrophoblasts in 1990 (28, 29). A number of studies have focused on the basic and clinical significance of HLA-G in foetal-maternal immune tolerance. Since then, immune receptors, including KIR2DL4, ILT-2, ILT-4, CD8, and CD160, have been discovered (30–34). HLA-G induced immune suppression has been extensively documented (including direct and/or indirect as well as long- and short-term), and the immunosuppressive functions of HLA-G have been well established (29, 35). The signalling between HLA-G and receptors KIR2DL4, ILT-2, ILT-4, CD8, and CD160 expressed on different types of immune cells is a fundamental prerequisite for the aforementioned immune suppressive functions of HLA-G (36). Recently, the immune inhibitory NKG2A/CD94 receptor, a well-known receptor for HLA-E, has been reported to be a new HLA-G allele-dependent receptor (37).

By directly binding to immune cell surface receptors ILT-2 or/and ILT-4, HLA-G can inhibit T cells, NK cells, and B cell proliferation, cytotoxicity, anti-inflammatory cytokines such as interferon-chemotaxis, immunoglobulin production, or MICA/NKG2D activation or cell senescence (22). HLA-G can also induce the generation of anergic and regulatory T cells (Treg), tolerogenic DCs, polarisation of M1 to M2, and Th2-type cytokine secretion (24). Moreover, HLA-G can suppress neutrophil reactive oxygen species production and the capacity for phagocytosis (38). Indirect immune suppression induced by HLA-G could be caused by multiple intracellular transfers of HLA-G from HLA-G-bearing cells to HLA-G-negative neighbouring cells or distant cells. Both allogeneic and autologous cell membranes containing HLA-G between/among cells through the process of trogocytosis have been observed,

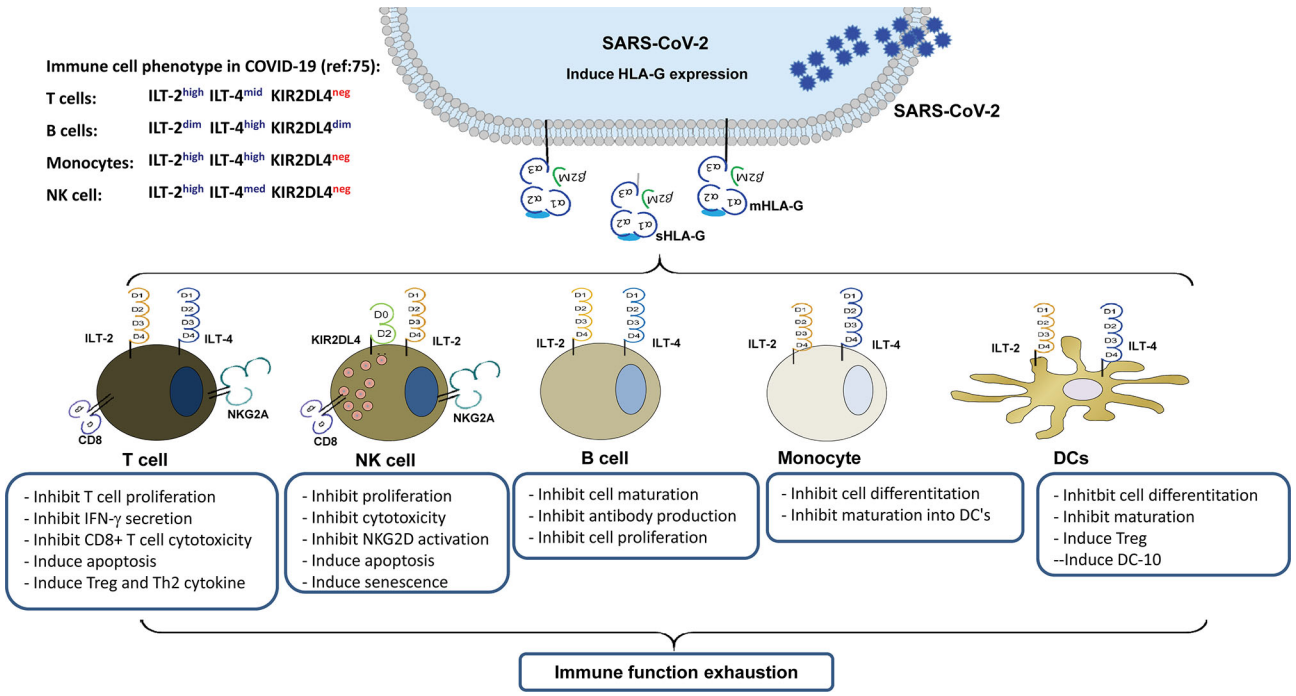


**FIGURE 1** | Immune suppression mediated by up-regulated HLA-G expression in virus infected cells and its interaction with immune receptors expressed on different types of immune cells during SARS-CoV-2 infection.

resulting in the activation of immune effector cells (T cells, NK cells, monocytes) into suppressor cells. In addition, HLA-G can be transferred by exosomes to long-distance cells, impairing the functions of immune-competent cells (39). Furthermore, indirect immune modulation induced by HLA-G could increase HLA-E expression, thus influencing the HLA-E/CD94/NKG2 receptor signalling pathway. Cell surface HLA-E expression is dependent on the binding of TAP-associated peptides derived from other HLA class I signal sequences (40). Among the different HLA class I signal peptides, the HLA-E/G-nonameric complex has a significantly higher affinity (41). However, different HLA-G isoforms may have different effects on the enhancement of HLA-E cell surface localization (42).

## INDUCTION OF HLA-G EXPRESSION IN INFECTIOUS DISEASES

HLA antigens are critical for both the innate and adaptive immune systems as they bind to T cell receptors to present HLA-restricted peptides to T cells and interact with NK cell receptors to modulate the functions of innate immune components, such as NK cells (43). Downregulation of the expression of the classical HLA class I antigens (HLA-A, -B, and -C) and HLA class II antigens (DP, DQ, and DR) is one of the most effective strategies for perpetuating viral infections, as it allows virus-infected cells to escape from the host immune attack led by virus-specific CD8+ T cells and blunt CD4+ T cells, which help B cells to produce virus-specific antibodies (44). The aberrant upregulation of the immune tolerant HLA-G expression is more common than the downregulation of the HLA-I and -II antigens during many infections, providing virus-infected cells a strategy to protect themselves from NK cell cytolysis (45, 46).

Mechanisms underlying the regulation of HLA-G protein expression is quite complex. HLA-G protein expression can be driven by specific *HLA-G* genetic polymorphisms, and extracellular and intracellular signals. *HLA-G* polymorphisms such as a rs66554220, a 14-bp insertion/deletion in the *HLA-G* exon 8 involved in the stability of *HLA-G* mRNA, where *HLA-G* mRNA is more stable with the 14-bp deleted allele and related to HLA-G protein expression (47–49). MicroRNAs miR-148a,miR-148b, miR-152, miR-133a, miR-628-5p, and miR-548q have been reported to regulate HLA-G expression (50). Among them, position +3142 (C>G, rs1063320) has high binding affinity to microRNAs miR-148a,miR-148b, andmiR-152, which is related to the suppression of HLA-G production (51). In addition to the microRNAs, specific RNA binding proteins, such as the heterogeneous nuclear ribonucleoprotein R (HNRNPR), which can bind *HLA-G* in 3' untranslated regions (UTR) and stablize the HLA-G transcripts, and increase HLA-G expression (52). HLA-G promoter region includes several specific regulatory elements. HLA-G promoter heat shock element can response to heat shock proteins, and dexamethasone and progesterone can interact with a unique progesterone response element to regulate HLA-G protein expression (53, 54). Also, extracellular

environmental factors such as cytokine IL-10, IFNs, indolamine 2, 3-dioxygenase (IDO), granulocyte-macrophage colony-stimulating factor (GM-CSF), hypoxia and demethylation condition affects HLA-G protein expression (48, 55–58). However, peripheral sHLA-G levels are not significantly different between male and female individuals (49, 59). Virus itself or viral gene products such as U94 viral gene of human herpesvirus 6 (HHV-6) can induce HLA-G activation by recognizing an *HLA-G* promoter consensus sequence (60). Through no specific mechanism for HLA-G up-regulation has been outlined during the SARS-CoV-2 infection, COVID-19 patients peripheral circulation highly increased cytokines such as IL-10, GM-CSF and IDO could be factors involved in the HLA-G expression modification (61, 62).

Upregulation of both virus-infected cell surface membrane-bound HLA-G and peripheral soluble HLA-G expression has been observed in various viral infectious diseases, such as human immunodeficiency virus type 1 (HIV-1), herpes simplex virus-1, rhabdovirus, human cytomegalovirus, hepatitis B and C virus, and influenza A virus (63). HLA-G expression in monocytes and T cells of HIV-1 infected individuals was much higher than that in healthy controls (64). Recent studies have shown that increased HLA-G expression on monocytes could be induced by highly active antiretroviral therapy (HAART), and that cell surface HLA-G expression is more stable than that of the other HLA molecules owing to its resistance to HIV-1 derived protein Nef degradation (65, 66). Moreover, soluble HLA-G could inhibit myeloid dendritic cell function, and higher peripheral circulating sHLA-G levels were shown to be linked to the rapid progression of HIV-1 infection (67, 68).

In the context of SARS-CoV-2 infection, there is currently limited information on the biological and clinical significance of HLA-G. In a previous study using global transcriptomic analysis, Josset et al. (26) found that SARS-CoV-2 and Middle East respiratory syndrome coronavirus (MERS-CoV) differentially activated *HLA-G* transcription in the human lung epithelial cell line Calu-3 *in vitro*. This study revealed that HLA-G transcripts could be specifically downregulated by MERS-CoV, whereas HLA-G was upregulated by SARS-CoV-2 infection. With a larger case/control cohort (2244/10220), a recent genome-wide association study (GWAS) by Pairo-Castineira et al. (69) showed that *HLA-G* (rs9380142) is a novel genetic locus, which is strongly associated with the severity of COVID-19. Association between *HLA-G* polymorphism and viral infection has been documented in a number of studies. *HLA-G**01:01:08 was reported to be a risk factor for HIV-1 infection in Zimbabwean while 3'UTR 14-bp In/14-bp In was a risk factor for HIV infection in South Africans of African ancestry women (70, 71). 14-bp del/14-bp del and *HLA-G*01:04:01*/14-bp del genotypes, and UTR-2 and UTR-3 haplotypes were found to be the risk factors for hepatitis C virus (72, 73).

In a patient recovered from COVID-19 for four weeks, induced HLA-G expression was observed in the intestinal mucosa epithelial cells and lymphocytes at the sites corresponding to SARS-CoV-2 positivity (74). In our case report, the dynamics of HLA-G and expression of its receptors ILT-2, ILT4, and KIR2DL4 on peripheral immune cell subpopulations in a critical COVID-19 case to convalescence were analysed. Our data showed that HLA-G expression in peripheral immune cells fluctuates along with the status of the disease that the percentage of HLA-G-positive T cells, B cells and monocytes presented a high-low-high pattern, while the percentage of receptors ILT2-, ILT4- and KIR2DL4-expressing immune cells remained relatively stable (75). Moreover, sHLA-G has been found to be significantly elevated in patients with COVID-19 and is related to disease severity (76). Notably, a very recent study by Bortolotti et al. (77) reported that an increased peripheral blood sHLA-G level was associated with an improved outcome in patients with COVID-19, which might be a result of reduced neutrophil adhesion to activated endothelia by sHLA-G as well as interaction with the receptor CD160 (**Table 1**).

However, the clinical significance of HLA-G and its receptor expression on immune cells among patients with COVID-19 remains largely unknown.

## IMPLICATION OF HLA-G/RECEPTOR-MEDIATED IMMUNE SUPPRESSION IN COVID-19

The marked manifestations of immunopathology during SARS-CoV-2 infection, particularly in patients with severe COVID-19, is a salient reduction in immune-competent cells and an overregulated production of pro-inflammatory cytokines and chemokines (78). Dramatically impaired antiviral cellular immune responses and uncontrolled pro-inflammatory humoral immunity lead to SARS-CoV-2 immune evasion and collateral local or systemic tissue damage (79). In severe COVID-19 cases, cellular immune functions are not compromised by dramatically decreased CD3+ lymphocytes, CD4+, CD8+ T, CD3+CD56+ NK T, B, and NK cells, but by the impaired and/or exhausted functions of these immune cells, which is accompanied by the expansion of myeloid-derived suppressor cells (80–82).

Cytotoxic lymphocytes, such as NK cells and CD8+ T lymphocytes, can directly target virus-infected cells, and virus-specific antibody-producing B cells are essential for viral clearance and infectious disease control. In severe COVID-19 cases, not only the absolute number but also the cytotoxicity of both NK cells and CD8+ T lymphocytes are remarkably reduced. Mazzoni et al. (16) found that peripheral circulating NK cell intracellular granzyme and perforin levels were dramatically lower in patients with severe COVID-19 than in healthy controls. At the same time, there are much higher frequencies of senescent phenotype TEMRA+ CD57+ CD8+ T cells but reduced antiviral cytokine production and cytotoxicity of CD4+, CD8+ T, and NK cells in patients with COVID-19. NK cell immune function impairment could be due to the significantly increased expression of the inhibitory receptor NKG2A, as reported by Zheng et al. (18). In that study, the functions of NK and CD8+ T cells were exhausted and exhibited lower

**TABLE 1** | Current available studies on HLA-G expression in patients with COVID-19.

| Study method, subjects and size | Results and Implication for HLA-G expression | Reference |
|---|---|---|
| A 50s male patient had a positive for SARS-CoV-2 4 days after the start of symptoms. After 4-week-negative, he was admitted due to stomach pain, and a histologic examination was performed after colonoscopy. | HLA-G expression was found in intestinal mucosa epithelial cells and in some lymphocytes, in correspondence with SARS-CoV-2–positive sites. In submucosa, HLA-G expression was detectable only in few lymphocytes. Induction of HLA-G expression at the site of SARS-CoV-2 infection might be a cause of the COVID-19-dependent bleeding. | (72) |
| A 55-year-old female patient with critical COVID-19 admitted seven days after the onset of symptoms. Dynamics of HLA-G and its receptors ILT2, ILT4 and KIR2DL4 expression in peripheral immune cells with flow cytometry, and the outcomes of the patient during the 23-day ICU treatment. | The percentage of HLA-G+ T cells (median: 6.29%; range: 1.18-11.2%), B cells (median: 5.93%; range: 2.38-10.50%) and monocytes (median: 9.73%; range: 5.51-12.20%) is of a high (at admission)–low (during hospitalization)–high ( convalescence) pattern, while the percentage of receptors ILT2-, ILT4- and KIR2DL4-expressing cells remained more stable. | (73) |
| 103 COVID-19 patients and 105 healthy controls were included in the case-control study. | sHLA-G were significantly increased in COVID-19 patients compared to controls (19.3 vs. 12.7 ng/mL; p <0.001). No statistical difference was observed between sHLA-G and gender, BMI, chronic disease, or ABO and Rh blood groups. Patients in the quartiles >50–75% and >75% of sHLA-G level were more likely to have COVID-19. | (74) |
| An investigator-initiated, prospective, single-center study. Fifty-four COVID-19 (moderate-to-severe) patients, 11 control patients that presented respiratory failure without SARS-CoV-2 infection), and 100 healthy control subjects. Serum sHLA-G were analyzed after enrollment (T1; Baseline), and every 7 ± 2 days for an additional 2 consecutive visits (T2 and T3). Correlation between sHLA-G and clinical outcomes was evaluated. | Higher sHLA-G in COVID-19 patients compared to controls with respiratory failure (165.87 vs. 49.54ng/mL; $p < 0.01$) and healthy controls ( 165.8 vs. 20.51ng/mL; $p < 0.001$) at T1. sHLA-G at T1 did not differ between COVID-19survivors and non survivors, but significantly decreased over time in non-survivors (p = 0.036 at T2; p = 0.04 at T3). In control patients, sHLA-G levels decreased in both survivors and non-survivors over time with no statistical differences. Increased severity of COVID-19 from T1 to T2 (but not T2 to T3) was associated with a significantly decreased sHLA-G (p = 0.012). Improved clinical conditions were associated with an increased sHLA-G between T1 and T2 (p = 0.01). Increased sHLA-G reduced neutrophil adhesion to the endothelial cells. | (75) |

CD107a, IFN-γ, IL-2, granzyme B, and TNF-α production, which were accompanied by highly increased expression of the inhibitory receptor NKG2A on both cells during SARS-CoV-2 infection. Importantly, in most patients with COVID-19, the reduced number of NK and CD8+ T cells was restored and the initially high level of NKG2A expression was reduced during the convalescent period after antiviral therapy.

Although detailed information on the significance of HLA-G/receptor signalling in SARS-CoV-2 infection is lacking, the multifaceted immune suppression induced by HLA-G engagement with the aforementioned receptors has provided accumulating evidence that HLA-G/receptor signalling induces immune impairment and exhaustion, and cytokine release could be of critical importance in COVID-19. Previous studies have described the profound immune suppression mediated by HLA-G interaction with ILT-2/4 in a wide range of contexts (83). The interaction and signalling could inhibit the cytotoxicity of NK and CD8+ T cells, allo-proliferation of CD4+ T cells, maturation of DCs, differentiation, proliferation, and immunoglobulin (IgA, IgG, and IgM) production by B cells (84–88). In line with this, the activation of DCs and B cells was hampered in patients with severe COVID-19, as indicated by Wang et al. (89). Moreover, HLA-G/CD8 interaction could induce the apoptosis of CD8+ T cells through the Fas/FasL pathway, which may also occur in subsets of CD8+ NK T cells (90, 91). In contrast, HLA-G/ILT-2/4 engagement could also induce the generation of CD8+CD28+ or CD4+CD25+CTLA-4+ regulatory T cells (Tregs), expansion of MDSCs, tolerogenic DC-10 induced adaptive type 1 regulatory T cells, and M2 type macrophages (92–95). A study by Tomić et al. (96) revealed that the expansion of PD-L1, ILT-3, and IDO-1-expressing monocytic MDSCs was related to the accumulation of regulatory B and T cells and poor T cell immune responses in patients with severe COVID-19. Other studies have shown that $CD4^{+}CD25^{+}CD127^{low}$ Treg cells were significantly increased in patients with both mild or severe COVID-19, regardless of recovery, and that the proportion of IL-10 producing Treg was significantly increased in patients with severe COVID-19 (97, 98). However, the expression status of HLA-G and its receptors on these immune cells remains to be investigated.

Moreover, HLA-G expression in swine endothelial cells can protect them from human macrophage-mediated cytotoxicity (99, 100). Based on our preliminary study on HLA-G receptor expression in circulating immune subpopulations in a critical patient with COVID-19, the data showed that T cells can be phenotyped as ILT-$2^{high}$ILT-4 $^{mid}$KIR2DL$4^{dim}$, B cells as ILT-2 $^{mid}$ILT-$4^{high}$KIR2DL$4^{dim}$, and monocytes as ILT-$2^{high}$ILT-$4^{high}$KIR2DL$4^{dim}$. However, the cell surface expression levels of these receptors remained relatively stable from the critical stage to convalescent stage and irrespective of the viral load in SARS-COV-2 infection (75). The marginal KIR2DL4 expression observed in T cells, B cells, and monocytes in our study is in agreement with previous reports and is mainly located intracellularly but detectable upon IL-2 activation of NK cells (101). The activation signal resulting from HLA-G/KIR2DL4 interaction not only initiates the production of robust pro-inflammatory cytokines and chemokines, such as IFN-γ, TNF-α, IL-1β, IL-6, IL-8, MIP-3α, MIP-1δ, MIP-1α, and MIP-2β, but also leads to cell senescence and cell cycle arrest in NK cells (102, 103).

HLA-G allelic products also affect the interaction with its receptors, caused by different amino acid residues, and consequently alter its biological functions. Celik et al. (104) indicated that a single amino acid difference in the α two domains of HLA-G could affect the lysis of target cells by NK

cells. The data showed that a much stronger immune suppressive function was observed for the HLA-G*01:04 allele than for the HLA-G*01:01 and HLA-G*01:03 alleles. It seems reasonable with a recent finding that the binding of the HLA-G*01:04 product to NKG2A/CD94 has a higher affinity than that of HLA-G*01:01 and HLA-G*01:03 products (37). As NKG2A expression is highly associated with the severity of COVID-19, these findings indicate that the genetic variation of HLA-G could be linked to susceptibility to disease and host immune response regulation during SARS-CoV-2 infection (105).

## CONCLUSIONS

Since the outbreak of the worldwide COVID-19 pandemic in December 2019, it has claimed more than 5,054,267 lives (4). More insights into the ever-increasing clinical characteristics and laboratory findings on COVID-19 are being reported, which show that immune-competent cell function impairment and/or exhaustion is one of the major features of COVID-19 pathogenesis (7). However, the mechanisms underlying immunological abnormalities remain largely unknown. As reported in previous studies, viruses have developed effective strategies to hide from host antiviral immune responses and survive during infection (106). One strategy successfully deployed by viruses for immune evasion is the impairment of the classical HLA class I and II antigens to hide infected cells from T cell recognition, and the induction of non-classical HLA class I antigen HLA-G, a ligand for immune inhibitory receptors differentially expressed on almost all subsets of immune cells. Consequently, differential alteration in HLA antigen expression by viral infection makes the host antiviral immune system vulnerable (22, 107).

Synergistic suppression effects induced by HLA-G/receptor signalling are well recognised. These effects include the inhibition of cell proliferation and differentiation and the induction of cell apoptosis and senescence, which could be involved in significant decrease or even exhaustion of immune-competent cells such as T cells, NK cells, B cells, and macrophages in patients with COVID-19. Other effects include the inhibition of T and NK cell cytotoxicity, antibody production by B cells, and induction of regulatory cells and expansion of MDSCs, which might be linked

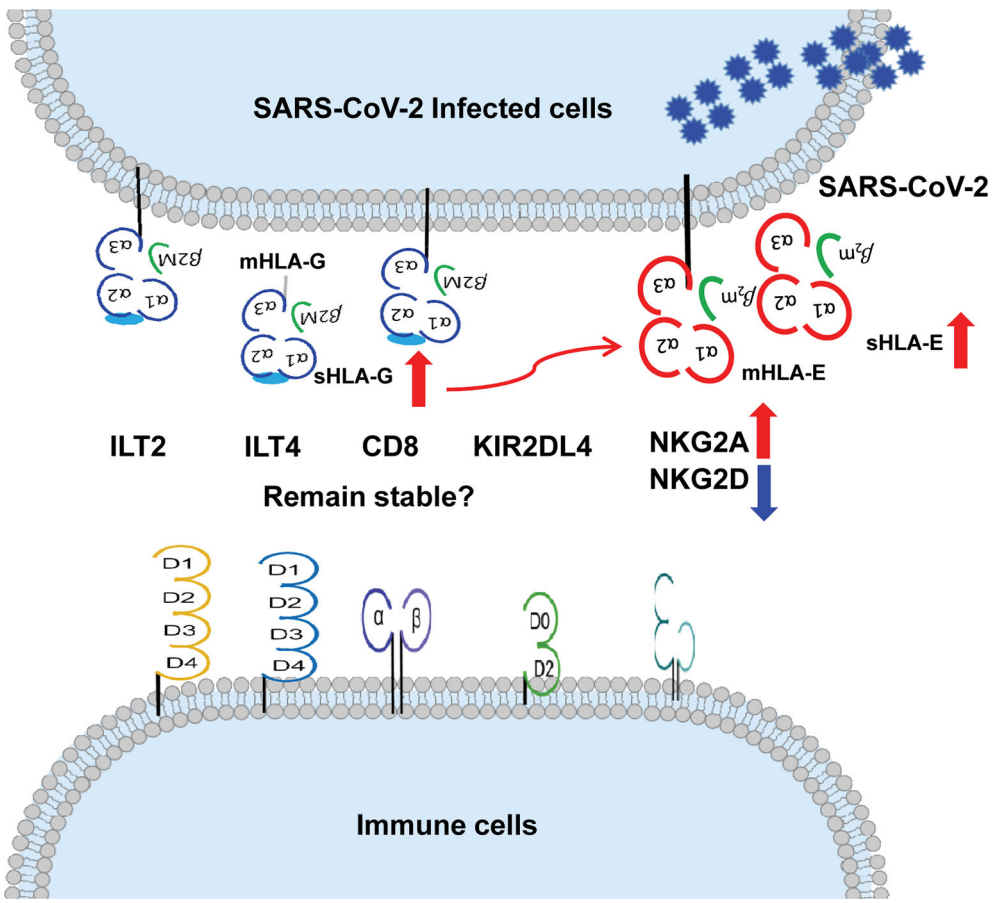


**FIGURE 2** | More evidence can be expected on the relationship between up-regulation of HLA-G and HLA-E expression and their immune receptors on immune cells during SARS-CoV-2 infection. ↑up-regulation. ↓down-regulation.

to the functional impairment of effector cells, such as T, NK, and B cells, during SARS-CoV-2 infection. However, more information on HLA-G and its receptor status is necessary for future clinical investigations and basic science studies.

## PERSPECTIVES

More evidence can be accumulated to solidify the basic and clinical aspects of HLA-G in COVID-19 progression and outcome. Aspects expected to be explored include (a) HLA-G expression is reported to be correlated with the progression of various infectious diseases (108, 109). We hypothesise that cell surface HLA-G and circulating soluble HLA-G levels are related to the severity, outcome, or viral load in patients with COVID-19. (b) The upregulation of HLA-G expression by cytokines, such as IFN-γ, IL-6, and IL-10, is dramatically increased in patients with severe COVID-19 (110, 111). We hypothesise that HLA-G expression is related to cytokines in patients with COVID-19. (c) Recently identified HLA-G allelic product-dependent receptor NKG2A has been observed to be dramatically increased in patients with COVID-19 (18). What is the status of other HLA-G receptors, such as ILT-2, ILT-4, and KIR2DL4, and their relationship with disease progression? and (d) Given HLA-E-CD94/NKG2A axis plays critical roles in COVID-19 and HLA-E cell surface expression depends other leader sequence peptides, particularly derived from HLA-G (112), what is the relationship between HLA-G and HLA-E expression? (**Figure 2**).

In this context, the clinical trial "HLA-G Immuno-Inhibitor Checkpoint Study in Patients With COVID-19 Infection: Molecular and Cellular Assessment (HLA-G-COVID) (NCT04613297)" has been started to evaluate the clinical significance of HLA-G and receptors ILT-2 expression on $CD4^+$ and $CD8^+$ lymphocytes, and the levels of peripheral sHLA-G and plasma HLA-G-bearing microvesicles among COVID-19 uninfected patients, non-hospitalized COVID-19 infected patients and hospitalized COVID-19 infected patients. With this clinical trial, further understanding of the significance of HLA-G and its receptors in COVID-19 patients can be expected.

The impaired immune functions of NK and T cells resulting from ILTs and NKG2A expression have been reported to be involved in virus immune evasion and related infectious disease progression (113–116). Fortunately, ILTs, NKG2A, and HLA-G targeted immunotherapy and signalling pathway blockades are already in development in clinical trials for cancer immunotherapy. In previous preclinical investigations, blocking tumour cell-expressed HLA-G or immune cell surface ILT2/4 with specific antibodies could restore the functions of NK cells or T cells against target cells (36). Furthermore, blocking NKG2A with monalizumab, an anti-NKG2A monoclonal blocking antibody, can significantly restore the cytotoxic function of NKG2A+ NK and T cells (117–119). Along with these findings, the application of ILTs and NKG2A targeted blocking antibodies could be an additional intervention to mitigate the severity of COVID-19. Finally, a clinical phase I trial with an HLA-G blockade antibody, TTX-80, was launched in July 2020 for patients with advanced solid cancer (120), which shed new light on the restoration of exhausted immune responses induced by HLA-G in diseases such as cancers or viral infections.

We hope that our review will provide a much better understanding of the immune pathogenesis of COVID-19, and thereby help in the development of immunointerventions to counteract SARS-CoV-2 infection.

## AUTHOR CONTRIBUTIONS

W-HY and AL conceived and designed the review. W-HY made the figures. AL and W-HY drafted and revised the manuscript and approved it for publication. All authors contributed to the article and approved the submitted version.

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# Skin Immunity and Tolerance: Focus on Epidermal Keratinocytes Expressing HLA-G

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Although the role of epidermal cells in skin regeneration has been extensively documented, their functions in immunity and tolerance mechanisms are largely underestimated. The aim of the present review was to outline the state of knowledge on resident immune cells of hematopoietic origin hosted in the epidermis, and then to focus on the involvement of keratinocytes in the complex skin immune networks acting in homeostasis and regeneration conditions. Based on this knowledge, the mechanisms of immune tolerance are reviewed. In particular, strategies based on immunosuppression mediated by HLA-G are highlighted, as recent advances in this field open up perspectives in epidermis-substitute bioengineering for temporary and permanent skin replacement strategies.

**Keywords: immunity, tolerance, human skin, keratinocytes, resident immune cells, HLA-G**

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## SKIN CELLS ENSURE TISSUE PROTECTION AND HOMEOSTASIS

The skin accounts for 15% of body weight, provides an exchange surface between organism and environment, and protects internal organs. It also helps to maintain homeostasis by preventing water loss and by regulating body temperature. The epidermis, the outermost layer of the skin, is composed of keratinocytes (90% of cells). It also contains melanocytes (5%) and rare Merkel cells. This barrier protects the underlying skin layers from injury, UV damage, harmful chemicals and infection by pathogens. The dermis, separated from the epidermis by the dermo-epidermal junction, is composed of extracellular matrix secreted by fibroblasts. It contains blood vessels, glands and nerve cells. Its main functions are to deliver oxygen and nutrients to the epidermis and to regulate body temperature.

Adult skin contains resident immune cells and recruits immune cells from the periphery in case of infection, burns or exposure to chemicals or radiation. Resident immune cells are found in all layers of the tissue, which therefore constitutes a reservoir of immune cells (1) (**Figure 1A**), and notably of T-cells. It was estimated that adult skin contains 20 billion T-cells, nearly twice as many as in the blood (2). In addition, resident non-hematopoietic skin cells have immune functions, which are not fully elucidated.

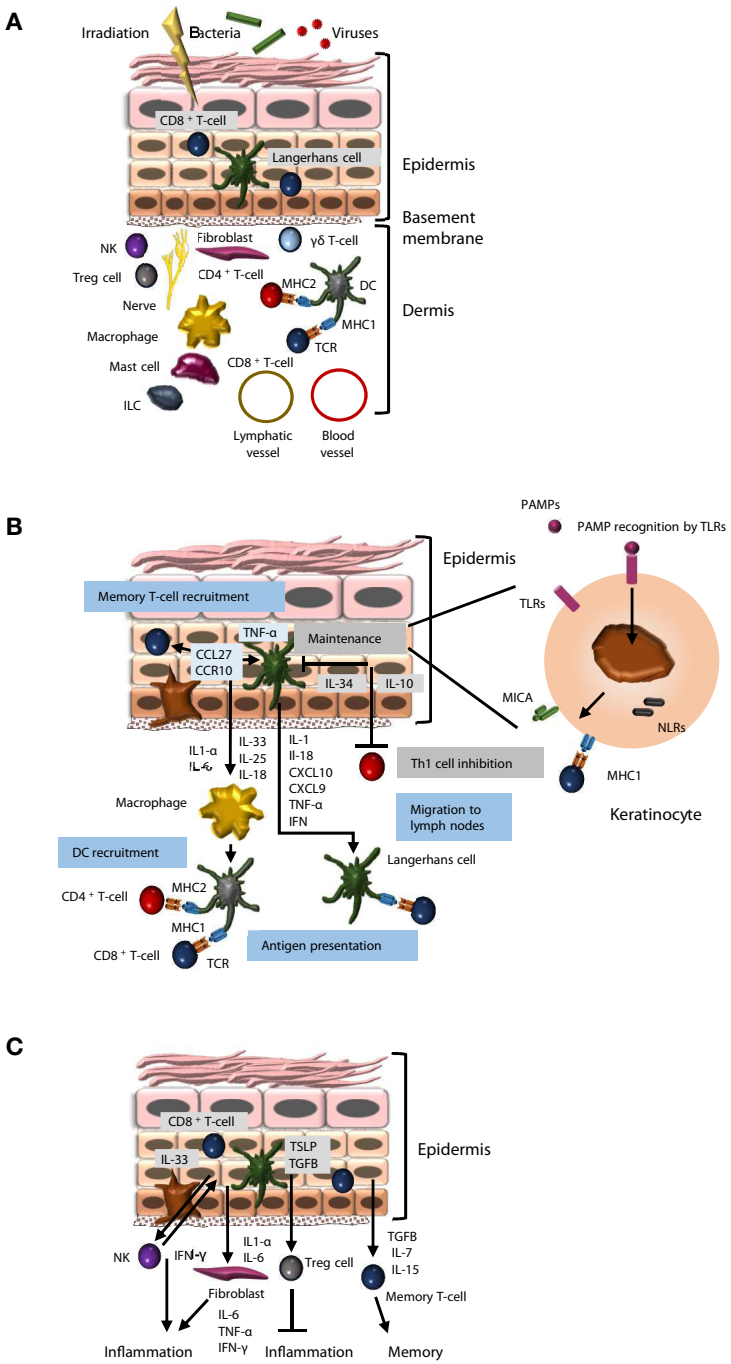


**FIGURE 1** | Immune properties of skin keratinocytes. **(A)** Cellular components of the skin immune system. The skin is a barrier that protects against injury, pathogens, chemicals and radiation. The local immune system includes resident immune cells and cells recruited from the periphery. The epidermis hosts effector cells, such as $CD8^+$ T-cells and Langerhans cells that migrate to lymph nodes to perform antigen presentation. In the dermis, Treg cells, NK, $CD4^+$ T-cells, mast cells, ILCs, macrophages and dendritic cells participate in immune activities. In addition, resident non-hematopoietic skin cells have immune functions, which are not fully elucidated. **(B)** Keratinocyte interactions with immune cells. Keratinocytes recognize pathogen-associated molecular patterns (PAMPSs) through TLR and NLR receptors and stimulate the recruitment of resident memory T-cells, dendritic cells and circulating T-cells through pro-inflammatory cytokine secretion. On the other hand, they are also able to promote tissue homeostasis through anti-inflammatory cytokine secretion (IL-34, IL-10). **(C)** Keratinocytes participate in the regulation of inflammation and the maintenance of skin immune memory. Keratinocytes promote inflammation through IL-1, IL-3 and IL-6 secretion, but also limit it through TSLP and TGFB secretion. Keratinocytes can promote the maintenance of immune memory through various mechanisms, including TGFB, IL-7 and IL-15 secretion.

# THE EPIDERMIS IS THE SEAT OF IMMUNE ACTIVITIES

## Immune Cells Reside in the Epidermis

As a direct interface with the environment, the epidermis hosts various immune cells, and notably tissue-resident memory αβ $CD8^+$ memory T-cells, dendritic epidermal gamma delta T-lymphocytes (γδ T-cells), and Langerhans cells (LCs, 5% of epidermal cells). LCs originate prenatally from erythromyeloid progenitors and permit antigen presentation to T-cells (3). They are in contact with keratinocytes *via* their dendrites and with dendritic cells below the dermo-epidermal junction. LCs produce inflammatory mediators such as interferons (IFNs), and enable presentation of antigens to other immune actors such as T-cells (4). LCs express CD1a (5), CD207 and MHC class II (MHC2), and have cytoplasmic Birbeck granules. In immune reactions, they express MMP proteases, translocate to the dermis, and migrate to lymph nodes, promoting T-cell recruitment (1), activation of $CD8^+$ T-cells, and differentiation of type 2 T-helper lymphocytes (Th2) (6). In short, LCs exert a regulatory role in the lymphocyte activation cascade. In case of tissue destruction, LCs are renewed by differentiation of monocytes recruited at the lesion site (7). LCs are able to divide and self-maintain, like macrophages, and to migrate to lymph nodes and stimulate T-cells, like dendritic cells (DCs), thus sharing both macrophage and DC properties.

Within the skin reservoir of resident T-cells, the most frequent subtype is αβ $CD8^+$ memory T-cells, localized in the basal and supra-basal layers next to LCs (8). These cells express cutaneous lymphocyte-associated antigen (CLA), a skin-homing receptor (2), and different chemokine receptors (CCR4, CCR8 and CCR10) (9). They also express the IL2 receptor (CD25) and HLA-DR (10). Tissue-resident innate lymphoid cells (ILCs) were recently described in epidermis, predominantly expressing ILC3/LTi-related genes or genes associated with ILC2s, but their role remains to be investigated (11).

## Molecular Effectors Related to Immune Functions Are Expressed by Keratinocytes

### Membrane Markers

Pathogens are identified *via* pathogen-associated molecular patterns (PAMPs), recognized by pattern recognition receptors (PRRs) (12). Major PRRs are toll-like receptors (TLRs), expressed as 8 isoforms in keratinocytes (13) (**Figure 1B**). Keratinocytes also express nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). NLR signaling leads to activation of the inflammasome *via* NLRP3, an intracellular sensor that detects microbial motifs, and the production of pro-inflammatory cytokines such as IL-1β and IL-18. NOD1 and NOD2 are expressed by keratinocytes, and drive inflammatory signals *via* the NF-κB and MAPK pathways [8]. TLRs and NLRs promote detection of alarmins released from or exposed at the surface of damaged cells (9). PAMP binding to TLRs leads to the production of cytokines (IL-1β, IL-18, CXCL9 and CXCL10) and recruitment of immune cells.

TLRs recognize different ligands, enabling efficient pathogen detection. TLR1, 2, 6 and 10 recognize lipoproteins, while TLR3, 4 and 5 respectively recognize RNA, bacterial LPS, and flagellin. TLR7, 8 and 9 recognize RNA and DNA. Most TLRs have a pro-inflammatory function, except TLR10 (14). NF-κB signaling is the major pathway driving TLR-mediated cellular response, leading to the production of IFNs (15). This cascade triggers LC migration to lymph nodes, inducing recruitment of T-cells expressing the skin diapedesis marker CLA (16). IFN-γ up-modulates TLR3 expression in keratinocytes, which in turn increase their secretion of IL-6, Il-8 and defensins, in the presence of the immune stimulant molecule polyinosinic: polycytidylic acid (poly I:C) (17). Poly I:C binding to TLR3 stimulates keratinocyte production of IL-1β and IL-18, promoting DC activation and T-cell differentiation into Th1 (9). The complex TLR3 signaling network in keratinocytes involves production of chemokines CXCL9 and CXCL10 and of cytokines CCL20 and CCL27, which respectively promote the recruitment of Th1 and memory T-cells (9).

Keratinocytes express MHC components, allowing antigen recognition, and notably MHC1-related cell-surface molecules (MICA, MICB and ULBP) that mediate interactions with CD8+ T and γδ T-cells through the natural-killer group 2D receptor (NKG2D). NKG2D is an immune reaction promoter expressed by resident or infiltrating immune cells in the skin (18). A key functional link is that ligand binding to TLRs induces expression of some HLA molecules (13). For example, poly I:C, a TLR3 ligand, induces expression of HLA-ABC and HLA-DR. Similarly, flagellin, a TLR5 ligand, induces expression of HLA-ABC, and LPS, a ligand of TLR4, induces expression of HLA-DR. MHC components are not present in all keratinocytes, as a basal sub-population expresses neither MHC1 nor MHC2 (19).

### Secreted Factors

Keratinocytes secrete antimicrobial peptides (AMPs), damage-associated molecular patterns (DAMPs) and the defensin peptides that ensure direct antimicrobial functions. Keratinocytes secrete antimicrobial proteins (S100) and peptides (cathelicidins) (9). AMP secretion by keratinocytes during infection increases following production of IL-17A and IL-22 by Th17-cells (20) and in response to IFN-γ (21), which amplifies the inflammatory response. Secreted mediators include pro- or anti-inflammatory cytokines, CXC and CC chemokines and growth factors (22). These mediators constitute the signaling network linking epidermal and dermal cells to resident immune cells to maintain skin homeostasis and defense against environment insults.

## Keratinocytes and Immune Cells Communicate Directly

### *Via* Cell-Cell Contact

Through MHC1 molecules, keratinocytes present antigens to memory CD8+ T-cells, inducing cytotoxic defenses and production of inflammatory cytokines (23), notably following stimulation by IFN-γ. Antigen presentation *via* MHC2 molecules was documented in a mouse skin model where interfollicular keratinocyte MHC2 drives the formation of Th1-cell clusters (24). Keratinocytes express adhesion molecules such as ICAM-1,

which, with its B7 costimulatory molecules, promotes lymphocyte recruitment (25).

Keratinocytes exert a role in the coordination of differentiation and functions of effector T-cells, natural-killer T (NKT) cells (26) and γδ T-cells (27). Target cell detection by NKT and γδ T-cells involves the NKG2D receptor, which recognizes the MICA and MICB proteins. NKG2D-mediated signaling leads to target-cell lysis (28). Overexpression of MICA and MICB in damaged keratinocytes drives a recognition signal by NKT and γδ T-cells, and their lysis (9). A major role of NKT cells is protection against microbial infection, through recognition of bacterial glycolipids (26).

### *Via* Secretion of Cytokines and Chemokines

Primary cytokines (IL-1, TNF-α) are secreted by keratinocytes in the initial stage of inflammatory reactions. Interleukin-1 exists in 3 isoforms (IL-1α, IL-1β and IL-1Ra), which bind to the same receptors (IL-1R1 and IL-1R2). Keratinocytes produce and store active IL-1α (29), and produce an inactive pro-IL-1β form that is cleaved and activated by caspase-1 synthesized by LCs, mast cells, monocytes, macrophages and neutrophils. IL-1α promotes its own expression and that of other pro-inflammatory cytokines (IL-6, IL-8, TNF-α). It induces expression of adhesion molecules promoting tissue infiltration by immune cells. Dysregulation of IL-1 signaling in keratinocytes was associated with mutations in the *NLRP1* inflammasome sensor gene in patients with inflammatory skin syndrome (30). Secretion of IL-1β induced *via* the TLR4-MAPK pathway in keratinocytes promotes early skin-wound healing (31). Production of IL-1 and IL-18 by keratinocytes following exposure to UV promotes recruitment of Th1 and Th2 cells, which themselves produce interleukins, TNF-α and CSF2 (32). IL-18 is produced by keratinocytes in an inactive form (33). Following UV exposure, inflammasome activation is driven by the stress sensor NLRP3, leading to pro-IL-18 cleavage by caspase-1 (34). Moreover, keratinocyte production of IL-6 promotes proliferation and differentiation of B-cells and cytotoxic T-cells (35).

Transforming growth factor beta (TGFB) is produced by epidermal keratinocytes, and plays an important role in skin remodeling after damage (36). TGFB inhibits macrophage differentiation, monocyte and $CD8^+$ T-cell activity, and presentation of antigens by LCs (37). In keratinocytes, TGFB inhibits proliferation of CD4+ T-cells through Smad3 signaling (38). In inflammatory contexts, TGFB promotes leukocyte adhesion and chemotaxis, and activates DC migration into lymph nodes, promoting T-cell recruitment in the skin (36).

IL-33 may either increase or inhibit inflammation. The inactive form, pro-IL-33, is located in the cell nucleus, where it suppresses transcription of pro-inflammatory cytokines (39). In keratinocytes, nuclear IL-33 inhibits epidermis differentiation genes in atopic dermatitis lesions, exacerbating skin barrier dysfunction (40). The active form after cleavage allows TH2 cytokine signaling *via* the IL-1 receptor ST2. In keratinocytes, IL-33 secretion is induced by cytokines and by pathogens such as S aureus (41). IL-25, like IL-33 and IL-1α, is stored in keratinocytes and secreted under the action of proteases during damage, contributing to immune response activation (42). Keratinocytes also produce the anti-inflammatory cytokine IL-10 (43), which may reduce formation of large scars (44).

Chemokines are of great importance for immune cell recruitment and mobility within tissue (45). They contribute to regulation of immune cell activation and differentiation (46). Keratinocytes express chemokines of both the CC and CXC families. CXC chemokines attract neutrophils during healing, while CC chemokines attract a wider range of leukocytes: basophils, eosinophils, T-cells and DCs (47).

In tissue injury, cytokines and chemokines contribute to skin repair through interaction with keratinocyte stem cells. In mouse skin, resident Tregs activate hair-follicle stem cells (HFSCs) by secreting the CXCL5-IL-17- IFN-γ signal. In response, HFSCs are recruited and migrate to the interfollicular epidermis, and contribute to on-site epithelial-barrier repair (48). Interactions between keratinocytes and effector T-cells have reciprocal impacts: keratinocytes promote effector T-cell recruitment within the skin, and these in turn produce growth factors such as CTGF, FGF9, KGF and IGF1, that promote healing (49).

### Other Communication Mechanisms

Keratinocytes communicate with other cells *via* secretion of extracellular vesicles containing cargoes of different types of molecule, including lipids, proteins and nucleic acids (50). Epigenetic mechanisms are also emerging, as illustrated by the study of the Mi-2β chromatin remodeler. In mouse skin, this factor directly controls regulatory T-cells by inhibiting pro-inflammatory TSLP secretion by keratinocytes (51).

## Keratinocytes Mediate Inflammation

Keratinocytes are involved in the initiation of inflammatory processes, through release of soluble mediators, including IFN-γ (**Figure 1C**). Keratinocytes are activated before 'true' immune cells at the onset of inflammation (25). IFN-γ has a central pro-inflammatory function in the skin, and a single intradermal injection of IFN-γ is sufficient to induce an inflammatory state, driven by a cytokine production cascade (52). Keratinocytes are the primary cellular actors in a positive loop, as, following exposure to IFN-γ, they increase their secretion of IL-33, which in turn increases their production of IFN-γ (53).

Studies in a mouse model showed that a subpopulation of keratinocytes expressing PD-L1 also promotes control of the extent of inflammation (54). Human keratinocytes promote local but not systemic inflammation, through expression of thymic stromal lymphopoietin (TSLP), a factor involved in Treg immune function coordination. By binding to its receptor on Tregs, TSLP maintains local inflammation while inhibiting lethal systemic inflammation (9). In addition, presentation of autoantigens by keratinocytes induces T-cell tolerance, and is a means of avoiding, rather than stimulating, autoimmune reactions in contexts of local inflammation (51).

## Keratinocytes Promote Immune Memory

The skin hosts resident memory T-cells that favor rapid response to infection by a pathogen to which the individual has already been exposed (55). A model of cutaneous immune response in three successive stages was proposed (**Figure 1C**) (56). First,

following pathogen entry into tissue, resident specific memory T-cells resulting from previous exposure react by transcriptional changes and secretion of activating factors. Second, circulating memory T-cells are recruited. And third, skin DCs presenting antigens of the pathogen migrate to lymph nodes, where they drive neo-production of specifically targeted effector T-cells, which are recruited in the infection site in the skin, within 24 to 72 hours (57). Keratinocytes promote long-term maintenance of a memory T-cell pool within the skin, through secretion of IL-7, IL-15 and TGFB, favoring a rapid defense response in case of new aggression by a previously encountered pathogen (9). A memory mechanism that does not require skin-resident macrophages or T cells has been identified in murine epidermal stem cells after acute inflammation (58). Stem cells maintain prolonged epigenetic memory to acute inflammation by maintaining chromosomal accessibility to stress response genes, which, in case of secondary stress, enables fast transcription of specific inflammasome genes, including the *Aim2* gene, which activates caspase-1 and IL-1β.

## Dysregulation of Immune Functions in Skin Pathophysiological Contexts

Immunity dysregulations are involved in various skin disorders, such as atopic dermatitis, psoriasis, and alopecia areata.

In atopic dermatitis, deficiency in E-cadherin expression by keratinocytes reduces intercellular junctions, which promotes the secretion of pro-inflammatory cytokines, notably IL-25, IL-33, TSLP, and PGD2, and then induces production of IL-13 and IL-5 by ILC2 (59). IL-13 and IL-4 stimulate activated B cells and T cell proliferation, and their overexpression is associated with allergies (60). Notably, a monoclonal antibody directed against the IL-4 receptor α subunit, blocking IL-4 and IL-13 signaling, has been evaluated in patients with atopic dermatitis, with significant improvement in disease severity (61).

Modified immune properties of keratinocytes have also been associated with the pathophysiology of psoriasis (62). Epidermal cells are renewed every 3 to 5 days in case of psoriasis instead of 28 to 30 days in healthy skin (63). This abnormally accelerated cell renewal rate is due to the premature maturation of keratinocytes, induced by an inflammatory cascade involving dendritic cells, macrophages, and T cells. Autocrine and paracrine secretion of IL-1β by keratinocytes auto-induces insulin-independent growth *via* activation of the p38 MAPK signaling pathway, which alters differentiation and consequently participates in the hyper-proliferative state of the epidermis (64). Knowing that HLA-G and PD-L1 are expressed in psoriatic skin, a possible regulatory link between keratinocyte hyper-proliferation and expression of immune checkpoints is a rational hypothesis, which should be investigated (65, 66).

Another example of skin pathology that involves an immune dysregulation is alopecia areata, which is characterized by hair loss in patch areas, notably but not exclusively in the scalp (67). Hair follicles are normally preserved from immune reactions, a phenomenon called immune privilege. The disruption of this immune privilege has been identified as one of the causes of alopecia areata (68). This pathophysiological process involves an abnormal infiltration of T-cells that causes local inflammation and the destruction of anagen hair follicles (67). Another aspect is the expression of MHC by keratinocytes, which promotes the maintenance of autoreactive T cells directed against hair follicles (69).

# REGULATION OF IMMUNITY AND TOLERANCE ARE KEY POINTS FOR IMPLEMENTING SKIN CELL AND GENE THERAPY

The high regenerative potential of adult keratinocyte stem cells underlay the development of skin replacement strategies based on autologous skin-substitute grafting, permanently reconstituting the skin in patients with third-degree burns affecting up to 90% body surface (70). Notably, a clinical trial using keratinocyte stem cells and gene therapy succeeded in regenerating the entire epidermis of a child suffering from epidermolysis bullosa (71). Preservation of functional keratinocyte stem cells during the successive steps of the process is a prerequisite for the long-term graft survival, a point that still requires intensive investigation (72), including of the immune properties of stem cells.

Alternatively, frozen cell banks of allogenic keratinocytes may be constituted for standardized skin substitute production, available immediately on demand. Currently, allogenic keratinocytes are only suited for the bioengineering of temporary cutaneous bio-dressings, as the problem of immune rejection limits any long-term reconstitution. Such temporary dressings are an option for the treatment of chronic venous leg ulcers and diabetic foot ulcers, where the living cells of the allogenic graft stimulate regenerative mechanisms and contribute to restoring the patient's skin healing functions (73). In addition to native keratinocytes obtained from skin biopsies, keratinocytes generated by differentiation of pluripotent embryonic stem cell (ESC) lines have been investigated as a source of allogenic cells for skin-substitute bioengineering (74).

Transplant rejection is explained by the allelic differences between donor and recipient at the level of the polymorphic loci of three classes of histocompatibility antigen: the ABO blood group, the major histocompatibility complex (MHC) and minor histocompatibility antigens (mHA) (75). In this regard, recognition of allo-HLA antigens by recipient T-cells is the central event initiating allograft rejection. Alloantigen recognition occurs *via* two mechanisms: the direct and indirect allorecognition pathways. Direct recognition consists in T-cell recognition of determinant peptides on intact donor MHC molecules displayed on the surface of the donor antigen-presenting cell (APC), while indirect recognition consists in recognition of determinant allo-peptides presented by the self-MHC on the recipient APC. Secretion of anti-HLA antibodies directed against the HLA donor system leads to graft rejection *via* recruitment of phagocytes or activation of the complement system (76). Experimental approaches have been developed to prevent allogeneic skin graft rejection. Rapamycin, an inhibitor

of T-cell proliferation, inhibited rejection in a mouse model (77) in association to IL-2, which controls Treg activity and promotes immune tolerance. There have been a few studies, in a small number of burn patients, using immunosuppressants (methylprednisolone, cyclosporine, prednisone, anti-thymocyte globulin and azathioprine), but this approach is still very limited (78).

## NEW STRATEGIES TO PROMOTE TOLERANCE FOR SKIN CELL AND GENE THERAPY ARE REQUIRED

### Reducing Antigen Presentation

HLA gene genome-editing has been implemented in induced pluripotent stem cells (iPSCs) to generate universal donor stem cells (79). One strategy consisted in producing pseudo-homozygous cells for the HLA class I genes, from heterozygous donors, by editing the targeted allele. A second approach, taking account of the pivotal role of HLA-C in the suppression of NK cells, consisted in suppressing HLA-A and HLA-B while retaining the HLA-C haplotype, increasing compatibility. In both cases, genome-edited cells were able to suppress T-cell and NK activity, while preserving HLA expression and antigen presentation. This strategy can be combined to MHC2 reduction by depletion of CIITA (**Figure 2**).

Alternatively, iPSC lines overexpressing the immune-suppressive molecule CD47, together with decreased MHC1 and MHC2 expression, have been bioengineered to generate hypo-immunogenic derivatives that attenuate rejection (80). In this strategy, genome-editing comprises overexpression of *CD47* cDNA, which inhibits phagocytosis and NK activity. It is combined with CRISPR directed against B2M to decrease MHC1 expression, and CRISPR directed against the CIITA regulator to decrease MHC2 expression.

### Over-Expressing Immunosuppressive or Immune Checkpoint Molecules

The programmed death-ligand 1 (PD-L1) immune checkpoint molecule is known to bind to the PD-1 receptor expressed at the surface of T-cells, inhibiting their activity (81) and autoimmune reactions (82). PD-L1 promotes tolerance when expressed on the keratinocyte cell surface, by activating IL-10-secreting T-cells (83) and limiting $CD4^+$ T-cell proliferation (84).

Bioengineering ESC lines expressing immunosuppressive molecules is an alternative approach for generating universal donor pluripotent stem cell sources. One approach was based on the immunosuppressive properties of CTLA4-Ig, a fusion protein between the extracellular domain of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and an immunoglobulin Fc portion that disrupts T-cell costimulatory pathways, combined to inhibition of T-cell activation *via* PD-L1 (85). High constitutive expression of both CTLA4-Ig and PD-L1 are needed to confer immune protection on hESCs and their derivatives, as these molecules are not sufficient individually.

### Targeting HLA-G Immune Checkpoint Molecules

Human leukocyte antigen-G (HLA-G) molecules are major candidates for implementing immunomodulation strategies since the initial demonstration of the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis (86). Application of HLA-G tolerogenic properties was demonstrated in murine models of allogenic tissue transplantation and is also supported by clinical data from transplanted patients. In lung transplant recipients, HLA-G was identified as a predictive marker of low chronic rejection risk (87). In heart transplant recipients, detection of HLA-G expression in sera and endomyocardial biopsies was associated with fewer rejection episodes and suppression of the allogeneic T-cell proliferation response (88). HLA-G was detected in only 20% of samples from patients with heart transplantation, but

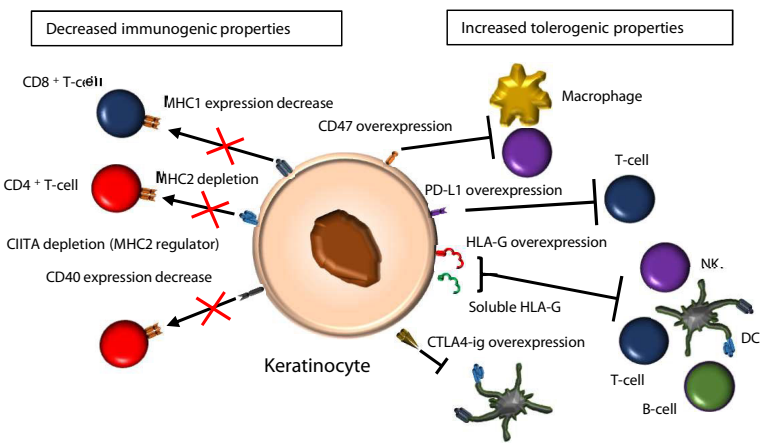


**FIGURE 2** | Candidate immunomodulatory strategies in epidermis. There are two main strategies to reduce keratinocyte immunogenicity for epidermis grafting. The first one is to decrease MHC1, MHC2 and co-stimulatory molecules such as CD40, which limits the ability of keratinocytes to present antigens to T-cells. The second one is to overexpress HLA-G and other immune checkpoint molecules (CD47, PD-L1, CTLA4), to increase tolerance.

86% of patients without acute rejection expressed HLA-G. Similar studies on liver, lung and kidney transplants have also shown a decrease in transplant rejection associated with the presence of HLA-G (89). Thus, low plasma levels HLA-G were proposed as a predictive marker of low risk of acute and chronic kidney rejection (90). Moreover, genomic studies highlighted markers of susceptibility to acute kidney rejection, as specific *HLA-G* gene polymorphisms were shown to participate in the lack of protection against a high risk of transplant rejection (91).

For skin regeneration, several approaches have been developed to use HLA-G as an immunoregulatory agent. A transgenic mouse model expressing HLA-G was designed to investigate immunosuppression in allogenic skin transplantation. In these mice, skin allograft survival was increased in response to HLA-G, which benefit was associated with attenuated T-cell activity (92). Human epithelial cells derived from the umbilical cord, which are known to promote epidermal reconstitution in organotypic models, express HLA-G and HLA-E, lack HLA-DR and several costimulatory molecules, and have a low capacity for presenting antigens. Interestingly, they exerted an inhibitory effect on alloproliferation of PBMCs, which suggested an immunosuppressive function (93). The immune modulatory properties of HLA-G were investigated in a cellular model of human adult skin keratinocytes, in which its expression could be modulated by a doxycycline-inducible construct. When HLA-G expression was induced, keratinocytes exhibited increased inhibition of $CD4^+$ T-cell proliferation (84). With the largest aim to generate universal donor pluripotent stem cell sources, human ESCs have been bioengineered to express a mutated form of HLA-G (mHLA-G) exhibiting enhanced mRNA expression and stability, and increased levels of cell-surface HLA-G protein (94). Expression of mHLA-G did not alter the capacity of ESCs to acquire keratinocyte markers in a culture condition directing epithelial orientation. In a mixed lymphocyte reaction assay, ESCs and their keratinocyte derivatives expressing mHLA-G restrained T-cell proliferation and cell lysis driven by allogeneic NK, demonstrating a decreased immunogenicity.

Another proposed approach consisted in using synthetic forms of HLA-G to inhibit transplant rejection. Producing HLA-G as a clinical grade molecule is notably impaired by its limited stability. Thus HLA-G-mediated promotion of immune tolerance was explored using HLA-G-derived synthetic polypeptides as a coating on microbeads suitable for intraperitoneal injection. Mice that received polypeptide-coated beads acquired tolerance to skin allografts, which resulted in prolonged graft survival (95). Thus, these different approaches point on the HLA-G research field as a promising domain for designing tools aiming at controlling the immunogenicity and the immunosuppressive properties of human keratinocytes.

Skin fibroblasts are also used in skin bioengineering approaches, and are thus concerned by the question of immune tolerance. The generation of fibroblasts expressing a stabilized form of HLA-G has been proposed to reduce their alloreactivity. To engineer a stable HLA-G molecule, mutated HLA-G1 was produced by modifying the endoplasmic reticulum retrieval motif, which allows its increased membrane expression, and the 3 ′UTR region miRNA binding site, which limits regulation by miRNAs. Dermal fibroblasts expressing this modified HLA-G1 were less sensitive to lysis by IL-2-stimulated NKs and reduced the proliferation of PBMCs following activation with PHA (96).

## HLA-G and the Risk of Post-Transplant Cancers

One point to take into account is to prevent the development of post-transplant cancer. Adult keratinocyte stem cells can drift into cancer cells, leading to cutaneous squamous cell carcinoma or basal cell carcinoma development (97, 98). Tumor growth is known to be enhanced by cancer cell ability to escape elimination by the immune system (99). HLA-G and PD-L1 inhibit different populations of T cells in cancer (100, 101), and therefore critically contribute to tumor escape from immunosurveillance. PD-L1 and HLA-G expression and targeting were particularly well documented in squamous cell carcinoma (102) and melanoma (103). It is therefore important to limit the development of post-transplant cancer, as HLA-G and PD-L1 may favor the immune escape of tumor cells. This point was investigated by HLA-G polymorphism matching in heart transplantation, in which recipient and donors were genotyped. Donor-recipient 14 bp polymorphism matching correlated with a limitation of the risk of tumor development post-cardiac transplant (104). It would be interesting to develop a similar approach in case of skin transplantation.

# CONCLUDING REMARKS

The role of epidermal cells in skin regeneration is well known; however, their functions in immunity and tolerance mechanisms are under-estimated. Keratinocytes are not merely a structural barrier against environmental insult, but also active members of the sophisticated immune ecosystem in the skin. They actively participate in protective immunity, are involved in the initiation of inflammatory processes, promote long-term maintenance of a memory T-cell pool, and develop their own epigenetic stress memory. As keratinocytes are key players in immune tolerance, they are major targets for overcoming graft rejection in the various strategies that aim to generate bioengineered banks of cells that could be used as universal donor cell sources. In these strategies, modulation of HLA-G expression and function is a promising means of controlling cell immunogenicity, and is being explored in native skin keratinocytes and keratinocytes generated by lineage-oriented differentiation of pluripotent stem cells.

# AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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# HLA-G in Allergy: Does It Play an Immunoregulatory Role?

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Allergy is an inflammatory process determined by a cascade of immune events characterized by T-helper 2 lymphocytes polarization leading to interleukin-4 upregulation, IgE secretion, and mast cell and eosinophil activation. HLA-G molecules, both in membrane-bound and in soluble forms, are known to play a key immunoregulatory role and their involvement in allergic diseases is supported by increasing literature data. HLA-G expression and secretion is specifically induced in peripheral blood mononuclear cells of allergic patients after *in vitro* incubation with the causal allergen. Elevated levels of soluble HLA-G molecules are detected in serum of patients with allergic rhinitis correlating with allergen-specific IgE levels, clinical severity, drug consumption and response to allergen-specific immunotherapy. HLA-G genetic polymorphisms confer susceptibility to allergic asthma development and high levels of soluble HLA-G molecules are found in plasma and bronchoalveolar lavage fluid of patients with allergic asthma correlating with allergen-specific IgE levels. Interestingly, allergic pregnant women have lower plasma sHLA-G levels than non-allergic women during the 3$^{rd}$ trimester of pregnancy and at delivery. Finally, in allergic patients with atopic dermatitis HLA-G molecules are expressed by T cells, monocytes-macrophages and Langerhans cells infiltrating the dermis. Although at present is difficult to completely define the role of HLA-G molecules in allergic diseases, it may be suggested that they are specifically expressed and secreted by immune cells during the allergic reaction in an attempt to suppress allergic inflammation.

**Keywords: HLA-G, soluble HLA-G, allergy, allergic rhinitis, allergic asthma**

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## INTRODUCTION

### HLA-G

Human leukocyte antigen-G (HLA-G) is an HLA class Ib antigen characterized by a restricted tissue expression, low polymorphism and 7 isoforms (HLA-G1 to HLA-G7) generated by alternative splicing of the primary HLA-G transcript (1, 2). Four of them, HLA-G1, -G2, -G3 and -G4, are bound to the cell surface, while the remaining three, HLA-G5, -G6 and -G7, are detectable in soluble

form (sHLA-G) (1). HLA-G, both membrane-bound and soluble form, exerts several immune-modulatory effects. In fact, it inhibits CD4+ T cells allogeneic proliferation (3), natural killer (NK) and CD8+ T cells cytotoxicity (4), dendritic cells (DC) maturation (5), and B cells activation (6). In addition, soluble HLA-G molecules (sHLA-G) trigger apoptosis in antigen specific CD8+ T lymphocytes (4, 7, 8).

Furthermore, HLA-G may induce immune tolerance leading to the development of tolerogenic DC with induction of anergic and immunosuppressive T cells promoting the expansion of CD4+ CD25+ FoxP3+ T regulatory lymphocytes (Tregs) and triggering the differentiation of CD4+ T-cells in suppressor cells (9). HLA-G seems also to be involved in the tuning of immune responses, as incubation of peripheral blood mononuclear cells (PBMC) with HLA-G-expressing cells favors a shift towards a Th-2 cytokine profile, whereas incubation with soluble HLA-G molecules may have a counterbalancing effect by creating an anti-inflammatory environment due to the release of interleukin (IL)-10 (10, 11). While originally described as restricted in its constitutive tissue expression (12–16), HLA-G expression can be induced in several pathologic conditions (17–24). Of note, cytokines such as interferon (IFN)-γ and IL-10 trigger the expression of HLA-G by PBMC. Particularly, IL-10 enhances HLA-G expression and down-regulates classical HLA class I and class II antigens expression on monocytes, thus regulating NK cells and T lymphocyte responses (25, 26).

Recently, a novel subset of thymus-derived T lymphocytes expressing HLA-G have been described as distinct population of Tregs (27). HLA-G+ Tregs can be differentiated from classical CD4+ Tregs because of the phenotype lacking Forkhead Box P3 (FoxP3), CD39 and CD25 expression (27) and since mediate their immunomodulatory functions through cell-to-cell contact independent mechanisms (28) whereas classical CD4+CD25+FoxP3+ Tregs act mainly *via* cell-to-cell mechanisms (29). HLA-G-expressing Tregs exert their regulatory activity through various tolerogenic soluble molecules such as sHLA-G5, IL-10, IL-35 and transforming growth factor (TGF)-β (30, 31). Besides thymus-derived HLA-G+ Tregs, normal CD4+ and CD8+ T cells may acquire the HLA-G1 molecule from antigen presenting cells (APCs) through trogocytosis thus modulating their function from effectors to regulatory cells capable to inhibit alloproliferative responses (32). Interestingly, the acquisition of HLA-G *via* trogocytosis mechanism has also been reported for NK cells and monocytes (33, 34). A non-cytolytic subset of NK cells expressing HLA-G (NK-ireg) can be generated *in vitro* from peripheral blood CD34+ hematopoietic progenitors. NK-ireg cells display a mature NK cell phenotype, release suppressive molecules (sHLA-G, IL-10, IL-21) and through these factors down-modulate DCs activity and NK cells cytotoxicity (35).

HLA-G+ immune cells are present in the peripheral blood of healthy subjects where they probably contribute to maintain immune tolerance. Conversely, increased percentages of circulating and tissue-infiltrating HLA-G+ immune cells (e.g., T and NK cells, monocytes, DCs, mast cells) can be observed in different pathological situations such as infections, cancers, transplants and autoimmune disorders suggesting a potential role for these cells in the pathogenesis of diseases in which immune system is strictly implicated (1, 2, 24, 36–38). Based on these findings, it has been proposed that HLA-G should be qualified as an 'immune checkpoint' molecule (39).

## Allergy

Allergic diseases are characterized by an IgE mediated antibody response to an environmental allergen. Both genetic and environmental factors contribute to the development of allergic disease. Exposure of a genetically predisposed individual to allergen results in uptake of the allergen by APC followed by intracellular digestion of the allergen into peptide fragments and display of the peptide fragments by HLA on the APC membrane. Allergen-specific T helper cells type 2 (Th2) interact with the APC and secrete cytokines like interleukin (IL)-4, IL-5 and IL-13 which induce mast cell, basophil and eosinophil proliferation and IgE production by B cells. In addition, it is now appreciated that other T cell types, such as Th17 and Th9 may be involved in allergy development (40). Cross-linking of FcεRI through allergen-IgE binding sensitizes mast cells and basophils to release biologically active mediators including histamine, serotonin, proteoglycans, tryptase, leukotrienes and prostaglandins causing the allergic reaction and tissue inflammation (40). Recent studies have identified a new type of Th cells localized in B-cell follicles in the secondary lymphoid organs, named follicular helper T (Tfh) cells, that produce IL-4 and IL-13 and regulate antibody isotype switching required for IgE production (41). Furthermore, a subset of Tregs has been identified within lymphoid follicles that counteract Tfh cells and suppress IgE production thus preventing allergic responses (41). Therefore, a tilted balance in the Tfh/Tregs axis may represent an essential feature of allergic diseases. From a clinical perspective, allergic diseases comprise allergic rhinitis, asthma, conjunctivitis and dermatitis, and food allergy.

# HLA-G AND ALLERGIC RHINITIS

Allergic rhinitis is sustained by mucosal IgE-dependent inflammation characterized by mast cell and eosinophil activation.

Our group investigated sHLA-G serum levels in adult allergic rhinitis patients allergic to seasonal and perennial allergens. Serum sHLA-G levels are significantly higher in allergic patients as compared to healthy controls and strongly correlate with allergen-specific IgE levels as well as with rhinitis clinical severity and anti-allergic drug consumption (**Table 1**). Of interest, serum sHLA-G levels are higher in patients with seasonal allergy than in those with perennial allergy (42–46, 51) Moreover, sHLA-G levels significantly decrease 3 months after the end of allergen-specific immunotherapy and correlate with the increased production of IFN-γ by peripheral blood mononuclear cells suggesting a successful shift from Th2 to Th1 immune response (52, 53).

**TABLE 1** | sHLA-G plasma levels in Allergic Rhinitis and Asthma.

| | Patients | Controls | *Ref. n.* |
|---|---|---|---|
| Allergic rhinitis | 35.86* | 12.79 | (42) |
| | 42.80 | 9.80 | (43) |
| | 35.38 | 9.76 | (44) |
| | 24.68 | 7.03 | (45) |
| | 46.36 | 6.75 | (46) |
| Asthma (Children) | 67.9** | n.a. | (47) |
| | 52 | 42 | (48) |
| | 179.3 | 35.2 | (49) |
| Bronchoalveolar lavage (adults) | 6.8 | 1.6 | (50) |

**ng/mL; **U/mL.*

These data agree with those recently published by another research group revealing that allergic rhinitis patients have significantly higher serum sHLA-G levels than normal subjects and that there is a highly significant and positive correlation between sHLA-G and specific IgE levels (54). Finally, elevated serum sHLA-G amounts have been also found in children with allergic diseases (49, 55) (**Table 1**).

## HLA-G AND ASTHMA

Allergic asthma is characterized by persistent airway inflammation, structural remodelling and bronchial hyperresponsiveness in lower airways driven by Th2 lymphocytes activation and IL-4, IL-5 and IL-13 release.

Genetic factors play a central role in asthma pathogenesis and over 100 genes have been implicated in asthma susceptibility. The potential involvement of HLA-G in asthma development has been suggested by pivotal studies indicating a linkage between asthma and chromosome 6p21 (56, 57). Particularly, HLA-G polymorphisms may confer susceptibility to airway hyperresponsiveness and asthma development. The G/G genotype at SNP -964 G/A in the promoter region is associated with asthma in the offspring of mothers with asthma or bronchial hyperresponsiveness, while the A/A genotype is associated with asthma in the offspring of asthma-free and hyperresponsiveness-free mothers (57).

In following years, a genome-wide association study (GWAS) performed in 6819 participants from the Framingham Heart Study identified potential susceptibility loci in the HLA-G gene regions as risk factors for IgE dysregulation and atopy (58).

Further studies analyzed the potential interaction among maternal asthma, microRNA regulation of soluble HLA-G in the airway and offspring subsequent risk for asthma. Variants in the HLA-G 3′ UTR including SNP +3142 C/G (rs1063320) that disrupts a target site for the microRNA (miR)-152 family were evaluated. Results indicated that +3142 genotypes were associated with elevated miR-148b and sHLA-G concentrations in BAL fluid among asthmatic subjects with an asthmatic mother but not among those with a non-asthmatic mother. These results are consistent with +3142 allele-specific targeting of HLA-G by the miR-152 family and support the hypothesis that miRNA regulation of sHLA-G in the airway is influenced by both the asthma status of the subject's mother and the subject's genotype (59, 60).

More recently, HLA-G haplotypes were characterized by next generation sequencing from position −1983 to +3447 and sHLA-G serum levels were quantified both in a cohort of 330 healthy subjects and in 580 asthmatic patients from a French multicenter cohort. HLA-G haplotypes displayed statistically significant differential distribution between healthy subjects and asthmatic patients and a significant association with eosinophil count as well as with history of near-fatal asthma and asthma exacerbations. By contrast, no association was found between sHLA-G serum level and genetic data suggesting the hypothesis that sHLA-G is not overexpressed as a systemic immune response to control local inflammation (61).

The potential role of HLA-G in asthma has been also reported in a Brazilian study evaluating the HLA-G untranslated region (3′UTR) in 115 asthmatic patients stratified according to disease severity (mild, moderate, and severe) and in 116 healthy individuals. The +3010C and +3142G alleles were overrepresented in mild asthma patients when compared to controls and the +3010G and +3142C alleles were overrepresented in severe asthma patients in comparison to patients with mild asthma. These results suggest that HLA-G 3′UTR segment variation sites were differentially associated according to asthma severity (62).

A role for HLA-G in asthma pathogenesis is further suggested by the demonstration of sHLA-G molecule expression in the airway epithelium and of increased levels of sHLA-G in plasma and bronchoalveolar lavage (BAL) fluid of children with atopic asthma (47–49) (**Table 1**). However, no significant association was observed between plasma sHLA-G, total IgE and allergen specific IgE levels. Moreover, sHLA-G levels were not significantly related to HLA-G 14-bp insertion/deletion polymorphism. Other studies indicated that, among subjects with asthma, BAL sHLA-G concentrations were inversely correlated with markers of inflammation in the airway. In particular, sHLA-G concentrations were highest in subjects with low BAL eosinophils, low fractional exhaled nitric oxide (FENO), a marker of airways inflammation, and low serum IgE suggesting that sHLA-G concentrations were highest in patients with low inflammatory endotype of asthma and best pulmonary function (50, 63) (**Table 1**). Interestingly, bronchial epithelial cells from patients with mild and severe asthma display impaired mRNA expression of HLA-G1, -G4, and -G5 functional isoforms and HLA-G expression is not affected by IL-13 supporting the hypothesis that an impaired expression of HLA-G isoforms in asthmatic patients could contribute to the loss of inflammation control and epithelium structural remodeling (64).

Of note, it has been reported that in infants with asthma sHLA-G plasma levels were significantly higher in subjects with persistent wheezing compared with subjects with transient wheezing. However, there was no significant difference in peripheral blood eosinophil count and total IgE level between the two groups. These results may suggest that the increased sHLA-G levels in infants with persistent wheeze may be able to be used to distinguish persistent from transient wheeze (47).

The potential role of pregnancy and labor on plasma sHLA-G levels was evaluated in allergic and non-allergic women (65). Plasma samples were obtained during the 3$^{rd}$ trimester of pregnancy, at delivery and at a non-pregnant state 2 years post-partum. Levels of

the sHLA-G1 isoform in plasma significantly increased during labor compared to levels detected during the 3rd trimester of pregnancy and two years after delivery. However, allergic women had lower plasma sHLA-G levels than non-allergic women during the 3rd trimester of pregnancy and at delivery. Interestingly, no significant differences were found in samples obtained 2 years after pregnancy. Finally, spontaneous production of sHLA-G by PBMCs resulted significantly higher in patients with isocyanate-induced asthma than in other groups of asthmatic patients (66).

HLA-G binding of KIR2DL4 (CD158d) receptor on NK cells induces the secretion IFN-γ, a cytokine critical for the generation of tolerogenic DC. As consequence it might be predicted that individuals with a functionally defective allele of KIR2DL4 would not be able to secrete IFNγ and might therefore be prone to Th2-biased immune responses and produce fewer tolerogenic DC. KIR2DL4 genotypes were analyzed in 2 cohorts of children at high risk for atopic disease and asthma. However, there was no significant relationship between KIR2DL4 genotype and the prevalence of atopy and asthma (67).

It has been suggested that infections may play a role in the pathophysiology of allergic diseases, in particular asthma. The relationship among allergy, infections and HLA-G is an intriguing question, however no data are currently available on this topic.

## HLA-G AND ATOPIC DERMATITIS

Atopic dermatitis (AD) is a chronic disease usually beginning in childhood. AD is characterized by increased production of IL-4, IL-13 and IgE. In AD biopsies, HLA-G positive cells were always found in the papillary and, less frequently, in the reticular dermis. HLA-G was expressed mainly by infiltrating T cells but also, to a lesser extent and less frequently, by monocytes-macrophages or Langerhans cells (20). It is noteworthy that topical administration of purified recombinant HLA-G1 ameliorate the AD-like skin lesions in the mice. In addition, serum levels of IgE, IL-13, and IL-17A are significantly reduced in HLA-G1-treated mice. Taken together, these observations suggest a potential role for recombinant HLA-G as novel therapeutic strategy for AD and other chronic inflammatory skin disorders (68).

## *IN VITRO* DATA

The *in vitro* expression and release of HLA-G molecules by PBMC after incubation with both allergenic and non-allergenic stimuli was evaluated in allergic rhinitis patients HLA-G membrane expression was specifically induced by incubation with the causal allergen, but not by incubation with non-causal allergens or non-specific stimuli. Monocytes and to a lesser extent CD4+ T cells, particularly Th2 cells, expressed HLA-G after allergenic challenge whereas CD8+ T lymphocytes, B lymphocytes, NK cells and Tregs did not show any detectable HLA-G expression after incubation with allergens. The exposure to the causal allergen seems to be the main factor inducing HLA-G expression. In fact, patients allergic to mites, evaluated during winter, when the exposure to mite was still present, showed the more intense membrane HLA-G expression, whereas grass pollen allergic patients, who were evaluated far from the pollen season, showed a very low increase of HLA-G expression. The measurement of sHLA-G in culture supernatants confirmed that high amounts of sHLA-G molecules are found when the causal allergen is used as stimulus. Soluble molecules detected in culture supernatants mainly belong to the HLA-G5 isoform suggesting that they are actively secreted by immune cells after incubation with allergen (69, 70).

## CONCLUSIONS

HLA-G molecules have a complex immune regulatory role in transplantation, cancer, viral infections, chronic inflammatory diseases and pregnancy (**Table 2**). In general, HLA-G is a tolerance-inducing molecule by inducing Treg cells, but it is also a pro-inflammatory molecule stimulating Th2 responses (**Figure 1**). Allergic diseases are driven by a Th2-polarized

**TABLE 2** | HLA-G in non-allergic diseases.

| Disease | Analyzed data | Pathophysiologic relevance | Ref. n. |
|---|---|---|---|
| Chron's disease | Polymorphisms | Increased disease susceptibility | (71) |
| | Elevated serum levels | Positive correlation with disease severity | |
| Rheumatoid arthritis | Polymorphisms | Increased disease susceptibility | (72) |
| | Elevated serum levels | Negative correlation with disease severity | |
| Systemic lupus erythematosus | Polymorphisms | Increased disease susceptibility | (73, 74) |
| | Elevated expression and serum levels | Positive correlation with disease severity | |
| Systemic sclerosis | Low serum levels | Negative correlation with disease severity | (75, 76) |
| | Elevated serum levels | No correlation with disease severity | |
| Multiple sclerosis | Polymorphisms | Increased disease susceptibility | (77, 78) |
| | Elevated dimer levels | Positive correlation with decreased inflammation | |
| Psoriasis | Polymorphisms | Positive correlation with treatment response | (79) |
| Toxoplasmosis | Elevated trophoblast release | Abnormal pregnancy | (80) |
| Malaria | Elevated cord blood levels | Low weight at birth and infection risk | (81) |
| *Helycobacter pylori* infection | Increased expression | Negative correlation with inflammation | (82) |
| HCV – HBV – HIV | Polymorphisms | Worse outcome and response to treatment | (83–85) |
| | Elevated serum levels | | |
| Tumors (Gastrointestinal, kidney, breast, lung, melanoma) | Increased expression and serum levels | Increased metastasis and worse outcome | (86–92) |

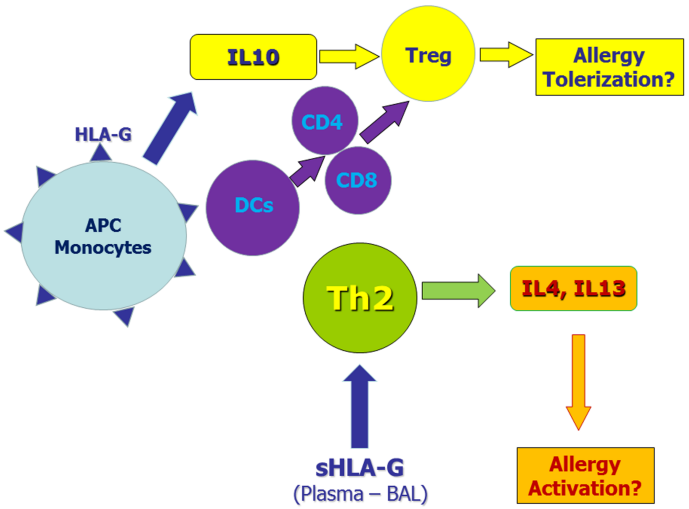


**FIGURE 1** | Potential role of membrane-bound and soluble HLA-G molecules in allergic diseases. Monocytes and antigen presenting cells (APC) expressing membrane-bound HLA-G molecules secrete IL-10 and induce tolerogenic dendritic cells. These mechanisms induce regulatory T cells (Tregs) that may exert tolerogenic effects on the allergic process. On the other hand, soluble HLA-G molecules in plasma and/or bronchoalveolar lavage (BAL) may facilitate Th2 polarization thus sustaining allergic responses.

inflammation and allergic patients display a defect in Treg cells which may be restored by specific immunotherapy. Taken together, the studies reported in this review suggest that: i) sHLA-G plasma levels are greater in atopic than in normal subjects and decrease after specific immunotherapy; ii) HLA-G is an asthma susceptibility gene; iii) HLA-G molecules are present in airway epithelium and BAL fluid of asthmatic subjects; iv) HLA-G is expressed and secreted by immune cells of atopic patients following *in vitro* allergenic challenge. At present, it remains unclear whether the presence of HLA-G is reactive in attempt to restore a proper balance in inflammatory cells and cytokines activated in allergic diseases or is a part of their pathogenesis by diverting the immune response towards a Th2 phenotype or by altering the presence and function of Treg cells. This latter hypothesis is supported by the finding that antigen presenting cells and monocytes expressing HLA-G molecules create a tolerogenic milieu enriched in IL-10 which, in turn, promotes Treg cells activity. In conclusion, it could be postulated that HLA-G molecules in allergy may be either compensatory or pathogenetic, but their precise mechanism of action is not yet completely known and needs further investigation.

## AUTHOR CONTRIBUTIONS

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

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# Systematic Evaluation of *HLA-G* 3'Untranslated Region Variants in Locally Advanced, Non-Metastatic Breast Cancer Patients: UTR-1, 2 or UTR-4 are Predictors for Therapy and Disease Outcome

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Despite major improvements in diagnostics and therapy in early as well as in locally advanced breast cancer (LABC), metastatic relapse occurs in about 20% of patients, often explained by early micro-metastatic spread into bone marrow by disseminated tumor cells (DTC). Although neoadjuvant chemotherapy (NACT) has been a successful tool to improve overall survival (OS), there is growing evidence that various environmental factors like the non-classical human leukocyte antigen-G (HLA-G) promotes cancer invasiveness and metastatic progression. HLA-G expression is associated with regulatory elements targeting certain single-nucleotide polymorphisms (SNP) in the *HLA-G* 3' untranslated region (UTR), which arrange as haplotypes. Here, we systematically evaluated the impact of *HLA-G* 3'UTR polymorphisms on disease status, on the presence of DTC, on soluble HLA-G levels, and on therapy and disease outcome in non-metastatic LABC patients. Although haplotype frequencies were similar in patients ($n = 142$) and controls ($n = 204$), univariate analysis revealed that the UTR-7 haplotype was related to patients with low tumor burden, whereas UTR-4 was associated with tumor sizes >T1. Furthermore, UTR-4 was associated with the presence of DTC, but UTR-3 and UTR-7 were related to absence of DTC. Additionally, increased levels of soluble HLA-G molecules were found in patients carrying UTR-7. Regarding therapy and disease outcome, univariate and multivariate analysis highlighted UTR-1 or UTR-2 as a prognostic parameter indicative for a beneficial course of disease in terms of complete response towards NACT or progression-free survival (PFS). At variance, UTR-4 was an independent risk factor for a reduced OS besides already known parameters. Taken into account the most common *HLA-G* 3'UTR haplotypes (UTR-1–UTR-7, UTR-18),

deduction of the UTR-1/2/4 haplotypes to specific SNPs revealed that the +3003C variant, unique for UTR-4, seemed to favor a detrimental disease outcome, while the +3187G and +3196G variants, unique for UTR-1 or UTR-2, were prognostic parameters for a beneficial course of disease. In conclusion, these data suggest that the *HLA-G* 3'UTR variants +3003C, +3187G, and +3196G are promising candidates for the prediction of therapy and disease outcome in LABC patients.

**Keywords: HLA-G, *HLA-G* UTR-4, breast cancer, *HLA-G* 3'UTR polymorphism, *HLA-G* UTR-2, *HLA-G* UTR-1**

## INTRODUCTION

Locally advanced breast cancer (LABC) is characterized by large tumor sizes, lymph node spread, and an unfavorable tumor biology in the absence of distant metastasis (1). Breast cancer (BC) classification according to the expression status of the hormone receptors for estrogen and progesterone as well as the amplification of the HER-2neu gene are crucial in the management of BC therapy (2). In case chemotherapy is indicated in early and especially in LABC, a neoadjuvant therapeutic regimen is preferred. Neoadjuvant chemotherapy (NACT) aims at reducing the tumor size prior to surgical resection, improving rates of breast conserving surgery, eliminating micro-metastases, and assessing response to chemotherapy quite early (1, 2). On the basis of large meta-analyses, a more favorable outcome has been demonstrated for patients who achieve a pathological complete remission (pCR). Depending on histological subtypes, pCR rates vary (3, 4) and therapeutic options in the post-neoadjuvant setting can be adapted according to pathological response to NACT. The evaluation of success to NACT is based on clinical assessment, imaging, and histology after surgery (5). Despite major improvements in diagnostics and therapy, metastatic relapse occurs in about 20% of patients, which is often explained by early micro-metastatic spread into bone marrow, mirrored by disseminated tumor cells (DTC). There is growing evidence that the occurrence of cancer depends on various environmental factors as well as genetic susceptibilities. In this regard, gene polymorphisms have been identified as a parameter in malignancies (6). As tumor immune evasion is a crucial step in carcinogenesis, there is evidence that post-transcriptional regulation of immune-modulatory molecules is an important event in this process (3, 4, 7–9). The immune checkpoint molecule human leukocyte antigen-G (HLA-G) as membrane-anchored or soluble molecule represents a compelling factor in the context of both development and progression of cancer. HLA-G belongs to the non-classical class I molecules characterized by restricted tissue distribution, low rate of polymorphism, and immunosuppressive properties (10). Aberrant expression of HLA-G and its soluble forms is considered to support tumor immune escape (11) and often correlates with adverse clinical courses (12). However, a discrepancy between HLA-G mRNA and protein expression has often been detected in human tumors indicating post-transcriptional control of HLA-G in malignancies (13). Posttranscriptional regulation of the *HLA-G* gene occurs through nucleotide variability at the 3' untranslated region (3'UTR) influencing HLA-G mRNA stability and/or microRNA targeting (14). Protein expression is constantly balanced by transcription levels and mRNA decay (14). The latter is dependent on the intrinsic stability of mRNA, which depends on the nucleotide sequence and the action of microRNAs (14). The *HLA-G* 3'UTR harbors several polymorphic sites (+3001C/T, +3003C/T, +3010C/G, +3027C/A, +3032C/G, +3035C/T, 3052C/T, +3092G/T, +3111A/G, +3121C/T, +3142C/G, +33177G/T, +3183A/G, +3187A/G, +3196C/G, and +3227A/G) and a 14-bp insertion/deletion (IN/DEL), which have been associated with differential HLA-G expression profiles or disease susceptibility (15–17). As these single-nucleotide polymorphisms (SNP) and the 14-bp IN/DEL are present in a short mRNA sequence and are just some nucleotides apart, it has to be considered that each polymorphic site in the *HLA-G* 3'UTR may not be independent of other polymorphic sites (14). A recent meta-analysis validated the *HLA-G* 3'UTR 14-bp IN/DEL polymorphisms as an eminent player in BC with the 14-bp IN allele assuming a protective role (6). Although the different mechanisms by which the various polymorphic sites influence HLA-G expression are still unknown, *in silico* studies revealed that several microRNAs target the *HLA-G* 3'UTR (18, 19). In this regard, HLA-G regulatory microRNAs have been identified that functionally target the *HLA-G* 3'UTR and bind with low or high affinity (13). As, for instance, binding of the key regulators depends on the presence of a Guanine at position +3142 (19), it is likely that polymorphisms in the *HLA-G* 3'UTR impact microRNA binding and thus HLA-G implications.

Here, we systematically evaluated the impact of HLA-G 3'UTR polymorphisms on disease status, on the presence of DTC, and known levels of soluble and vesicular HLA-G (20), and on therapy as well as disease outcome in 142 non-metastatic LABC patients.

## MATERIALS AND METHODS

### Patients' Characteristics

A total of 142 patients diagnosed between November 2007 and June 2012 at the Department of Gynecology and Obstetrics, University Hospital Essen, with histologically confirmed early and LABC were analyzed. Clinical characteristics of the patients are documented in **Table 1**. The median follow-up time for progression-free survival (PFS) was 66 months and that for OS

**TABLE 1** | Patient characteristics.

| | | Pre-NACT *n* (%) | Post-NACT *n* (%) |
|---|---|---|---|
| **Total** | 142 | | |
| **Age, years** | Median: 50 (18–83) | | |
| **Follow-up, months** | 71 (4–138) | | |
| **Menopausal status** | Premenopausal | 67 (47.2) | |
| | Perimenopausal | 19 (13.4) | |
| | Postmenopausal | 56 (39.4) | |
| **Nodal status**[a] | Node-negative (c/pN-) | 71 (50.4) | 86 (61.9) |
| | Node-positive (c/pN+) | 70 (49.6) | 53 (38.1) |
| **Tumor size**[a] | ≤ c/pT1a-c | 36 (25.7) | 49 (35.5) |
| | c/pT2 | 84 (59.2) | 40 (29.0) |
| | > c/pT2 | 20 (14.1) | 49 (35.5) |
| **Tumor grading**[a] | G1 | 9 (6.5) | |
| | G2 | 66 (47.5) | |
| | G3 | 64 (46.0) | |
| **Histological finding**[a] | Ductal | 103 (73.6) | |
| | Lobular | 18 (12.9) | |
| | Other | 19 (13.6) | |
| **Tumor subtype** | ER-, PR-, HER2- | 26 (18.3) | |
| | ER-, PR-, HER2+ | 10 (7.0) | |
| | ER+/PR+, HER2- | 77 (54.2) | |
| | ER+, PR+, HER2+ | 29 (20.4) | |
| **NACT regimen** | CTX | 96 (68.1) | |
| | CTX + Trastuzumab | 22 (15.6) | |
| | CTX + Trastuzumab + Lapatinib | 8 (5.7) | |
| | CTX + Avastin | 5 (3.5) | |
| | HTX | 10 (7.1) | |
| **DTC**[a] | Pos/total | 31/117 | |
| **Pathological response**[a] | Complete response | | 29 (21.2) |
| | Partial response | | 98 (71.5) |
| | No response | | 10 (7.3) |
| **Survival (OS)**[a] | Alive | | 122 (85.9) |
| | Dead | | 20 (14.1) |
| **Progression-free survival (PFS)** | Alive | | 109 (76.8) |
| | Relapsed | | 33 (23.2) |

[a]*Clinical parameters could not be determined for all patients; CTX, chemotherapy; DTC, disseminated tumor cells; ER, estrogen receptor; HTX, anti-hormonal therapy; NACT, neoadjuvant chemotherapy; PR, progesterone receptor.*

was 70 months (range: 4 to 138 months). Thirty-three out of 133 patients experienced a disease relapse and 21/121 patients died of BC. The patients' eligibility criteria, NACT treatment regimens, and definition of NACT response have already been described in detail elsewhere (20).

A total of 204 healthy controls (HC), namely, 122 females and 82 males, served as a control panel for genotyping. At time of blood sampling, the median age of HC was 52 years, ranging from 21 to 73 years. Written informed consent was obtained by all participants, and the study was approved by the Local Ethics Committees (Essen 05-2870 and 17-7495) and was performed according to the Declaration of Helsinki.

## Detection Evaluation of DTC

DTC were analyzed as described before (21). In short, DTC were analyzed by immunocytochemistry using the pan-cytokeratin antibody A45-B/B3.

## Isolation of Extracellular Vesicles and Assessment of HLA-G levels

Extracellular vesicles (EV) were isolated from plasma samples by the use of ExoQuick™ (SBI Systems Bioscience Inc., Mountain View, VA, USA) as described previously (20). Plasma samples and the corresponding EV suspensions were diluted 1:2 in PBS and analyzed for the content of HLA-G molecules as described before (20, 22). For quantification of HLA-G, purified HLA-G1 (23) served as standard reagent. Levels were determined by four-parameter curve fitting. ELISA detection limit of HLA-G was 0.25 ng/ml.

## *HLA-G* 3'UTR Analysis

After genomic DNA extraction from Cytospin preparations containing single-cell suspensions derived from liquid biopsies using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, *HLA-G* 3'UTR typing was performed by polymerase chain reaction (PCR) as previously described (24, 25). To verify differences regarding haplotype frequencies among the patient cohort ($n$ = 142) and controls ($n$ = 204), a residuals analysis was performed.

## Statistical Analysis

Allele and genotype frequencies of polymorphic sites were calculated by using Fisher's exact test. Contribution of haplotypes and allelic variants to clinical parameters was

evaluated by either two-sided Chi-square test or Fisher's exact test. Stepwise multivariate Cox regression according to proportional hazards assumption or binomial logistic regression was used to identify prognostic factors for PFS/OS or therapy outcome towards NACT, respectively. *p*-values <0.05 were considered statistically significant. Statistical analyses were performed by using SPSS 25.0 software (SPSS Inc., Chicago, IL, USA) or GraphPad Prism V8.4 software (GraphPad Software, San Diego, CA, USA). OS and PFS analyses were assessed by the *survminer* package implemented in the R package version 0.4.3 using the method of Kaplan–Meier and compared using log-rank test (https://CRAN.R-project.org/package=survminer).

## RESULTS

### *HLA-G* 3'UTR Haplotype Analysis in LABC Patients and HCs

To determine *HLA-G* 3'UTR haplotypes, 15 SNPs of the *HLA-G* 3'UTR were sequenced and analyzed in 142 well-defined, non-metastatic LABC patients and 204 healthy donors (HC). Haplotype analysis revealed 8 out of 18 haplotypes with a frequency >1%. As expected (19), UTR-1 (30% and 35%) and UTR-2 (31% and 30%) displayed the most frequent haplotypes in both LABC patients and HC, respectively, accounting for roughly 60% of the haplotypes (Additional File 1). Of note, examination of the haplotype or genotype distribution revealed that distribution of both the haplotypes and genotypes was similar among LABC patients and HC and did not reach significance (Additional File 2).

### *HLA-G* 3'UTR Haplotypes UTR-4 and UTR-7 Associate With High or Low Tumor Burden Pre-NACT in LABC Patients

Concerning the clinical status of LABC patients, presence of UTR-4 was positively associated with a tumor size >T1 ($p = 0.03$, relative risk [RR]: 2.48, 95% confidence interval [CI]: 1.11–5.9) as 33.7% (35/104) of patients having a tumor size >T1 carried UTR-4, whereas only 13.9% (5/36) of patients with T1 were positive for UTR-4 (**Figure 1A**). In contrast to UTR-4, presence of UTR-7 seemed to be associated with lower tumor stage ($p = 0.03$, RR: 0.45, 95% CI: 0.27–0.87): 22.2% (8/36) of the LABC patients with T1 carried UTR-7, while only 7.7% of patients with $T > 1$ were positive for this haplotype (**Figure 1B**). None of the *HLA-G* 3'UTR haplotypes were associated with high/low tumor burden or other clinical disease status pre-NACT.

### *HLA-G* 3'UTR Haplotype UTR-4 and UTR-3/7 Associate With the Presence or Absence of DTC Pre-NACT in LABC Patients

Next, we asked whether *HLA-G* 3'UTR haplotypes were associated with the presence or absence of DTC pre-NACT in LABC patients. Fifteen out of 31 LABC patients (48%) with DTC pre-NACT carried the UTR-4 haplotype, whereas only 22 out of 86 patients (22%) without detectable DTC were positive for

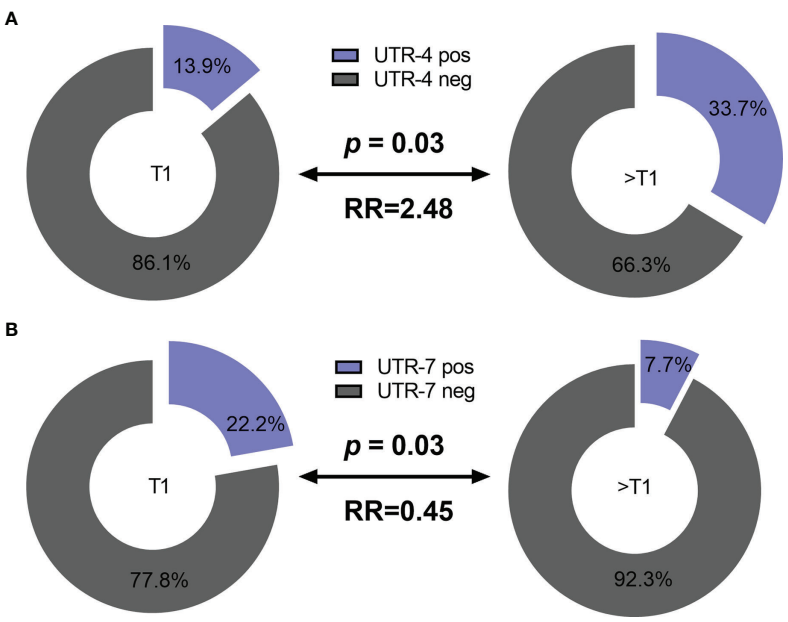


**FIGURE 1** | Association of the *HLA-G* 3'UTR haplotype UTR-4 and UTR-7 carrier status with tumor size pre-NACT in LABC patients. Higher frequencies of UTR-4 haplotype were observed in LABC patients with tumor size >T1 compared to patients with stage T1 pre-NACT **(A)**. Frequencies of UTR-7 haplotypes were significantly reduced in UTR-7-positive patients compared to UTR-7-negative ones **(B)**. Blue or gray color indicated frequencies of UTR-4/7-positive and UTR-4/7-negative patients, respectively. RR: relative risk.

UTR-4 (**Table 2**, $p$ = 0.02, RR: 2.03, 95% CI: 1.13–3.65). At variance to UTR-4 haplotype, the UTR-3 and UTR-7 haplotype were significantly ($p$ = 0.01 and $p$ = 0.02, respectively) associated with the absence of DTC pre-NACT (**Table 2**, RR: 0.11, 95% CI: 0.00–1.70 and RR: 0.13, 95% CI: 0.00–1.99, respectively). None of the remaining *HLA-G* 3'UTR haplotypes were associated with the presence or absence of DTC.

## *HLA-G* 3'UTR Haplotype UTR-7 Associates With Increased Levels of Soluble HLA-G Molecules in LABC Patients

For 116 LABC patients, the levels of total amount of soluble HLA-G (sHLA-G) and of vesicular HLA-G (HLA-GEV) were available (20). To investigate the impact of *HLA-G* 3'UTR haplotypes on pre-NACT sHLA-G/HLA-GEV levels, the patients were stratified according to the presence and absence of a certain haplotype. As shown in **Figures 2A, B**, pre-NACT levels of sHLA-G as well as HLA-GEV were significantly higher in UTR-7-positive LABC patients ($p$ = 0.009; $n$ = 14) than in UTR-7-negative ones ($n$ = 102). None of the remaining *HLA-G* 3'UTR haplotypes were associated with sHLA-G/HLA-GEV.

## *HLA-G* 3'UTR Haplotype UTR-1 Is an Independent Prognostic Co-Variate for Complete Response to NACT in LABC Patients

Concerning therapy outcome, LABC patients were stratified into patients with pathological complete response (pCR, $n$ = 29) and in patients with pathological partial response or with no response (pPR/pNR, $n$ = 108). The presence of UTR-1 in LABC patients seemed to be a beneficial factor for the response to NACT ($p$ = 0.003, RR: 0.76, 95% CI: 0.02–1.28): 75.9% (22/29) of the LABC patients with pCR to NACT carried UTR-1, while the response to NACT was insufficient in only 43.5% (47/108) of LABC patients positive for this haplotype (**Figure 3**). None of the remaining *HLA-G* 3'UTR haplotypes were associated with the response to NACT. Multivariate analysis including age (<60 vs. >60), menopausal status (premenopausal vs. peri/postmenopausal), pre-NACT nodal status (N0 vs. >N0), pre-NACT tumor size (T1 vs. >T1), NACT regimen (CTX vs. CTX + Trastuzumab vs. CTX + Trastuzumab + Lapatinib, vs. CTX + Avastin vs. HTX), UTR-1 haplotype carrier status and levels of vesicular HLA-GEV (<15.8 vs. >15.8 ng/ml (20), revealed that the UTR-1 haplotype carrier status was exclusively an independent prognostic factor correlating with pCR remission in LABC patients ($p$ = 0.003; HR: 0.221; 95% CI: 0.081–0.604; $n$ = 110).

## *HLA-G* 3'UTR Haplotypes UTR-2 and UTR-4 Are Independent Prognostic Co-Variates for Beneficial Progression-Free or Inferior Overall Survival of LABC Patients

Concerning disease outcome, Kaplan–Meier probabilities of PFS ($p$ = 0.01; log-rank Hazard Ratio [HR]: 0.41, 95% CI: 0.20–0.82) were significantly prolonged for patients carrying UTR-2 haplotype with undefined median PFS time compared with patients being UTR-2 negative with a median PFS of 115 months (**Figure 4A**). Similarly, the presence of UTR-2 haplotype in LABC patients showed an improved OS in comparison to UTR-2-negative ones ($p$ = 0.046, log-rank HR: 0.40, 95% CI: 0.17–0.98; **Figure 4B**). In contrast to UTR-2-positive patients, combined Kaplan–Meier analysis and log-rank testing revealed that UTR-4-positive LABC patients had a significantly deteriorated PFS ($p$ = 0.014, HR: 2.31, 95% CI: 1.01–5.27; **Figure 4C**) as well as OS ($p$ = 0.0081, HR: 3.01, 95% CI: 1.11–8.45, **Figure 4D**) compared to UTR-4-negative LABC patients.

The UTR-1, UTR-2, and UTR-4 carrier status, the age (<60 vs. >60), menopausal status (premenopausal vs. peri/postmenopausal), post-NACT nodal status (N0 vs. >N0), post-NACT tumor size (≤T1 vs. >T1), Triple-Negative Breast Cancer (TNBC, yes vs. no), pathological response (pCR vs. pPR/NR), and levels of vesicular HLA-GEV (<15.8 vs. >15.8 ng/ml) were enrolled as co-variates in the multivariate analysis for PFS and OS. The optimal HLA-GEV cutoff value for PFS and OS was defined earlier (20). Among these co-variates, the UTR-2 haplotype appeared to be exclusively an independent factor indicative for a beneficial disease outcome in terms of PFS ($p$ = 0.007, HR: 0.3 95% CI: 0.1–0.7), while a positive post-NACT nodal status ($p$ = 0.009, HR: 29, 95% CI: 1.3–6.7), HLA-GEV levels >15.8 ng/ml ($p$ = 0.003, HR: 4.0, 95% CI: 1.6–10.1), and a TNBC tumor subtype ($p$ = 0.021, HR: 3.0, 95% CI: 1.2–7.6) were predictors for a reduced PFS (**Table 3A**). At variance to UTR-2, the UTR-4 haplotype was an independent prognostic risk factor for an inferior OS ($p$ = 0.005, HR: 4.9, 95% CI: 1.6–15.1) besides a positive post-NACT nodal status ($p$ = 0.015, HR: 4.3, 95% CI: 1.3–14.2, **Table 3B**).

## *HLA-G* 3'UTR SNP Variants +3187G, +3196G, and +3003C as Distinct Features of UTR-1/2, and UTR-4

Next, we elucidated whether certain allelic variants are responsible for the antagonistic effects observed for the UTR-1/

**TABLE 2** | Association of *HLA-G* 3'UTR haplotypes with the presence of DTC pre-NACT in LABC patients.

| | DTC pos *n* (%) | DTC neg *n* (%) | *p* | RR (95% CI) |
|---|---|---|---|---|
| **UTR-4 pos** | 15 (48) | 22 (26) | 0.02 | 2.03 (1.13–3.65) |
| **UTR-4 neg** | 16 (52) | 64 (74) | | |
| **UTR-3 pos** | 0 (0) | 15 (17) | 0.01 | 0.11 (0.00–1.70) |
| **UTR-3 neg** | 31 (100) | 71 (83) | | |
| **UTR-7 pos** | 0 (0) | 13 (15) | 0.02 | 0.13 (0.00–1.99) |
| **UTR-7 neg** | 31 (100) | 73 (85) | | |

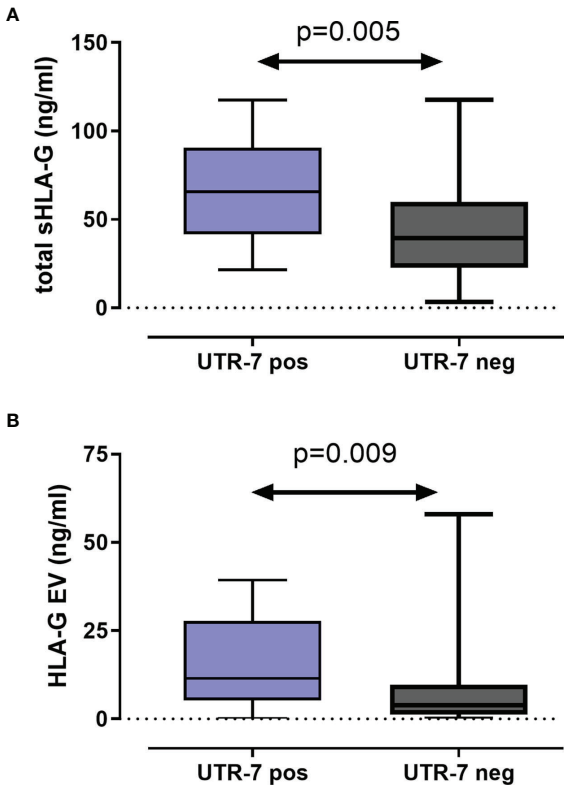


**FIGURE 2** | Association of the *HLA-G* 3'UTR haplotype UTR-7 with increased levels of soluble HLA-G molecules pre-NACT in LABC patients. Levels of total amount of soluble HLA-G [sHLA-G; **(A)**] and of vesicular HLA-G [HLA-EV; **(B)**] were increased in UTR-7 carrier (blue) compared with UTR-7-negative patients (gray). Data are presented as median with the minimum and maximum. Statistical significance was determined by Mann–Whitney test.

UTR-2 and UTR-4 haplotypes in LABC patients. Therefore, we analyzed the arrangement of the 15 SNP for the UTR-1, UTR-2, and UTR-4 haplotypes regarding differences and consensus in the most common 3'UTR haplotypes (UTR-1 to UTR-7 and UTR-18) in a Venn diagram (**Figure 5**). Obviously, the +3196G variant was unique for the UTR-2, whereas the +3187 variant was a distinctive feature of the UTR-1 haplotype. Thus, these variants seem to be of prognostic parameters for a beneficial course of disease. At contrast, the deduction of UTR-4 to a specific SNP revealed the +3003C variant as a unique feature, which seemed to be responsible for a detrimental disease outcome of LABC patients.

## DISCUSSION

Various studies have suggested that the *HLA-G* 3'UTR may act as a predictor for the genetic predisposition of an individual to certain immune-mediated diseases (6, 24, 26–33). Thus, we evaluated the impact of *HLA-G* 3'UTR variants arranged as *HLA-G* 3'UTR haplotypes on the clinical status and outcome of non-metastatic LABC patients. Indeed, our study demonstrates the following: (i) Although HC and LABC patients did not differ in their UTR haplotypes' frequencies, the UTR-4 haplotype was associated with unfavorable disease status regarding tumor size and the presence of DTC, whereas UTR-7 or UTR-3 were related to low tumor sizes, absence of DTC, and high soluble HLA-G levels, respectively. (ii) Concerning therapy and disease outcome, univariate and multivariate analysis displayed UTR-1 or UTR-2 as prognostic parameters indicative for a favorable course of disease in terms of complete response towards NACT or PFS. At variance, UTR-4 was an independent risk factor for an inferior OS besides already known clinical parameters. (iii) Taken into account the most common *HLA-G* 3'UTR haplotypes (UTR-1 to UTR-7, UTR-18) with haplotype frequencies > 1% (Additional file 1), the attribution of the UTR-1/2/4 haplotypes to specific SNPs revealed that the +3003C variant, unique for UTR-4, seems to support an adverse disease outcome, whereas the +3187G and +3196G variants, unique for UTR-1 or UTR-2, are prognostic parameters for a beneficial course of disease.

At variance to our study, in which all *HLA-G* 3'UTR haplotype frequencies were very similar in LABC patients and HC, the UTR-4 haplotype frequency was increased in prostate cancer patients compared to controls. Additionally, this haplotype was more frequent in women with an uneventful pregnancy and less frequent in women with recurrent miscarriage (32).

Regarding the clinical and prognostic significance of *HLA-G* 3'UTR haplotypes as indicators for a favorable or deleteriously

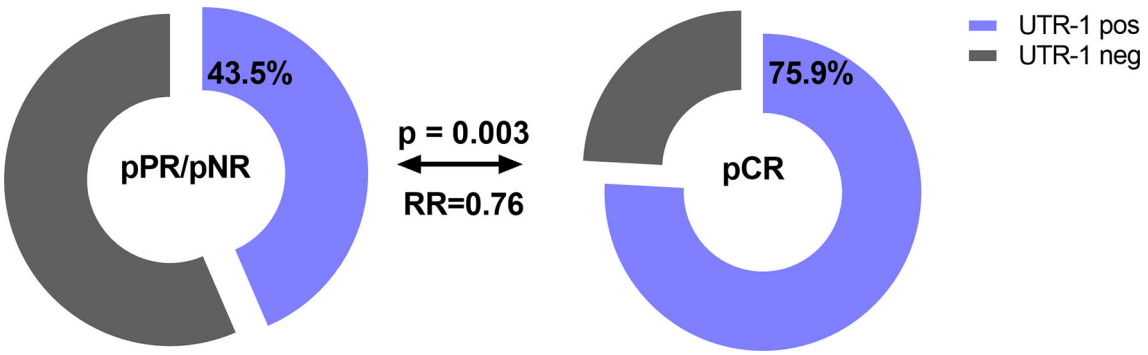


**FIGURE 3** | Association of the *HLA-G* 3'UTR haplotype UTR-1 carrier status with pathological complete response to NACT in LABC patients. Higher frequencies of UTR-1 haplotype were observed in LABC patients with pCR compared to patients with insufficient therapy response. Clinical data were available for all patients. Blue or gray color indicated frequencies of UTR-1-positive and UTR-1-negative patients, respectively. pCR: pathological complete remission, pPR: pathological partial response, pNR: pathological no response, OR: odds ratio.

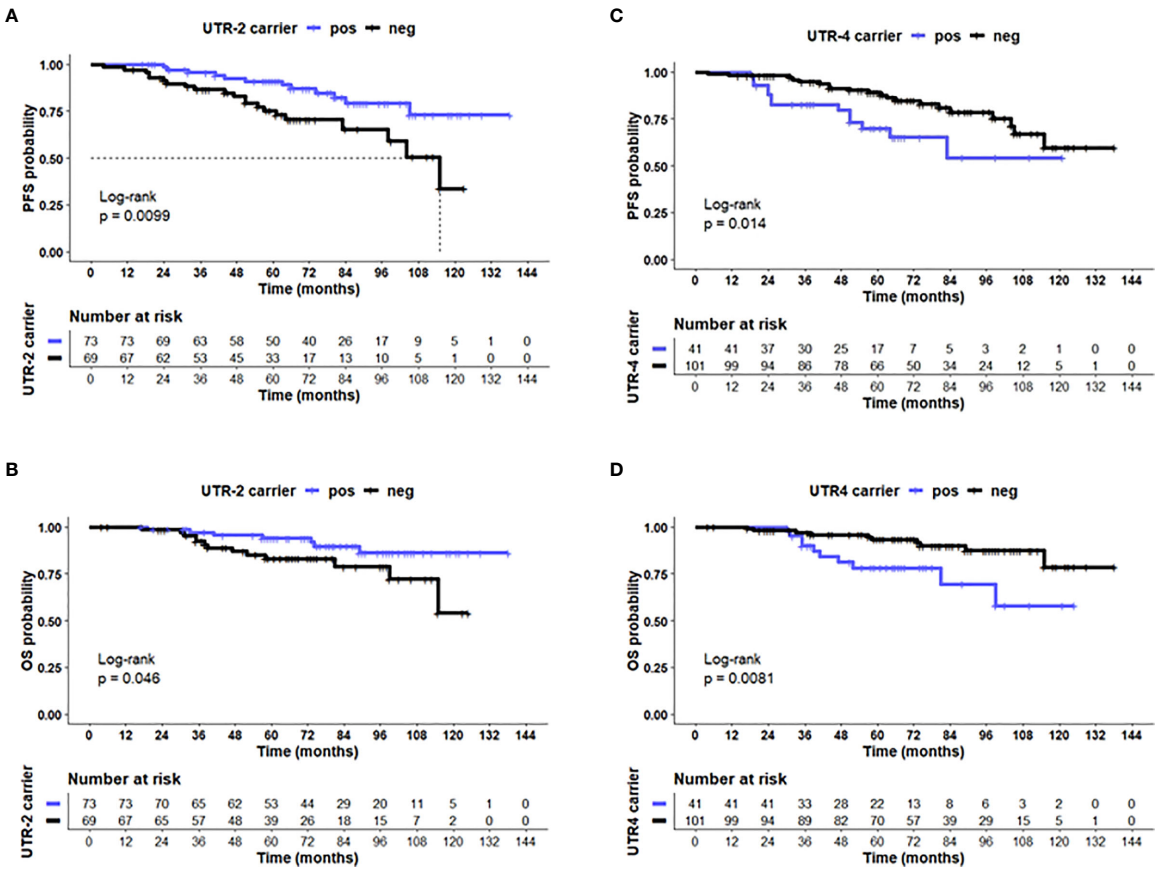


**FIGURE 4** | UTR-2 and UTR-4 haplotypes of the *HLA-G* gene are associated with PFS and OS in LABC patients. Kaplan–Meier plot analysis combined with log-rank test revealed that LABC patients carrying the **(A)** UTR-2 haplotype of the HLA-G 3'UTR had a significantly improved PFS **(B)** compared with UTR-2-negative patients. UTR-4-positive patients had a reduced PFS **(C)** and OS **(D)** compared to UTR-4 patients. The blue line indicates patients positive for either the **(A, B)** UTR-2 or **(C, D)** UTR-4 haplotype, whereas the black line illustrates the equivalent negative patients. Dotted line reveals median survival time where applicable. Tables under Kaplan–Meier plots show corresponding numbers at risk.

**TABLE 3A |** Multivariate analysis to predict progression-free survival in LABC patients.

| Co-variate | | *N* = 111[a] | *p*-value | HR (95% CI) |
|---|---|---|---|---|
| **UTR-1** | Pos | 52 | 0.349 | 0.7 (0.3–1.6) |
| | Neg | 59 | | |
| **UTR-2** | Pos | 61 | **0.007** | **0.3 (0.1–0.7)** |
| | Neg | 50 | | |
| **UTR-4** | Pos | 37 | 0.267 | 1.7 (0.7–4.6) |
| | Neg | 74 | | |
| **Age, years** | <60s | 79 | 0.280 | 1.5 (0.2–1.7) |
| | >60 | 32 | | |
| **Menopausal status** | Premenopausal | 49 | 0.421 | 1.5 (0.5–4.5) |
| | Peri/postmenopausal | 62 | | |
| **Post-NACT tumor size** | ≤T1 | 71 | 0.691 | 0.8 (0.3–2.0) |
| | >T1 | 40 | | |
| **Post-NACT nodal status** | N0 | 72 | **0.009** | **2.9 (1.3–6.7)** |
| | >N0 | 39 | | |
| **TNBC** | Yes | 22 | **0.021** | **3.0 (1.2–7.6)** |
| | No | 89 | | |
| **Therapy response** | pCR | 102 | 0.222 | 2.7 (0.5–14.6) |
| | pPR/NR | 9 | | |
| **HLA-GEV (ng/ml)** | <15.8 | 96 | **0.003** | **4.0 (1.6–10.1)** |
| | >15.8 | 15 | | |

**TABLE 3B |** Multivariate analysis to predict overall survival in LABC patients.

| Co-variate | | *N* = 111[a] | *p*-value | HR (95% CI) |
|---|---|---|---|---|
| **UTR-1** | Pos | 52 | 0.600 | 1.3 (0.4–4.7) |
| | Neg | 59 | | |
| **UTR-2** | Pos | 61 | 0.440 | 0.6 (0.1–2.3) |
| | Neg | 50 | | |
| **UTR-4** | Pos | 37 | **0.005** | **4.9 (1.6–15.1)** |
| | Neg | 74 | | |
| **Age, years** | <60s | 79 | 0.483 | 0.6 (0.1–2.6) |
| | >60 | 32 | | |
| **Menopausal status** | Premenopausal | 49 | 0.535 | 1.6 (0.4–7.1) |
| | Peri/postmenopausal | 62 | | |
| **Post-NACT tumor size** | ≤T1 | 72 | 0.939 | 1.0 (0.3–3.6) |
| | >T1 | 39 | | |
| **Post-NACT Nodal status** | N0 | 71 | **0.015** | **4.3 (1.3–14.2)** |
| | >N0 | 40 | | |
| **TNBC** | Yes | 22 | 0.070 | 3.0 (0.9–10.3) |
| | No | 89 | | |
| **Therapy response** | pCR | 102 | 0.222 | 2.7 (0.5–14.6) |
| | pPR/NR | 9 | | |
| **sHLA-GEV (ng/ml)** | <15.8 | 96 | 0.441 | 1.9 (0.3–10.3) |
| | >15.8 | 15 | | |

[a]*Clinical parameters could not be determined for all patients; NACT, neoadjuvant chemotherapy; TNBC, Triple-Negative Breast Cancer; pCR, pathological complete remission; NR, no response; pPR, pathological partial remission; HLA-GEV, vesicular-bound soluble HLA-G. Significant p-values are shown in bold.*

disease status and outcome, conflicting results were reported: An age-adjusted logistic regression established UTR-4 as a risk factor in prostate cancer (34). In contrast to our study, the UTR-2 haplotype was reported to be a risk factor for colorectal cancer, whereas the UTR-4 haplotype resumed a protective role. In epithelial ovarian cancer, the homozygous UTR-1 genotype could be associated with metastases formation, whereas in our study, the presence of UTR-1 in LABC patients seems to be a beneficial factor for the response to NACT (24). In non-malignant situations, UTR-4 was reported to resume a protective role for the development of chronic kidney disease. In living-donor kidney transplantation, UTR-4 haplotype in donors and recipients was associated with occurrence of BK polyomavirus replication or nephropathy and with protection of antibody mediated rejection, whereas the donor UTR-2 haplotype was related to acute cellular or antibody-mediated rejection (28). Moreover, in our LABC patients' cohort, the UTR-7 haplotype could be associated with high levels of circulating soluble as well as vesicular-bound HLA-G molecules, whereas in a recent study the UTR-5 and UTR-7 haplotypes were associated with low sHLA-G levels in French and Brazilian healthy individuals (31). Although high levels of HLA-GEV were found to be an independent parameter indicative for early relapse in LABC, the UTR-7 haplotype itself could not be established as a prognostic co-variate in LABC patients. This supports a recent observation that

| Position | SNP |
|---|---|
| +2961 | INS / DEL |
| +3001 | C / T |
| +3003 | C / T |
| +3010 | C / G |
| +3027 | C / A |
| +3032 | C / G |
| +3035 | C / T |
| +3052 | C / T |
| +3092 | T / G |
| +3111 | A / G |
| +3121 | C / T |
| +3142 | C / G |
| +3187 | A / G |
| +3196 | C / G |
| +3227 | A / G |

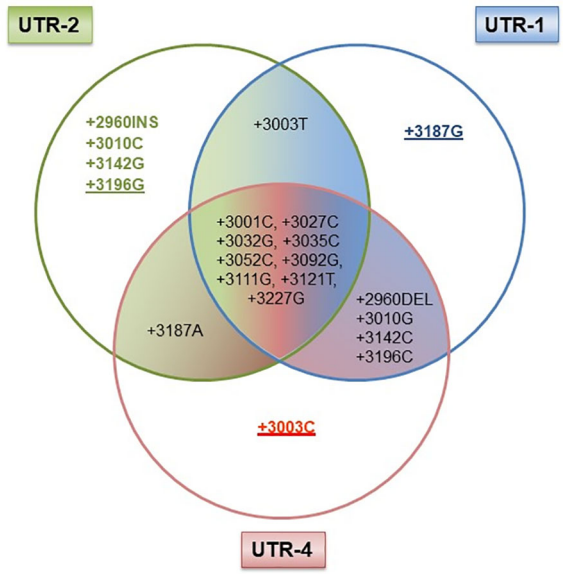


**FIGURE 5** | Dissection of the *HLA-G* 3'UTR haplotypes UTR-1, UTR-2, and UTR-4. Table shows the positions of the 15 single-nucleotide polymorphisms (SNP) with the HLA-G 3'UTR. UTR-1 (blue), UTR-2 (green), and UTR-4 (red) share 9 out of 15 SNP as shown in the intersection, while six SNP were different (+2960INS/DEL, +3003C/T, +3010G/C, +3142C/G, +3187A/G, and +3196C/G) among these UTR haplotypes. Underlines in allelic variants (+3187G, +3196G, and +3003C) represent the unique feature of the UTR-1, UTR-2, or UTR-4 haplotype in our patient cohort. The allelic variants +2960INS, +3003T, +3010C, and +3142G of the UTR-2 haplotype can also be found in other HLA-G 3'UTR haplotypes.

inhibition of effector immune cells *via* HLA-GEV is not restricted to cells expressing the ILT-2 receptors (35). Thus, all these partly contradictory associations of UTR haplotypes in physiological and pathological conditions underline a recent thesis that mechanisms regulating HLA-G expression can be distinctly active in different diseases (17). Indeed, the biologic function of the *HLA-G* 3'UTR haplotypes clearly depends on the presence or absence of regulatory elements targeting certain single nucleotides within the *HLA-G* 3'UTR. Thus, it is likely that, in cancer, the tumor location, phenotype, and heterogeneity may affect the functional consequence of certain haplotypes.

Concerning DTC, a recently published pooled analysis including 10.307 early BC patients has strengthened their prognostic significance as independent prognostic markers for OS, PFS, and distant disease-free survival. We recently demonstrated a phenotypic heterogeneity of DTC in early BC patients with regard to the expression of the chemokine receptor type 4 (CXCR4) as well as the transcription factor JUNB. Patients who harbored double-positive DTC (CXCR4- and JUNB-positive) showed an unfavorable clinical outcome (36). However, not all of the DTC have metastatic potential and not every patient with detectable DTC has a higher risk of relapse. Consequently, the additional analysis of factors that promote cancer invasiveness and metastatic progression, like the *HLA-G* 3'UTR haplotypes UTR-4, UTR-3, and UTR-7, might help to identify patients at a higher risk or with a favorable course more precisely. From the clinical point of view, it is particularly important that molecular genetic analysis of *HLA-G* 3'UTR haplotype analysis is less invasive than a bone marrow aspiration since it can be performed in blood and in the follow-up of the disease.

By the deduction of *HLA-G* 3'UTR haplotype to specific SNP, it became evident that the +3003C variant reflects the impaired clinical course of disease in UTR-4 carriers. This is in line with a study analyzing the potential use of *HLA-G* 3'UTR for prostate cancer prediction, in which the +3003C variant was suggested as a tag SNP for prostate cancer risk (33). Strikingly, two microRNAs, namely, miR-628-5p and miR-548q, have already been identified to target the segment encompassing the +3003 variant (13, 34). Binding of these microRNAs is most likely influenced by the presence of a certain nucleotide at this locus. In contrast, the protective role of UTR-2 could be deduced to the +3196G variant. In a study investigating kidney transplant recipients, stable allograft function was linked to the +3196CC genotype (37), suggesting an immunosuppressive role for this genotype. Of note, the +3196G variant is also characteristic for the rare UTR-8 as well as for UTR-10 haplotypes. However, both *HLA-G* 3'UTR haplotypes were not present at all in our cohort of LABC patients, whereas in the group of HC, the frequencies of these haplotypes were below 1% (0.2% and 0.4%, respectively).

The relevance of the gene polymorphisms of the *HLA-G* 3'UTR associated with LABC course/outcome is supported by the correlation between exosomal microRNA expression and disease stage of NACT-treated localized breast cancer (38). Here, distinct exosomal microRNAs allowed discrimination of localized from distant breast cancer patients and from healthy women (38). A combined study encompassing gene polymorphisms of the *HLA-G* 3'UTR and exosomal

microRNAs could contribute to a better understanding of diagnosis, prognosis, and treatment response in breast cancer.

A limitation of the study might be the small number of patients included in this study to statistically discriminate between the different BC subtypes and the detected haplotypes. However, the results obtained for this group of LABC patients strengthen the fact that worse outcome seems to be multifactorial, underlining the requirement to perform a comprehensive analysis of the primary tumor as well as the liquid biopsy, which also includes circulating HLA-GEV to identify patients at higher risk to allow secondary treatment options. Regarding HLA-G and its cognate immune checkpoint receptor ILT-2 as targets for immunotherapeutic intervention, HLA-G-specific CAR-T cells are under development (39). Furthermore an anti-ILT-2 blocking antibody has been established and currently approved for a Phase I clinical trial (NCT04717375).

Combined, these data give evidence that the UTR-4 haplotype with its characteristic variant +3003C as well as the UTR-1 and UTR-2 haplotypes reflected by the +3187G or +3196G variant represent promising factors for the prognosis of disease outcome for LABC patients. Generally, OS and PFS in the cohort of patients presented here are improved, not only due to advanced therapeutic applications for these patients but also due to the fact that our patients were recommended an additional therapy with oral clodronate (2 × 520 mg per day for at least 2 years) in case of DTC positivity at primary diagnosis resulting in better outcomes (26). Thus, it has to be emphasized that the *HLA-G* 3'UTR variants or haplotypes may serve as a further promising parameter in the outcome of non-metastatic LABC patients.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Ethic Committee of the University Hospital in Essen, Germany. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

VR, ES, and SK-B conceived and designed research, performed the experiments, interpreted data, performed statistical analysis, wrote the initial draft, and read and approved the final article. LG performed experiments. RM and PH interpreted data, and read and approved the final article. A-KB, HR, RK, and OH collected and provided clinical data, interpreted data, and read and approved the final article. 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.2021.817132/full#supplementary-material

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# The Association of *HLA-G* Gene Polymorphism and Its Soluble Form With Male Infertility

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Successful reproduction depends on many factors. Male factors contribute to infertility in approximately 50% of couples who fail to conceive. Seminal plasma consists of secretions from different accessory glands containing a mixture of various cytokines, chemokines, and growth factors, which together can induce a local immune response that might impact on a male's as well as a female's fertility. Human leukocyte antigen (HLA)-G expression has been suggested as an immunomodulatory molecule that influences pregnancy outcome. The *HLA-G* gene encodes either membrane-bound or/and soluble proteins. The aim of this study was the evaluation of HLA-G polymorphisms and their impact on soluble HLA-G (sHLA-G) production. We tested the *HLA-G* polymorphism in three positions: rs1632947: c.-964G>A; rs1233334: c.-725G>C/T in the promoter region; rs371194629: c.∗65_∗66insATTTGTTCATGCCT in the 3′ untranslated region. We tested two cohorts of men: 663 who participated in *in vitro* fertilization (test material was blood or sperm), and 320 fertile controls who possessed children born after natural conception (test material was blood). Since 50% of men visiting assisted reproductive clinics have abnormal semen parameters, we wondered if men with normal sperm parameters differ from those with abnormal parameters in terms of *HLA-G* polymorphism and secretion of sHLA-G into semen. We found that certain rs1632947-rs1233334-rs371194629 *HLA-G* haplotypes and diplotypes were associated with male infertility, while others were protective. Normozoospermic men with the A-C-del haplotype and A-C-del/A-C-del diplotype secreted the most sHLA-G into semen (574.1 IU/mL and 1047.0 IU/mL, respectively), while those with the G-C-ins haplotype and G-C-ins/G-C-ins diplotype – the least (80.8 IU/mL and 75.7 IU/mL, respectively). Men with the remaining haplotypes/diplotypes secreted sHLA-G at an intermediate level. However, only in one haplotype, namely G-C-ins, did we observe strong significant differences in the concentration of sHLA-G in the semen of men with teratozoospermia compared to men with normal sperm parameters ($p = 0.009$). In conclusion, fertile men differ in the

profile of *HLA-G* polymorphism from men participating in IVF. Among all *HLA-G* haplotypes, the most unfavorable for male fertility is the G-C-ins haplotype, which determines the secretion of the lowest concentration of the soluble HLA-G molecule. This haplotype may reduce sperm parameters.

**Keywords: HLA-G polymorphism, sHLA-G, male infertility, *In vitro* fertilization, semen**

## INTRODUCTION

Successful reproduction depends on many factors. Male factors contribute to infertility in approximately 50% of couples who fail to conceive (1). This is a growing problem observed around the world and in Poland (2). Decreased semen quality has been observed over the years and may be caused by endocrine disrupting chemicals (3, 4) but it can also result from anatomical or genetic abnormalities, systemic or neurological diseases, infections, trauma, iatrogenic injury, gonadotoxins and the development of sperm antibodies. Male infertility may be due to testicular and post-testicular deficiencies. However, in 30–40% of male infertility cases, no cause is identified (idiopathic male infertility) (5). Moreover, male factors may have an influence upon fertilization and embryo development failure, the increase in the risk of idiopathic recurrent miscarriages, autosomal dominant diseases and neurobehavioral disorders in their offspring (6).

Seminal plasma consists of secretions from different accessory glands, such as the epididymis, seminal vesicle, prostate, and bulbourethral gland. Seminal plasma contains a heterogeneous mixture of various cytokines, chemokines, and growth factors, which together can induce a local immune response that might impact on a male's as well as a female's fertility. These molecules are considered crucial for spermatocyte transport, function and protection against sperm damage (7–11).

So far, only a few studies have revealed that seminal fluid contains varying amounts of soluble HLA-G (sHLA-G) protein (12–16). The level of sHLA-G depends on the *HLA-G* genotype and haplotype (16, 17). The *HLA-G* gene encodes either membrane-bound and/or soluble proteins due to alternative splicing of its transcript: HLA-G1 to HLA-G4 are membrane bound, while HLA-G5 to HLA-G7 soluble. The most important isoforms are the full-length membrane bound HLA-G1 and the full-length secreted HLA-G5. Soluble HLA-G1 (sHLA-G1) is generated by the shedding of membrane-bound HLA-G1 molecules (18).

*HLA-G* gene, 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) (19, 20). The 14 bp insertion/deletion polymorphism (rs371194629 c. ∗ 65_ ∗ 66insATTTGTTCATGCCT) is between positions +2961 and +2974. A 14 bp insertion is considered an ancestral allele and a small fraction of the transcripts have been reported to undergo further alternative splicing leading to the removal of 92 bases (including 14 bases) from the 5' end of the previously named "exon 8". The 92 bases deleted transcripts are more stable than the longer ones (21). The insertion allele is associated with lower levels or absence of sHLA-G in plasma (19, 22–24). HLA-G expression is determined by the combination of multiple single nucleotide polymorphisms (SNPs) (24, 25). Therefore, in addition to the 14-bp insertion/deletion in 3′UTR, we tested other polymorphic positions in the *HLA-G* gene promoter region, which could affect the level of HLA-G expression, rs1632947: c.-964G>A and rs1233334: c.-725G>C/T, and to correlate them with the level of soluble HLA-G secreted into the plasma of semen. The presence of an adenine at -964 position (CpA dinucleotide) destroys a potentially methylated CpG dinucleotide (26), and the G variant at position -725 (C>G, T) creates a CpG dinucleotide influencing the transcriptional activity of the gene (27). However, Ober et al. revealed a significantly higher expression level of the promoter haplotype containing the -725G allele compared with those containing the -725C or -725T alleles (28).

The aim of this study was the evaluation of the role of sHLA-G and its gene polymorphism in male infertility. Since 50% of men visiting assisted reproductive clinics have abnormal semen parameters, we were curious if men with normal sperm parameters differed from those with abnormal parameters in terms of secreting sHLA-G into semen.

## MATERIAL AND METHODS

### Study Design

In our research, we tested a total of 993 men. Six hundred and sixty three males were patients who, together with their female partners, underwent *in vitro* fertilization (IVF), while a group of 320 fertile men constituted the control group. From the IVF group, we collected blood from 480 men, and semen from 183 men. Patients were qualified at the Gameta Assisted Reproduction Clinic in Rzgów, certified by the European Society for Human Reproduction and Embryology (ESHRE). Patients were also recruited from the Department of Surgical, Endoscopic and Oncologic Gynecology and the Department of Gynecology and Gynecologic Oncology, Polish Mothers' Memorial Hospital–Research Institute in Łódź and Gynemed. The men were of mean age 35.06 years ± 5.08 (age range 19-53). The semen samples were obtained by masturbation after 2-7 days of sexual abstinence. The patients' ejaculate samples were analyzed and categorized according to the nomenclature of the WHO (World Health Organization) from 2010 using Sperm Class Analyzer CASA System (29). Normozoospermia means the total number of sperm cells, their concentration, progressively motile and morphology above or equal reference values (N ≥ 15 mln/mL of sperm cells); OS – oligozoospermia; Moderate OS (5 < N < 15 mln/mL); Severe OS (1-5 mln/mL), very severe OS (N < 1 mln/mL); AS – azoospermia (lack of sperm cells in

ejaculate); Asthenozoospermia – number of sperm cells with progressive motility below reference values; Teratozoospermia – number of morphologically normal sperm cells below reference values.

The control group was recruited mainly from the 1st Department of Obstetrics and Gynecology, Medical University of Warsaw. These men and their female partners had at least one healthy child from natural conception. The men were of mean age 34.15 years ± 6.29 (age range 25-70). All tested males were of Polish origin. Men from the control group differ in age from IVF men ($p = 0.0003$).

Experimental protocols were approved by Local Ethics Committees (the agreement of Medical University of Wrocław and Polish Mothers' Memorial Hospital–Research Institute in Łódź) and informed consent was obtained from all individual participants included in the study.

## DNA Preparation and Genotyping

Genomic DNA was isolated from venous blood and semen using the Invisorb Spin Blood Midi Kit (Invitek, Germany) or QIAamp DNA Blood Mini Kit (Qiagen, Germany) or NucleoSpin Blood, Mini kit for DNA from blood (Macherey-Nagel, Germany) according to the manufacturer's instructions. We tested HLA-G polymorphism in three positions: rs1632947: c.-964G>A, rs1233334: c.-725G>C/T in the promoter, and rs371194629: c.*65_*66insATTTGTTCATGCCT in 3′UTR. Polymorphisms in the *HLA-G* gene were performed in all qualified men by PCR-SSP method or using TaqMan assays in Real-Time PCR according to Bylinska et al. and Nowak et al. (17, 30).

## sHLA-G Measurement

We had access to 183 semen samples taken from patients. Samples were stored at −80°C until the time of assay. The concentration of sHLA-G (IU/mL) in semen plasma was tested with a sandwich enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (Exbio/Biovendor, Czech Republic). Standard curve measured the concentration of HLA-G1 (which are shed from the cell surface by proteolytic cleavage) and sHLA-G5 isoforms from 3.91 to 125 IU/mL. The limit of sHLA-G detection in this test was 0.6 IU/mL. Samples in which the sHLA-G concentration exceeded 125 IU/mL were retested after diluting them by a factor 1:4.

## Statistical Analysis

We used the two-tailed Fisher exact test (R program version 3.4.3) for the estimation of allelic and genotypic frequencies. Deviation of the genotype counts from Hardy-Weinberg equilibrium was analyzed using the chi-square test with one degree of freedom. All genotype frequencies were in Hardy-Weinberg equilibrium both in control and in patient groups. A $p < 0.05$ was considered significant. The odds ratio (OR) and its 95% CI were computed as the measure of effect size. For multiple comparison tests, the Bonferroni correction was done. Haplotypes were generated by FAMHAP 19 (http://famhap.meb.uni-bonn.de). Statistical analyses concerning sHLA-G concentration measured in the seminal plasma of patients were performed using Mann-Whitney or T-student test (GraphPad Prism 5 software). All parameters of statistical analyses concerning sHLA-G (numbers, medians, means, standard deviation and errors, min, max, and 25-75% percentiles) are part of **Supplementary Tables 6–8**. The power calculation for the Mann-Whitney test was estimated using the available free calculator https://www.benchmarksixsigma.com/calculators/sample-size-calculator-for-mann-whitney-test/. This calculator allowed us to determine the number of samples needed to compare two population medians with at least 80% test power and a confidence level of 95%.

# RESULTS

## *HLA-G* Polymorphism in Fertile Men and Men Participating in IVF

We did not find differences in the frequencies of the individual *HLA-G* genotypes (**Supplementary Table 1**). Instead, we observed differences in the frequencies of haplotypes. Among controls and patients, 9 haplotypes in the following order rs1632947–rs1233334–rs371194629 were generated. Out of 9 detected haplotypes, 2 of them (A-C-ins and G-G-del) were statistically significantly more frequent in the group of fertile men compared to men participating in *in vitro* fertilization ($p < 0.0001/p_{corr.} = 0.0004$, OR = 0.653, 95% CI = 0.53-0.80; $p = 0.005/p_{corr.} = 0.042$, OR = 0.648, 95% CI = 0.48-0.88, respectively, **Table 1**). Two haplotypes (A-G-del and G-C-ins) were also observed to be carried more frequently by men from the IVF group than men from the control group ($p < 0.0001/p_{corr.} < 0.0001$, OR = 3.535, 95% CI = 1.73-8.14; $p < 0.0001/p_{corr.} < 0.0001$, OR = 3.035, 95% CI = 2.11-4.47, respectively, **Table 1**)

We also estimated 23 different diplotypes: 19 of them were present in the control group, while 21 were present in the IVF group. To determine the diplotypes, we adopted the same order of tested SNPs as in the case of haplotypes (**Table 2**). For 4 diplotypes, we found differences in frequencies between IVF and controls. Diplotypes A-C-del/A-G-del and G-C-ins/G-C-ins were more prevalent in patients than controls ($p = 0.0001/p_{corr.} = 0.002$, OR = 4.857, 95% CI = 1.91-15.81; $p < 0.0001/p_{corr.} = 0.001$, OR = 3.066, 95% CI = 1.72-5.82, respectively, **Table 2**). Two diplotypes (A-C-ins/A-C-ins and G-G-del/G-C-del) were protective and present in higher frequencies in the control group than in comparison to the IVF group ($p < 0.0001/p_{corr.} < 0.0001$, OR = 0.273, 95% CI = 0.16-0.46; $p < 0.0001/p_{corr.} < 0.0001$, OR = 0.191, 95% CI = 0.08-0.42, respectively, **Table 2**). Additionally, we found a significant association for the G-C-del/G-C-ins ($p = 0.024/p_{corr.}$ = ns, OR = 2.396, 95% CI = 1.08-6.02, **Table 2**). A protective effect was observed for A-C-ins/A-C-del ($p = 0.039/p_{corr.}$ = ns, OR = 0.474, 95% CI = 0.22-1.00) and G-T-ins/A-C-del ($p = 0.035/p_{corr.}$ = ns, OR = 0.000, 95% CI = 0.00-1.18, **Table 2**). However, statistical significance in these analyses was lost when the correction for multiple comparisons was applied.

When we compared diplotypes which differed only in the insertion allele (G-C-del/G-C-del vs. G-C-del/G-C-ins vs. G-C-

**TABLE 1** | *HLA-G* haplotype frequencies in men from Control and IVF group.

| Haplotype* | Fertile control (%) | IVF men (%) | IVF men vs. Fertile control | | |
|---|---|---|---|---|---|
| | 2N = 638 | 2N = 1308 | $p/p_{corr.}$ | OR | 95% CI |
| ACdel | 90 (14.11) | 216 (16.51) | 0.185 | 1.204 | 0.92-1.59 |
| ACins | 229 (35.89) | 350 (26.76) | **<0.0001/0.0004** | 0.653 | 0.53-0.80 |
| AGdel | 9 (1.41) | 63 (4.82) | **<0.0001/0.0001** | 3.535 | 1.73-8.14 |
| ATdel | 1 (0.16) | 9 (0.69) | **0.181** | 4.412 | 0.61-193.59 |
| GCdel | 170 (26.65) | 315 (24.08) | **0.220** | 0.873 | 0.70-1.09 |
| GCins | 38 (5.96) | 211 (16.13) | **<0.0001/<0.0001** | 3.035 | 2.11-4.47 |
| GGdel | 86 (13.48) | 120 (9.17) | **0.005/0.042** | 0.648 | 0.48-0.88 |
| GGins | 0 (0.00) | 3 (0.23) | 0.555 | – | – |
| GTins | 15 (2.35) | 21 (1.61) | 0.283 | 0.678 | 0.33-1.42 |

**Haplotypes were estimated in the following order: rs1632947:-964G>A; rs1233334:-725G>C/T; rs371194629:insATTTGTTCATGCCT/del. Values in bold indicate significant differences. IVF, in vitro fertilization; p, probability; $p_{corr.}$, probability after Bonferroni correction for 9 possible haplotypes; OR, odds ratio; 95% CI, confidence interval from two-sided Fisher's exact test; ns, not significant.*

**TABLE 2** | *HLA-G* diplotype frequencies in men from Control and IVF groups.

| Diplotype* | Fertile control (%) | IVF men (%) | IVF men vs. Fertile control | | |
|---|---|---|---|---|---|
| | N = 319 | N = 654 | $p/p_{corr.}$ | OR | 95% CI |
| AGdel/AGdel | 2 (0.63) | 8 (1.22) | 0.512 | 1.962 | 0.39-19.07 |
| ACdel/AGdel | 5 (1.57) | 47 (7.19) | **0.0001/0.002** | 4.857 | 1.91-15.81 |
| ACdel/ACdel | 15 (4.70) | 45 (6.88) | 0.204 | 1.497 | 0.80-2.94 |
| ACins/ACdel | 17 (5.33) | 17 (2.60) | **0.039/ns** | 0.474 | 0.22-1.00 |
| ACins/ACins | 45 (14.11) | 28 (4.28) | **<0.0001/<0.0001** | 0.273 | 0.16-0.46 |
| GGdel/GGdel | 5 (1.57) | 3 (0.46) | 0.123 | 0.290 | 0.04-1.50 |
| GGdel/GCdel | 24 (7.52) | 10 (1.53) | **<0.0001/0.0001** | 0.191 | 0.08-0.42 |
| GCdel/GCdel | 20 (6.27) | 28 (4.28) | 0.207 | 0.669 | 0.36-1.27 |
| GCdel/GCins | 8 (2.51) | 38 (5.81) | **0.024/ns** | 2.396 | 1.08-6.02 |
| GCins/GCins | 15 (4.70) | 86 (13.15) | **<0.0001/0.001** | 3.066 | 1.72-5.82 |
| ACdel/GCdel | 22 (6.90) | 45 (6.88) | 1.000 | 0.998 | 0.57-1.78 |
| ACdel/GGdel | 13 (4.08) | 16 (2.45) | 0.165 | 0.591 | 0.26-1.35 |
| ACins/ATdel | 1 (0.31) | 8 (1.22) | 0.285 | 3.934 | 0.52-175.16 |
| ACins/GCdel | 75 (23.51) | 164 (25.08) | 0.634 | 1.089 | 0.79-1.51 |
| ATdel/ACdel | 0 (0.00) | 1 (0.15) | 1.000 | – | – |
| GCins/ACins | 0 (0.00) | 1 (0.15) | 1.000 | – | – |
| GGdel/ACins | 37 (11.60) | 87 (13.30) | 0.475 | 1.169 | 0.76-1.82 |
| GGdel/GGins | 0 (0.00) | 1 (0.15) | 1.000 | – | – |
| GGins/GTins | 0 (0.00) | 2 (0.31) | 1.000 | – | – |
| GTins/ACdel | 3 (0.94) | 0 (0.00) | **0.035/ns** | 0.000 | 0.00-1.18 |
| GTins/ACins | 9 (2.82) | 17 (2.60) | 0.834 | 0.919 | 0.38-2.37 |
| GTins/GCdel | 1 (0.31) | 2 (0.31) | 1.000 | 0.975 | 0.05-57.71 |
| GTins/GGdel | 2 (0.63) | 0 (0.00) | 0.107 | 0.000 | 0.00-2.59 |

**Diplotypes were estimated in the following order: rs1632947:-964G>A; rs1233334:-725G>C/T; rs371194629:insATTTGTTCATGCCT/del. Values in bold indicate significant differences. IVF, in vitro fertilization; p, probability; $p_{corr.}$, probability after Bonferroni correction for 23 possible diplotypes; OR, odds ratio; 95% CI, confidence interval from two-sided Fisher's exact test; ns, not significant.*

*Chi-square for trend: ACdel/ACdel vs. ACins/ACdel vs. ACins/ACins: $p < 0.0001$; GCdel/GCdel vs. GCdel/GCins vs. GCins/GCins $p = 0.0005$.*

ins/G-C-ins), we observed a strong association with male infertility expressed by a higher frequency in samples from the IVF group (p = 0.0005; Chi-square test for trend). The insertion allele in these diplotypes was disadvantageous because the odds ratios increased from protective 0.669 in G-C-del/G-C-del men to predisposing 2.396 in GC-del/G-C-ins men and 3.066 in G-C-ins/G-C-ins men. However, the insertion allele in diplotypes A-C-ins/A-C-del and A-C-ins/A-C-ins was protective, and the odds ratios ranged from 1.497 in A-C-del/A-C-del patients to 0.474 in A-C-ins/A-C-del and to 0.273 in A-C-ins/A-C-ins patients (p < 0.0001, Chi-square test for trend; **Table 2**).

We can conclude that fertile men differ in the profile of *HLA-G* polymorphism from men participating in IVF.

## *HLA-G* Polymorphism and Stratification of Patients According to Sperm Parameters

When patients were stratified by sperm count, i.e., normozoospermia, moderate, severe and very severe oligozoospermia, we did not observe differences in haplotype frequencies with the exception of the G-T-ins haplotype, which was more common in the groups with a low sperm count and no sperm (p = 0.013/$p_{corr.}$ = ns, OR = 3.140, 95% CI = 1.18-9.26, p = 0.006/$p_{corr.}$ = ns, OR = 4.068, 95% CI = 1.33-13.01

respectively, **Supplementary Table 2**). By dividing the patients into those with all normal parameters and those with abnormal parameters, we found differences in the frequency of G-C-ins haplotype ($p = 0.009/p_{corr.}$ = ns, OR = 1.525, 95% CI = 1.10-2.12, **Supplementary Table 3**). In particular, a stronger association for this haplotype was observed when comparing the male with teratozoospermia and normozoospermia. ($p = 0.0005/p_{corr.} = 0.004$, OR = 2.000, 95% CI = 1.34-2.99, **Supplementary Table 3**).

After analysis of diplotype frequencies, only G-T-ins/A-C-ins was more frequent in the group with a reduced sperm count and no sperm than in men with normal number of sperm cells ($p = 0.011/p_{corr.}$ = ns, OR = 3.829, 95% CI = 1.24-14.06, $p = 0.012/p_{corr.}$ = ns, OR = 4.500, 95% CI = 1.20-18.35, respectively, **Supplementary Table 4**). Some associations were found in diplotype analysis of patients with reduced sperm motility or morphology (**Supplementary Table 5**). Namely, the A-C-ins/A-C-ins and G-C-del/G-C-del diplotypes were more common in men with normal sperm parameters than in men with teratozoospermia ($p = 0.026/p_{corr.}$ = ns, OR = 0.135, 95% CI = 0.00-0.90 and $p = 0.044/p_{corr.}$ = ns, OR = 0.157, 95% CI = 0.00-1.07, respectively). Conversely, the G-C-ins/G-C-ins diplotype was more common in men with teratozoospermia ($p = 0.008/p_{corr.}$ = ns, OR = 2.224, 95% CI = 1.19-4.15, **Supplementary Table 5**).

## Impact of HLA-G Haplotypes/Diplotypes on Soluble HLA-G Level in Semen of Patients Participating in IVF

We tested 183 semen samples for sHLA-G secretion. Regardless of the haplotype in the *HLA-G* gene, men with normozoospermia secreted more sHLA-G (median 288.9 IU/mL) than men with abnormal sperm parameters (median 227.9 IU/mL). This difference was not statistically significant (**Supplementary Table 6**).

**Table 3** shows the level of sHLA-G measured in semen depending on the presence of haplotypes and diplotypes in men with normal and abnormal semen parameters. Due to the insufficient number of samples having A-T-del and G-T-ins haplotype, the sHLA-G level in these samples cannot be interpreted. Normozoospermic men with the A-C-del haplotype secreted the most sHLA-G into semen (574.1 IU/mL), while those with the G-C-ins haplotype – the least (80.8 IU/mL). Men with the remaining haplotypes secreted sHLA-G at an intermediate level. When analyzing men with regards to diplotype, we can conclude that the carriers of the diplotype A-C-del/A-C-del secreted the most (1047.0 IU/mL), while the carriers of G-C-ins/G-C-ins – the least (75.7 IU/mL).

Differences in secretion of sHLA-G depending on the haplotype are also shown in **Figure 1** and **Supplementary Table 7**. The greatest differences in sHLA-G concentration were observed when comparing the G-C-ins haplotype (due to being the one that determines lowest secretion) with the remaining haplotypes in men with normozoospermia (G-C-ins vs. A-C-del $p < 0.0001$, G-C-ins vs. A-C-ins $p = 0.0004$, G-C-ins vs. A-G-del $p < 0.0001$; G-C-ins vs. G-C-del $p < 0.0001$). This regularity is also visible for men with abnormal sperm parameters. We did not observe differences in individual haplotypes between men with normozoospermia and men with abnormal sperm parameters, as well as after dividing patients into asthenozoospermic and teratozoospermic groups. Only in one haplotype, namely G-C-ins, did we observe strong significant differences in the concentration of sHLA-G in the semen of men with teratozoospermia compared to men with normal sperm parameters ($p = 0.009$). Men with the G-C-ins haplotype with asthenozoospermia also secreted less sHLA-G, but this difference lost significance ($p = 0.058$) (**Figure 2**).

**TABLE 3** | The level of secreted HLA-G in semen dependent on the haplotype/diplotype in normozoospermic men and men with sperm abnormalities.

| Haplotype/diplotype* | Normozoospermia | | Abnormal sperm | |
|---|---|---|---|---|
| | N | median [IU/mL] | N | median [IU/mL] |
| ATdel | 2 | 847.1 | 1 | 594.9 |
| ACdel | 22 | 574.1 | 47 | 650.0 |
| ACins | 23 | 391.2 | 50 | 261.7 |
| GCdel | 25 | 347.7 | 47 | 351.5 |
| AGdel | 12 | 315.2 | 5 | 395.3 |
| GGdel | 7 | 145.9 | 21 | 200.7 |
| GCins | 28 | 80.8 | 70 | 67.5 |
| GTins | 1 | 31.3 | 3 | 178.8 |
| ACdel/ACdel | 6 | 1 047.0 | 16 | 743.4 |
| ACins/ATdel | 2 | 847.1 | 1 | 594.9 |
| ACins/GCdel | 13 | 720.1 | 29 | 495.9 |
| AGdel/AGdel | 2 | 582.2 | 0 | – |
| ACdel/GCdel | 2 | 537.9 | 6 | 459.4 |
| ACdel/AGdel | 8 | 315.2 | 5 | 395.3 |
| GCdel/GCdel | 1 | 160.6 | 1 | 91.1 |
| GGdel/ACins | 7 | 145.9 | 17 | 99.8 |
| GCdel/GCins | 8 | 112.5 | 10 | 149.7 |
| GCins/GCins | 10 | 75.7 | 30 | 59.6 |
| GTins/ACins | 1 | 31.3 | 3 | 178.8 |
| ACdel/GGdel | 0 | – | 4 | 282.8 |

**Haplotypes/diplotypes were estimated in the following order: rs1632947:-964G>A; rs1233334:-725G>C/T; rs371194629:insATTTGTTCATGCCT/del. Normozoospermia – total number of sperm cells, their concentration, progressive motility and morphology above or equal reference values; Men with abnormal sperm – men with at least one parameter of semen below reference value.*

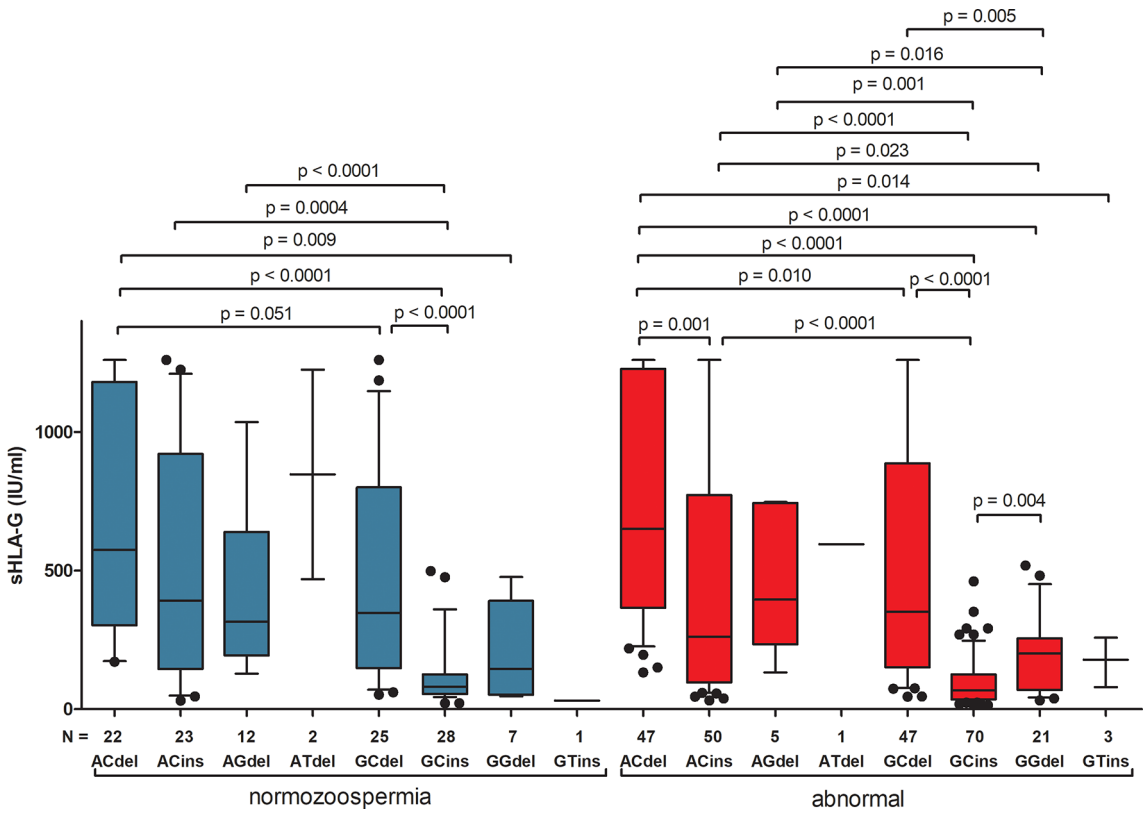


**FIGURE 1** | Concentration of soluble HLA-G per milliliter of plasma (IU/ml) measured in semen samples according to *HLA-G* haplotypes. Haplotypes were estimated in the following order: rs1632947:–964G>A; rs1233334:–725G>C/T; rs371194629:insATTTGTTCATGCCT/del. Blue boxes represent the level of sHLA-G measured in normozoospermic men and red boxes – in men with abnormal semen parameters. Boxes are drawn from the first quartile (25th Percentile) to the third quartile (75th Percentile). Black lines in boxes are medians. Whiskers represent 10-90 percentiles. N is the number of patients. P-values are calculated by Mann-Whitney test or unpaired t test.

For most analyses of sHLA-G secretion depending on diplotypes, the results cannot be interpreted due to insufficient numbers of individual diplotypes, which is visible in **Figure 3** and **Supplementary Table 8**. As for haplotypes, there are significant differences in sHLA-G secretion between individual diplotypes but within one group of men, not between men with normozoospermia and men with abnormal sperm parameters. However, when we compared secretion of sHLA-G in patients with rs1632947G allele, who differed only by the insertion allele (G-C-del/G-C-del vs. G-C-del/G-C-ins vs. G-C-ins/G-C-ins), we found significant differences among these diplotypes in men with abnormal sperm parameters ($p = 0.031$, Kruskal-Wallis test), while in comparisons of diplotypes A-C-del/A-C-del vs. A-C-ins/A-C-del vs. A-C-ins/A-C-ins, such differences were not observed (**Supplementary Table 8**, **Figure 3**).

## DISCUSSION

We tested two cohorts of men: those who participated in *in vitro* fertilization (test material was blood or sperm), and fertile controls who had children from natural conception (test material was blood). We found that certain rs1632947-rs1233334-rs371194629 *HLA-G* haplotypes and diplotypes were associated with male infertility, while others were protective. A-C-ins and G-G-del haplotypes were significantly more common in the fertile control group compared to men participating in *in vitro* fertilization, while the opposite trend was observed for haplotypes – A-G-del and G-C-ins. The role of G-C-ins haplotype seems to be crucial due to the fact that this haplotype determines the lowest secretion of sHLA-G and its association has been observed in men with teratozoospermia. However, the test power calculator indicated that we need 318 male samples with the G-C-ins haplotype (additionally 270 samples) to achieve a test power of 80% and a confidence level of 95% to compare the medians of men with normozoospermia and asthenozoospermia. When comparing men with normozoospermia and teratozoospermia ($p = 0.009$), we need 240 persons to achieve this power. Assuming a G-C-ins haplotype frequency in IVF men of 16.13% (**Table 1**), we should still have 1,928 samples to find 318 with this haplotype.

According to previous data regarding HLA-G linkage disequilibrium obtained in Brazil and other countries: A-C-del haplotype corresponds to G*01:04 allele, G0104 and UTR-3; A-C-ins haplotype – G*01:01:02, G*01:06 or G*01:05N, G010102 promoter, UTR-2; haplotype G-C-del – G*01:01:01:01 or

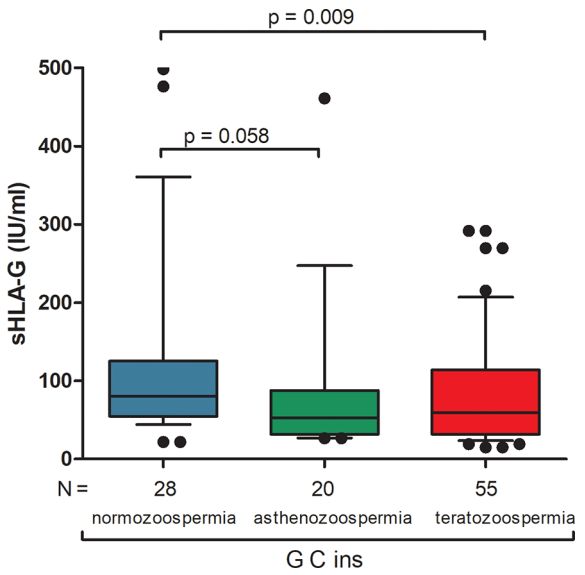


**FIGURE 2** | Concentration of soluble HLA-G per milliliter of plasma (IU/ml) measured in semen samples of G-C-ins haplotype carriers. Haplotype was estimated in the following order: rs1632947:–964G>A; rs1233334:–725G>C/T; rs371194629:insATTTGTTCATGCCT/del. Blue box represents the level of sHLA-G measured in normozoospermic men, green box – in men with asthenozoospermia, red box – in men with teratozoospermia. Boxes are drawn from the first quartile (25th Percentile) to the third quartile (75th Percentile). Black lines in boxes are medians. Whiskers represent 10-90 percentiles. N is the number of patients. P-values are calculated by Mann-Whitney test.

G*01:01:01:04, UTR-1 or UTR-6; haplotype G-G-del – Promo G010101b and G010101c, G*01:01:01:04 and G*01:01:01:05, UTR-4 and UTR-6; haplotype G-T-ins, G*01:03, UTR-5, Promo G0103 (22, 24). According to Castelli et al. (24) G-C-ins haplotype corresponds to the G010101 (a, b, c, d, f), G010102a, G010104 (a, b) and UTR-2, UTR-5, UTR-7 alleles. G-G-ins and A-G-del haplotypes are very rare in general population, including G-C-ins (below 1%). By contrast, G-C-ins haplotype in our fertile male population are at the level of 5.96%. In fertile women, this percentage is 0.31% (only 2 women out of 320 controls, for details see reference 17). This points to gender differences. Our control groups (women and their partners) do not, however, reflect the randomly selected population group in which there are both fertile and infertile subjects. Our groups were specifically selected for research into fertility and human reproductive diseases. Whether we isolate DNA from blood or sperm should not affect the genotype of a given person and should not affect the results, although in our semen isolates we have more G-C-ins haplotype than would be expected compared to the frequency for the world population. Testing another semen cohort could help to overcome this issue and, as previously mentioned, would enhance the power of the test.

Our genetic and protein studies suggest the involvement of sHLA-G in seminal plasma in the creation of an immune environment suitable for maintaining the proper parameters of sperm required for optimal oocyte fertilization. There are reports showing the HLA-G molecule is important for facilitating fertilization of the oocyte and promoting successful embryo implantation (7, 12, 14). The presence of HLA-G mRNA was found in unfertilized oocytes and in early embryos. Expression of HLA-G mRNA was associated with an increased cleavage rate, when compared to embryos lacking the HLA-G transcript (31–34). If the paternal G-C-ins haplotype is inherited by the embryo, this may result in lower sHLA-G production. Our unpublished data indicate that embryos whose transfer to the uterus resulted in pregnancy secreted twice as much sHLA-G as those embryos whose transfer ended in no pregnancy or miscarriage.

Several studies have suggested an immunoregulatory role of HLA-G in the male reproductive system and in seminal plasma. Larsen et al. detected sHLA-G protein in seminal plasma, and HLA-G expression in normal testis and in epididymal tissue of the male reproductive system but not in the seminal vesicle (12). In the testis, HLA-G might have a role as an immunosuppressive factor, and thereby avoiding recognition of 'self' sperm cells, which can be perceived as autoantigens by the immune system. On the other hand, paternal sHLA-G in the seminal plasma, may be associated with the induction of tolerance in the mother to paternal antigens. This induction may be important for the success of the pregnancy. Costa et al. enrolled couples undergoing assisted reproduction treatment and couples who conceived naturally. In that study the haplotype HLA-G/01:01:01b/HLA-G/01:01:01 showed a significant higher frequency in control groups and protection against infertility (35). Moreover, the HLA-G UTR-4 haplotype (possessing -964G, -725G, 14 bp del) was associated with a shorter time to achieving pregnancy in an infertility treatment setting when both female and male partners were carriers (36).

One of the conclusions of our study is that polymorphisms in the promoter region of the *HLA-G* gene and the 3'UTR influence the expression and secretion of the soluble form of HLA-G. We found many statistical differences in the sHLA-G concentration in semen between particular haplotype and diplotype carriers. Other studies also indicate the dependence of sHLA-G secretion on its gene polymorphism. Low levels of sHLA-G expression have been associated with HLA-G/01:01:03 and HLA-G/01:05N alleles, intermediate levels are associated with HLA-G/01:01:08 and HLA-G/01:04b alleles, while HLA-G/01:04:01 and HLA-G/01:01g have been known as high secretor alleles (37). A-C-del haplotype is mostly associated with G*01:04/UTR-3 alleles, and this allele has been associated with higher sHLA-G production. A-C-ins haplotype is mostly associated with G*01:01:02, G*01:06, G*01:05, UTR-2, which in turn has been associated with fertility problems (38).

Expression of HLA-G is mainly restricted to the trophoblast cells in the placenta, where it participates in maintaining tolerance at the fetal-maternal interface (39). However, HLA-G can be *de novo* expressed in pathological conditions such as tumors, chronic infections, or after allogeneic transplantation (40, 41). It should be emphasized that HLA-G expression and sHLA-G secretion in different fluids/tissues may depend on various factors, e.g., changes in gene methylation (42, 43) as well as post-transcriptionally by genetic variations in the 3′UTR, which contains several sites for miRNAs binding (41). Moreover, the HLA-G promoter region interacts with specific transcription

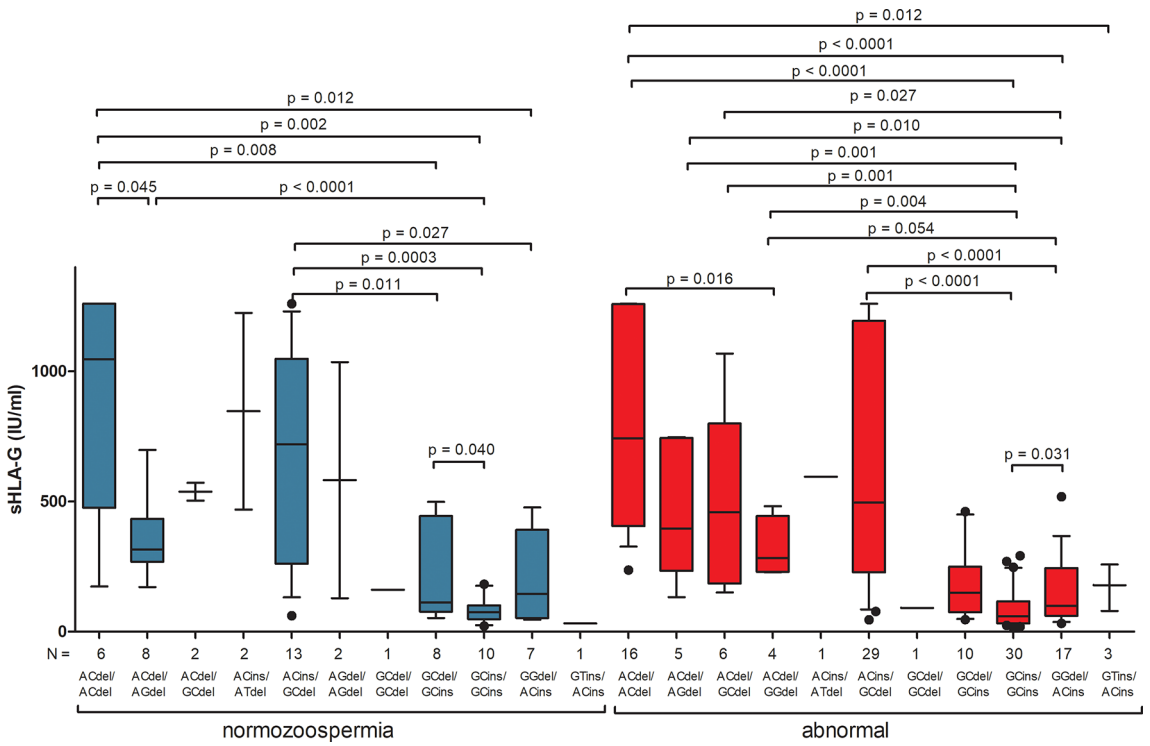


**FIGURE 3** | Concentration of soluble HLA-G per milliliter of plasma (IU/mL) measured in semen samples according to *HLA-G* diplotypes. Diplotypes were estimated in the following order: rs1632947:–964G>A; rs1233334:–725G>C/T; rs371194629:insATTTGTTCATGCCT/del. Blue boxes represent the level of sHLA-G measured in normozoospermic men and red boxes – in men with abnormal semen parameters. Boxes are drawn from the first quartile (25th Percentile) to the third quartile (75th Percentile). Black lines in boxes are medians. Whiskers represent 10-90 percentiles. N is the number of patients. P-values are calculated by Mann-Whitney test or unpaired T-test.

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 (41).

Dahl et al. (2014a) correlated sHLA-G levels in seminal plasma with a 14 bp insertion/deletion (ins/del) polymorphism in the 3'UTR of the *HLA-G* gene in couples attending a fertility clinic (13). The concentration of sHLA-G in seminal plasma samples was significantly associated with the HLA-G 14 bp ins/del genotype of the men. Moreover, the del/del genotype showed the highest level of sHLA-G, and the ins/ins genotype showed the lowest level. Considering the combination of the 14 bp insertion with the rs1632947 G allele and the rs1233334 C allele, our results are consistent with the studies by Dahl et al., however in the A-C-ins haplotype (G*01:01:02, G*01:06 or G*01:05N, G010102 promoter, UTR-2), we have the rs1632947 A allele and the opposite is true (13). This haplotype was responsible for the secretion of high levels of sHLA-G. Therefore, the presence of the 14 bp insertion is not always associated with lower sHLA-G secretion. Other polymorphic sites that influence the expression of the *HLA-G* gene are also important, such as rs1632947 and rs1233334. They appear in the promoter region, which are close to regulatory elements and CpG sites, and could alter the binding of transcription factors or promoter methylation and therefore impact the rate of transcription (44). We also reached such conclusions in our previously published studies on sHLA-G tested in blood plasma of women with recurrent implantation failure (17). Significant differences between the levels of sHLA-G in male seminal plasma in relation to HLA-G 3'UTR diplotypes and ins/del HLA-G 14 bp genotypes were also found in the research by Nillson et al. (45). There was a significant difference in semen sHLA-G concentrations between male who were heterozygous for the 14 bp insertion/deletion (del/ins), homozygous for the 14 bp deletion (del/del), or homozygous for the 14 bp insertion (ins/ins) ($p = 0.0005$). Male homozygous for UTR-2 had significantly lower concentrations of seminal plasma sHLA-G compared with male subjects with the UTR-1/UTR-1 diplotype and the UTR-1/UTR-3 diplotype, respectively. However, they did not find any differences in sHLA-G levels when they compared men with normal sperm parameters and with reduced sperm quality (45).

According to our research, carriers of the A-G-del haplotype and A-C-del/A-G-del diplotype had approximately 3.5 times and 4.9 times (respectively) greater chance of not having children after natural fertilization. Their influence is difficult to explain, because the A-G-del haplotype and the A-C-del/A-G-del diplotype determine a reasonably high sHLA-G secretion, approx. 315 ng/mL in men with normozoospermia. May an excess of sHLA-G be disadvantageous? The HLA-G molecule is considered the immune checkpoint. Its function can therefore be beneficial when expressed by the fetus or transplant and then

protects them against rejection, or harmful when expressed by the tumor protecting it from anti-tumor immunity. In fact, higher sHLA-G concentration was associated with progression of several cancers and a poor prognosis of cancer patients (46–48). The upregulation of HLA-G in tumors might be due to proteins associated with inflammation and secreted into the tumor microenvironment. These include IL-6, IL-8 and TNF-α as well as the immune suppressive cytokines IL-10 and TGF-β, which increase HLA-G expression on tumor cells resulting in promotion of evasion from immune cells (49).

Unfortunately, many diplotype analyses cannot be interpreted due to the small number of samples per diplotype, despite the fact that we had nearly 200 semen samples for testing. However, we can comment regarding the secretion of sHLA-G for the most common diplotypes. Normozoospermic men with the A-C-del haplotype and A-C-del/A-C-del diplotype secreted the most sHLA-G into semen (574.1 IU/mL and 1047.0 IU/mL, respectively), while those with the G-C-ins haplotype and G-C-ins/G-C-ins diplotype – the least (80.8 IU/mL and 75.7 IU/mL, respectively). Men with the remaining haplotypes/diplotypes secreted sHLA-G at an intermediate level. Our current studies, as well as data previously published by other researchers (26, 50), provide the evidence of balancing selection acting on the HLA-G promoter, suggesting that the promoters were maintained with high heterozygosity. Thus, divergent HLA-G haplotypes/diplotypes are associated with differential HLA-G expression as was mentioned by Castelli et al. and Rebmann et al. (25, 37). In our study, heterozygous diplotypes account for approximately 74% of all diplotypes. This is probably due to possible better adaptation of people bearing both high and low expression promoters. Heterozygous promoters ensure also the optimal amount of sHLA-G. From an evolutionary point of view, it is beneficial for the male population to have optimal sHLA-G secreted into semen for their reproductive success. On the other hand, the G-C-ins/G-C-ins diplotype amongst men is least beneficial. Chi-square test for trend indicated the insertion allele in this diplotype as being disadvantageous because the odds ratios increased from protective 0.669 in G-C-del/G-C-del men to predisposing 2.396 in G-C-del/G-C-ins men and 3.066 in G-C-ins/G-C-ins men. However, the insertion allele in diplotypes A-C-ins/A-C-del and A-C-ins/A-C-ins was protective, and the odds ratios ranged from 1.497 in A-C-del/A-C-del patients to 0.474 in A-C-ins/A-C-del and to 0.273 in A-C-ins/A-C-ins patients (**Table 2**). The Kruskal-Wallis test confirmed the risk of abnormal sperm parameters from the insertion allele only in the diplotype G-C-ins/G-C-ins, which determined the secretion of a reduced amount of sHLA-G (**Supplementary Table 8**). Unfortunately, in the semen samples we have collected we did not detect A-C-ins/A-C-del and A-C-ins/A-C-ins diplotypes, therefore we could not estimate the level of sHLA-G in these diplotypes.

One of the limitations of our work was the lack of sperm samples from healthy men who have children from natural insemination. Since they have no fertility problems, they have no reason to come to an assisted reproductive clinic and therefore it was not possible to collect material for testing. We could only compare those patients who, despite good sperm parameters, came to the clinic because they cannot have children with their female partners. Moreover, we have only tested sHLA-G once and therefore we could not conclude whether sHLA-G concentration varies in different samples from one male, as was the case in the studies by Nilsson et al. (45). The semen levels of sHLA-G in the samples were fairly consistent over time in the individual males. We had also no data on the exact days of abstinence before the sample was collected for testing, therefore we could not infer the effect of the number of days of sexual abstinence on this concentration.

Nevertheless, in the context of our previously published data on female patients participating in IVF (17), as well as the results currently presented on their partners, *HLA-G* polymorphism and sHLA-G level can affect both female and male infertility.

## CONCLUSIONS

We can conclude the following statements:

i. Male infertility is associated with *HLA-G* polymorphism.
ii. Polymorphisms in the *HLA-G* promoter region and 3'UTR influence expression and secretion of its soluble protein.
iii. Among all *HLA-G* haplotypes and diplotypes, the most unfavourable for male fertility is the G-C-ins haplotype and G-C-ins/G-C-ins diplotype, which determine the secretion of the lowest concentration of the soluble HLA-G molecule.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local Ethics Committees of University of Wrocław and Polish Mothers' Memorial Hospital – Research Institute in Łódź. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

IN conceived and designed the experiments. IN, KP, AT, and AW performed the experiments. IN, KP, AT, and AW analysed the data. PR, MR, JW, AM, and RK contributed to patients and control recruitments. IN and KP wrote the paper. 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.2021.791399/full#supplementary-material

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# HLA-G: Too Much or Too Little? Role in Cancer and Autoimmune Disease

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HLA-G is a non-classical HLA class I molecule with immunomodulatory properties. It was initially described at the maternal-fetal interface, and it was later found that this molecule was constitutively expressed on certain immuneprivileged tissues, such as cornea, endothelial and erythroid precursors, and thymus. The immunosuppressive effect of HLA-G is exerted through the interaction with its cognate receptors, expressed on immunocompetent cells, like ILT2, expressed on NK, B, T cells and APCs; ILT4, on APCs; KIR, found on the surface of NK cells; and finally, the co-receptor CD8. Because of these immunomodulatory functions, HLA-G has been involved in several processes, amongst which organ transplantation, viral infections, cancer progression, and autoimmunity. HLA-G neo-expression on tumors has been recently described in several types of malignancies. In fact, tumor progression is tightly linked to the presence of the molecule, as it exerts its tolerogenic function, inhibiting the cells of the immune system and favoring tumor escape. Several polymorphisms in the 3'UTR region condition changes in HLA-G expression (14bp and +3142C/G, among others), which have been associated with both the development and outcome of patients with different tumor types. Also, in recent years, several studies have shown that HLA-G plays an important role in the control of autoimmune diseases. The ability of HLA-G to limit the progression of these diseases has been confirmed and, in fact, levels of the molecule and several of its polymorphisms have been associated with increased susceptibility to the development of autoimmune diseases, as well as increased disease severity. Thus, modulating HLA-G expression in target tissues of oncology patients or patients with autoimmune diseases may be potential therapeutic approaches to treat these pathological conditions.

**Keywords: HLA-G, Cancer, autoimmunity, immunoediting, checkpoint, ILT2, therapy, polymorphisms**

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## 1 HLA-G

HLA-G is a non-classical HLA class I gene that encodes a molecule with tolerogenic properties (1). This molecule shows restricted tissue expression pattern, and was initially observed in extravillous cytotrophoblasts, where it plays an important role in the maintenance of fetal-maternal immune tolerance (2); it has also been observed in few healthy immune-privileged tissues, as cornea (3) and thymic medulla (4, 5). However, HLA-G expression has also been reported in some pathological conditions, such as cancer and autoimmunity.

The *HLA-G* gene has a genetic structure similar to other classical HLA class I genes, although, in contrast, the sequence of the *HLA-G* gene is highly conserved (6).

The entire HLA-G molecule consists of a heavy chain, encoded on chromosome 6, non-covalently associated with β2 microglobulin, encoded on chromosome 15. Like classical HLA genes, the *HLA-G* gene has 7 introns and 8 exons. Exon 1 encodes the signal peptide, exons 2, 3 and 4 the extracellular domains α1, α2 and α3, respectively, and exons 5 and 6 the transmembrane and cytoplasmic domain, respectively (7) (**Figure 1A**).

Compared to classical class I molecules, HLA-G has a shortened cytoplasmic domain due to the presence of a premature stop codon in exon 6. Exon 7 is always absent from the mature mRNA and, as a consequence of this stop codon, exon 8 will never be present in the final protein. However, exon 8 forms the 3′ untranslated region (3'UTR), which is key in the regulation of *HLA-G* gene expression (8).

In total, seven isoforms of HLA-G mRNA generated by alternative splicing have been described, including four membrane-bound isoforms (HLA-G1, -G2, -G3 and -G4), and three soluble isoforms (HLA-G5, -G6 and -G7) (9) (**Figures 1B, C**).

## 2 *HLA-G* GENE POLYMORPHISMS

The constitutive and inducible expression of HLA-G is primarily controlled by its promoter, which has unique structural characteristics such as enhancer A, interferon-stimulated regulatory element (ISRE) and SXY modulator, which contains regulatory sequences common to class I and II HLA genes (10, 11).

HLA-G expression may be controlled, as in any other gene, by regulatory regions both in the 5'URR and 3'UTR regions of the gene. The role of the 5'URR region in the expression of the HLA-G molecule and, thus, its involvement in pathology, is not as well known as that of the 3'UTR region, with scarce publications so far. Moreover, there are studies that question the impact of the 5'URR region on the expression of the HLA-G molecule and suggest that it alone cannot predict soluble HLA-G (sHLA-G) expression *in vivo* (12). This review will then focus on the 3'-UTR region of the *HLA-G* gene. HLA-G expression is highly regulated by its 3′UTR region, which has a high variability compared to the coding region, in contrast to classic class I HLA molecules (13).

Population studies have found nine polymorphic sites in the 3'UTR region of the *HLA-G* gene. Among them, the 14 base pair (14bp) INS/DEL (rs371194629), +3142C/G (rs1063320) and +3187A/G (rs9380142) polymorphisms are implicated in HLA-G expression (14) (**Figure 2**).

Regarding the rs371194629 polymorphism, it consists of the presence (insertion, INS) or absence (deletion, DEL) of a 14bp

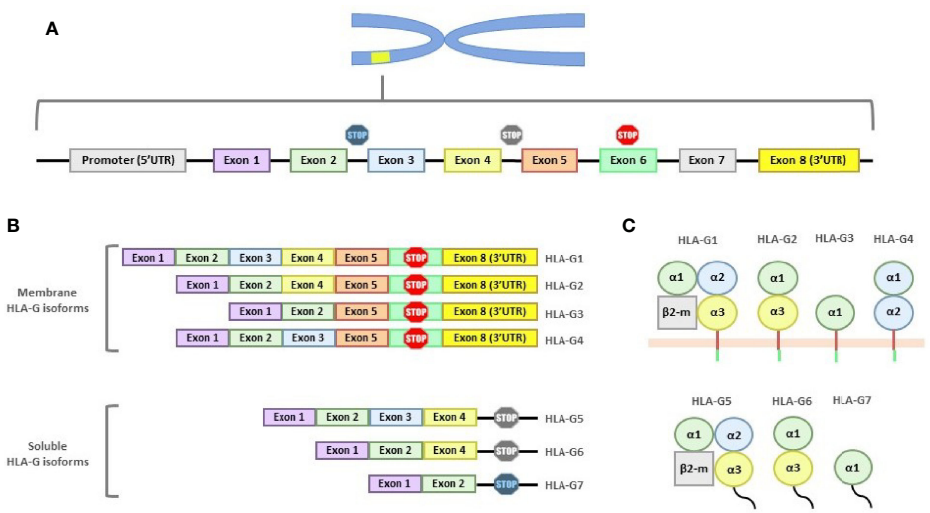


**FIGURE 1** | Structure of the HLA-G gene and its products. **(A)** HLA-G gene is composed of 7 introns and 8 exons. Exon 1 encodes the signal peptide; exons 2, 3 and 4 encode the α1, α2 and α3 subunits; exon 5 encodes the transmembrane region; and exon 6 the cytoplasmic region. Exons 7 and 8 are always transcribed, although exon 7 is never present in the mature mRNA and exon 8 (3'UTR region) is not translated due to the stop codon present in exon 6. **(B)** Mature mRNA of the different HLA-G isoforms. All isoforms show exon 1 and 2. Soluble isoforms are generated by alternative splicing which eliminates the exons involved in membrane binding. **(C)** The seven HLA-G isoforms, grouped whether they are membrane-bound or soluble, are shown.

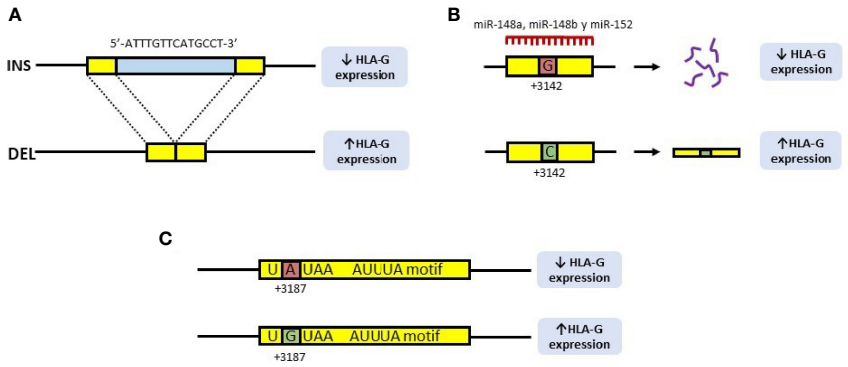


**FIGURE 2** | Polymorphisms of the 3'UTR region of the HLA-G gene involved in the expression of the HLA-G molecule. **(A)** 14bp INS/DEL polymorphism is characterized by the insertion (INS) or deletion (DEL) of a 14bp fragment at position +2960 of the *HLA-G* gene. Insertion is associated with decreased expression of HLA-G, due to a longer and unstable transcript, while deletion is associated with increased expression. **(B)** In the +3142C/G polymorphism, the presence of a guanine (G) at position +3142 of the *HLA-G* gene increases the affinity of the microRNAs, miR-148a, miR148b and miR-152 for this region, thus decreasing the availability of the mRNA. In contrast, the presence of a cytosine (C), produces an increase in the expression of HLA-G. **(C)** In the +3187A/G polymorphism, the presence of an adenine (A) at this position modifies an AU-rich motif in the gene mRNA, decreasing its stability, while G is associated with an increase in HLA-G production.

fragment (7, 8). This fragment (5'-ATTTGTTCATGCCT-3') is located at position +2960 of the 3'UTR region, and has been associated with both, the splicing and the stability of the mRNA (8, 15, 16), as it contains an AUUUG domain putatively exerting an AU-pentamer-like effect, decreasing mRNA stability (17). Therefore, the DEL allele provides a higher stability of the mRNA (15), associated with a high expression of HLA-G (16) (**Figure 2A**).

The rs1063320 polymorphism (+3142C/G), consists of the transversion of a cytosine (C) to guanine (G) at position +3142 of the 3'UTR region. The presence of a G increases the affinity of the microRNAs miR-148a, miR148b and miR-152 for this region, thus decreasing the availability of the mRNA by degradation of the primary transcript, as well as by the suppression of its translation (18). Should a C be found at this position, miRNAs affinity will decrease, increasing the mRNA availability and the production of HLA-G (8) (**Figure 2B**).

Finally, the +3187A/G polymorphism (rs9380142) is also implicated in the stability of HLA-G mRNA: the presence of an adenine (A) at this position modifies an AU-rich motif in the corresponding mRNA, decreasing its stability, while the G allele is associated with increased production of HLA-G (19) (**Figure 2C**).

## 3 HLA-G FUNCTION: CONTROLLING THE IMMUNE RESPONSE

Several evidences have supported the role of HLA-G as a tolerance-inducing molecule, playing an important role in the suppression of the immune response. This molecule is able to exert this function by means of different strategies:

### 3.1 HLA-G Receptors

The HLA-G molecule is able to bind to different inhibitory receptors present on cells of the immune system (**Figure 3**). The immunoglobulin-like transcription receptor type 2 (ILT2/LILRB1/CD85j) is expressed on T cells, B cells, natural killer (NK) cells and antigen presenting cells (APCs), whereas type 4 (ILT4/LILRB2/CD85d) is unique to APCs. Both ILT2 and ILT4 recognize HLA class I molecules; however, they bind with higher affinity (3- to 4-times fold) to the HLA-G molecule, which contributes mostly to the functional inhibition of cells expressing these receptors (20).

Another receptor that recognizes HLA-G is the killer cell immunoglobulin-like receptor (KIR) 2DL4 (CD158d), exclusive to NK cells. This receptor specifically recognizes HLA-G and has been associated with both activating and inhibitory functions. The mechanisms by which KIR2DL4 produces the activation of these cells are not clearly understood. However, it has been reported that it is limited to increasing interferon γ (IFNγ) production but not cytotoxic activity of non-activated NK cells (21). This increase in IFNγ would concomitantly lead to an increase in HLA-G expression (22).

All these receptors have in common that they display immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic tails, whereby HLA-G exerts its inhibitory functions. Upon HLA-G binding to its cognate receptors, the ITIM motifs recruits mainly the protein tyrosine phosphatase SHP-1, and also SHP-2 (20, 23), which dephosphorylate key points of the activating signaling pathways, causing distinct inhibitory effects on target cells.

In addition, the CD8 co-receptor is also able to recognize and bind to HLA-G. It has been described that the ILT2 receptor, expressed on CD8+ T lymphocytes, competes with this co-receptor for binding to HLA class I molecules, and therefore HLA-G is able to modulate the activation of cytotoxic T lymphocytes by blocking CD8 binding (20).

#### 3.1.1 T Cells

The binding of HLA-G to its receptors on T cells causes modifications in their function (**Figure 3A**). In general, they inhibit the proliferation of these cells (24) and also the cytotoxic

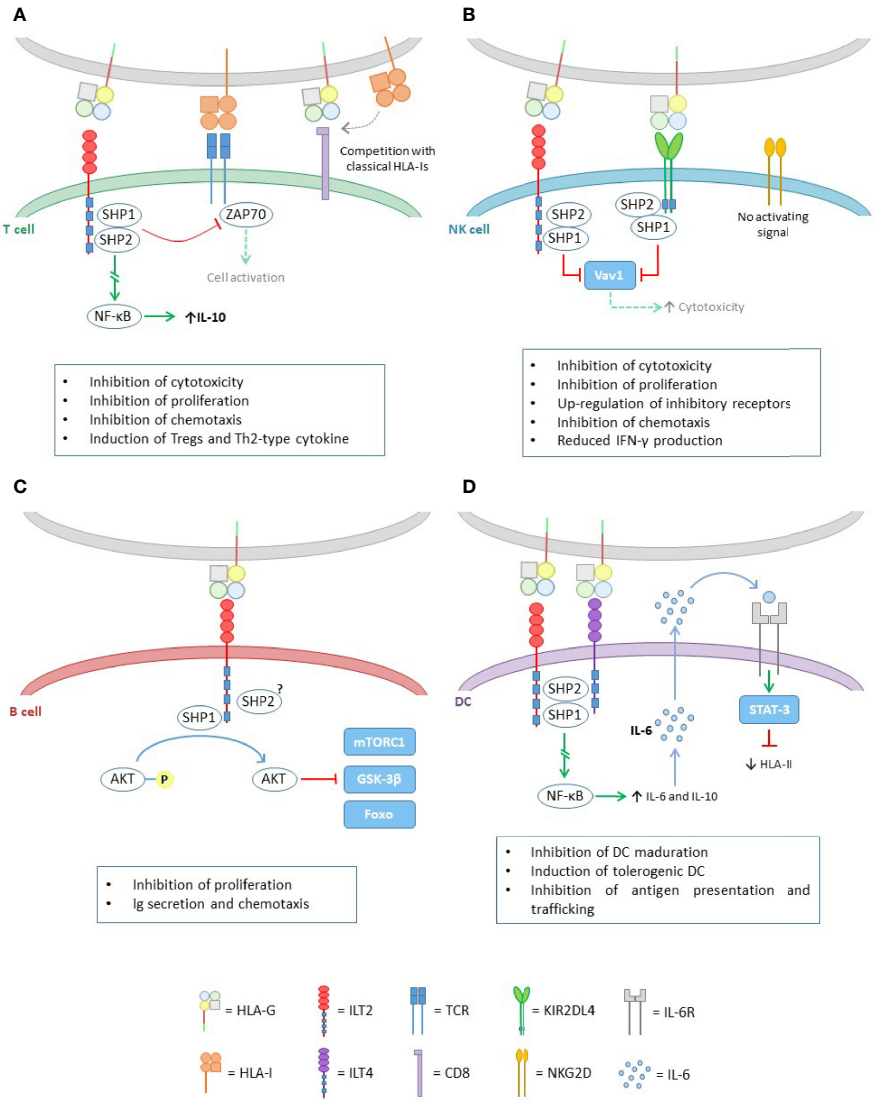


**FIGURE 3** | HLA-G effect in the different cells of the immune system. **(A)** Inhibitory effect of HLA-G on T cells. The binding of HLA-G to ILT2, activates the ITIM domains of the latter and recruits the SHP-1 and SHP-2 phosphatases. On the one hand, these proteins are capable of inhibiting the TCR signal, through ZAP70 dephosphorylation. On the other hand, they trigger a signaling pathway leading to NF-kB activation, which enhances IL-10 expression. HLA-G also exerts its inhibitory function through competition for the union of classical HLA class I molecules to the CD8 coreceptor in CD8+ T cells, and to the TCR. **(B)** HLA-G binds to its cognate receptors ILT2 and KIR2DL4, both containing inhibitory domains ITIM, which upon activation, recruits SHP-1 and SHP-2, leading to Vav1 inactivation, and thus downregulating the cytotoxicity capacity of NK cells. **(C)** In B cells, HLA-G works *via* ILT2, where SHP-1 mainly, is responsible for the dephosphorylation of AKT leading to mTORC1, GSK-3β and Foxo inactivation. **(D)** Like in T cells, the pathway initiated by HLA-G through ILT2 and ILT4 leads to NF-kB activation. This factor causes an increase in IL-6 and IL-10 expression. IL-6 is capable of binding to IL-6 receptors on DCs and, through the STAT-3 activation factor, inhibits HLA class II assembly.

function of CD8+ T cells (25), as well as the alloreactivity of CD4+ T cells (26). In addition, it has been reported that HLA-G can induce apoptosis of CD8+ cells (27, 28).

Also, the expression of HLA-G regulates the balance between T helper (Th) 1 and Th2 cells, promoting polarization to the Th2 subset. In fact, HLA-G decreases the production of IFNγ and tumor necrosis factor α (TNF-α) but increases the production of interleukin (IL)-3, IL-4 and IL-10 by Th2. IL-4 promotes differentiation to Th2 while IL-10 increases HLA-G expression in macrophages and monocytes activated by a feedback mechanism. Thus, HLA-G may have important implications in controlling the development of Th1- and Th2-mediated diseases (29).

### 3.1.2 NK Cells

HLA-G exerts its effect on NK cells through ILT2 and KIR2DL4 (**Figure 3B**). Upon binding to these receptors, HLA-G is able to block certain NK signaling pathways, resulting in inhibition of

the cytotoxic function (30) and transendothelial migration (31) of these cells. In addition, HLA-G promotes the up-regulation of the inhibitory receptors ILT2 and KIR2DL4 (32) and the secretion of pro-angiogenic factors, such as VEGF and angiopoietin-1 and -2, which allow early remodelling of the maternal vasculature during pregnancy, favoring the development of the fetus (33). This mechanism can be exploited by tumor cells, favoring tumor progression.

HLA-G can also enhance the surface expression of other non-classical HLA molecules, such as HLA-E, in target cells (34), which, through the CD94/NKG2A receptor, can also exert inhibitory effects on NK cells (35). Therefore, HLA-G allows an indirect inhibition of the NK cells cytotoxic function through stabilization of cell-surface HLA-E.

#### 3.1.3 B Cells

Like in T and NK cells, HLA-G exerts its immunosuppressive effect on B cells through the ILT2 receptor, leading to tolerance (**Figure 3C**).

HLA-G inhibits the response of both naive and memory B cells (36). It impairs cell proliferation and differentiation, as HLA-G causes key pathways for these processes to be inactivated and induces G0/G1 cell cycle arrest. Chemotaxis is also affected, as HLA-G causes a decrease in the expression of both CXCR4 and CXCR5, negatively influencing cell trafficking. Finally, the HLA-G/ILT2 pathway has been shown to inhibit antibody production induced by both T cell-dependent and -independent responses.

#### 3.1.4 Antigen Presenting Cells (APCs)

APCs are key cells in the activation of different immune cell types, influencing both innate and adaptive responses.

These cells have ILT-2/4 receptors which, when interacting with HLA-G, alters their behavior (**Figure 3D**). Generally, dendritic cells (DC) are the most commonly affected. HLA-G acts by inhibiting the differentiation and maturation of DCs, and also inducing the production of tolerogenic DCs.

The interaction with HLA-G also interferes with the assembly and transport of HLA class II molecules to the cell surface, decreasing the presentation of antigens to other cells of the immune system, thus disrupting the characteristic function of DCs (37).

Altogether, these induced tolerogenic DC are able to generate anergy in the CD4+ T population, in addition to favoring the appearance of CD4+CD25+CTLA-4+ and CD8+CD28- cells with a high capacity of IL-10 production, thus having suppressive and regulatory properties.

Because of the tolerogenic function of HLA-G in DCs, some works have tried to use these cells as a therapeutic approach to induce tolerance in some pathologies (38), such as multiple sclerosis (39).

Finally, as seen in the case of NK cells, HLA-G also induces up-regulation of inhibitory receptors on APCs (32).

### 3.2 Long-Term Tolerance Generation (Treg)

Regulatory T cells (Treg) are key cells in the maintenance of normal immune homeostasis. It has been observed that the HLA-G molecule, which is characterized by its tolerogenic function, promotes the differentiation of naïve T cells, both CD3+CD4low and CD3+CD8low, to suppressor T cells that, unlike natural regulatory T cells, do not express the Foxp3 transcription factor (40). This HLA-G-induced regulatory T-cell subset does not depend on HLA-G expression to exert its regulatory function. This suppressor T cells rely on the secretion of inhibitory cytokines, such as IL-10, which apart from inhibiting various immune cells populations, also up-regulates HLA-G expression (41) and enhances other cell-to-cell mechanisms that induce anergy in several cell types, such as CTLA-4/B7 (42), PDL1/PD1 (43), etc.

In addition, a population of regulatory T cells that constitutively expresses HLA-G has been described (44). These cells also mediate their immunosuppressive function through various tolerogenic cytokines such as IL-10, IL-35 and transforming growth factor (TGF)-β which, like IL-10, up-regulates HLA-G expression (45).

### 3.3 Short-Term Tolerance Generation

Trogocytosis is a process in which the rapid uptake of membranes and their associated molecules occurs through cell-to-cell contact. This phenomenon has been described in T and B lymphocytes, NK cells, APCs and even tumor cells (46, 47).

Some of these membrane-associated molecules are the various membrane-bound HLA-G isoforms. LeMaoult et al. (48) have found that, when contact occurs between an APC and a T lymphocyte, the T cell may acquire the HLA-G from the APC membrane and incorporates it into its membrane through this trogocytosis phenomenon. These HLA-G expressing T cells are then able to adopt a short-term regulatory function and, unlike Tregs, do not require specific cell maturation.

The same happens when contact occurs between a tumor cell expressing HLA-G on its membrane and a NK cell. When NK cells incorporate the membrane-bound isoforms of HLA-G, they stop proliferating, limit their cytotoxic activity and behave as suppressor cells. In addition, these cells can inhibit the cytotoxic function of other NK cells, contributing to tumor escape (49).

## 4 HLA-G AND PATHOLOGY

As a consequence of this tolerogenic role, it has been postulated that HLA-G might be implicated in a wide variety of processes:

### 4.1 HLA-G and Transplant

The immunosuppressive role of HLA-G in transplants is directly related to graft survival, due to the induction of tolerance that this molecule exerts on the immune system (50). In transplanted patients, HLA-G expression may allow escape from recognition and destruction of the graft by the cytotoxic activity of T lymphocytes and NK cells (51), which are one of the main reasons for graft rejection (52), suggesting the potential role of HLA-G in the protection of the allograft.

The mechanism whereby HLA-G is expressed *de novo* in transplanted organs remains to be elucidated. Several factors, relevant in this context have been shown to induce *de novo* HLA-G

expression *in vitro*, such as steroids used in acute rejection episodes, and cytokines expressed in allogeneic immune responses, like the pro-inflammatory cytokine IFNγ and the anti-inflammatory cytokine IL-10 (53).

Historically, the relationship between HLA-G and graft acceptance/rejection was first observed in heart transplantation (54), and, over time, information on this subject has been expanded. These studies reported the presence of HLA-G in biopsies of transplanted heart tissue, where HLA-G was especially prevalent in patients with no or low rejection scores (55). In HLA-G-positive patients, the incidence of acute or chronic rejection was significantly decreased ($p<0.001$ and $p<0.032$, respectively) when compared with HLA-G-negative patients (54).

Similar findings were reported in lung, liver and kidney transplant recipients (50, 55). Altogether, these results provided evidence of the immune inhibitory effect of HLA-G and pointed to its potential role as a biomarker of cellular rejection status.

As mentioned in the "HLA-G" section, this molecule presents both membrane and soluble isoforms. As membrane HLA-G and soluble HLA-G (sHLA-G) structures exhibit the same receptor specificity, both are potent molecules modulating the innate and adaptive immune response, with sHLA-G having the advantage of being free in the extracellular medium, being able to exert its effect in a paracrine or even endocrine fashion upon extravasation into the bloodstream.

This feature regarding sHLA-G is very important, since detection in blood is less invasive than detection of HLA-G in biopsies. In fact, some articles have seen that, after kidney transplantation, sHLA-G levels were detected in the blood of these patients, and patients with chronic rejection belong in the sHLA-G negative group (56). Thus, elevated levels of sHLA-G in blood of transplant patients are associated with better graft acceptance and a higher survival rate after transplantation (54).

### 4.2 HLA-G in Chronic Viral Infections

It is known that viral infections lead to a decrease in the expression of HLA class I molecules, including HLA-G, allowing NK cells to detect the infected cells and thus be able to perform their cytotoxic function on them. However, some viruses, such as herpes simplex virus type I (HSV-1) or the rabies virus (RABV), have developed resistance mechanisms based on the induction of the overexpression of HLA class I molecules, both classical and non-classical (such as HLA-G), which has an immunosuppressive effect on NK lymphocytes (57). Therefore, an increase in the HLA-G expression induced by the virus itself or by the inflammatory environment, can aggravate the morbidity or mortality of the infection (58, 59).

### 4.3 HLA-G and Cancer

Tumor cells present, anchored to MHC class I molecules, certain tumor-derived antigens on their surfaces that can be recognized by the patient's immune system. However, even in an immunocompetent organism, neoplastic cells are able to grow and progress, leading to aggressive and malignant lesions. In this context, immunoregulatory molecules, like HLA-G, play an important role in the progression of cancer.

In addition to its localization and physiological function, HLA-G expression has been observed in different types of tumors, such as gastric (60), colorectal (61) and breast (62) cancer, among others, where it favors tumor progression by inhibiting the immune system surveillance (63).

### 4.4 HLA-G and Autoimmunity

Autoimmune diseases comprise a very heterogeneous group of pathologies, whose main feature is the exacerbation of the immune response against self-antigens of the organism.

Therefore, the presence of tolerogenic mechanisms is fundamental, such as the AIRE transcription factor, which allows negative selection of developing thymocytes. According to this, Melo-Lima et al. (5) have been able to prove that this factor up-regulates HLA-G expression in thymic cells, limiting autoimmune diseases (59).

Due to the relevant implications of HLA-G in cancer and autoimmunity, we will describe in detail the most relevant studies of HLA-G in different tumor types and autoimmune diseases in the next sections of this review.

## 5 CANCER

Tumors are complex tissues composed not only of tumor cells, but also a repertoire of immune cells (microenvironment) in a continuous crosstalk with the malignant cells. The immune microenvironment can recognize tumor cells as "foreign" and initiate mechanisms to eliminate tumor cells, while malignant cells are able to prevent the action of the immune cells, by releasing immune suppressing extracellular signals and taking advantage of several molecular mechanisms to frustrate immune-mediated death.

Thus, we know as "immunosurveillance" the extrinsic mechanism of cancer suppression that eliminates emerging tumors. Therefore, escape from this immunosurveillance represents an essential step in the development of neoplastic diseases.

One of the molecules that tumors use to their advantage for this purpose is HLA-G which, as mentioned previously, has tolerogenic capabilities that could benefit tumors. In fact, over the years, many studies have reported on the expression of HLA-G in many different types of cancers, which supports the idea that this molecule participates in cancer development.

Thanks to the ability of tumors to escape from the immune system, the mechanism of "cancer immunoediting" arises, by which immunoresistant tumor variants are generated and in which molecules such as HLA-G participate (63–66) (**Figure 4**). This process is divided into three phases:

- Elimination: It corresponds to the immunosurveillance mechanism, and involves the elimination of tumor cells by cells of innate (NK, NKT, LTγδ, DC, etc) and adaptive (antigen-specific LT and LB) immunity. In this phase, the activity of tumor infiltrating lymphocytes (TILs) and NK cells is associated with cytotoxicity and with the production of Th1 profile cytokines (IFNγ), that increase the cell surface

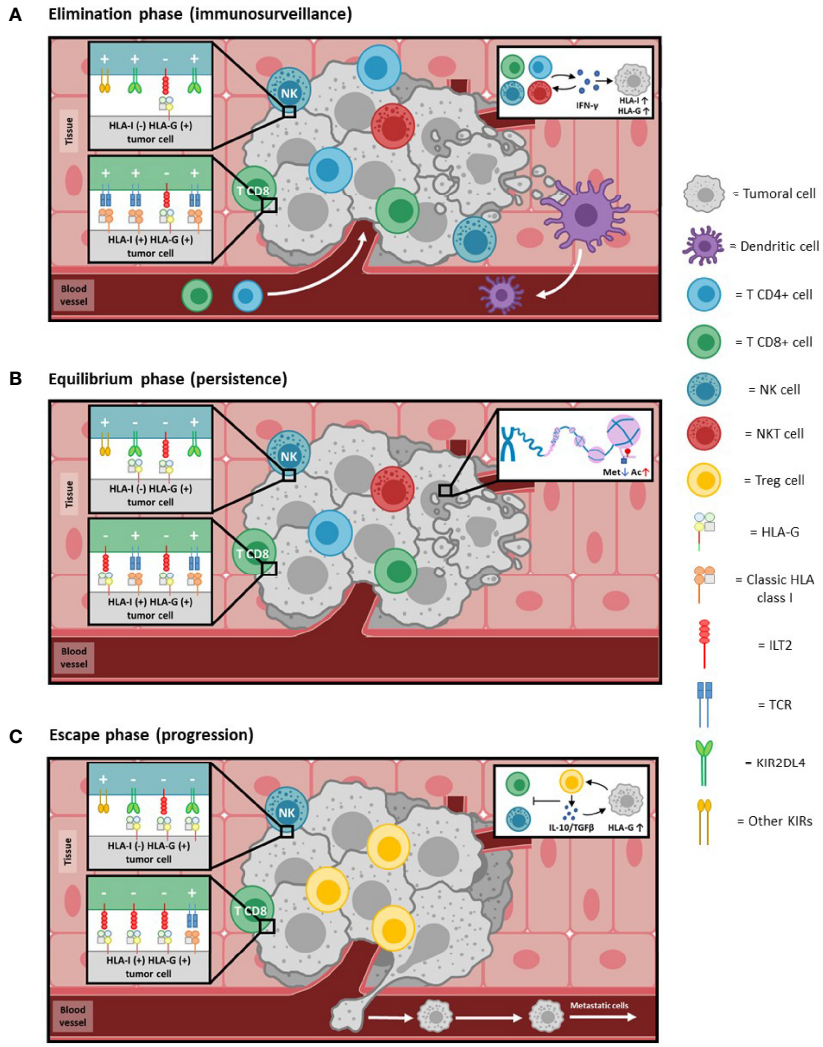


**FIGURE 4** | HLA-G and cancer immunoediting. Immunoediting of cancer, a phenomenon whereby cancer and immunocompetent cells interplay, leading in some instances to tumor overgrowth. Consists of three phases (see references 63-66 for details): **(A)** Elimination phase: In reply to the neoplastic process, immunocompetent cells extravasate and infiltrate the tumoral tissue. The combined action of both innate and adaptive immunity effectors detects and kills malignant cells. The production of cytokines, such as IFNγ (see insert 4A), induces an increase in the expression of HLA class I molecules. Cells that present tumor antigens through HLA class I molecules [HLA-I (+)] are targeted mainly by CD8+ T lymphocytes. Those that do not present class I molecules on their surface [HLA-I (-)] are eliminated by NK cells. The expression of HLA-G can also be induced [HLA-G (+)] by IFNγ, although to such low levels compared to HLA class I that its inhibitory activity (-) is negligible compared to the activating (+) capacity, and thus the tumor is controlled by the immune system. **(B)** Equilibrium phase: As adaptive immunity continues to eliminate tumor cells, it simultaneously exerts selective pressure leading to the generation of less immunogenic malignant cells (e.g., selective or complete loss of HLA class I molecules and/or tumor antigens). Genetic instability resulting from defective intracellular control mechanisms, as well as frequent epigenetic changes, further contribute to the development of a non-immunogenic phenotype. Histone demethylation and acetylation processes (insert 4B) lead to epigenetic activation of HLA-G, allowing its expression to increase to levels comparable to the HLA class I molecules present at that time. Activation (+) and inhibition (-) signals will be balanced, leading to an equilibrium process in which the immune system becomes progressively less able to eliminate the tumor. **(C)** Escape phase: Tumor cells that have lost molecules relevant for immune recognition [HLA-I (-)] now tend to express higher levels of HLA-G on their surface [HLA-G (+)]. HLA-G creates peripheral tolerance by inducing anergy, immunosuppressive cells (Treg) and production of immunosuppressive cytokines (IL-10 and TGFβ; insert 4C). In addition to the local effects of membrane-bound HLA-G, secreted soluble HLA-G could also have systemic inhibitory activity due to its distribution through the blood circulation. Inhibiting signals (-) overcome activating (+) ones in immunocompetent cells. Taken together, this allows complete escape by the tumor from the action of the immune system, which favors its development and processes such as metastasis.

expression of HLA class I molecules, including HLA-G (**Figure 4A**).

- Equilibrium: The immune system exerts a selective pressure on tumor cells which, together with genetic instability and frequent epigenetic changes, leads to the generation of clones with less immunogenicity, resistant to the action of the immune system. An example of this process is the selective loss of classical HLA class I molecules, which prevents the

presentation of tumor antigens and thus avoids the attack by adaptive immunity.

Also, during this phase, epigenetic mechanisms lead to a process of histone hypomethylation and acetylation that favors the expression of HLA-G, which competes with the antigenic presentation by classical histocompatibility molecules, promoting a process of tumor tolerance (**Figure 4B**).

- Escape: In this phase, high increase of HLA-G expression, together with other mechanisms takes place; this allows complete evasion of the immune system by the tumor. HLA-G induces anergy and apoptosis of immune effector cells, and promotes the production of Treg cells and immunosuppressive cytokines (IL-10).

With this, malignant cells are able to evade the immune response, extravasate into the circulation and generate long-distance metastases (**Figure 4C**).

The presence of HLA-G in tumors has been described in several studies, some of them emphasizing that it is only present in tumor and not in distal/healthy tissue (60, 61, 67, 68), a finding that would reinforce the idea of the participation of HLA-G in tumor development and progression.

To describe the effect of HLA-G in the context of tumor progression, we refer here some of the most studied types of cancer:

## 5.1 Gastric Cancer

As previously mentioned, HLA-G expression is highly dependent on different polymorphisms, especially those present in the 3'UTR region, which affect the stability of HLA-G mRNA. For this reason, analyzing the genetic association of HLA-G polymorphic variants and the development or progression of pathologies dependent on HLA-G inhibitory activity could establish HLA-G polymorphism as a risk factor for several diseases.

In fact, in a study performed by our group in a cohort of 107 patients with gastric cancer and 58 controls in which we analyzed the 14bp INS/DEL and +3142C/G polymorphisms (69), the implication of the DEL allele in gastric cancer (70.0% in patients vs 57.0% in controls, p=0.025) was assessed, as well as that of the haplotype formed by the combination of the 14bp DEL/+3142C variants, also increased in patients compared to controls (54.1% vs 44.4%, p=0.034).

In addition, Kaplan-Meier analysis revealed that 14bp DEL/DEL patients showed lower 5-year life-expectancy than INS/DEL or INS/INS (p=0.041). Therefore, we concluded that the 14bp INS/DEL and +3142C/G polymorphisms of the *HLA-G* gene mediate gastric cancer risk and survival, becoming important risk factors to be taken into account.

Although the effect of these polymorphisms on the mRNA and protein expression are well established, a limitation for the majority of this kind of studies is that the correlation between polymorphisms and HLA-G expression is not analyzed. In our case, we have detected HLA-G in tissue (immunohistochemistry) and sHLA-G in plasma (ELISA) (69), although that correlation was not explored.

As mentioned, in addition to genetic studies, determination of HLA-G expression is key to understanding the involvement of this molecule in cancer. Wan et al. (60) observed both HLA-G expression in 61.2% of their patients and that it correlated with tumor invasion depth (p=0.012), lymph node metastasis (p=0.015) and clinical stages (p=0.001). In addition, patients with HLA-G-positive (+) tumors had a worse prognosis than HLA-G-negative (–) patients. Importantly, they found that the number of tumor-infiltrating NK cells was significantly lower in HLA-G (+) tumors compared to HLA-G (–) tumors (p<0.001), emphasizing the inhibitory role of this molecule.

The study by Murdaca et al. (70) reinforces what was observed for gastric cancer. The presence of HLA-G in tumor tissue (25.5% of patients) was also reported, and the presence of HLA-G correlated with worse patient survival (p<0.0001).

## 5.2 Colorectal Cancer

In addition to analyzing the three most classic polymorphisms of the *HLA-G* gene (14bp INS/DEL, +3142C/G and +3187A/G), Garziera et al. (71) analyzed the +3035 C/T polymorphism, among others, which also affect HLA-G expression. As for the 14bp INS/DEL polymorphism, carriers of the DEL/DEL allele (higher HLA-G expression) had lower disease-free survival rate, lower overall survival rate and higher risk of recurrence compared to the other genotypes.

Similar results were found for the +3187G/G genotype, which increases the HLA-G mRNA stability and, therefore, HLA-G molecule expression, thus favoring tumor progression, as expected. A similar finding was also observed for the +3035C/C genotype of the +3035T/C polymorphism. Altogether, authors concluded that +3035C>T and, in particular, +2960 14bp INS/DEL and +3187A/G polymorphisms were prognostic biomarkers in determining survival outcome in colorectal cancer (CRC) (71).

The study by Ye et al. (61), in addition to proving the expression of HLA-G in 64.6% of CRC patients, allowed to establish a relationship between HLA-G expression and the risk of developing this disease. A statistically significant correlation was found between HLA-G protein levels and clinicopathological factors of depth of invasion, histological grade, host immune response, nodal status and clinical stages of the disease. They also found that the presence of HLA-G negatively affected in a significant manner the 3-year survival of patients (96.0% in HLA-G (-) vs 51.0% in HLA-G (+), p=0.001).

Interestingly, Lázaro-Sánchez et al. (72) found that patients with CRC have significantly higher sHLA-G levels in saliva than control subjects (18.8 U/ml vs 6.3 U/ml, p = 0.036). In addition, higher levels of sHLA-G were observed in the saliva of patients with CRC in more advanced stages, compared with patients in early stages (24.2 U/ml vs. 8.1 U/ml, p = 0.019). Therefore, sHLA-G is a potential new biomarker for this type of cancer, which, furthermore, can be detected in a non-invasive way.

## 5.3 Breast Cancer

Regarding breast cancer, Eskandari-Nasab et al. (73) found a statistically significant difference in the frequency of the DEL allele in breast cancer patients compared to the control group (56.4% vs 46.5%, respectively. p=0.004). Likewise, the prevalence

of the HLA-G 14bp DEL/DEL genotype was higher in breast cancer patients than in the control group (33.9% vs 24.1%, respectively, p=0.006), suggesting that the 14bp INS/DEL polymorphism could be a genetic risk factor mediating susceptibility to breast carcinoma.

As for the +3142C/G polymorphism, Zidi et al. (74) observed that both the G allele and the G/G genotype have a protective effect for breast cancer risk (p=0.0004 and p=0.0005, respectively), as they are less prevalent in patients compared to controls. This is consistent with the fact that the +3142G variant implies lower mRNA stability and lower HLA-G production, and therefore lower tolerogenic capacity in carriers.

An interesting research was carried out by He et al. (62). They found HLA-G expression in 66.0% of their patients. This expression significantly correlated with tumor size, nodal status, and clinical disease stage (p=0.0001, 0.012, and 0.0001, respectively). In addition, sHLA-G levels were higher in patients compared to controls (p<0.001). HLA-G expression also correlated with survival, as the patients with higher expression had a worse outcome (p=0.028). They also correlated HLA-G expression with the host immune response (measured as the number of tumor infiltrating lymphocytes), finding that the higher the HLA-G expression the less lymphocytes (p=0.011).

Altogether, these results suggest that HLA-G may have potential clinical implications in diagnosis and prognosis of patients with breast cancer.

## 5.4 Esophageal Cancer

Chen et al. (75) found that individuals carrying the 14bp DEL/DEL genotype had a 2.69-fold increased risk of suffering esophageal cancer compared with those carrying the 14bp INS/INS genotype (p=0.04). Further, individuals carrying the 14bp DEL/DEL and +3142C/C genotypes (DEL/C haplotype, which combines HLA-G-enhancing variants) had a 2.82-fold increased risk of esophageal cancer compared with individuals carrying the INS/C haplotype (p=0.04).

Regarding HLA-G expression in this type of cancer, Zheng et al. (68) observed that in 70.0% of the patients analyzed a positive HLA-G staining was achieved (p<0.05), and this correlated with cancer cell differentiation (p=0.033) and lymph node metastasis (p=0.035). A significant difference in plasma sHLA-G levels was also found between patients (15.04 U/mL) and healthy controls (6.81 U/mL, p<0.01). In addition, HLA-G-positive patients showed a poor prognosis. Interestingly, IL-10, an inhibitory cytokine whose expression is enhanced by HLA-G, has its levels increased in patients compared to controls (23.86 pg/mL vs. 12.81 pg/mL, p<0.01).

Hence, HLA-G is a potential predictive biomarker of esophageal cancer, and the modification of HLA-G transcription or expression may be of benefit in the prevention and treatment of this type of cancer.

## 5.5 Lung Cancer

There is also evidence of a link between HLA-G and lung cancer. Yie et al. (76) analyzed a cohort of 106 patients, finding HLA-G overexpression in 75.0% of cases. This expression significantly correlated with lymph nodal metastasis and clinical stages of the disease (p=0.0001 in all instances), and they observed lower presence of infiltrating lymphocytes in the areas where HLA-G was abundant compared to regions with scarce HLA-G (p=0.027), thus emphasizing the inhibitory role of HLA-G. Again, the overall survival rate of patients with HLA-G (-) tumors was significantly higher when compared to those with HLA-G (+) tumors (50.0% vs 22.0%; p=0.001).

HLA-G has also been correlated with worse prognosis and survival in other malignances such as hepatocellular cancer (77, 78), renal cancer (79) and pancreatic cancer (80).

## 5.6 Limitation of HLA-G Expression Analysis in Tissues

It is now widely accepted that HLA-G is a critical marker of immunotolerance in cancer immune evasion and is strongly associated with disease progress and prognosis in cancer patients. However, not all published works are equally consistent and, although they show HLA-G expression in cancer, do not allow to correlate HLA-G with certain types of cancer (81).

The reason for this discrepancy lies mainly in two points: inter- and intra-tumor heterogeneity and the different methodological approaches used for detecting HLA-G.

Regarding heterogeneity, multiple transcriptional, epigenetic, post-transcriptional and environmental mechanisms are involved in modulating protein expression in cancer. This also affects HLA-G, in addition to its genetic background (polymorphisms), which leads to the discrepancies mentioned earlier (82, 83).

The other problem lies in the methodological approaches employed. The vast majority of HLA-G detection studies use immunohistochemistry as the main method, but each laboratory uses different antibodies and variable experimental conditions (incubation times, antibody concentration, etc), even when analyzing the same type of tumor (81–83). In addition, some of these antibodies used, such as the 4H84 clone, appear to be poorly specific for HLA-G detection (84). This complicates the ability to interpret and compare different studies. It would be advisable to establish common experimental procedures to assess which of the available antibodies should be used in all the studies.

# 6 AUTOIMMUNITY

In recent years, several studies have shown that HLA-G plays an important role in the control of autoimmune and inflammatory diseases, caused by the uncontrolled activation of immune system cells (85). The ability of HLA-G to limit the progression of these diseases has been confirmed and, in fact, the levels of the molecule or the distribution of the polymorphisms of the gene modulating them, have been associated with an increased susceptibility to the development and severity of these diseases (86).

## 6.1 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune inflammatory disease that involves many organs and systems. It has been described that patients with this disease show a significant increase in the frequency of the 14bp INS/INS

genotype of the 14bp INS/DEL polymorphism, compared to healthy controls (87). In addition, patients with SLE have a significant increase in the +3142G allele and the +3142G/G genotype of the +3142C>G polymorphism, associated with a lower expression of HLA-G due to increased degradation of the primary transcript, as well as by suppression of its translation (18, 88). In fact, a significant decrease of sHLA-G has been found in the plasma of these patients compared to healthy controls, which is usually associated with the 14bp INS/INS genotype (17, 89).

### 6.2 Multiple Sclerosis

Another disease in which the HLA-G molecule is implicated is Multiple Sclerosis (MS), the most common disabling neurological disorder in young adults (90). Studies to date have revealed that MS patients have a significantly higher frequency of the 14bp INS allele of the 14bp INS/DEL polymorphism than healthy controls and, in addition, the 14bp INS/INS genotype has been correlated with decreased plasma sHLA-G in these patients (91). On the other hand, an increase in the frequency of the +3142G allele of the +3142C>G polymorphism has also been observed in these patients compared to healthy controls and, in fact, the +3142C/C genotype is considered protective for this disease, appearing more frequently in the group of healthy controls (92).

### 6.3 Rheumatoid Arthritis

The HLA-G molecule is also one of the factors that contribute to the development of Rheumatoid Arthritis (RA), which involves chronic inflammation of the synovial membrane (93). A case-control study revealed that RA patients had a significant increase in the +3142G/G genotype of the +3142C/G polymorphism. In addition, patients with this genotype had significantly higher plasma sHLA-G levels than healthy controls (94). However, in contrast to other autoimmune diseases, there is some controversy in the association of the 14bp INS/DEL polymorphism and the risk or protection of this disease. Nevertheless, the possibility that RA is associated with this polymorphism cannot be ruled out due to lack of statistical power, considering that sample size is a critical factor in this type of study (95). Further research in this pathology is needed to unveil the implication of HLA-G in the development or severity of RA.

### 6.4 Type 1 Diabetes Mellitus

Some of the *HLA-G* gene polymorphisms and molecule levels have also been correlated with Type 1 Diabetes mellitus (T1D), a chronic and genetically complex disease characterized by pancreatic beta-cell destruction, mediated by humoral and cellular immune responses. The 14bp INS allele of the 14bp INS/DEL polymorphism appears significantly more frequent in patients than in healthy controls (96). Considering that the HLA-G molecule is constitutively expressed in the pancreas, where it protects the organ against cytotoxic cells, decreased expression level should be detrimental in individuals genetically prone to producing less HLA-G (97).

### 6.5 Psoriasis

In the case of psoriasis, a chronic inflammation in which environmental and genetic factors are involved, patients with the 14bp DEL allele and the DEL/DEL genotype of the 14bp INS/DEL polymorphism respond better to treatment with acitretin (98); so the analysis of this polymorphism could contribute to the development of personalized treatments for these patients.

### 6.6 Uveitis

Uveitis is a disorder characterized by inflammation of the uvea. Regarding this pathology, Crabtree et al. (99) have described that an increase of HLA-G induced in an animal model of experimental autoimmune uveitis (both soluble and membrane-bound), reduces the severity of the pathology in these mice, thus reiterating the benefit of the tolerogenic role of the HLA-G molecule in autoimmune/inflammatory diseases.

Uveitis is one of the possible clinical manifestations in patients suffering from Behçet's syndrome, a systemic condition. Patients suffering from this syndrome show a significant increase in the frequency of the INS 14bp allele compared to controls and, in fact, patients with homozygous INS/INS genotype have twice the risk of suffering from this disease compared to healthy controls (100).

Furthermore, Park et al. (101) confirmed that the HLA-G*01:01:02 allele significantly increases the risk of the Behçet disease, since it contains the insertion of the 14bp fragment of 14bp INS/DEL polymorphism in 3'UTR region, which is associated with lower HLA-G expression. Also, the G*01:05N allele, that is a non-functional allele and does not encode either soluble isoforms or the membrane-bound G1 isoform, is significantly more frequent in patients compared to controls, thus being associated with higher risk to develop Behçet disease.

## 7 HLA-G AND THERAPY

### 7.1 Cancer

As shown in the present review, HLA-G expression has been reported in a wide variety of tumors, correlating significantly with the clinical outcome of patients. Both genetic (polymorphisms) and protein expression studies confirm that HLA-G overexpression in cancer is related to faster disease progression and worse clinical outcome. In fact, some of these studies (60, 62), clearly show a decrease in immunocompetent cells in an HLA-G rich tumor microenvironment, emphasizing the tolerogenic role of this molecule. Therefore, HLA-G and the downstream signaling pathways upon interaction with its cognate receptors can be soundly considered a new target for immune-based anti-tumor therapy.

One approach for anti-HLA-G therapy is downregulating HLA-G expression with miRNAs. This has been proposed by Kaminski et al. (102) in a clinical setting different from cancer (pregnancy). They propose to deliver miR-148a and miR-152 microRNAs, carried by liposomes, into the target cells, where they would interfere with the production of HLA-G (as mentioned in the "*HLA-G* gene polymorphisms" section).

Fu et al. (103) found that HLA-G expression was directly regulated by miR-152, and also that an aberrant expression of miR-152 influenced tolerance to, in this case, NK cell cytolysis *in vitro*. The cell lysis rate increased significantly when overexpressing miR-152 in the target cells (A549 cells), because, as a consequence of this, they had less HLA-G to protect themselves from NK cell-mediated lysis. They also did *in vivo* studies where they inoculated HLA-G expressing or HLA-G devoid (transfected with si-HLA-G) A549 cells in mice, finding that, in the latter case, the size of the tumors generated was significantly smaller than in the former. Thus, HLA-G expression was correlated with the growth and immune escape ability of tumoral (A549) cells *in vivo* and *in vitro*.

Based on the two aforementioned works (102, 103), it seems interesting to develop therapies with miRNAs aimed at reducing HLA-G expression in cancer, in order to restore the anti-tumor activity of cells restrained by this molecule.

On the other hand, the immune checkpoint inhibitor (ICI) therapy merits further attention. This type of therapy has been a major breakthrough in cancer treatment. However, despite the good achievements of therapeutic approaches such as anti PD1/PDL1, they have limitations, and there are still patients who do not benefit from this type of treatments (104). This is why there is a need to continue searching for new alternatives.

A recent study by Dumont et al. (105) lays the foundations to consider the HLA-G/ILT2 pathway as a new checkpoint. They characterized the CD8+ILT2+ lymphocyte population as antigen-experienced cells and highly cytotoxic, more so than CD8+PD1+ (the main target of ICI).

CD8+ILT2+ cells markedly upregulated cytotoxicity-related genes, consistent with the expression of KLRG1, perforin, and granzyme B, observed by flow cytometry. When CD3 activation assays were performed, in comparison with CD8+ILT2- and CD8+PD1+ cells, CD8+ILT2+ cells exhibited the highest degranulation rate (measured by CD107a and IFNγ). Therefore, these cells exhibit a high anti-tumor capacity.

Importantly, they assessed that these CD8+ILT2+ cells were specifically inhibited by the presence of HLA-G. When cocultured with HLA-G-expressing cells, a reduction in degranulation and IFNγ production was observed, thus reducing the anti-tumoral capacity of these cells. However, when using antibodies that interfere with the interaction between HLA-G and ILT2, CD8+ILT2+ cell activity was completely restored, blocking the immunosuppressive capacity of HLA-G. Accordingly, as with PD1/PDL1, an anti-HLA-G/ILT2 therapy could be effective for cancer treatment.

Additionally, Fu et al. (103) performed blockade studies of the HLA-G/ILT2 pathway in NK cells, also finding *in vitro* that using anti-ILT2 antibodies restored the cytotoxic capacity of these cells.

Also, and similar to the experiments mentioned above, *in vivo* studies were performed by transfecting mice with A549 cells and injecting them in the presence or absence of anti-ILT2 antibodies. Although no significant differences were clearly observed, tumors of the mice treated with anti-ILT2 were, nevertheless, smaller than in untreated mice, suggesting a blocking effect on the anti-tumor capacity of HLA-G. Consequently, blocking ILT2 maintained the lytic effect of NK cells in the presence of HLA-G, making the use of this anti-ILT2 a promising therapy for cancer.

Because PD1/PDL1 and HLA-G/ILT2 seem to work as independent mechanisms, a combination of therapies would cover a wider range of cells to restore the anti-tumor activity of the immune system.

Still, further investigation is needed to establish whether targeting HLA-G and its receptors is truly a robust therapy. More studies, such as those of Dumont et al. (105) and Fu et al. (103) are needed to fully elucidate the relevance of the HLA-G/ILT2 pathway, along with all other HLA-G mediated signaling pathways, involving pertinent immune cells.

### 7.2 Autoimmune Diseases

Currently, no HLA-G-based therapies have been developed for the treatment of autoimmune and autoinflammatory diseases. Despite the lack of studies in patients, a study performed in a murine model of experimental uveitis found that increasing HLA-G levels in these mice, significantly improved the clinical manifestations of this pathology, which confers this molecule a therapeutic potential for patients suffering from this type of diseases (99).

In addition, some of the HLA-G gene polymorphisms have been associated with response to certain autoinflammatory disease treatments. For example, the DEL/DEL genotype of the 14bp INS/DEL polymorphism has been associated with a better response to treatment with methotrexate in patients with rheumatologic diseases (59).

## 8 CONCLUSION

Given the tolerogenic function of HLA-G, this molecule plays a key role in modulating the immune response in several pathologies. Measuring the protein levels (whether membrane bound or soluble) or analyzing the gene polymorphisms involved in its expression, is of interest in cancer susceptibility and progression and in autoimmune diseases.

To the best of our knowledge, no HLA-G variants are currently used as diagnostic markers in current clinical settings. While no HLA-G allele can be used as a clear susceptibility marker, it holds true that some markers identify early on cancer patients who might present with worse clinical evolution and shorter life expectancy of the disease, as has been reported in gastric adenocarcinoma (69), colorectal cancer (61, 71) and breast cancer (73), among others.

In depth knowledge of the function of HLA-G opens the possibility of establishing new immunomodulatory therapeutic approaches in cancer (new immune checkpoint inhibitors) or in autoimmune diseases (modulating the levels of this molecule).

## AUTHOR CONTRIBUTIONS

JMM-V: Literature research, writing and revision. CV-Y and MM-A: Experimental work, literature research, writing and figure

drawing. IJ: Experimental work, literature research, writing and revision. FS-T, AL-N, JP-G, LB-G, EF-C, and CR-S: writing and revision. AA-V: Literature research, writing and revision.

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REVIEW
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# Roles of HLA-G/KIR2DL4 in Breast Cancer Immune Microenvironment

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Human leukocyte antigen (HLA)-G is a nonclassical MHC Class I molecule, which was initially reported as a mediator of immune tolerance when expressed in extravillous trophoblast cells at the maternal-fetal interface. HLA-G is the only known ligand of killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4), an atypical family molecule that is widely expressed on the surface of NK cells. Unlike other KIR receptors, KIR2DL4 contains both an arginine–tyrosine activation motif in its transmembrane region and an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail, suggesting that KIR2DL4 may function as an activating or inhibitory receptor. The immunosuppressive microenvironment exemplified by a rewired cytokine network and upregulated immune checkpoint proteins is a hallmark of advanced and therapy-refractory tumors. Accumulating evidence has shown that HLA-G is an immune checkpoint molecule with specific relevance in cancer immune escape, although the role of HLA-G/KIR2DL4 in antitumor immunity is still uncharacterized. Our previous study had shown that HLA-G was a pivotal mediator of breast cancer resistance to trastuzumab, and blockade of the HLA-G/KIR2DL4 interaction can resensitize breast cancer to trastuzumab treatment. In this review, we aim to summarize and discuss the role of HLA-G/KIR2DL4 in the immune microenvironment of breast cancer. A better understanding of HLA-G is beneficial to identifying novel biomarker(s) for breast cancer, which is important for precision diagnosis and prognostic assessment. In addition, it is also necessary to unravel the mechanisms underlying HLA-G/KIR2DL4 regulation of the immune microenvironment in breast cancer, hopefully providing a rationale for combined HLA-G and immune checkpoints targeting for the effective treatment of breast cancer.

**Keywords: HLA-G, KIR2DL4, breast cancer, immune microenvironment, immunotherapy**

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## INTRODUCTION

Breast cancer is the most common cancer diagnosed in women worldwide, with an estimated 2.3 million new cases and 0.7 million deaths in 2020 (1). Breast cancer is a very heterogeneous disease, both at molecular and histological levels. Five intrinsic subtypes of breast cancer were initially identified: Luminal-A, Luminal-B, $HER2^+$, triple negative/basal like (TNBC) and normal like, which is based on the gene expression of estrogen receptor (ER), progesterone receptor (PR), and human

epidermal growth factor receptor 2 (HER2) (2). The character of each subtype was associated with incidence, treatment response, rate of disease progression and metastasis. The conventional treatment options for patients with breast cancer include surgery, chemotherapy, radiotherapy, hormonal therapy and targeted therapy (3). Increasing evidence has showed that breast cancer microenvironment is not only composed of tumor cells but also of other different cell types, including endothelial cells, several stromal cell types, and immune cells (4). Due to the potential of the immune system in various new treatment strategies, immunotherapy has attracted the attention of researchers, including adoptive cell therapy, oncolytic virus and the most noteworthy immune checkpoint blockade therapy (5). Monoclonal antibodies against PD-1/PD-L1 and CTLA-4 have now entered clinical trials for the clinical treatment of triple negative breast cancer (6). Our recent study has showed that human leukocyte antigen-G (HLA-G) was a pivotal mediator of HER2 positive breast cancer resistance to trastuzumab and blockade of HLA-G can improve the antitumor activity of NK cells significantly (7).

HLA-G is a non-classical HLA Class I molecule that is first found specifically expressed in extravillous trophoblasts (EVTs) and plays a major regulatory role in maternal-fetal immune tolerance (8). During pregnancy, the fetus expresses paternal HLA, which are foreign antigens for maternal tissue, yet the fetus is neither rejected nor attacked by the maternal immune system (9). This phenomenon is due to the immune tolerance elicited by interaction between EVTs expressing HLA-G and leukocytes expressing inhibitory receptors (8). In 1998, Paul and his colleagues first reported the abnormal HLA-G expression in melanoma lesions rather than adjacent normal tissues (10). Accumulated evidence have showed that HLA-G is abnormally highly expressed in a variety of tumor cells and is involved in immune escape of tumors (11). All these findings suggest that HLA-G might be an important immune checkpoint in breast cancer immunotherapy.

Unlike the classical HLA Class I molecule, such as HLA-A, HLA-B, and HLA-C, the gene of HLA-G displays limited polymorphism (12). Seven different transcripts of *HLA-G* gene, namely HLA-G1 to G7, have been identified. HLA-G1 can be translated into both membrane-binding isoform and soluble isoform; HLA-G2, G3, and G4 transcripts can be translated into membrane-binding proteins; HLA-G5, G6, and G7 transcripts are templates for soluble proteins (11). Different HLA-G isoforms might underlie the diverse functions in cancer immunotherapy.

According to the recent studies, there are multiple receptors of HLA-G, including immunoglobulin-like transcript (ILT)-2, ILT-4, and killer inhibitory receptor (KIR) 2DL4, which are differentially expressed on immune cells (11, 13). ILT-2 and ILT-4 are the type I transmembrane glycoproteins with four extracellular immunoglobulin like domains, a transmembrane region, and an intracellular tail, which have four or three immunoreceptor tyrosine-based inhibitory motifs (ITIMs). ILT-2 and ILT-4 are expressed on T cells, natural killer cells (NK), and dendritic cells (DC) (14). Based on the structure of the intracellular tail, ILT-2/ILT-4 can initiate inhibitory signaling after binding to HLA-G, leading to immune suppression. KIR2DL4 is a member of the killer cell immunoglobulin (Ig)-like receptor (KIR) family, with two atypical extracellular domains, a positively charged arginine residue in the transmembrane region and an ITIM domain in its intracellular tail. The charged arginine residue of KIR2DL4 can recruit activation adaptors that contain immunoreceptor tyrosine-based activation motifs (ITAMs) (14). KIR2DL4 has both the activation and inhibitory signaling domains, suggesting that it can function as both activating and inhibitory receptor.

Our recent study found that KIR2DL4 synergizes with FcRγ to enhance NK cell activation and degranulation, while HLA-G binding to KIR2DL4 impairs the cytotoxicity of NK cells in HER2 positive breast cancer microenvironment (7). These findings suggested that KIR2DL4 might provide a switch for NK cell activity *via* its association with HLA-G. Increasing evidence has showed that HLA-G is involved in both innate and adaptive immune responses required for immune escape. With the unique structure, whether KIR2DL4 participates in cancer immunotherapy remains to be explored. In this review, we aim to summarize the roles of HLA-G/KIR2DL4 in breast cancer immune microenvironment.

## MOLECULAR STRUCTURE OF HLA-G AND KIR2DL4

There are 8 exons and 7 introns in *HLA-G* gene. Most of the full-length HLA-G transcripts contains 7 exons, as exon 7 is usually removed by splicing. Compared with classical HLA Class I molecules, HLA-G is relatively short, the full length of which is only about 340 amino acids. The signal peptide was translated from exon 1; the extracellular α1, α2, and α3 domains were translated from exon 2-4, respectively; the transmembrane domain was generated by exon 5; and the intracellular cytoplasmic tail was generated by exon 6 (15). Accumulated evidences have shown that there are 7 isoforms of HLA-G. Each HLA-G isoform has its unique molecular structure due to different transcript splicing. HLA-G1 has both membrane-binding and soluble isoforms with extracellular α1, α2, and α3 domains, while HLA-G2/-G3/-G4 were membrane-binding isoforms. HLA-G2 has extracellular α1 and α3 domains; HLA-G3 has the only α1 domain; and HLA-G4 has α1 and α2 domains. HLA-G5/-G6/-G7 were soluble isoforms. HLA-G5 has extracellular α1, α2, and α3 domains; HLA-G6 has α1 and α3 domains; and HLA-G7 has the only α1 domains (11). The α1 and α2 domains contain the peptide-binding sites and the α3 domain can bind to β2-microglobulin ($\beta_2$m) non-covalently. Recent studies have shown that a novel isoform of HLA-G without α1 domain can be detected in colorectal cancer patients and clear cell renal cell cancer patients, which might suggest distinct clinical significance (16, 17).

Killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4) is the only KIR receptor recognized by HLA-G. KIR2DL4 belongs to the killer cell Ig-like receptors (KIR) family, which is expressed

mainly on NK cells (7). KIRs can recognize both classical and nonclassical HLA Class I molecules. The *KIR* genes are located in chromosome 19q13.4, with each spanning 10,000-15,000 bp and separated by 1,000 bp. KIRs are encoded by up to nine exons: the leader peptide was encoded by the first two exons (exon 1 and exon 2), the two or three extracellular Ig-like domains (2D or 3D) by exons 3-5, the stem structure by exon 6, the transmembrane region by exon 7, and the cytoplasmic tail by exons 8-9 (18). Generally, KIRs have two or three extracellular immunoglobulin domains that were named KIR2D or KIR3D. Inhibitory KIRs including KIR2DL or KIR3DL contain a long cytoplasmic tail (L) with two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Activating KIRs like KIR2DS or KIR3DS contain a short cytoplasmic tail (S) and a positively charged residue in the transmembrane region, which can recruit adaptors that contain the immunoreceptor tyrosine-based activation motif (ITAM) (19). Unlike the conventional KIR2DL receptors, KIR2DL4 contains only one ITIM instead of two in its cytoplasmic tail and a positively charged arginine in its transmembrane region, which can recruit activation adaptors (19–21). Based on the unique structure, both activating and inhibitory functions have been described for KIR2DL4 on NK cells. Rajagopalan et al. found that KIR2DL4 is an activating receptor that can induce IFN-γ production by but not cytotoxicity of resting NK cells (22). Yusa and her colleagues showed that the single ITIM of KIR2DL4 can inhibit cytotoxic response of NK cells efficiently, which depends on SHP-2, but not SHP-1, and phosphorylated tyrosine (20). Our recent study has showed that HLA-G can desensitize breast cancer cells to trastuzumab by binding to the NK cell receptor KIR2DL4. Unless engaged by HLA-G, KIR2DL4 promotes ADCC function and forms a regulatory circuit with the IFN-γ production pathway, in which IFN-γ upregulates KIR2DL4 *via* JAK2/STAT1 signaling, and then KIR2DL4 synergizes with the CD16 to increase IFN-γ secretion by NK cells (7). Recent studies have showed that the IFN-γ production can impair the function of immune cells by upregulating PD-L1 on tumor cells (23–25). Consistent with previous reports, we observed that IFN-γ significantly increased the level of PD-L1 in breast cancer cells. Blockade of PD-L1 increased the cytotoxicity of NK cells against trastuzumab-treated HER2-overexpressing breast cancer cells (7). These findings suggested that since KIR2DL4 functions as either an activating receptor or an inhibitory receptor, it can affect the outcome of immunotherapy through the complicated cross-talk between different immune checkpoints and cytokines in the breast cancer immune microenvironment.

## HLA-G/KIR2DL4 EXPRESSION IN BREAST CANCER IMMUNE MICROENVIRONMENT

HLA-G expression in cancer microenvironment was regulated by several intracellular and extracellular mechanisms mediated by microRNAs, RNA-binding proteins, heat shock proteins, cytokines, et al. There are several microRNAs were correlated with the expression of HLA-G, such as miR-133a, miR-148a, miR-148b, miR-152, miR-199b-5p, miR-548q and miR-628-5p *et.al*, mainly investigated *in vitro* (26–28). Reches A et al. found that the RNA-binding protein, the heterogeneous nuclear ribonucleoprotein R (HNRNPR) can regulate the expression of HLA-G by binding to the 3'UTR of the HLA-G transcripts (29). Heat shock proteins was also reported to induce the HLA-G expression *via* the heat shock transcription factor 1 binding to the heat shock element of HLA-G promoter (30). The expression of HLA-G was regulated not only by intracellular post-transcriptional regulation, but also by extracellular mediators. HLA-G expression was found to be induced by progesterone *via* the interaction between progesterone receptor and progesterone response element (PRE) in the HLA-G promoter (31). Cytokines were also found to regulate the expression of HLA-G, such as GM-CSF, IL-10, TGF-β and IFN-β et al. (7, 32–34). All these findings indicated that the HLA-G expression in cancer microenvironment might be a key factor to investigate the progression of disease.

Many studies have shown that HLA-G/KIR2DL4 expression in immune microenvironment were correlated with the prognosis and progression of breast cancer. In 2002, Lefebvre et al. found that HLA-G was up-regulated at high frequencies in human breast cancer and it may impair anti-tumor immunity by recognizing inhibitory receptors, ILT-2 (35). Meanwhile, Urosevic and colleagues made an editorial comment, proposing that HLA-G and related killer cell inhibitory receptor might represent another mechanism for tumor immune escape (36). However, a recent study has showed that HLA-G expression did not significantly correlate with poor clinical outcome of cancer patients, which indicated that HLA-G expression might not necessarily participate in immunosuppression in carcinogenesis (37). Palmisano et al. analyzed the HLA-G expression at both RNA and protein levels in 25 breast cancer patient tissues. They failed to detect HLA-G expression in breast cancer tissues and cell lines, which was later found attributed to stromal cell contamination in tissue samples (38). In 2003, a comparative study reported that soluble HLA-G can be detected in the malignant ascites of breast cancer patients (39). In the same year, Korkola JE et al. Found in microarray assays that HLA-G were differentially expressed in lobular versus ductal breast cancer (40). Later investigations have established that HLA-G is an important marker of tumor immune escape (41, 42). Several studies have shown that the expression of HLA-G is significantly associated with progression and poor prognosis in a variety of tumors including aggressive breast carcinoma (43–45). Based on previous studies, whether HLA-G can be used as a marker for clinical diagnosis and treatment of breast cancer patients has also been extensively investigated (46, 47). In 2010, a comparative study reported that estradiol/progesterone-induced HLA-G expression can inhibit allo-cytotoxic lymphocyte response to human breast cancer MCF-7 cells (48). Chen et al. found that high soluble HLA-G levels was significantly correlated with the increased infiltration of Treg in breast cancer patients, which indicated that HLA-G might play an important role in the immunosuppressive breast cancer microenvironment (49, 50). HLA-G expression was also

associated with subtypes of breast cancer. Provatopoulou X et al. found that HLA-G expression was increased in co-existing ductal and lobular breast cancer patients, compared to those with pure ductal cancer or pure lobular cancer alone (51). Yang's team reported that there were more cases with high expression of HLA-G in non-luminal than in luminal subtypes, and HLA-G expression was associated negatively with the density of tumor-infiltrating lymphocytes (52). The mRNA stability of HLA-G was found associated with a 14-bp insertion/deletion in exon 8 of the 3' untranslated region. A series of studies have shown that *HLA-G* polymorphism could be a diagnostic and prognostic marker for the susceptibility and pathogenesis of breast cancer in populations from different regions (53–59). MicroRNAs were also found to be involved in the regulation of HLA-G expression in breast cancer. Tao et al. found that G protein-coupled estrogen receptor (GPER) mediated the regulation of HLA-G by miR-148a, which was induced by estrogen (E2) in 2 human breast cancer cell lines, MCF-7 and MDA-MB-231 (60). It has been shown that HLA-G is expressed on immune cells such as NK cells and T cells (61, 62).

KIR2DL4 is the only killer cell Ig-like family receptor recognized by HLA-G. In 1999, Long EO et al. first reported that KIR2DL4, which is expressed at the surface of all NK cells including activated NK cells and resting NK cells, can bind to cells expressing HLA-G (22, 63). Goodridge et al. found that there are two KIR2DL4 alleles with either 9 or 10 consecutive adenines (9A or 10A) in exon 6, which encodes the transmembrane domain (64). The 9A alleles can produce a secreted receptor due to the splicing out of the transmembrane region, which might cause a lack of KIR2DL4 expression (65). In contrast, the "10A" alleles encode a membrane-expressed receptor that is constitutively expressed on resting $CD56^{bright}$ and $CD56^{dim}$ NK cells (65). The It has been described that the interaction of the KIR2DL4 receptor with the HLA-G molecule is mediated by the α1 domain, which indicated that all the seven identified isoforms of HLA-G can be recognized by KIR2DL4 (66). In breast cancer immune microenvironment, HLA-G can bind to its receptor, such as KIR2DL4, to induce immunosuppression. Ueshima C et al. found that human mast cells expressing KIR2DL4 can promote invasion of HLA-G-expressing malignant cells and the subsequent metastasis of breast cancer and choriocarcinoma (67). Recently, we have found that HLA-G expression can predict a low trastuzumab response in HER2 positive breast cancer patients (7). We also detected abundant KIR2DL4 expression on infiltrating NK cells in HER2 positive breast cancer tissues. These findings indicated that HLA-G/KIR2DL4 in immune microenvironment might play an important role in the progression of breast cancer and become a new target for breast cancer treatment.

## HLA-G/KIR2DL4 AS THE NOVEL TARGETS IN BREAST CANCER IMMUNOTHERAPY

So far, the role of HLA-G/KIR2DL4 in breast cancer immunotherapy has been progressively elucidated. Roberti et al. found that blockade of ILT-2 with antibodies can restore cetuximab-mediated ADCC in triple-negative breast cancer patients and revert immunosuppression mediated by HLA-G (68). In 2016, Ishibashi and colleagues found that an MHC Class II-binding peptide HLA-G26-40 can elicit tumor-reactive $CD4^+$ T cell responses effectively (69). With the wide application of immunotherapy in cancer, the relationship between immunological signature and progression of breast cancer has also been intensively investigated. A study reported that the expression of HLA-G was associated with both improved relapse-free survival (RFS) and overall survival (OS) of basal-like breast cancer, which might indicate a better status of lymphocyte infiltrating (70). Zhang's team reported that overexpression of HLA-G in breast cancer cells was induced by abnormal DNA methylation modification, which was mediated by DNA methyltransferase (DNMT) and ten-eleven translocation (TET) (71). Therefore, TET inhibitor can prevent aberrant HLA-G expression *via* maintenance of DNA methylation, which provides a novel potential target for cancer immunotherapy (71). Jørgensen et al. also found that HLA-G expression was regulated partially by DNA methylation since the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine, induced HLA-G expression, suggesting the feasibility of manipulating HLA-G expression for immunotherapy in breast cancer (72). In 2020, Schwich et al. reported that soluble HLA-G can induce a an immunosuppressive/exhausted phenotype, and the purified soluble HLA-G1 protein or extracellular vesicles derived from an HLA-G1-positive human breast cancer cell line, SUM149, can affect the anti-tumor function of $CD8^+$ T cells by binding to ILT-2 (73). Our recent study reported that KIR2DL4, an alternative receptor of HLA-G, might be a novel target in breast cancer immunotherapy (7). We found that HLA-G impaired trastuzumab-triggered ADCC by binding to KIR2DL4 on NK cells, and blockade of HLA-G/KIR2DL4 can enhance the antitumor activity of trastuzumab *in vivo*. However, unless engaged by HLA-G, KIR2DL4 can promote ADCC and form a regulatory circuit with the IFN-γ production pathway *via* JAK2/STAT1 signaling in NK cells. In addition, paracrine TGF-β and IFN-γ in breast cancer microenvironment can induce PD-1/PD-L1 expression on NK cells and tumor cells, which might further enhance intercellular signaling that leads to immunosuppression. These findings demonstrated the applicability of combined HLA-G/KIR2DL4 and PD-1/PD-L1 targeting in the treatment of trastuzumab-resistant breast cancer (**Figure 1**).

## CONCLUSIONS

The immune system played an important role in the occurrence and progression of breast cancer. Many immunotherapy approaches have been investigated for breast cancer, including antibodies against tumor antigens, immune checkpoint blockade and CAR-T cells (4). Recent studies have shown that the combination of conventional chemotherapy or radiotherapy with immunotherapy contributes to improved outcome of

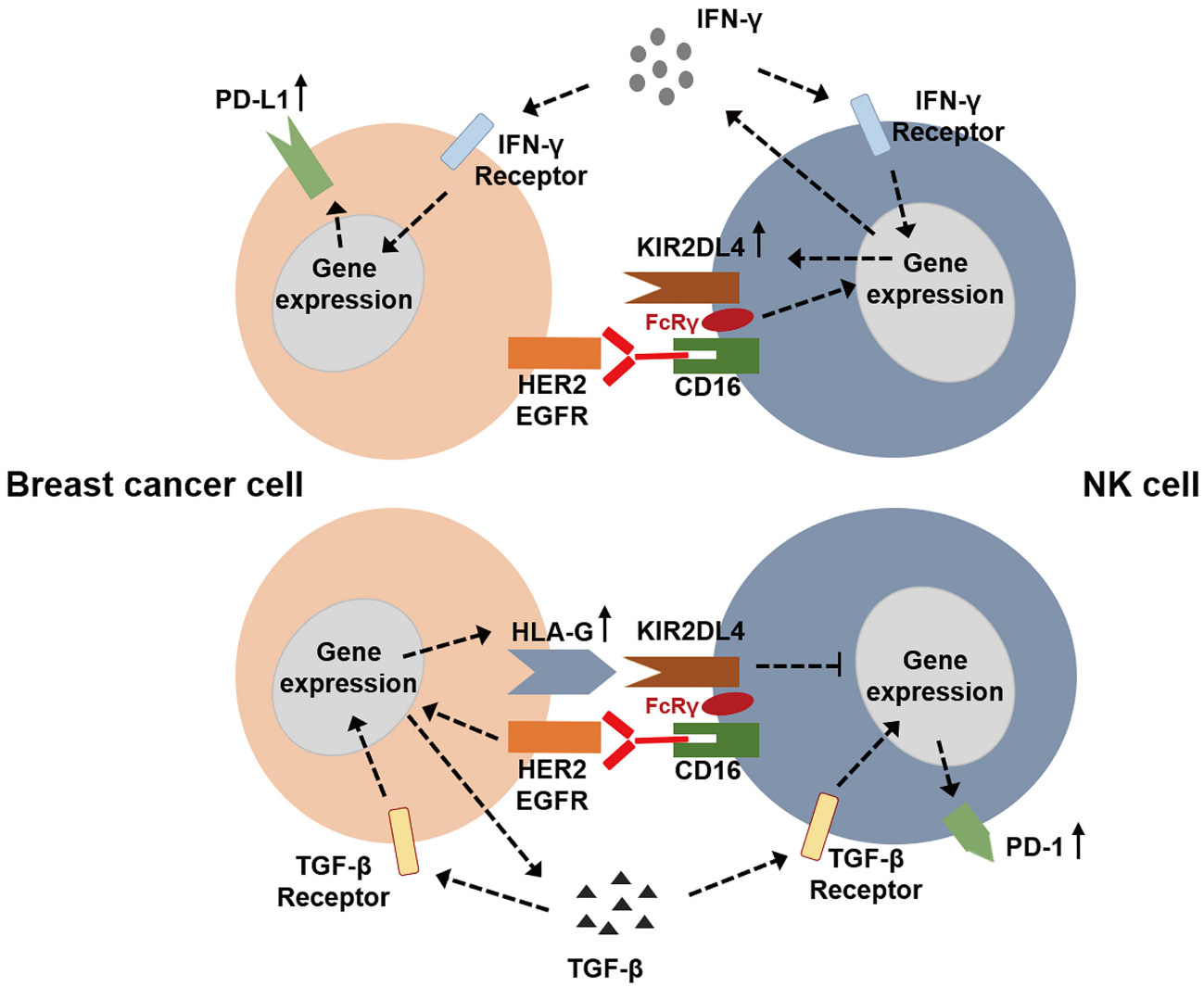


**FIGURE 1** | The potential roles of HLA-G/KIR2DL4 signaling in the NK cell mediated immunotherapy of breast cancer.

breast cancer patients (74, 75). The therapeutic strategies for breast cancer have shifted from cytotoxic therapy to priming anti-tumor immune responses. Nonetheless, only a small part of breast cancer patients can currently benefit from immunotherapy. The relatively low responsive rate and the immunosuppressive tumor microenvironment even in the responding populations have limited the benefit of immunotherapy for breast cancer patients. The abnormal expression of immune checkpoint molecules is the main cause of immune escape. Thus, immune checkpoint blockade represents an important approach to reversing the immunosuppressive status of tumor microenvironment.

In this review, we summarized the special structural features of HLA-G and KIR2DL4. Then, we reviewed the expression of HLA-G/KIR2DL4 in breast cancer immune microenvironment and the potential of HLA-G/KIR2DL4 application in breast cancer immunotherapy. HLA-G, a non-classical HLA Class I molecule, was highly expressed in breast cancer tissues and associated with tumor progression and poor prognosis of patients. HLA-G engagement of its cognate receptors, ILT-2, ILT-4 and KIR2DL4 expressed on immune cells, can significantly induce immunosuppression and result in tumor immune escape. KIR2DL4 is the only KIR receptor that binds to HLA-G. KIR2DL4 contains an ITIM domain in its cytoplasmic tail and a positively charged arginine in its transmembrane region, which can recruit activation adaptors containing a ITAM domain. Based on its special structure, KIR2DL4 can mediate a complicated cross-talk between immune checkpoint and cytokines in breast cancer microenvironment, and dictate distinct outcome of immunotherapy depending on whether or not HLA-G is engaged. A deep understanding of the regulatory role of HLA-G/KIR2DL4 in the immune microenvironment of

breast cancer might provide new ideas for the treatment of breast cancer.

## AUTHOR CONTRIBUTIONS

Conceptualization, funding acquisition: A-GY. Writing original draft, review and editing: GZ and LJ. All authors contributed to the article and approved the submitted version.

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REVIEW
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# The Human Leukocyte Antigen G as an Immune Escape Mechanism and Novel Therapeutic Target in Urological Tumors

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The non-classical human leukocyte antigen G (HLA-G) is a potent regulatory protein involved in the induction of immunological tolerance. This is based on the binding of membrane-bound as well as soluble HLA-G to inhibitory receptors expressed on various immune effector cells, in particular NK cells and T cells, leading to their attenuated functions. Despite its restricted expression on immune-privileged tissues under physiological conditions, HLA-G expression has been frequently detected in solid and hematopoietic malignancies including urological cancers, such as renal cell and urothelial bladder carcinoma and has been associated with progression of urological cancers and poor outcome of patients: HLA-G expression protects tumor cells from anti-tumor immunity upon interaction with its inhibitory receptors by modulating both the phenotype and function of immune cells leading to immune evasion. This review will discuss the expression, regulation, functional and clinical relevance of HLA-G expression in urological tumors as well as its use as a putative biomarker and/or potential therapeutic target for the treatment of renal cell carcinoma as well as urothelial bladder cancer.

**Keywords: HLA-G, renal cell carcinoma, epithelial bladder cancer, immune evasion, immunotherapy, immune cell infiltration**

## INTRODUCTION

During the last two decades it has been generally accepted that altered immune responses and immune evasion strategies are characteristic hallmarks of cancer. It has been demonstrated that the immune system within a tumor undergoes changes in its cellular composition, functionality and localization (1). While effector cells are often precluded from the invasive margin of tumors, immune suppressive effector cells are frequently found in this localization. Tumor extrinsic factors, like immune suppressive cells, soluble immune modulatory molecules, e.g. prostaglandin, arginase, metabolites or (anti-inflammatory) cytokines, will alter either the composition or the activity of

tumor infiltrating lymphocytes (TILs) and promote tumor growth and metastasis (2). Further changes of the tumor microenvironment (TME) include an altered metabolism resulting in a low pH, hypoxia and chronic inflammation, which are predisposing factors and implicated in modulating the immune cell repertoire and contributing to immunological dysfunction (3, 4). Defects of immune sensing mediated by the expression of inhibitory immune checkpoint receptors (ICP-R), such as e.g. the program death-1 receptor (PD-1), the CTL-associated antigen-4 (CTLA-4), T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT), T cell immunoglobulin 3 (TIM-3), V-domain Ig suppressor of T cell activation (VISTA) and the lymphocyte activation gene (LAG-3) expressed on T and/or natural killer (NK) cells, represent so far known major immune escape mechanisms (5, 6). Thus, there is an urgent need for appropriate strategies in order to revert the immune suppressive TME to a more stimulatory milieu. In addition, tumor cells themselves are constantly developing strategies to escape immune surveillance, e.g. by altering the expression of classical and non-classical human leukocyte antigens (HLAs) and immune checkpoint ligands (ICP-L) and/or ICP-R (7). This will also lead to a reduced reactivity to innate and adaptive immune responses. Thus, a plethora of distinct features of tumor and immune cells limit the treatment efficacy and clinical outcome to cancer (immuno)therapies.

Novel cancer immunotherapies, such as immune checkpoint (ICP) inhibitors and adoptive cell therapy, have been developed during the last decade and demonstrated a therapeutic efficacy in hematologic tumors, but also in some solid cancers. However, the response rates to these therapies, in particular to solid tumors, still need to be improved and only a few patients achieve durable response rates due to intrinsic and/or acquired resistances mediated by immune evasion strategies of the tumor cells. This could be driven by both cellular and molecular suppressive networks within the TME, but also within the tumor, such as e.g. the loss of tumor antigens, downregulated expression of HLA class I molecules and antigen processing machinery (APM) and interferon (IFN) pathway components as well as upregulation of various immune checkpoint (ICP) molecules, like PD-L1, B7-H4, B7-H6, LAG-3, TIM-3, VISTA, HLA-E or HLA-G (8–10).

**Abbreviations:** AIRE, autoimmune regulator; APC, antigen presenting cell; APM, antigen processing machinery; β2-m, β2-microglobulin; BC, bladder carcinoma; BCG, Bacillus Calmette-Guerin; bp, base pair; CAR, chimeric antigen receptor; ccRCC, clear cell renal cell carcinoma; CRC, colorectal carcinoma; CREP1, cAMP-responsive element binding protein 1; CTL, cytotoxic T lymphocyte; CTLA4, cytotoxic T lymphocyte-associated protein-4; DC, dendritic cell; EV, extracellular vesicle; HC, heavy chain; HIF, hypoxia inducible factor; HLA, human leukocyte antigen; ICP, immune checkpoint; ICP-L, immune checkpoint ligand; ICP-R, immune checkpoint receptor; IFN, interferon; IHC, immunohistochemistry; ILT, immunoglobulin-like transcript; IL-10; LAG3, lymphocyte activation gene 3; mAb, monoclonal antibody; MHC, major histocompatibility complex; miRNA, microRNA; MMP2, matrix metalloproteinase 2; NK, natural killer; OS, overall survival; PD1, programmed cell death-1; PD-L1, programmed death ligand 1; PFS, progression-free survival; RBP, RNA-binding protein; RCC, renal cell carcinoma; sHLA-G, soluble HLA-G; TAP, transporter associated with antigen processing; TGF-β, transforming growth factor β; TIGIT, T cell immune receptor with immunoglobulin and ITIM domain; TIL, tumor infiltrating lymphocyte; TIM-3, T cell immunoglobulin and mucin domain 3; TM, transmembrane; TME, tumor microenvironment; Treg, regulatory T cell; UTR, untranslated region; VEGF, vascular endothelial growth factor; VHL, Von Hippel-Lindau; VISTA, V-domain Ig suppressor of T cell activation; WB, Western blot.

## GENERAL FEATURES OF HLA-G - GENE STRUCTURE, EXPRESSION, REGULATION, PHYSIOLOGIC FUNCTION

HLA-G is a non-classical HLA class Ib molecule with a length of 4144 base pairs (bp) and is localized like the classical HLA class Ia molecules within the cluster of the major histocompatibility complex (MHC) on the short arm of chromosome 6 at region 6p21.3. However, it has distinct properties to HLA class Ia molecules (HLA-A, -B, -C) such as a highly restricted and tightly regulated expression, which is under physiological conditions limited to immune-privileged tissues/organs with confined local immune and inflammatory responses (11) preventing irreversible tissue damages. HLA-G expression was found on the cornea, but also on conjunctival and retinal pigment epithelial cells (12–14), insulin- and glucagon-positive cells within the endocrine islets of the pancreas (15), medullary thymic epithelial cells (16) and on extravillous trophoblasts of the placenta (17–19) protecting the fetus with its paternal antigens from maternal immune rejection (20–22). In these tissues, HLA-G contributes to immunological tolerance by acting as a ligand to inhibitory receptors expressed on several immune effector cells (23). Thus, HLA-G belongs to the few immunomodulatory proteins, whose main function lies in the mediation of a sufficient immunological tolerance even to foreign antigens. Although HLA-G is not physiologically expressed in most adult tissues, neoexpression/(re)expression of HLA-G is a frequently observed phenomenon in different cancers thereby inducing an immunological tolerance and suppression of immune surveillance (18, 19, 24). Due to alternative splicing, the HLA-G protein can exist as membrane-bound isoforms and soluble forms (25–27), which bind to inhibitory receptors of immune effector cells thereby inhibiting immune responses (28, 29): (i) leukocyte immunoglobulin-like receptor, subfamily B, member 1/LILRB1 (synonym: Ig-like transcript 2/ILT2; CD85J), (ii) leukocyte immunoglobulin-like receptor, subfamily B, member 2/LILRB2 (synonym: Ig-like transcript 4/ILT4; CD85D), (iii) killer cell immunoglobulin-like receptor, two domains long cytoplasmic tail, 4/KIR2DL4 (CD158D), (iv) killer cell lectin-like receptor, subfamily c, member 1/KLRC1 (synonym: natural killer cell lectin; NKG2A) and (v) natural killer cell receptor By55 (CD160). LILRB1 is expressed on monocytes, dendritic cells (DCs), B cells, NK cells and T cells; LILRB2 on cells of myeloid origin, KIR2DL4 on NK cells, KLRC1 on approximately 50% of NK cells and on a subset of $CD8^+$ T cells and CD160 is expressed on NK cells. Furthermore, HLA-G can be expressed and secreted from non-cancer cells, such as human mesenchymal stem cells (hMSCs). These hMSCs can inhibit both NK cell cytotoxicity and T lymphocyte alloproliferation (30). In addition, the HLA-G receptor LILRB2

(ILT4) has been shown to be expressed on hematopoietic stem cells supporting their stemness through binding to angiopoietin-like proteins (29).

The expression of LILRB1/ILT2 and LILRB2/ILT4 on different immune effector cells, endothelial cells and tumor cells and the effects of the interaction with HLA-G have been recently summarized by Carosella and co-authors (31), while the different affinities of the HLA-G receptors to HLA-G as ligand and the exact binding positions have been reviewed elsewhere (26).

Another different feature of HLA-G compared to the HLA class Ia molecules is the low number of HLA-G alleles. To date (October 2021) the IPD and IMGT/HLA database (32) enlists 88 different HLA-G alleles compared to the several thousands of different HLA-A/B/C alleles. Furthermore, alternative splicing is commonly found within the human *HLA-G* gene leading to at least seven different HLA-G protein encoding splice variants (HLA-G1-HLA-G7) reviewed by Hviid, 2006 (33). Next to the primary transcript of HLA-G (HLA-G1), containing the α-1, -2 and -3 domains as well as a transmembrane (TM) domain resulting in a membrane-bound protein, several other membrane-bound and soluble HLA-G isoforms have been described (34–37) (**Table 1**).

While the isoforms HLA-G1 to HLA-G4 encode for membrane-bound proteins, the isoforms HLA-G5 to HLA-G6 contain the unspliced intron 4, HLA-G7 the unspliced intron 2 resulting in an early stop codon and leading to the loss of the transmembrane domain of HLA-G. As a consequence, the proteins HLA-G5 to HLA-G7 are soluble and secreted thereby contributing to changes in the local and peripheral microenvironment (38). The most abundant HLA-G proteins are HLA-G1 and HLA-G5, which both consist of a heavy chain with three domains (α1, α2, α3). In both cases the heavy chains are associated with the $\beta_2$-microglobulin ($\beta_2$-m) and these complexes can even present a limited and cell type-specific peptide repertoire towards $CD8^+$ cytotoxic T lymphocytes (CTL) (39), which is not a prerequisite for their inhibitory functions towards immune effector cells.

In contrast, the other HLA-G isoforms are not bound to $\beta_2$-m lacking one or two α domains, e.g. HLA-G2 (has α1 and α3), HLA-G3 (α1), HLA-G4 (α1 and α2), HLA-G6 (α1 and α3), and HLA-G7 (α1) (33). Not only the alternative splicing, but also the proteolytical shedding of membrane-bound HLA-G proteins mediated by the matrix metalloproteinase (MMP) 2 leads to the generation of sHLA-G protein isoforms (40, 41). It is noteworthy that in addition to the functional role of membrane-bound and soluble HLA-G, the knowledge of the splicing pattern must be considered for the choice of respective antibodies for quantification and/or identification of the cellular localization. Recently, a number of antibodies directed against the different HLA-G isoforms have become available for appropriate protein detection and quantification as summarized by Krijgsman and co-authors (42). Their use in combination with antibodies staining HLA-G receptors demonstrated a heterogeneous expression leading to a fine-tuned network regulating the HLA-G-mediated immune interaction (42).

HLA-G expression is tightly regulated at different levels (**Figure 1**), such as transcriptional, posttranscriptional as well as epigenetic control. Indeed, a HLA-G promoter methylation can be frequently found in HLA-G-negative cells, which could be reversed by demethylating agents thereby inducing HLA-G transcription (43, 44). DNA methylation of its promoter is based on the covalent binding of a methyl ($-CH_3$) group to a cytosine residue in CpG dinucleotides enriched CpG islands (45). The degree of CpG methylation at the HLA-G promoter is associated with HLA-G expression (46). Chromatin immunoprecipitation demonstrated a differential histone acetylation status of $HLA\text{-}G^+$-expressing vs. HLA-G-non-expressing cells. Treatment with histone deacetylation inhibitors (HDACi), like sodium butyrate or trichostatin A (TSA), induced histone hyperacetylation, which was associated with a reversion of HLA-G repression (47). In addition to epigenetic mechanisms, HLA-G expression is transcriptionally regulated. In this context, several HLA-G relevant transcription factors have been reported. In the HLA-G expressing medullary thymic epithelial cells, the transcription factor AIRE has been identified to increase HLA-G transcription (48), but it has to be mentioned that the AIRE expression dramatically decreases by age-related thymic involution (49, 50), which could affect indirectly the HLA-G expression. Recently, the cAMP-responsive element binding protein (CREB)1 and IRF1 have been demonstrated to bind HLA-G promoter sequences leading to an increased HLA-G transcription (51–53). In contrast, RREB-1 and LINE1 repressed HLA-G gene transcription (54, 55). Furthermore, several stress stimuli have been reported to increase HLA-G transcription, including heat stress, heavy metal stress, viral/bacterial/parasitic infections [as reviewed by L. Amiot (56)] and several cytokines, such as IL-10, IL-6 and IFN-γ (46, 57, 58).

**TABLE 1** | Overview of all described HLA-G isoforms.

| HLA-G isoforms | Alternative Splicing | Effect | Reference |
|---|---|---|---|
| HLA-G1 | wild type | membrane-bound heavy chain (HC) with α-1, α-2, α-3 domain and transmembrane domain (TM) | (34–36) |
| HLA-G2 | no exon 3 | membrane-bound lack of α-2 | (34) |
| HLA-G3 | no exon 3 and 4 | membrane-bound lack of α-2 and α-3 | (34) |
| HLA-G4 | no exon 4 | lack of α-3 | (34) |
| HLA-G5 | includes intron 4 translation stops after exon 4 | no TM domain; soluble; HC with α-1, α-2, α-3 | (35) |
| HLA-G6 | includes intron 4 translation stops after exon 4 no exon 3 | no TM domain; soluble; lack of α-2 | (35) |
| HLA-G7 | includes intron 2 translation stops after exon 1 | no TM domain; soluble; lack of α-2 and -3 | (35) |
| HLA-G1L | 5 additional N-terminal amino-acids (MKTPR) | membrane-bound | (36) |

*TM, transmembrane domain; intron 5 was previously known as intron 4 according to the IMGT/HLA nomenclature.*

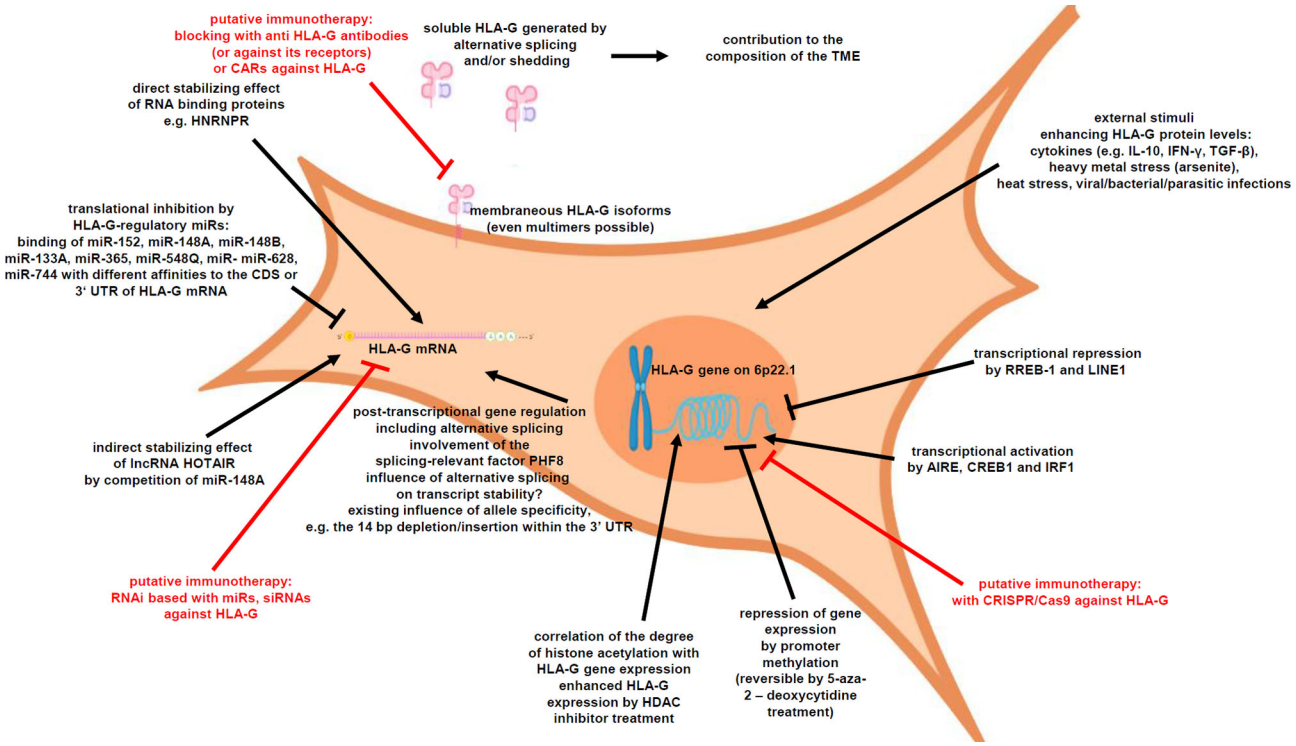


**FIGURE 1** | Underlying mechanisms of altered HLA-G expression.

Next to the transcriptional regulation, HLA-G expression is posttranscriptionally controlled by microRNAs (miRNAs), RNA-binding proteins (RBPs) and long non-coding RNAs (lncRNAs) (59–61). A number of miRNAs have been reported to directly bind to the HLA-G mRNA and interfere with the HLA-G translation leading to HLA-G mRNA decay: miR-152, miR-148A, miR-148B, miR-133A, miR-365, miR-548q, miR-628, and miR-744 (57, 62–66). These miRNAs usually bind either to the 3'-untranslated region (UTR) or in the case of miR-744 to the coding sequence (CDS) of HLA-G. An allele specificity is the 14 nt deletion/insertion within the 3'-UTR of the HLA-G mRNA in regard to the binding of regulatory factors including miRNAs (67). Recently, a distinctive site only present in the 3' UTR of HLA-G was identified, which binds RBPs and miRNAs (68). In addition, the RBP HNRNPR has been identified to regulate HLA-G, but also classical HLA class I antigens (60), while the lncRNA HOTAIR modulates HLA-G expression by competitively binding the HLA-G regulatory miR-148A and miR-152 (66, 69).

## HLA-G EXPRESSION IN SOLID AND HEMATOPOIETIC TUMORS AND ITS CLINICAL RELEVANCE

It has been recently demonstrated that HLA-G is crucial for tumor immune evasion and is also associated with malignant transformation (29). A pathophysiological expression of HLA-G was frequently detected on the cell surface of solid and hematopoietic malignancies (70). These include breast cancer, non-small cell lung cancer, esophageal squamous cell carcinoma, gastric cancer, colorectal cancer, hepatocellular carcinoma, oral squamous cell carcinoma, cervical cancer, ovarian cancer, bladder cancer, pancreatic cancer, glioma, renal cell carcinoma, and thyroid cancer as well as leukemia and lymphoma (**Table 2**). In contrast, HLA-G expression in adjacent healthy tissues has not been detected. Next to membranous HLA-G expression, sHLA-G isoforms were often elevated in plasma or serum samples of tumor patients (85, 86). In addition, HLA-G has been detected in extracellular vesicles (EV) in the supernatant of tumor cells including melanoma cells and might play a role in cancer immune escape by inhibiting immune cells in the TME even at distal sites (87).

Since HLA-G is frequently associated with an advanced tumor stage and a poor prognosis of patients, a diagnostic and prognostic potential has been suggested for HLA-G in different cancer types.

## FREQUENCY OF HLA-G EXPRESSION IN RENAL CELL CARCINOMA

### Characteristic Features of RCC

Renal cell carcinoma (RCC) is the most common kidney tumor with an incidence of 2% of tumors in the Western world (88). Based on the histology several RCC subtypes were classified, with

**TABLE 2 |** Summary of pathological HLA-G neoexpression in solid and hematopoietic malignancies.

| Tumor Entity | Cell Lines/tumor Samples/ Plasma Samples; Number of Samples | Method/Applied Antibody | Frequency of HLA-G Expression | Correlation of the HLA-G with Clinical Parameters | References |
|---|---|---|---|---|---|
| breast cancer | 235 primary breast cancer lesions; 44 plasma samples of breast cancer patients and 48 plasma samples of healthy controls | IHC (mAb HGY), ELISA | 66% HLA-G positive breast cancers; sHLA-G: 0.74 µg/mL in stage I patients, sHLA-G: 0.78 µg/mL in stage II patients; sHLA-G: 0.43 µg/mL in healthy donors | statistically significant correlation tumor size ($p = 0.0001$), nodal status ($p = 0.012$), disease stage ($p = 0.0001$), HLA-G positive patients with lower survival rate ($p < 0.028$); elevated sHLA-G levels in plasma of breast cancer patients ($p < 0.001$) | (71) |
| breast cancer | 677 early breast cancer lesions | IHC (mAb 4H84) | 60% HLA-G positive breast cancers | predictor for breast cancer patients | (72) |
| cervical cancer | 22 normal cervical tissues, 14 cervical intraepithelial neoplasia patients, 129 patients with squamous cell cervical cancer | IHC (mAb 4H84) | 0% in normal cervical tissues, 35.7% in cervical intraepithelial neoplasia, 62.8% in squamous cell cervical cancer patients | association with disease progression | (73) |
| cervical cancer | 22 normal cervical tissues, 119 primary cervical lesions; 172 plasma samples of patients with cervical cancer and 20 plasma samples of healthy controls | IHC (mAb 4H84), ELISA (MEM-G/9) | 0% in normal cervical tissues, 45% in primary cervical lesions; statistically significant higher sHLA-G levels in plasma of cervical patients (median 191.4 U/ml) versus plasma of healthy controls (median 45.18 U/ml, $p < 0.001$) | significant correlation ($p < 0.05$) to size of the main lesion, parametrical invasion and lymph node metastasis | (74) |
| colorectal cancer (CRC) | 457 primary colorectal cancer (CRC) (colon = 232, rectal = 225 lesions) | IHC (mAb 4H84) | 70.7% HLA-G positive CRC specimen | significant association with worse prognosis ($p = 0.042$) | (75) |
| colorectal cancer | 144 plasma samples of CRC patients, 60 plasma samples of healthy controls | ELISA (MEM-G/9) | statistically significant ($p < 0.01$) increased sHLA-G plasma levels in CRC patients (median 124.3 U/ml) than in healthy controls (median 25 U/ml) | no correlation | (76) |
| gastric cancer | 94 unselected patients with gastric adenocarcinoma (stage I to III) | IHC (mAb, 4H84) | 25.5% HLA-G positive gastric adenocarcinoma specimen | significant association with ($p < 0.0001$), worse survival | (77) |
| glioblastoma | 108 glioblastoma specimen | IHC (mAb, MEM-G/02) | 60.2% HLA-G positive glioblastoma specimen | negative effects on the survival rate | (78) |
| hepatocellular carcinoma | 74 primary hepatocellular carcinoma specimen | IHC (mAb, 4H84) | 31% HLA-G positive hepatocellular carcinoma specimen | no correlation | (79) |
| ovarian cancer | 169 primary ovarian carcinoma lesions with type II, high grade serous and undifferentiated | IHC (mAb 4H84) | 47.9% HLA-G positive primary ovarian cancer specimen | significant correlation with a favorable prognosis ($p = 0.038$) | (80) |
| ovarian cancer | 33 primary ovarian carcinoma lesions, 13 normal ovarian tissues | IHC (mAb 4H84) | 66.7% HLA-G positive primary ovarian cancer specimen, 0% of the normal ovarian tissues | protection from NK cell lysis (*in vitro*) | (81) |
| pancreatic carcinoma | 42 primary pancreatic carcinoma specimen | IHC (mAb, 4H84) | 66% HLA-G positive pancreatic carcinoma specimen | significant correlation with grade ($p=0.007$), stage ($p=0.038$) and poor prognosis | (79) |
| testicular cancer | 34 primary testicular cancer patients | IHC (mAb 4H84) | 20.6% HLA-G positive samples | no correlation | (82) |
| chronic myeloid leukemia | 68 chronic myeloid leukemia | ELISA (sHLA-G1, HLA-G5) | association of sHLA-G with HLA-G alleles | reduced event free survival | (83) |
| chronic lymphocytic leukemia | 45 chronic lymphocytic leukemia | flow cytometry (MEM-G/9) | 1 – 12% positive | independent prognostic factor | (84) |

clear cell (cc)RCC as major subtype with an incidence of 75 % of all RCCs, followed by papillary and chromophobe RCC with an incidence of 10 % and 5%, respectively and other subtypes accounting for less than 1 % (89). Hypertension, smoking, diabetes mellitus, chronic kidney diseases, kidney cysts, kidney transplantation are known risk factors for this disease with a clear gender imbalance (1 female patient to 1.8 male patients) (90). In addition, genetic predispositions, like mutations in tumor suppressor genes, e.g. the von Hippel Lindau (VHL) and PTEN gene, have been reported (88, 91).

During the last decades, the therapeutic options of RCC improved starting from unspecific cytokine treatment using high doses of IL-2 for lymphocyte activated killer cell generation (92), followed by application of receptor tyrosine kinase inhibitors, like sorafenib, sunitinib and axitinib, or the mTOR inhibitors temsirolimus and everolismus. More recently, therapeutic mAbs directed against ICP axes, namely the PD1 and PD-L1 axis (e.g. nivolumab, durvalumab) as well as the CTLA-4 and B7-1/B7-2 axis (e.g. ipilimumab) were developed and introduced into the treatment regimens. Actual research focuses on the identification of novel ICPs as well as the development of mAbs directed against LAG-3, TIM-3, TIGIT and other ICP-R and/or ICP-L for mono- or combination therapy (93).

It is noteworthy that HLA-G neoexpression and/or LILRB2/ILT4 expression in ccRCCs has been recently linked to aberrant expression of the vascular endothelial factor (VEGF)A and VEGFC (94). Thus, receptor tyrosine kinase inhibitors, which block predominantly the neoangiogenesis by inhibiting the tyrosine kinase domain of the VEGF receptors, might be used in combination with anti-HLA-G therapies.

## Frequency of HLA-G Expression in RCC

While HLA-G expression could not be detected in healthy renal tissue, the pathophysiological HLA-G expression in RCC lesions ranged between 30 and 60% and was found at the cell surface as well as in the cytoplasm. Furthermore, the HLA-G plasma levels were statistically significant increase in RCC patients (**Table 3**). In ccRCC, a high frequency of HLA-G mRNA and protein expression has been described, which is age and sex independent, while in other RCC subtypes HLA-G expression was not detected. **Table 3** summarizes the frequency of HLA-G expression in RCCs, which was determined by different methods including immunohistochemistry (IHC), Western blot (WB) analysis and/or PCR for HLA-G detection as well as its clinical relevance.

Omitting early reports applying only RCC cell lines, studies analyzing RCC patient cohorts could demonstrate a statistically significant correlation between HLA-G expression and higher tumor grading and staging in RCC patients using IHC and ELISA. These data suggest that HLA-G might be an interesting prognostic marker for RCC.

Furthermore, a link between the pathophysiologic HLA-G expression and the frequency and composition of the immune cell infiltration was reported (57). The HLA-G expression in RCC appears to be also associated with an altered immune cell repertoire in the TME. These TILs did not express CD25 and CD69 activation markers within the HLA-G positive group confirming the hypothesis that HLA-G expression might contribute to the immune evasion of the tumor cells by inhibition of immune effector cells. Interestingly, the HLA-G receptors LILRB2/ILT4 were detected in stromal macrophages, plasma cells and infiltrating lymphocytes of RCC samples suggesting the presence of an immune-tolerant microenvironment.

Analysis of mRNA and protein levels revealed a discordant HLA-G mRNA and protein expression pattern frequently occurred suggesting a post-transcriptional regulation of HLA-G in this tumor entity (70, 98). Furthermore, a loss of HLA-G mRNA and cell surface expression of ccRCC cells was observed during cell culture, which might be explained by the absence of TFs modulated by the hypoxic microenvironment, the lack of cytokines, such as IFN-γ, IFN-α and IL-10, or an increasing promotor methylation (96).

# HLA-G EXPRESSION IN BLADDER CANCER

Urothelial bladder cancer (BC) is the 10$^{th}$ most common tumor worldwide with a high incidence in the Western world. It is more prevalent in male than female (99). The initiation and progression of BC is a multifactorial process and comprises of immune surveillance, immune balance and immune escape (100). There exists accumulated evidence that BCs evade immune surveillance by modulating immune suppressive networks in the TME and upregulating co-inhibitory molecules like PD-L1 and HLA-G (101, 102). A heterogeneous pathophysiological neoexpression of HLA-G has been demonstrated in various stages of urothelial BC ranging from a frequency between 16.7 to 68 %, whereas adjacent normal urothelium lacks HLA-G expression (103, 104). In contrast, sHLA-G levels in serum of bladder cancer patients and healthy controls did not differ. Furthermore, higher levels of HLA-G transcripts than HLA-G protein were found in bladder cancer suggesting a posttranscriptional control comparable to that of RCC lesions. However, HLA-G-regulating mRNAs have not been yet investigated in bladder cancer (104).

In addition, repeated applications of Bacillus Calmette Guerin (BCG) can induce HLA-G neoexpression extrinsically leading to acquired resistance to further BCG-based instillation therapy (105). Few studies also demonstrated that patients with non-muscle-invasive urothelial carcinoma have an increased prevalence of peripheral blood T cells that are susceptible to HLA-G-mediated immunosuppression through co-expression of ILT2 and ILT4 (106). High peripheral prevalence of T helper cells and CTL expressing ILT2 is also associated with an increased risk of recurrence of non-muscle invasive urothelial carcinoma (107). However, no other published data currently exist on the significance of HLA-G in urothelial BC. Due to the specific immunological function of HLA-G and the overall sobering results of conventional ICP-oriented immunotherapies, further research on the role and potential therapeutic target ability of HLA-G in local and advanced stages of urothelial BC is required.

To best of our knowledge meaningful studies with sufficient BC or RCC patient cohort sizes in regard to elevated HLA-G

**TABLE 3 |** Frequency of HLA-G expression and its clinical relevance in RCCs.

| Cell Lines/tumor Samples/plasma Samples; Number of Samples | Method/Applied Antibody | Frequency of HLA-G Expression | Clinical Relevance | Study |
|---|---|---|---|---|
| 18 primary RCC lesions with adjacent renal tissue | IHC (4H84) | primary RCC: 61.1% | none | (95) |
| 37 primary RCC lesions with adjacent renal tissue; | WB (mAbs MEM-G/9 and MEM-G/1) | primary RCC lesions: 27% RCC cell | none | (96) |
| 24 RCC cell lines and 8 autologous normal kidney cells | qPCR | lines: 12.5% mRNA positive, RCC cell lines: 8.3% protein positive | | |
| 14 RCC cell lines | WB (mAb 4H84), qPCR | mRNA positive: 57%<br>protein positive: 43% | n.a. | (43) |
| 109 primary RCC lesions,<br>34 adjacent tumor negative renal tissue,<br>16 plasma samples of RCC patients | IHC/WB (mAb 4H84);<br>ELISA (MEM-G/9) | primary RCC lesions: 47.7%<br>ccRCC: 49.5%<br>chromophobe: 50% (n: 2/4)<br>collecting duct RCC: 50% (n: 3/6) RCC<br>sHLA-G in RCC patients:<br>39.5 U/ml<br>normal controls: 19.2 U/ml (P = 0.002) | none | (97) |
| 453 primary RCC lesions | IHC (mAb 4H84) | RCC samples: 49.9%<br>membranous: 38.1% cytoplasmic expression | higher frequency of stronger cytoplasmic HLA-G staining in grade 3 tumors than lower grade tumors (p = 0.014) | (57) |
| 33 plasma samples of RCC patients and healthy control group | ELISA (MEM-G/9) | sHLA-G levels in RCC (46.6 U/ml) than in HC (18.3 U/ml); (p = 0.41) | correlation of higher sHLA-G levels with advanced tumor stage and progression | Rodrigo et al., 2016 (DOI: 10.1200/JCO.2016.34.15_suppl.e16066 Journal of Clinical Oncology 34, no. 15_suppl) |

*n.a., not analyzed.*

protein levels in urine samples and its suitability as a potential prognostic marker has not yet been performed.

## INTRATUMORAL HETEROGENEITY OF HLA-G EXPRESSION IN RCC AND BC

A strong intratumoral heterogeneity exists in RCC for the expression of ICPs, such as PD-L1, B7-H3, PD-L2 and HLA-G, in primary RCC lesions with a highly variable HLA-G expression either between patients' tumor samples or at different areas within a tumor tissue (36, 108). Tronik-Le Roux and co-authors showed that ccRCC tumors were strongly, diffusely positive or negative using an antibody (ab) directed against the HLA-G alpha-1 domain (36). However, using an antibody specific for amino acids of intron 4 recognizing HLA-G without a transmembrane domain, IHC staining results were highly variable ranging from weak, negative to strong staining (36). Due to the assumption that the HLA-G α-1 domain is present in all HLA-G isoforms, the results of HLA-G expression would have been negative for patient tumors according to the α-1 specific antibody, but were strongly positive for the intron 4 (=5) specific antibody (36). Another study also showed intratumoral heterogeneity in ccRCC patients (108) with very heterogeneous staining pattern for HLA-G ranging between 37 – 70% of HLA-G throughout one tumor using IHC as well as for ILT4, which preferentially binds HLA-G. In contrast, a different RCC tumor lack HLA-G expression, but exhibit heterogeneous staining of ILT4. Interestingly, the HLA-G staining was inversely correlated to the PD-L1 staining (108). A larger study with 109 mixed RCC lesions demonstrated HLA-G expression in different RCC subtypes except for papillary RCC and control tissues. Soluble HLA-G in plasma of RCC patients showed higher expression compared to controls (97).

Immunohistochemical analysis of 75 primary bladder transitional cell carcinoma (TCC) lesions demonstrated a HLA-G expression in 51 of 75 tumors while the normal bladder lacks HLA-G expression. However, the expression varied from negative to 100% positive (103). A similar intratumoral heterogeneity of HLA-G expression was also shown in colorectal cancer (CRC) using different HLA-G specific antibodies (109).

## CLINICAL RELEVANCE OF HLA-G EXPRESSION IN RCC AND BLADDER CANCER

Due to the frequent pathophysiological HLA-G expression in solid and hematopoietic tumor entities a clinical relevance has been suggested. In **Table 2**, selected studies investigating the HLA-G expression in solid tumor entities with meaningful patient cohort sizes are summarized and correlations to clinical parameters are highlighted.

As shown in **Table 2**, a correlation of HLA-G expression with disease progression, tumor size and in some cases also with prognosis of RCC exists. It is noteworthy that the expression intensity of the HLA-G protein had an impact on clinical relevant parameters, such as tumor staging/grading, disease progression and patients' survival. In addition to alternative splicing of HLA-G transcripts, deletions, insertions and nucleotide polymorphisms of the *HLA-G* gene have been described, which are important parameters for cancer and clinical correlations (110). In a study with 56 metastatic RCC, the 14 bp insertion/deletion polymorphism in the 3' UTR was analyzed (111). A trend towards better patients outcome was demonstrated in the presence of the homozygosity for the 14 bp deletion allele, while a better patients' survival for RCC with heterozygotic T/C vs. homozygotic T/T nucleotide polymorphism at p3003 was detected. Other allelic groups of HLA-G (G*0104 and G*0103) were found to be associated with the susceptibility to urinary bladder papillary transitional carcinoma (112). Next to the HLA-G isoform expression, it will be important to analyze, whether HLA-G isoforms are present as monomers, dimers or even as homotrimers (113) associated with or without $\beta_2$-m (20) and with distinct immune suppressive activities. In particular, for the HLA-G dimer an efficient inhibition of $CD8^+$ T cells and granzyme B was shown (114). The formation of HLA-G multimers again affects the antibody-based recognition, since some mAbs are detecting the respective HC, but only in complex with the $\beta_2$-m.

In summary, HLA-G has multiple splicing mechanisms leading to different HLA-G isoforms, which are not all detected with commonly used HLA-G antibodies. A recommendation could be to use variable HLA-G antibodies in order to detect most HLA-G isoforms and draw conclusions with clinical parameters. It will also be imperative to understand the function of the different HLA-G domains and subsequently of the HLA-G multimers. These data highlight that the HLA-G protein is an interesting target for therapy based attempts for its downregulation and to target tumor cells in analogy to the anti-PD-L1 mAbs.

## ROLE OF HLA-G FOR CANCER IMMUNOTHERAPY

Due to its interactions with numerous immune effector cell populations, the HLA-G neoexpression has rekindled interest as an immune checkpoint inhibitor and suggested as a potential target. In healthy human renal tissues about 47% (+/- 12%) of the immune cells were $CD3^+$ T cells, divided in 44% $CD4^+$ and 56% $CD8^+$ T cells. About 10% of total immune cells were $CD14^+$ and $CD16^+$ myeloid cells. The frequency of NK and B cells in the kidney epithelium was 18.2% ± 10.5% and 1.4% ± 1.2%, respectively (115).

Furthermore, a large effect of the HLA-G expression significantly influenced on the immune cell infiltration of RCC. When compared to HLA-G-negative RCCs, HLA-G-positive RCCs had a statistically significant higher infiltration of $CD3^+$ and $CD8^+$ cells and a non-statistically significant higher number of $CD4^+$ and $CD56^+$ cells. However, the T cell activation markers CD69 and CD25 did not show a statistically significant difference between HLA-G-negative and HLA-G-positive RCC samples (57).

Based on these characteristics, HLA-G is postulated as a novel potential immune checkpoint in different malignancies (29) raising the question how HLA-G expression influences existing cancer immunotherapies including checkpoint inhibitors?

This question is of increasing interest and therefore currently addressed by various research groups independent of urological malignancies (31). This is due to the HLA-G-mediated inhibition of various immune effector cells of the innate and adaptive immune system.

ICPs physiologically protect against an overreaction of the immune system. Tumor cells use ICPs to escape from immune surveillance. ICPs are e.g., the programmed cell death protein 1 (PDCD-1, PD-1) expressed on T cells, its ligand CD274, the programmed cell death protein ligand 1 (PD-L1; B7H1) expressed on tumor cells and immune cells (T cells, B cells, DCs, NKs, macrophages), the cytotoxic T-lymphocyte-associated antigen (CTLA-4) expressed on T cells (T helper cells, cytotoxic T cells and regulatory T cells) and its immunological counterparts B7-1/B7-2 (CD80/CD86) expressed on antigen presenting cells (APCs: DCs, monocytes, macrophages and B lymphocytes). Several ICP inhibitors targeting CTLA4 and the PD1/PD-L1 axes have been recently approved by the EMA and/or the FDA. Their application can result in an activation of different immune cells accompanied by tumor cell elimination, leading to the remarkable success of this therapeutic approach in different cancers including RCC and BC (116–121). Recently, multiple ICPs are investigated as novel targets in experimental tumor models or in clinical trials, like LAG3, TIM-3, TIGIT, BTLA and/or agonists of the co-stimulatory receptors GITR, OX40, 41BB and ICOS (93).

Next to HLA-G and PD-L1 (B7-H1) *in silico* analyses of RCC TCGA data also identified B7-H3, B7-H5, HVEM, CD40, CD70 and ILT2 (on tumor cells) as putative novel ICP axes (122). Indeed, different innovative immunotherapies are in the clinical development for the treatment of patients with RCC. These include inhibitors of ICP, costimulatory agonists, modified cytokines, metabolic modulators, cell therapy and therapeutic vaccines (123).

However, the currently available ICPi are mostly restricted to T cells, since CTLA-4 and PD-1 are expressed on T cells (29). During pregnancy trophoblasts express and secrete members of the B7 family (B7H1/CD274/PD-L1 and B7H3/CD276) as well as HLA-G as immune suppressive proteins (124). Due to the abilities of HLA-G to inhibit various immune effector cell populations including NK cells, $CD8^+$ CTL, $CD4^+$ T helper cells, B cells and other APC, HLA-G appears to be a potent candidate for further anti-tumor immunotherapy aiming on inhibition of immune tolerance/suppression/evasion exerted by HLA-G expressing tumors.

In numerous *in vitro* studies, the downregulation of HLA-G protein levels, e.g. by overexpression of HLA-G regulatory miRs (63), by HLA-G-specific CRISPR/Cas9 systems (125) or by simple inhibition of HLA-G with antibodies (96), resulted in an increased lysis of tumor cells by immune effector cells. Recently, also chimeric antigen receptors (CARs) directed against HLA-G have been published (126).

Beside the advantages of blocking HLA-G alone or in combination with other immunotherapies including antibodies directed against the checkpoint axes PD-L1/CTLA4 the annual costs per patient receiving ICPi treatment has to be considered. Combination of an anti-HLA-G antibody or antibodies directed against the HLA-G receptors with ICPi might be challenging for financial reasons. The other molecular biological approaches modulating HLA-G expression still requires a long time before successful translation into the clinics, but might offer less expensive alternatives. Another limitation of a possible down-regulation of the HLA-G are putative side effects in regard of the physiological HLA-G expression within the immune privileged tissues. Would a long-term anti-HLA-G therapy increase the risk for irreversible tissue damages by any inflammatory reactions? What are the adverse effects by combining anti-HLA-G therapies with other immunotherapies?

Regarding antibody-based therapies, PD-L1 glycosylation has been shown to lower the binding affinity of respective therapeutic antibodies (avelumab, durvalumab, atezolizumab) (127). It is known that also HLA-G as well as ILT2 can be glycosylated (128, 129), but so far no information exists about their glycosylation pattern in RCCs or BCs, which might be associated with a possible negative effect on the affinity of anti-HLA-G antibodies.

## WHAT ARE THE FUNCTIONAL CONSEQUENCES OF HLA-G EXPRESSION FOR IMMUNE CELLS?

Soluble HLA-G released by tumor cells interacts with NK cell receptors and $CD8^+$ T cell receptors and even may cause apoptosis of immune effector cells as well as the functional inhibition of immune effector cells. HLA-G suppresses proliferation of $CD4^+$ T lymphocytes (130, 131). In addition, sHLA-G alters $CD4^+$ and $CD8^+$ cells resulting in a loss of their capacity to respond to antigenic stimulation and to their differentiation into immune tolerant Tregs (25). Tregs, DCs and tumor cells can produce and release the anti-inflammatory cytokine interleukin 10 (IL-10), which can promote the expression of HLA-G (29). Membrane-bound HLA-G can affect immune effector cells by trogocytosis, a rapid intercellular transfer of membrane fragments and their associated molecules at intercellular contact (132). In this way, HLA-G can be transferred from tumor cells to activated NK cells or to monocytes. Since transferred HLA-G remains functional, the immune effector cells with acquired HLA-G on their surface do not attack the tumor cells and even gain the capability to inhibit other immune effector cells (29). Beside HLA-G localization on tumor cells, it can also occur in EVs, e.g. in exudates or serum/plasma from cancer patients (133).

## HLA-G AND THE IMMUNE CELL INFILTRATION OF TUMORS

It has been suggested that HLA-G expression can be involved in the immune editing process, which is defined by three distinct

stages of immune responses and the interaction between tumor cells with their microenvironment: the elimination, equilibrium and escape (134). During the elimination phase, HLA-G can inhibit T and B cell activation, proliferation, cytotoxic function of T and NK cells as well as DC function. In the equilibrium phase, HLA-G can downregulate MHC class I expression and induce suppressive myeloid cells (MDSC) as well as regulatory T cells (Tregs) (135–137). The escape phase is characterized by an increased cell proliferative rate and a hypoxic environment. HLA-G has been shown to be induced by the hypoxia inducible factor (HIF)-1 and the vascular epidermal growth factor (VEGF) (94). Furthermore, immune suppressive cytokines, such as IL-10 and TGF-β, are often secreted by tumor cells. For example, in non-small cell lung carcinoma (NSCLC), a loss of classical HLA class I antigens was found to be associated with an upregulation of HLA-G as well as IL-10 expression. In ovarian cancer, HLA-G expression correlated with an elevated expression of tumor marker CA-125 and a combination of both serum markers could improve the clinical screening and diagnosis (138). Multiple studies revealed a broad immune regulatory role of HLA-G affecting innate and adaptive immune responses. Overall, the immune inhibitory mechanisms mediated by HLA-G can be summarized in three main categories: (i) direct inhibition of effector cells and antigen presenting cells, (ii) indirect immune inhibition through induction of regulatory cells and (iii) other mechanisms.

Interestingly, $CD8^+$ $ILT2^+$ T cells in the TME (tumor infiltrating lymphocytes, TIL) show a more mature and aggressive CTL phenotype with a higher cytolytic capacity compared to ILT2-negative peripheral blood precursors or $CD8^+$ $PD\text{-}1^+$ TIL. Since HLA-G is able to nearly completely block their activity, this cell pool is an interesting target to release their cytolytic capacity by therapeutic HLA-G inhibition in HLA-G expressing tumors (139). This observation might be particularly interesting for cancer entities, where HLA-G neoexpression has been associated with concomitant high immune infiltration levels, such as Ewing sarcoma and RCC (140).

Indirect immunosuppressive mechanisms are usually based on the induction of durable immune suppressive effects through the generation or induction of Tregs and/or accumulation of myeloid suppressor cells (135). HLA-G is able to impair T helper cell alloproliferation, the regular function of $CD4^+$ T cells and to induce their differentiation into Tregs to indirectly increase

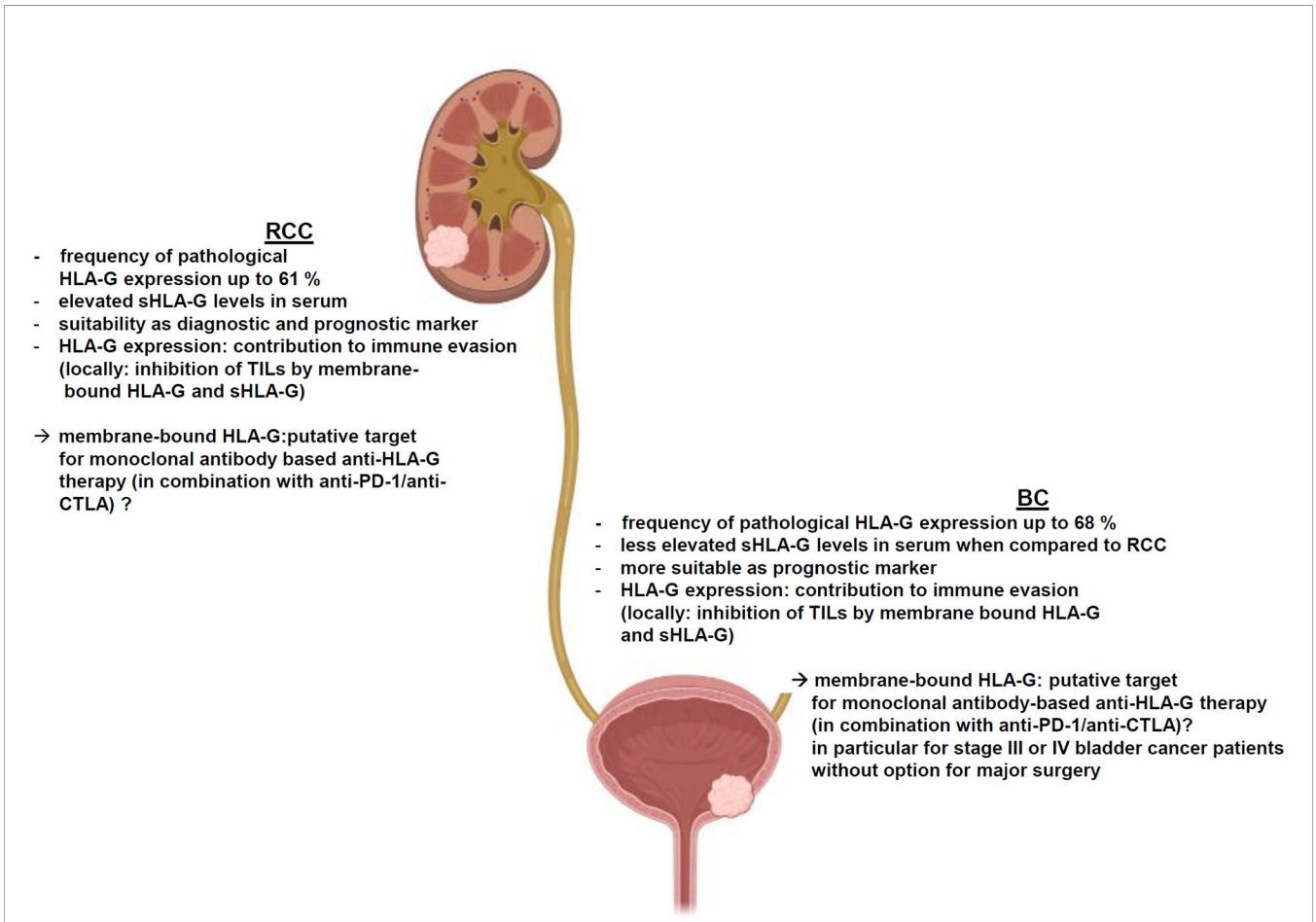


**FIGURE 2** | HLA-G-mediated immune escape of RCC and bladder cancer and its clinical relevance.

immunosuppressive effects in the TME (141). Interestingly, HLA-G-induced Tregs are dependent on HLA-G during the differentiation, but not for their immunosuppressive function (142). Transient immunosuppressive effects *via* HLA-G can also be mediated by NK cells, CTLs and monocytes/macrophages, which acquired HLA-G expressing membrane components from other cells *via* trogocytosis (132). Furthermore, HLA-G mediates immune suppression *via* so called "DC-10" dendritic cells (dendritic cells characterized by IL-10 production), which are characterized by high expression of HLA-G and other tolerogenic molecules, such as ILT2 and ILT4 (143). DC-10 cells are known as potent stimulators of allospecific type 1 Tregs, which play a crucial role in promoting and maintaining durable immune tolerance and immune suppression (58, 143). Other immune suppressive mechanisms of HLA-G include the induction of apoptosis of effector cell populations *via* sHLA-G, upregulation of immune inhibitory receptors (including KIR2DL4, ILT2 and ILT4), the suppression of IFN-γ release by NK cells (131, 144–147) and inhibition of NK cells and CTL *via* indirect induction of HLA-E expression, which can activate the inhibitory immune checkpoint NKG2A expressed on T and NK cells (148, 149). Furthermore, the HLA-G expression may be induced after infection with human papilloma viruses (150), which are known to have moderate effects on urological malignancies including RCCs and BCs (151).

## CONCLUSIONS

HLA-G is frequently, but heterogeneously expressed in both RCC and BC, which is dependent on the tumor subtype, tumor grading or staging as well as the composition of the immune cell infiltration. Unlike other regulatory ICPs, HLA-G exhibits its immune regulatory and immune suppressive functions at multiple levels of the immune response and it is able to either inhibit or stimulate key immune cell populations involved in immune responses to induce potent long-term immune suppression. Despite little information exists regarding the functional link between HLA-G expression and immune responses, an impaired NK cell- and CTL-mediated recognition of HLA-G-expressing RCC cell lines has been shown, but deserves further investigations in RCC and BC. As summarized in **Figure 2**, HLA-G contributes to the immune escape of both RCC and BC by inhibiting TIL activity due to its high frequency of expression and clinical relevance in both diseases. However, the immune regulatory and immune suppressive functions of HLA-G are considered to be much more profound and complex than those of individual co-inhibitory ICPs, such as PD-L1, CTLA-4 or PD-1, which currently serve as common targets for clinically approved immunotherapies. Recent knowledge offers insights into the underlying molecular mechanisms of HLA-G neoexpression demonstrating a role of HLA-G regulatory miRNAs in RCC. Furthermore, the TME consisting of immune suppressive cytokines secreted by either RCC and BC cells or by different immune cells might impair immune effector responses. In this context, HLA-G might serve as diagnostic and/or prognostic marker or as novel therapeutic target for both malignancies.

Therefore, investigations are urgently required to monitor membranous and sHLA-G in both malignancies in general, but also during immunotherapies of RCC and BC. This will give insights into the potential of HLA-G to serve as target for diverse immunotherapies of HLA-G-expressing tumors than singular inhibition of a less significant ICP – especially in the view of combination therapies.

## AUTHOR CONTRIBUTIONS

BS and SJ-B designed the study. BS, SJ-B, ME, HT, SW, CF, RS, and AH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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# Immunosuppressive Properties of Epidermal Keratinocytes Differ According to Their Immaturity Status

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Preservation of a functional keratinocyte stem cell pool is essential to ensure the long-term maintenance of epidermis integrity, through continuous physiological renewal and regeneration in case of injury. Protecting stem cells from inflammation and immune reactions is thus a critical issue that needs to be explored. Here, we show that the immature CD49f$^{high}$ precursor cell fraction from interfollicular epidermis keratinocytes, comprising stem cells and progenitors, is able to inhibit CD4$^{+}$ T-cell proliferation. Of note, both the stem cell-enriched CD49f$^{high}$/EGFR$^{low}$ subpopulation and the less immature CD49f$^{high}$/EGFR$^{high}$ progenitors ensure this effect. Moreover, we show that HLA-G and PD-L1 immune checkpoints are overexpressed in CD49f$^{high}$ precursors, as compared to CD49f$^{low}$ differentiated keratinocytes. This potency may limit immune reactions against immature precursors including stem cells, and protect them from exacerbated inflammation. Further exploring this correlation between immuno-modulation and immaturity may open perspectives in allogenic cell therapies.

**Keywords: immunosuppression, keratinocyte stem cells, HLA-G, PD-L1, CD4$^{+}$ T-cell proliferation**

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## INTRODUCTION

Stem cells are essential for the maintenance and renewal of tissues, thus survival of this cellular pool is critical (1). Here, we explored the question whether the degree of keratinocyte 'stemness' in the interfollicular epidermis is associated with a particular immune status through expression of immune checkpoints. Understanding the immune properties of these adult stem cells will provide information on the mechanisms protecting these cells from immune reactions, and contributing to the maintenance of skin tissue in a stress environment.

Human epidermal keratinocytes can be fractionated into functional subpopulations according to cell-surface phenotypes. Basal keratinocytes can be first selected from the total keratinocyte population based on high integrin α6 (CD49f) expression (2) or rapid adhesion on collagen (3). Then subpopulations enriched in stem or progenitor cells can be separated according to cell surface

**Abbreviations:** CD49f, Integrin α6 (ITA6); EGFR, EGF receptor; KLF4, Kruppel-like factor 4; MHC, major histocompatibility complex; HLA-G, human leucocyte antigen G; PD-L1, programmed death-ligand 1.

receptors. A first described phenotype used the level of transferrin receptor (TFR) to distinguish quiescent stem cells ($TFR^{low}$) from cycling progenitors ($TFR^{high}$) (2). The present work used the EGF receptor (EGFR) for this purpose (3). The most immature [stem cell-enriched] subpopulation ($EGFR^{low}$) was functionally characterized as endowed with a higher long-term growth and epidermis reconstruction potential, as compared to that of the less immature [progenitor-enriched] subpopulation ($EGFR^{high}$) which potential declined after short-term growth (3).

Several molecular effectors have been involved in the maintenance or loss of the stem cell state in human keratinocytes, including the transcription factor KLF4, whose expression level is critical for this cellular fate (4). More generally, a complex gene network has been linked to the regulation of stemness in human keratinocytes, involving shutdown of differentiation signals together with induction of self-renewal-promoting effectors (5). Of note, the reprogramming of human keratinocytes into iPSCs through vector-driven expression of the transcription factors KLF4, cMYC, OCT3/4 and SOX2 constituted a good model of stemness promotion (6).

Epidermis regeneration involves both immune and cell renewal properties. For example, the stemness gene *KLF4* also regulates anti-inflammatory genes in murine keratinocytes *via* an interaction with the glucocorticoid receptor (GR) (7). This regulation operates *via* the mediators of inflammation Tsc22d3 and Zfp36. The interaction between KLF4 and GR therefore plays on the balance between keratinocyte differentiation *versus* proliferation. In mice, in case of skin injury altering intercellular junctions, resident epithelial stem cells also modify their gene expression profile and recruit immune cells (DCs and Treg cells) that will in turn stimulate the proliferation of stem cells (8).

In human skin, we have recently demonstrated that keratinocytes from interfollicular epidermis inhibit allogenic $CD4^+$ T-cell proliferation, notably through the secretion of TGFB1 and the cell-surface expression of HLA-G1 and PD-L1 immune checkpoints (9). Several precursor cells, such as mesenchymal stem cells and hair follicle stem cells (10), have developed immune escape mechanisms and exhibit immunosuppressive properties likely to help ensure their survival in deleterious conditions. The present work aimed at investigating such immunosuppressive mechanisms in keratinocyte precursors of the human interfollicular epidermis. Studies in a mouse model have shown that keratinocytes expressing the immune checkpoint PD-L1 reduce the proliferation and effector function of T cells at inflammatory sites (11). Notably, PD-L1 expression was reduced in human psoriatic epidermis, which could promote the activity of effector T cells (12). PD-L1 binds to PD-1, expressed on the surface of T cells, inhibits their activity (13) and limits autoimmune reactions (14). The non-classical HLA class I molecule HLA-G is another immune checkpoint molecule originally described as permitting maternal-fetal tolerance (15). Notably, transplanted patients expressing HLA-G were significantly less prone to acute and chronic transplant rejection in solid organ transplantation such as in heart, kidney, liver and lung transplantation (16–19). HLA-G inhibits the cytolytic function of NK and T cells, the alloproliferative response of $CD4^+$ T cells, the antibody production by B cells and the antigen-presenting function of dendritic cells (20). It also induces the emergence of regulatory cells such as Tregs and MDSC. In cancer, HLA-G and PD-L1 expressed by tumor cells have been shown to inhibit different populations of T cells (21, 22).

The present work aimed at investigating whether keratinocyte stem and progenitor cells from the human interfollicular epidermis develop specific immunosuppressive properties by modifying the expression of HLA-G1 and PD-L1 immune checkpoints.

# MATERIALS AND METHODS

## Human Tissues and Cells

The present study was approved by the review board of the iRCM (Institut de Radiobiologie Cellulaire et Moléculaire, CEA (Atomic Energy Commission), Fontenay-aux-Roses, France), and is in accordance with the scientific, ethical, safety and publication policy of the CEA (CODECO number DC-2008-228, reviewed by the ethical research committee IDF-3). PBMCs were collected from healthy donors in the French Blood Bank (EFS) at Saint Louis Hospital (Paris) after informed consent was obtained. Human skin tissue from healthy adult donors was collected in the context of breast reduction surgery, after obtaining informed consent. Epidermal keratinocytes and dermal fibroblasts were extracted as previously described (4). Briefly, enzymatic treatment with a solution containing (v/v) 3/4 grade II dispase 2.4 U mL−1 (Roche Molecular Biochemicals, Mannheim, Germany) and 1/4 trypsin 0.25% (Gibco) was conducted for 24 h at 4°C. We used two categories of cells in this study: cells directly extracted from the tissue and not amplified (tissue keratinocytes), and cells extracted from the tissue, amplified and used between passage 1 and 3 in culture (amplified keratinocytes).

## Cell Culture

Amplified adult epidermal keratinocytes were obtained as follows: bulk cultures were generated in a serum-containing medium, in the presence of a feeder layer of human dermal fibroblasts growth-arrested by 60 Gy γ irradiation (23). All cultures were performed in plastic flasks coated with type-I collagen (BioCoat, Becton-Dickinson, Le Pont de Claix, France). The composition of the serum-containing medium consisted of DMEM and Ham's F12 media (Gibco, ThermoFisher, Les Ulis, France) (v/v, 3/1 mixture), 10% fetal calf serum (Hyclone, Fisher Scientific, Illkirch, France), 10 ng/mL epidermal growth factor (EGF) (Chemicon, Fisher Scientific, Illkirch, France), 5 μg/mL transferrin (Sigma, Saint-Quentin Fallavier, France), 5 μg/mL insulin (Sigma, Saint-Quentin Fallavier, France), 0.4 μg/mL hydrocortisone (Sigma, Saint-Quentin Fallavier, France), 180 μM adenine (Sigma, Saint-Quentin Fallavier, France), 2 mM tri-iodothyronine (Sigma, Saint-Quentin Fallavier, France), 2 mM L-glutamine (Gibco, ThermoFisher, Les Ulis, France), and 100 U/mL penicillin/streptomycin (Gibco, ThermoFisher, Les Ulis, France). The medium was renewed 3 times a week. For cell amplification, keratinocytes were seeded at 1000 cells/cm2 and sub-cultured weekly. Feeder cells were seeded at 5000 cells/cm2.

## Flow Cytometry Analysis

For analysis of cell-surface immune marker expression, keratinocytes were processed as single-cell suspensions and stained for 1 h at room temperature with monoclonal antibodies. The staining monoclonal antibodies used were: phycoerythrin (PE) conjugated rat anti-human CD49f (ITA6) (clone GoH3, BD Pharmingen, Le Pont de Claix, France), Alexa Fluor 405 conjugated rat anti-human EGFR (clone ICR10, Novus Biologicals, Lille, France), PE-cy7-conjugated mouse anti-human PD-L1 (clone MIH1, Thermo Fisher, Les Ulis, France), Alexa 700-conjugated mouse anti-human HLA-G (clone 87G, Novus Biologicals, Lille, France), FITC conjugated mouse anti-human TGFB (clone 1D11, R&D Systems), PE conjugated mouse anti-human IL-10 (clone B-S10, Diaclone). Non-reactive antibodies of similar species and isotype, coupled with the same fluorochromes, were used as isotypic controls. CD49f, EGFR, PD-L1, HLA-G, TGFB and IL-10 expression profiles were analyzed using an Astrios cell-sorter (Beckman Coulter, Villepinte, France) or Attune NxT (Thermo Fisher, Les Ulis, France) or MACSquant (Miltenyi, Paris, France) analyzer. Data were analyzed using FlowJo software (BD Biosciences, Le Pont de Claix, France).

## Cell Sorting

Adult epidermal keratinocytes were sorted according to CD49f and EGFR expression, using phycoerythrin (PE)-conjugated rat anti-human CD49f (ITA6) monoclonal antibody (clone GoH3, BD Pharmingen, Le Pont de Claix, France) and Alexa Fluor 405 conjugated rat anti-human EGFR monoclonal antibody (clone ICR10, Novus Biologicals, Lille, France). Appropriate isotype controls were systematically used. Cells were sorted using a FACS Aria 3 sorter (BD Biosciences, Le Pont de Claix, France).

## High-Content Imaging and Screening

Keratinocytes were plated in 96 well plates (TPP, Trasadingen, Switzerland) at a concentration of 3000 cells/cm$^2$. After 1 week of growth, cells were stained for 1 hour at room temperature using the following monoclonal antibodies: phycoerythrin (PE)-conjugated rat anti-human CD49f (ITA6) (clone GoH3, BD Pharmingen, Le Pont de Claix, France), APC-conjugated mouse anti-human PD-L1 (clone MIH1, Thermofisher, Les Ulis, France), Alexa700-conjugated mouse anti-human HLA-G (clone 87G, Novus Biologicals, Lille, France). Appropriate isotype controls were systematically used. Nuclei staining was performed using Hoescht (Thermofisher, Les Ulis, France). CD49f, PD-L1, CD40, MHC1 and HLA-G expression profiles were analyzed using AnalysisCellInsight CX7 High-Content Screening (HCS) Platform (Thermofisher, Les Ulis, France).

## Flow Cytometry-Based Analysis of CD4$^+$ T-Cell Proliferation

Keratinocytes were seeded at various ratios in 96-well culture plates (collagen-1 96-well, BD BioCoat, Le Pont de Claix, France) and incubated for 4 h at 37°C, in 5% CO2. Then, PBMCs were incubated for 1 h at 37°C, in 5% CO2 in 100 µL RPMI medium (Sigma, Saint-Quentin Fallavier, France) supplemented with 20% FCS, enriched in streptomycin and glucose. PBMCs were incubated for 20 min with a proliferation dye (eBioscience Cell Proliferation Dye eFluor 450, Thermo Fisher, Les Ulis, France), then stimulated or not by anti-CD2:anti-CD3:anti-CD28-coated beads (T-Cell Activation/Expansion Kit, Miltenyi, Paris, France, one bead per cell), and seeded at 100,000 cells per well with or without keratinocytes. PBMC proliferation was quantified by reduction in cell dye intensity after 7 days. Analysis was performed on day 7, using a flow cytometer. The ability of keratinocytes to modulate CD4$^+$ T-cell proliferation was analyzed by comparing CD4$^+$ T-cell dye intensity decrease in the presence versus absence of keratinocytes. For this, cells were stained for 20 minutes at room temperature using Viobright FITC conjugated mouse anti-human CD4 (clone REA623, Miltenyi).

## Statistics

Significant differences were assessed *via* 2-tailed Mann–Whitney U-test or t-test. All data are presented as mean ± SEM. Differences were considered significant for $p < 0.05$; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$.

# RESULTS

## Tissue Keratinocyte Precursors Inhibit CD4$^+$ T-Cell Proliferation

We investigated whether the degree of immaturity of keratinocytes modulates their immunomodulatory properties. To this end, we analyzed whether adult keratinocytes at three differentiation stages (stem cells, progenitors, or differentiated) could differentially inhibit CD4$^+$ T-cell proliferation. For this, tissue keratinocytes were sorted by flow cytometry according to their immaturity level (**Figure 1A**). Keratinocyte stem cells were defined as CD49f$^{high}$ EGFR$^{low}$, keratinocyte progenitor cells as CD49f$^{high}$ EGFR$^{high}$, and differentiated keratinocytes as CD49f$^{low}$. Isotypic controls for CD49f and EGFR are shown. Sorted cells were then incubated with activated PBMC pre-labeled with a proliferation dye (**Figure 1B**). We observed differences in CD4$^+$ T-cell proliferation inhibition according to the keratinocyte immaturity level (mean ± SEM, $p<0.05$, n=3) (**Figure 1C**). As can be seen, keratinocyte stem cells and progenitors (i.e. keratinocyte precursors) inhibited CD4$^+$ T-cell proliferation at low numbers, whereas differentiated keratinocytes did not. This CD4$^+$ T-cell proliferation inhibition increased in proportion to the number of keratinocyte precursors present (**Figure 1C**). We noticed that no effect was observed in the condition with differentiated keratinocytes, contrarily to conditions with immature keratinocytes, although responder cells and beads were the same in every condition. This result rules out steric hindrance as a mechanism for responder cell proliferation inhibition. Tissue keratinocyte precursors were therefore more effective than differentiated keratinocytes in suppressing CD4$^+$ T-cell proliferation.

Next, we determined whether keratinocyte precursors still inhibited CD4$^+$ T cell-proliferation after *in vitro* amplification. For this, we cultured tissue keratinocytes *in vitro* for 1 to 3 weeks (1 passage per week), and sorted CD49f$^{high}$ keratinocyte precursors (stem + progenitors) and CD49f$^{low}$ differentiated keratinocytes (2). (**Figure 1D**). Sorted keratinocytes were then incubated with

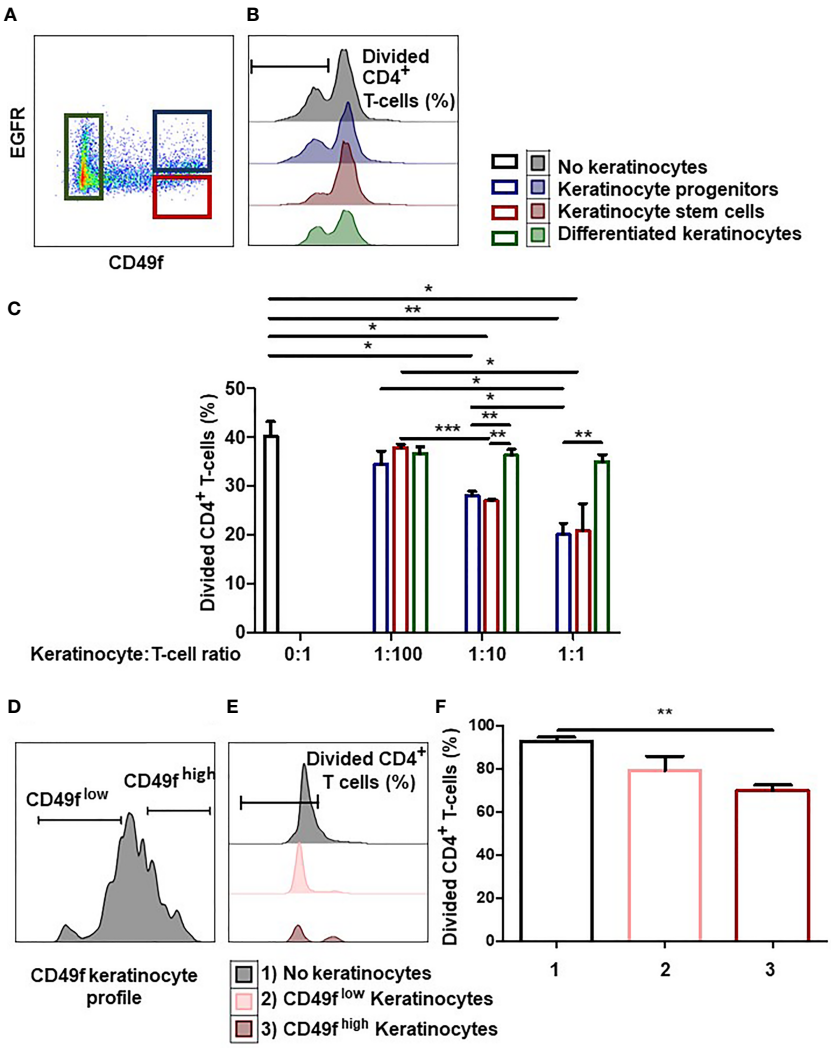


**FIGURE 1** | Keratinocyte precursors limit CD4⁺ T cell proliferation. **(A)** Representative flow cytometry profiles of keratinocytes directly extracted from the tissue and sorted according to their immaturity level visualized thanks to CD49f and EGFR stainings. **(B, C)** 1,000, 10,000 or 100,000 tissue keratinocytes (adult stem cells, progenitors or differentiated cells) from one representative donor were incubated with 100,000 PBMC for 7 days. PBMCs were pre-marked with a proliferation dye. PBMC were activated by CD3⁺ CD28⁺ beads. PBMC proliferation was quantified by dye decrease at day 7. **(B)** Representative flow cytometry profiles at day 7. **(C)** CD4⁺ T-cell proliferation inhibition according to the keratinocyte number and immaturity level (mean ± SEM, $p<0.05$, n=3). **(D)** Representative flow cytometry profiles of amplified keratinocytes sorted according to their CD49f level. **(E, F)** 10,000 amplified keratinocytes, sorted according to their CD49f expression after amplification for 7 days, were incubated with 100,000 PBMC during 7 days. PBMCs were pre-marked with a dye. PBMC were activated by CD3⁺ CD28⁺ beads. PBMC proliferation was quantified by dye decrease at day 7. **(E)** Representative flow cytometry profiles at day 7. F CD4⁺ T-cell proliferation inhibition according to the keratinocyte CD49f expression (mean ± SEM, $p<0.05$, n=3). Exact p-values were determined according to the t-test * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

activated PBMC pre-labeled with a proliferation dye (**Figure 1E**). We observed that only keratinocyte precursors suppressed CD4⁺ T-cell proliferation (mean ± SEM, $p<0.05$, n=3) (**Figure 1F**). Keratinocyte precursors therefore retained their ability to reduce CD4⁺ T-cell proliferation after *in vitro* amplification.

## Keratinocyte Precursors Overexpress the Immune Checkpoints HLA-G and PD-L1

We then investigated the mechanisms by which keratinocyte precursors inhibit CD4⁺ T-cell proliferation. By high content single-cell image analysis, we observed that 7-days amplified keratinocytes expressed HLA-G and PD-L1 immune checkpoints (**Figures 2A, C**). When HLA-G and PD-L1 were analyzed according to CD49f expression, we found that amplified keratinocytes that had the highest levels of CD49f overexpressed both HLA-G and PD-L1 (mean ± SEM, $p<0.0001$, n=3) (**Figures 2B, D**). These data were confirmed by flow cytometry (**Figures 2E, G**). Furthermore, keratinocytes were analyzed for expression of the immune suppressive cytokines IL-10 and TGFB. No IL-10 expression was observed. TGFB expression was found in all keratinocyte subpopulations, but no difference in expression levels was observed.

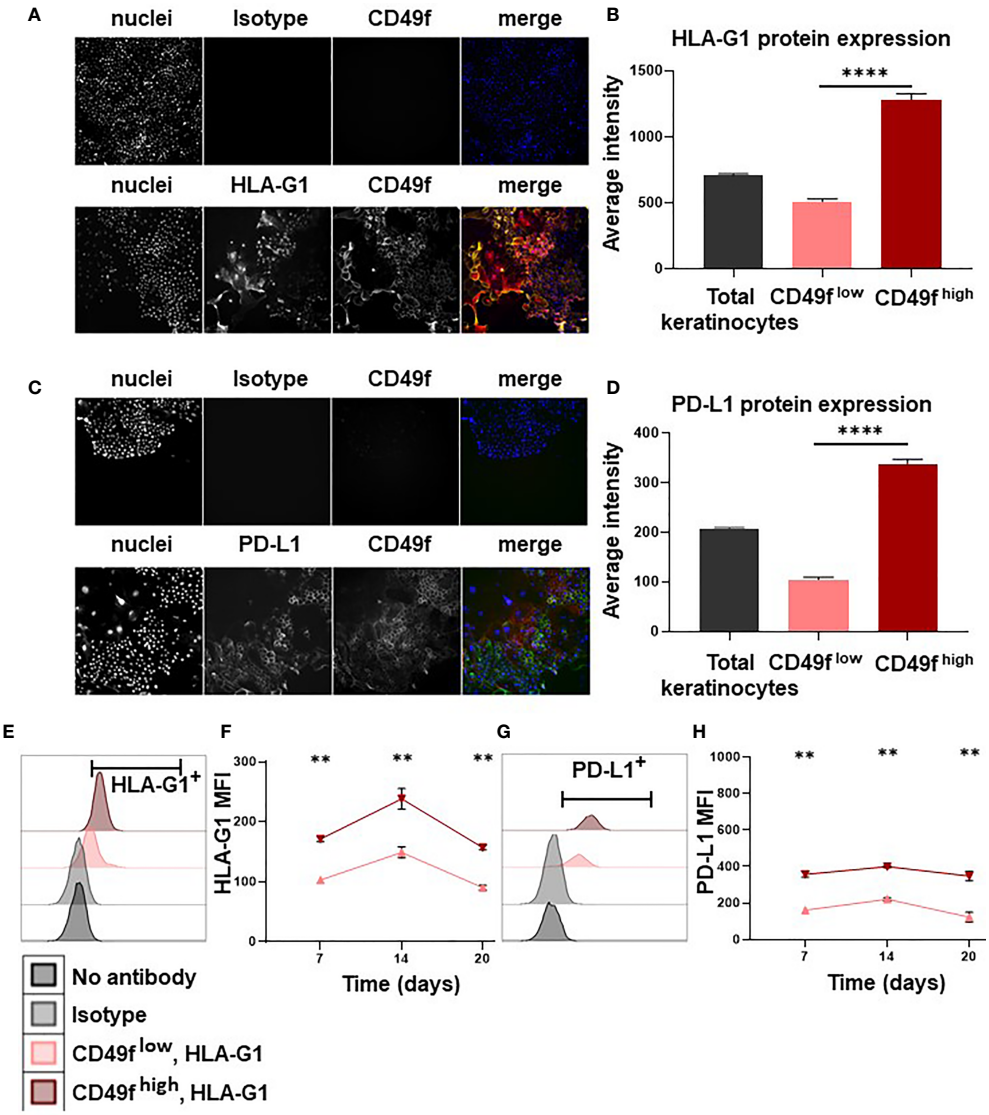


**FIGURE 2** | Keratinocyte precursors overexpress the immune checkpoints HLA-G1 and PD-L1. Cells from one representative donor were cultivated for 7, 14 or 20 days in a culture medium with serum and a layer of feeder cells. Analysis by high content single-cell image analysis. **(A, C)** Representative profiles of HLA-G1 and PD-L1 expression according to CD49f expression on keratinocytes amplified for 7 days. **(B, D)** High content single-cell image analysis of HLA-G1 and PD-L1 levels (Average intensity of fluorescence) (mean ± SEM, p<0.0001, n=1,000 cells*3 culture replicate). **(E, G)** Representative flow cytometry profiles of HLA-G1 and PD-L1 expression on keratinocytes amplified for 7 days. **(F, H)** Analysis by flow cytometry of HLA-G1 and PD-L1 expression (mean ± SEM, n=6) according to CD49f level on keratinocytes amplified for 7/14/20 days. Exact p-values were determined according to the Mann-Whitney U-test ** = $p < 0.01$ and **** = $p < 0.0001$.

(**Supplementary Figure 1**). This phenotype was stable, as $CD49f^{high}$ keratinocytes that had been amplified for 3 weeks (1 passage per week) still displayed the highest expression levels of HLA-G and PD-L1 (mean ± SEM, n=6) (**Figures 2F, H**).

## DISCUSSION

Keratinocytes obtained from donor skin samples could be separated into stem cells with an $CD49f^{high}$ $EGFR^{low}$ phenotype, progenitors with an $CD49f^{high}$ $EGFR^{high}$ phenotype, or differentiated keratinocytes with an $CD49f^{low}$ phenotype (2, 3). We show here that these populations exhibited distinct immune profiles. Compared to differentiated keratinocytes, progenitors and stem cells, whether tissue extracted or amplified in cell culture, exert immunomodulatory properties by inhibiting $CD4^+$ T-cell *in vitro* proliferation.

We have recently shown that human keratinocytes inhibit allogenic $CD4^+$ T-cell proliferation through secretion of soluble factors and cell-surface expression of HLA-G and PD-L1 immune checkpoints (9). In the present study, we refine these results by considering keratinocyte populations according to

their level of immaturity, and show that keratinocyte precursors with high levels of cell-surface CD49f overexpress HLA-G and PD-L1. Studies in mice indicated that keratinocytes expressing PD-L1 reduced the proliferation and effector function of T cells at local inflammatory sites (11). PD-L1 expression also correlated with a higher presence of Tregs in the mice skin (24). PD-L1 binds to PD-1 expressed on the surface of T cells, inhibits their activity (13), and limits autoimmune reactions (14). HLA-G inhibits the cytolytic function of NK and T cells, the alloproliferative response of $CD4^+$ T-cells, the antibody production by B cells and the antigen-presenting function of dendritic cells (20, 25). Notably, HLA-G expression was also described in other immature cell types, such as mesenchymal stem cells, osteoblasts, chondroblasts, erythroid progenitors and hepatic stem cells (26). It has been proposed that stem cells are preserved from immune attacks, a phenomenon called immune privilege (10). This is in accordance with a recent study demonstrating that, unlike mature endothelial cells, their progenitors [Endothelial progenitor cells (EPCs) or Endothelial Colony-Forming Cells (ECFCs)] were immunosuppressive and that this was linked with HLA-G, IL-10 and TGFB expression (27, 28). Overexpression of PD-L1 and HLA-G on keratinocyte precursors may support this immune privilege by protecting them against a prolonged abnormal immune response, such as in auto-immunity and chronic inflammation (9).

In this regard, murine hair follicle stem cells downregulate Nlrc5 and MHCI in their quiescent state (10). Expression of Nlrc5 upregulates MHC1 on murine hair follicle stem cells, so its downregulation explains that of the MHC1. The alteration of this immune privilege is one of the causes of alopecia areata (29). This pathological context was described as an autoimmune disorder, in which an abnormal infiltration of T cells causes local inflammation and destruction of anagen hair follicles (30). Expression of MHC by keratinocytes promotes the maintenance of autoreactive T cells directed against hair follicles (31). In psoriasis, another autoimmune pathology, epidermal cells are renewed every 3 to 5 days rather than 28 to 30 days in normal conditions (32). It would be interesting to investigate if this high renewal is linked to immune checkpoints expression, knowing that HLA-G and PD-L1 are expressed in psoriatic skin (33, 34). Investigating the expression of immune checkpoints on keratinocyte precursors in hair follicles could therefore provide a better understanding of their immune privilege and autoimmune-associated pathologies.

The immunosuppressive properties exerted by keratinocyte progenitors may also be involved in the development of skin cancer. Adult keratinocyte stem cells can drift into cancer cells, leading to cutaneous squamous cell carcinoma or basal cell carcinoma development (35). Tumor growth is known to be enhanced by cancer cell ability to escape elimination by the immune system (36). HLA-G and PD-L1 inhibit different populations of T cells in cancer (21, 22), and therefore critically contribute to tumor escape from immunosurveillance. PD-L1 expression and targeting were particularly well documented in squamous cell carcinoma (37). As immunotherapies are increasingly used to direct the body's immune system against tumor (38), overexpression of PD-L1 and HLA-G by keratinocyte precursors should be considered in understanding the function of skin tumor initiator cells.

Finally, the immunosuppressive properties of keratinocyte precursors could be beneficial for skin repair therapy. Human keratinocytes derived from the hair follicle can promote healing (39) or be used to treat leg ulcers (40). We postulate that the use of keratinocyte precursors from the interfollicular epidermis, engineered to express high levels of HLA-G and PD-L1, could promote skin graft tolerance and open perspectives for their use in allogeneic settings for cell therapy (9). Indeed, the generation of allogeneic skin grafts presents a double stake. On the one hand, the maintenance of epidermal stem cells with high regenerative potential, and on the other hand, obtaining an immunocompatible graft.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethical research committee IDF-3. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

GM designed the study, performed experiments, analyzed data, wrote the manuscript. EDC, NR-F, MM, NF, and JL designed the 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.2022.786859/full#supplementary-material

**Supplementary Figure 1 |** Expression of immunomodulatory cytokines by keratinocyte precursors. Analysis by flow cytometry. **(A)** Expression of IL-10 by keratinocytes. **(B)** Expression of TGFB by keratinocytes according to their immaturity level (mean ± SEM, n=6). Exact p-values were determined according to the Mann-Whitney U-test.

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# Role of HLA-G in Viral Infections

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The human leukocyte antigen (HLA)-G is a non-classical HLA class I molecule, which has distinct features to classical HLA-A, -B, -C antigens, such as a low polymorphism, different splice variants, highly restricted, tightly regulated expression and immune modulatory properties. HLA-G expression in tumor cells and virus-infected cells, as well as the release of soluble HLA-G leads to escape from host immune surveillance. Increased knowledge of the link between HLA-G expression, viral infection and disease progression is urgently required, which highlights the possible use of HLA-G as novel diagnostic and prognostic biomarker for viral infections, but also as therapeutic target. Therefore, this review aims to summarize the expression, regulation, function and impact of HLA-G in the context of different viral infections including virus-associated cancers. The characterization of HLA-G-driven immune escape mechanisms involved in the interactions between host cells and viruses might result in the design of novel immunotherapeutic strategies targeting HLA-G and/or its interaction with its receptors on immune effector cells.

**Keywords: human leukocyte antigen G, viral infection, immune escape, virus-induced tumors, interleukin 10**

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## INTRODUCTION

Accumulating evidence exists that immune suppressive mechanisms play a critical role in promoting viral infections by either suppressing the capacity of infected host cells to overcome viral infection or by preventing the elimination of virus-transformed cells by immune effector cells. A common mechanism to escape immune surveillance is a loss or downregulation of classical HLA class Ia antigens and the neoexpression of non-classical HLA class Ib antigens, such as HLA-E, -F and –G (1–3). While the expression of HLA class Ia antigens leads to a T cell-mediated control of host immune responses mediated by antigen presentation and recognition, neoexpression of HLA-G has immune modulatory properties by inhibiting both innate and adaptive immune responses thereby leading to an immune escape of virus-infected cells. Although diverse viruses exploit HLA-G to establish persistent infections, the underlying molecular mechanisms tremendously differ. In

**Abbreviations:** APC, antigen presenting cells; $\beta_2$-m, $\beta_2$-microglobulin; CDS, coding region, coding sequence; CTL, cytotoxic T lymphocyte; DC, dendritic cell; HC, heavy chain; HCMV, human cytomegalo virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HHV, human herpesvirus; HLA, human leukocyte antigen; HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; HTLV-1, human I-lymphotropic virus type II; IAV, influenza A virus; IL, interleukin; MDSC, myeloid-derived suppressor cell; miRNA, microRNA; MSC, mesenchymal stem cells; NK, natural killer; NPC, nasopharyngeal carcinoma; PD1, programmed death receptor 1; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SNP, single nucleotide polymorphism; TGF-β, transforming growth factor β; Treg, regulatory T cell; ULBP, UL16-binding protein; URR, upstream regulatory region; UTR, untranslated region.

this review, the expression, regulation, and clinical impact of HLA-G neoexpression in the context of viral infections as well as the underlying molecular mechanisms will be summarized.

## FEATURES OF THE HLA-G

The non-classical HLA-G gene is located on the short arm of chromosome 6 and consists of 8 exons, 7 introns, a 5' upstream regulatory region (URR) and a 3' untranslated region (UTR). The primary HLA-G transcript is alternatively spliced into at least 7 mRNAs, which encode 4 membrane-bound (HLA-G1, -G2, -G3, -G4) and 3 soluble (sHLA-G; HLA-G5, -G6, -G7) protein isoforms (4). Each HLA-G isoform contains 1-3 extracellular domains (α1, α2, α3), which are encoded by exon 2, 3 and 4; exons 5 and 6 encode the transmembrane and cytoplasmic domain of the heavy chain (HC), while the presence of intronic sequences are highly variable (5). The HLA-G1 and sHLA-G5 isoforms have a comparable structure to classical HLA class Ia antigens (HLA-A, -B, -C) and contain the HLA class I HC, which is non-covalently linked to $\beta_2$-microglobulin ($\beta_2$-m) (6–9). Furthermore, a peptide is bound in the cleft of the α1 and α2 domains, while the α3 domain of both the membranous and the sHLA-G is bound by the CD8 co-receptor (10). Similar to the classical HLA class Ia molecules, HLA-G is capable of presenting a broad repertoire of peptides and requires peptide binding to be efficiently presented on the cell surface (11–13). The other HLA-G isoforms lack one or two extracellular domains, are smaller and not associated with $\beta_2$-m. HLA-G1 to HLA-G4 are membrane-bound and have a transmembrane region. In contrast, HLA-G5 and HLA-G6 are soluble isoforms with the presence of intron 4, which contains a premature stop codon thereby preventing the translation of the transmembrane and cytoplasmic residue. HLA-G7 is also a soluble isoform due to the presence of intron 2 containing a pre-mature stop codon. Recently, a number of novel HLA-G isoforms have been identified, which could be generated by skipping exon 6 and by a deletion of the α1 domain (8, 14). It should be noted that sHLA-G1 could be generated by a metalloproteinase-dependent shedding (15). Furthermore, membrane-bound HLA-G can also circulate within the blood stream, in particular in form of extracellular vesicles (10).

## PHYSIOLOGICAL EXPRESSION AND IMMUNE SUPPRESSIVE ACTIVITY OF HLA-G

HLA-G expression is generally restricted to immune privileged tissues, such as cytotrophoblasts, cornea and pancreatic islets (16–18). However, HLA-G neoexpression was detected under pathophysiological conditions, such as cancers, inflammatory diseases, auto-immune diseases and pathogen infections including viruses (19). It is known that HLA-G has immune suppressive properties by interacting with different inhibitory receptors, namely ILT2/LILRB1, ILT4/LILRB2 and KIR2DL4, which are expressed on various immune cell subpopulations (20–22). Other receptors have been recently discovered, which are able to bind HLA-G, in particular NKG2A (23), CD160 (24) and CD8 (25) thereby interacting with the α3 domain of the respective HLA-G isoforms. The structures of the HLA-G isoforms, their receptors including their HLA-G-binding domains and the cell types expressing these receptors are summarized in **Figure 1**. The interaction of these receptors with HLA-G leads to the inhibition of the cytolytic function of natural killer (NK) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), macrophage-mediated cytotoxicity, allo-proliferative response of $CD4^+$ T cells and of the maturation as well as function of dendritic cells (DCs).

However, it needs to be underlined that the interactions of HLA-G with immune effector cells are even much more diverse and complex as summarized so far, since also indirect immune modulatory effects of HLA-G on immune effector cells are known. Recently, it could be demonstrated that the non-classical HLA-E molecule can present peptides derived from HLA class Ia, but also from HLA-G leader sequences (26–30). HLA-E is a ligand for the inhibitory CD94/NKG2A,-B receptors and for the activating CD94/NKG2C receptor expressed on NK cells (31). This indirect mechanism modulates the immune effector mechanisms depending on the context of the engaged receptors. For example, the HLA-G-derived nonamer VMAPRTLFL presented on HLA-E molecules caused an enhanced NK cell-mediated lysis in an *in vitro* experiment of transfected 721.221 cells naturally lacking HLA class Ia/b molecules thereby representing valuable targets for NK cells. However, it needs to be addressed in much more detail whether this effect also plays a role *in vivo*, especially in the context of sHLA-G molecules within the TME of solid tumors inhibiting immune effector cells even prior to tumor infiltration.

In addition, immune suppressive cells are stimulated to secrete cytokines, like transforming growth factor (TGF)-β and interleukin (IL-10), which are able to increase HLA-G expression (32–35). Furthermore, the interaction of HLA-G with its receptors could lead to long-term immune modulatory effects by inducing and/or accumulating regulatory T cells (Tregs) (36), mesenchymal stem cells (MSCs) (37, 38) and myeloid-derived suppressor cells (MDSCs) (39, 40). In addition to the direct interaction of HLA-G with its appropriate receptors, the HLA-G-mediated immune suppression could be also caused by intercellular transfer mechanisms, such as trogocytosis, exosomes or tunneling nanotubes, which also leads to escape from the destruction by the host immune system.

## POLYMORPHISMS AFFECT THE EXPRESSION OF HLA-G

So far, more than 88 different HLA-G alleles (41) have been discovered. Within its coding region, HLA-G shows a limited protein variability compared to classical HLA class Ia, but both the 5' URR and the 3' UTR contain a multitude of polymorphic sites affecting gene regulation (42). The major HLA-G

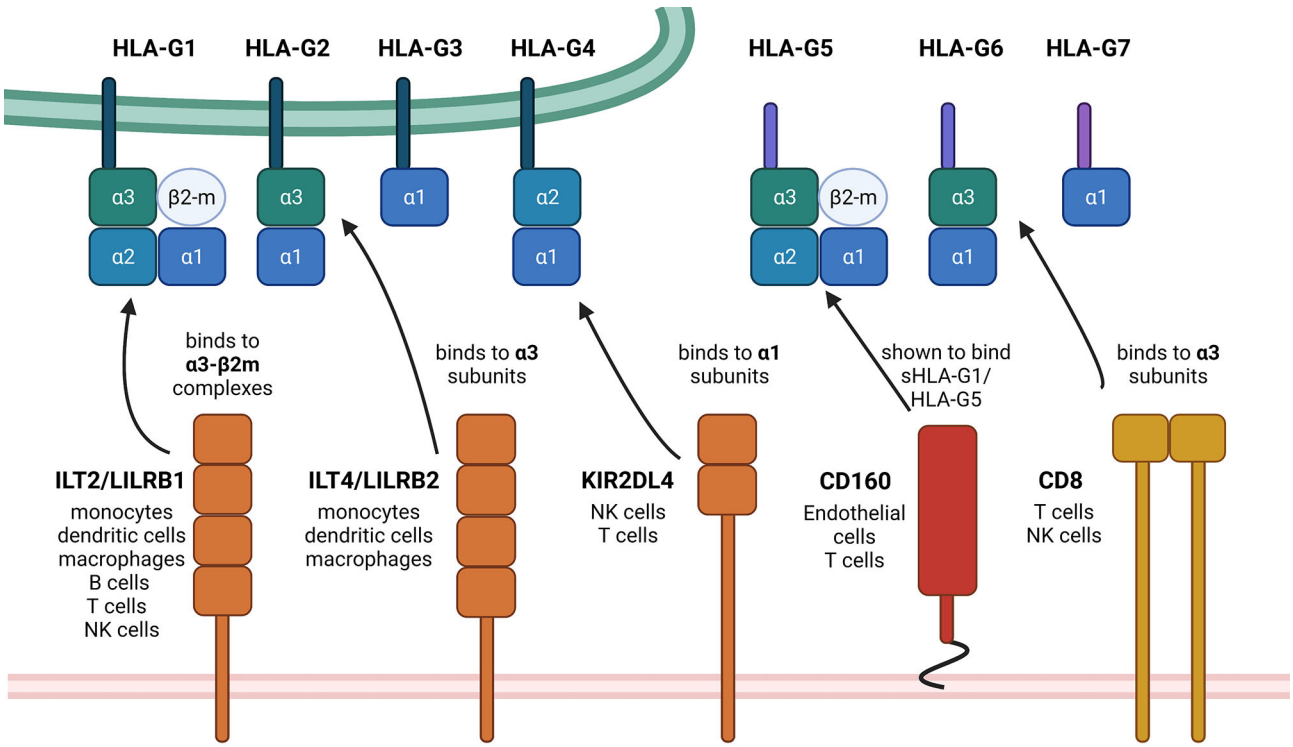


**FIGURE 1** | Summary of HLA-G isoforms with their binding positions for the so far known receptors. This schematic summary of the structural differences of HLA-G isoforms shows the so far known HLA-G receptors and also designates the exact binding position. The black arrows show exemplarily, but not exclusively possible interactions, depending on mentioned binding positions on the HLA-G ligands. There exist first evidence of a possible interaction between NKG2A with HLA-G, which still needs to be investigated in more detail. Therefore, NKG2A is not yet mentioned within this scheme. Created with BioRender.com.

polymorphisms associated with viral infections and their role in virus susceptibility are summarized in **Table 1**. Response elements for diverse transcriptional inducers have been identified, like the progesterone response elements (PRE), heat shock elements (HSE), interferon-stimulated response elements (ISRE) and elements responding to cyclic AMP (CRE) (50–54). In addition, negative regulators have been identified like the ras-responsive elements (RRE) (55). Many polymorphisms and single nucleotide variants within the regions of such response elements have been described (56, 57). *In vitro* evaluations of the effects of the most frequent 5' URR haplotypes have indeed shown differential transcriptional regulation of HLA-G (58, 59). In addition, to the 5' UTR, diverse polymorphic sites exist also in the 3' UTR of the HLA-G transcript (60); many of them have been shown to change the affinity of gene-targeted sequencing for transcriptional or post-transcriptional factors, affecting splicing (61), microRNA (miRNA) binding (62) and RNA turnover (63). A variation of major significance for HLA-G expression is a 14 bp insertion/deletion (ins/del, rs66554220) in the 3' UTR, which is associated with the alternative splicing of HLA-G and miRNA stability. Furthermore, the region downstream of the 14 bp ins/del polymorphic site has been

**TABLE 1** | Impact of HLA-G polymorphisms on viral infections and virus-mediated diseases.

| Virus | Polymorphism/allele | Regulatory impact |
|---|---|---|
| HPV | +14 bp/+14 bp | significant association with an increased risk for HPV18 infection (OR = 12.95, P < 0.01) (43) |
| | +14 bp/-14 bp | increased risk for HPV58 infection (OR = 5.55, P < 0.05) (43) |
| | +14 bp/G | significant increase in HPV18-infected patients (60.0% vs 27.3%, OR = 4.00, Pc < 0.05) (43) |
| | frequency of the allele +3142 C | significant decrease in HPV-infected patients compared with normal controls (43) |
| | frequency of the genotype +3142 CC | significant decrease in HPV-infected patients compared with normal controls (43) |
| | 130C del | association with an increased progression and reduced overall survival of NPC patients (44) |
| CMV | +3142 CC genotype | association with a higher susceptibility to CMV infection after kidney transplantation (45) |
| HIV | HLA-G*010108 allele | association with a 2.5-fold increased risk of HIV-1 infection (46) |
| | 14 bp del/del | significantly reduced rates of perinatal HIV transmission (47) |
| HTLV-1 | 14 bp del/del | higher proviral load (48) |
| SARS-CoV-2 | SNP rs9381042 | correlation with severe COVID-19 infections (49) |

suggested to be a target for HLA-G-specific miRNAs thereby leading to a direct downregulation of HLA-G. However, there exist controversial data regarding the role of the HLA-G 14 bp ins/del polymorphism and susceptibility to viral infections (64). Of note, population studies suggest that HLA-G haplotypes are strongly shaped by selective pressure throughout evolution thereby preserving protein-coding sequences and enabling divergence in regulatory sequences (42, 65).

## REGULATION OF HLA-G EXPRESSION AND VIRAL INFECTIONS

There exist more than 220 viral species able to infect humans (66) and annually even more human pathogenic viruses are identified or even emerge including the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (67). In 2008, before the SARS-CoV-2 pandemic started, Woolhouse and co-authors generated *in silico* prediction tools for estimating the emergence of novel human pathogenic viruses concluding that the formation/discovery of novel human viruses must be anticipated in public health planning and the future should prove this statement (68).

Infection with intracellular pathogens, such as viruses, could lead to the presentation of pathogen-relevant peptides, e.g. *via* HLA class I molecules to T cells resulting in T cell activation and elimination of the infected host cell, although numerous other molecular mechanisms also contribute to anti-viral immune surveillance. These include the induction of the MICA/MICB molecules as well as the UL16-binding proteins (ULBP)1-6, which are physiologically not expressed, but induced by cellular stress and viral infections and act as ligands for the activating NKG2D receptor present on NK cells and CTLs (69). In addition, intracellular, membranous and even secreted pattern recognition receptors exert a strong role in anti-viral immune surveillance. A prerequisite for appropriate immune effector cell functions is the recruitment of these immune effector cells to the virus-infected cells, a process, which involves a functional chemokine signaling. Indeed, the authors recently reviewed the distinct viral mechanisms targeting the above mentioned strategies involved in immune recognition and elimination of viral infections (70).

However, several human pathogenic viruses can successfully develop immune evasion by various molecular mechanisms, including the induced expression of immune checkpoints, like PD-L1 and HLA-G, in the virus-infected host cells (1, 71–73). In this review, we will summarize known examples of human viruses able to induce immunological tolerance towards host immune effector cells by HLA-G induction. Therefore, an overview of the tightly regulated HLA-G expression will be provided.

HLA-G is predominantly expressed in immune-privileged tissues, in particular placental and eye tissues (17, 74), but also insulin- and glucagon-positive cells within the endocrine islets of the pancreas (18) and the medullary thymic epithelial cells (75) and locally induce immunological tolerance thereby preventing tissue damages due to inflammatory reactions. The HLA-G gene expression itself is regulated at multiple levels, including the grade of promoter methylation (76), the acetylation of histones (77), the existence of transcriptional activators (CREB1, AIRE) (78, 79) as well as the lack of transcriptional repressors (RREB-1, LINE1) (55, 80). In addition to the transcriptional control, several factors contribute to a complex posttranscriptional gene regulation of HLA-G including many miRNAs, such as miR-148 family members with the highest affinity to the 3' UTR of the HLA-G transcript and miRNA directed against the coding sequence (CDS) of HLA-G (81, 82) as well as long non-coding RNAs like HOTAIR (83). Furthermore, RNA-binding proteins (RBPs), like e.g. HNRNPR, have been reported with regulatory potential for the HLA-G mRNA (84).

Several cytokines, but also certain stress stimuli are known to enhance the HLA-G levels, like interferon (IFN)-γ, IL-10, TGF-β (76), hypoxia and heat stress (52, 85).

This complex regulatory network for the control of the HLA-G expression can be altered to induce or enhance the immune evasion of tumor cells, but also of infectious pathogens, e.g. viruses, bacteria and parasites (86). It is noteworthy that a number of viruses have an oncogenic potential and independently of viral infections a pathophysiological HLA-G neoexpression, which can be detected with high frequencies in virus-associated malignancies as well as independently of viral infections in solid and hematopoietic tumor diseases (87–90).

## ROLE OF HLA-G IN VIRAL INFECTIONS – EXPRESSION, MECHANISMS AND CLINICAL RELEVANCE

### HLA-G and Human Papillomavirus

Human papillomavirus (HPV) infection is a common infection and has been linked to epithelial cancers including in particular cervical and head and neck cancers (91–93). HLA-G has an impact on the clinical course of persistent HPV infections, epithelial cell transformation, tumor growth, metastasis, formation and therapy resistance. In HPV-associated tumors like cervical cancer and head and neck squamous cell carcinoma (HNSCC), a role of HLA-G in HPV infections and in the initiation and progression has been described (94, 95) mediated by polymorphisms, methylation and deregulation of HLA-G (44).

HLA-G polymorphisms are genetic susceptibility and/or disease-relevant factors for cervical HPV infections and viral persistence of cervical cancers (**Table 1**). Most of the studies focused on the polymorphisms in the 3' UTR of the HLA-G gene rather than on its promoter region or in its CDS. The HLA-G +14/G and +3142G alleles are risk factors for HPV infections and increase the risk of high-grade cervical lesions and are associated with cervical carcinogenesis (43). Interestingly, the HLA-G promoter methylation was not associated with the HPV infection status (96). Moreover, a codon 130C deletion was associated with an increased progression and reduced overall survival of patients with nasopharyngeal carcinoma (NPC) (97).

In comparison to HPV-negative HNSCC, higher HLA-G expression levels were found in HPV-positive HNSCC with HLA-G7 as the most frequent isoform (98). In addition to various HLA-G polymorphisms, which have been associated with the susceptibility of HPV infection, an influence of HLA-G on the immune modulation of HPV-positive HNSCC has been described. Furthermore, high levels of IFN-γ, but lower levels of IL-10, TGF-β, SOCS1/3 and programmed death receptor 1 (PD1) were found in HPV-positive HNSCC (98).

## HLA-G and Hepatitis Virus

Infections with hepatitis B (HBV) and C virus (HCV) are major health threats worldwide (99, 100). Chronic HBV/HCV infection is followed by chronic hepatitis, which might lead to liver cirrhosis and hepatocellular carcinoma (HCC) (101). Despite the host immune response is crucial for the control of HBV and HCV, both viruses have developed different strategies to escape immune surveillance including HLA-G neoexpression (102). Indeed, a high frequency of HLA-G expression was found in tissues of HCV-infected patients. In addition, elevated sHLA-G serum levels were detected in patients with chronic HCV infection (103, 104). Furthermore, HLA-G expression was more prominent in high fibrosis specimens. In association with the increased HLA-G expression in liver, an enhanced inflammatory activity and liver fibrosis were demonstrated, which might have implications for progression and prognosis of liver diseases. HLA-G expression in the liver upon chronic HCV infection might be due to the composition of the inflammatory infiltrate, in particular of mast cells known to induce fibrosis by stimulating the proliferation of TGF-β-secreting hepatic stellate cells as demonstrated by Amiot and co-authors (105). In addition, hepatic stellate cells also secrete IL-10, which is able to upregulate HLA-G expression. Thus, the liver microenvironment including cytokines affecting the inflammatory response and fibrosis, such as IL-10 and TGF-β, are responsible for the induction of liver HLA-G expression in chronic hepatitis-diseased patients (104).

As described for HCV-infected tissues, HLA-G is expressed at a high frequency in HBV-infected liver biopsies, but not in control hepatocytes (106). Furthermore, sHLA-G levels were also significantly higher in HBV-infected patients when compared to healthy controls (15, 107, 108). The HBV-mediated induction of HLA-G expression in hepatocytes might be caused by modulation of the hepatocyte expression of miRs, such as downregulation of the HLA-G-regulating miR-152 representing the most HLA-G mRNA affine member of the miR-148 family leading to neoexpression of HLA-G (109). These data further suggest that HLA-G and HLA-G-specific miRs are involved in HBV-induced HCC.

## HLA-G and Human Cytomegalovirus

Human cytomegalovirus (HCMV) causes a life-long human infection, which may be life-threatening for immune-suppressed patients (110, 111). Like other viruses, HCMV has developed different strategies to escape immune surveillance including the modulation of HLA-G expression (112). Membrane-bound HLA-G was shown to be expressed in macrophages and monocytes undergoing lytic infection, while sHLA-G levels are increased in serum of patients with acute HCMV infection (113–115). Furthermore, an enhanced HLA-G expression has been associated with allograft tolerance after kidney transplantation (116). The single nucleotide polymorphism (SNP) (3142C>G) in the HLA-G gene of the recipient, but not in the transplant donor was associated with a higher susceptibility to HCMV infection after kidney transplantation. In addition, sHLA-G levels were associated with a higher susceptibility to HCMV infection (**Table 1**). These data suggested that both the recipient HLA-G+3142CC phenotype and sHLA-G levels represent predictive risk markers for HCMV infection (45). Recently, an association between other HLA-G 3' UTR variants and kidney graft outcomes has been reported. In recipients with stable allograft function, significantly higher sHLA-G levels were found in patients who were +3010GG, +3142CC, +3187GG and +3196CC carriers in comparison to acute rejection recipients (117). Thus, there exists a direct association between this HLA-G 3' UTR variants and sHLA-G levels in kidney recipients leading to graft acceptance. Therefore, monitoring of sHLA-G levels prior to transplantation might serve as suitable marker to predict kidney graft outcome. This was confirmed by a recent study demonstrating that the acute and chronic rejection rate of kidneys increased 1.06 times and 1.14 times, respectively, in kidney transplant recipients with low serum sHLA-G levels. The frequency of acute rejection was lower in patients with a 14 bp del/del polymorphism than that of ins/ins and ins/del polymorphisms. Based on these results, the HLA-G 3' UTR polymorphism and the sHLA-G levels might represent useful markers for the prediction of rejection in kidney transplants (118).

## HLA-G and Human Herpesvirus 6

Human herpesvirus (HHV)-6 is a β-herpesvirus comprising of the two viruses HHV-6A and HHV-6B (119–121) that cause both productive and life-long latent infections (122). HHV-6A/B induce HLA-G expression in mesothelial cells leading to impaired NK cell functions against virus-infected cells (123). HHV-6A/B express the viral protein U94, which has key functions in the viral life cycle and elicits immune responses. Furthermore, U94 has been shown to induce HLA-G expression by upregulating the expression of the transcription factor ATF3, which activates HLA-G expression and release (124). In line with these findings, patients suffering from systemic sclerosis showed elevated sHLA-G levels when being co-infected with HHV-6A. Furthermore, viral load correlated to NK cell dysfunction and disease severity (125).

## HLA-G and Epstein-Barr Virus

Another member of the HHVs, the Epstein-Barr virus (EBV), has also been reported to induce the HLA-G expression by yet undefined molecular mechanisms (126). It could be speculated that the EBV-encoded viral IL-10 (vIL10), a known agonist of the human IL-10, might be an inducer of the HLA-G expression. Interestingly, EBV infections also affect the epigenetic control within the host cell genome, including altered DNA methylation patterns (127) as well as aberrant histone modifications (128),

both mechanisms known as important regulators of HLA-G gene expression. It is noteworthy that EBV infections can be linked to various tumor entities, such as NPC, gastric adenocarcinoma (GC), classical Hodgkin lymphoma (cHL) and Burkitt lymphoma (BL) (129), which have been shown to exhibit pathophysiological HLA-G neoexpression.

## HLA-G and Human Immunodeficiency Virus

The human immunodeficiency virus (HIV) is a RNA virus. Several studies suggested elevated sHLA-G in serum samples of untreated HIV patients, but membrane-bound HLA-G is also detected on immune cells of patients. Furthermore, HIV infection is not only inducing HLA-G on monocytes, macrophages and T lymphocytes (130), but also causes an increased HLA-E expression in infected $CD4^+$ T helper cells (131), which exerts immune modulatory functions comparable to that of HLA-G thereby contributing to immune evasion of HIV-infected cells (131). HLA-E is the ligand for the inhibitory NK cell receptors CD94/NKG2A, -B and for the activating -C expressed on NK cells and CTL (132). HIV infection also induces the expression of the immune modulatory ligands PD-L1 and PD-L2 on macrophages (133), which interact with their receptor PD-1. The PD-L1/PD-1 interaction causes an inhibition of CTL activity, but also an increased IL-10 secretion, which contributes to immune evasion of HIV-infected $CD4^+$ T helper cells (134).

The HIV-related induction of HLA-G expression has been currently well characterized. Certain HLA-G alleles increase the risk for heterosexual transmission of HIV in African women. Indeed, the HLA-G*010108 allele was associated with a 2.5-fold increased risk of HIV-1 infection and the two HLA-G*010108 alleles containing genotypes HLA-G*010108/010401 and G*010101/010108 were associated with an elevated risk of HIV-1 infection (**Table 1**) (46). Furthermore, children carrying the homozygous HLA-G genotype with the 14 bp deletion within the HLA-G 3'-UTR exhibited significantly reduced rates of perinatal HIV transmission (47, 135).

The overexpression of HLA-G in HIV-infected individuals may be secondary due to an increased release of IL-10. The HIV-encoded protein gp41 has been identified to induce IL-10 secretion in monocytes (136) thereby revealing a possible mechanism for the occurrence of HLA-G.

In addition, HIV infection causes a downregulation of the classical HLA class Ia molecules HLA-A and HLA-B (137), a known mechanism for (tumor) immune evasion, clearly demonstrating a deregulation of appropriate HLA class Ia/b signaling upon HIV infection.

## HLA-G and Human T Cell Lymphotropic Virus Type I

The human T cell lymphotropic virus type 1 (HTLV-1) known to induce very aggressive adult T-cell lymphoma (ATL) also upregulates HLA-G expression, mainly HLA-G1 and HLA-G5 (138), and in analogy to HIV, the homozygous HLA-G genotype −14-bp/−14-bp genotype has a higher proviral load than the +14-bp/−14-bp and +14-bp/+14-bp genotypes (**Table 1**) (48). However, a detailed mechanism for the HTLV-1-mediated HLA-G increase has not yet been identified, but an IL-10-based mechanism analogous to the HIV-dependent HLA-G induction might be likely.

## HLA-G and Influenza A Virus

Influenza A virus (IAV) causes acute respiratory infections (139). Infections with IAV are known to induce HLA-G mRNA and protein expression in alveolar epithelial cells (140), which is a major inhibitory molecule of host immune responses to IAV infections. Although the detailed underlying mechanisms have not yet been determined, the study suggested an involvement of cytokines, in particular IFNs, which could cause the HLA-G neoexpression mediated by the viral encoded NS1 protein.

## SARS-CoV-2 and HLA-G

In 2019, a novel corona virus emerged that caused a worldwide pandemic and lead to the potentially life-threatening severe acute respiratory syndrome (SARS-CoV-2) (67). In patients with severe infection, high serum levels of sHLA-G were observed (141) and HLA-G was also found on the cell surface of diverse immune cells during the course of infection. HLA-G was detected on monocytes, T and B cells and showed a high-low-high pattern, probably correlating with infection, replication and clearance phase (142). However, these results were only compiled from a single patient and require further validation. Another clinical study found a correlation between high sHLA-G levels and improved disease outcome probably due to immune-dampening effects of HLA-G that suppress an excessive tissue-damage, possibly mediated by reduced neutrophil infiltration to the sites of infection (143). Disease outcome was not clearly linked to HLA-G variants despite a meta-analysis listed the HLA-G 3'UTR SNP rs9381042 as a candidate variant that was slightly overrepresented in a UK cohort of critically ill patients due to COVID-19 infection compared to the general population (**Table 1**) (49).

# CONCLUSIONS

As summarized in this review, multiple viral infections are known to induce membranous HLA-G expression as well as its secretion in infected host cells. In some cases, the role of HLA-G in infections has been characterized to contribute to immune evasion of the infected host cells by inhibition of immune effector cells. So far, the detailed molecular mechanisms of this HLA-G neoexpression have not yet been identified. In many of these studies a correlation of the HLA-G neoexpression with elevated IL-10 levels is reported. Indeed, IL-10 is a known inducer of the HLA-G expression and has also been described in tumor cells independently of viral infections as inducer of HLA-G expression. Therefore, the very likely IL-10-based hypothesis of the virus-driven HLA-G induction is summarized in **Figure 2**.

Physiologically, IL-10 exerts its functions as an anti-inflammatory cytokine in preventing inflammatory and autoimmune pathologies by inhibition of certain immune effector cells thereby preventing inflammation-induced and

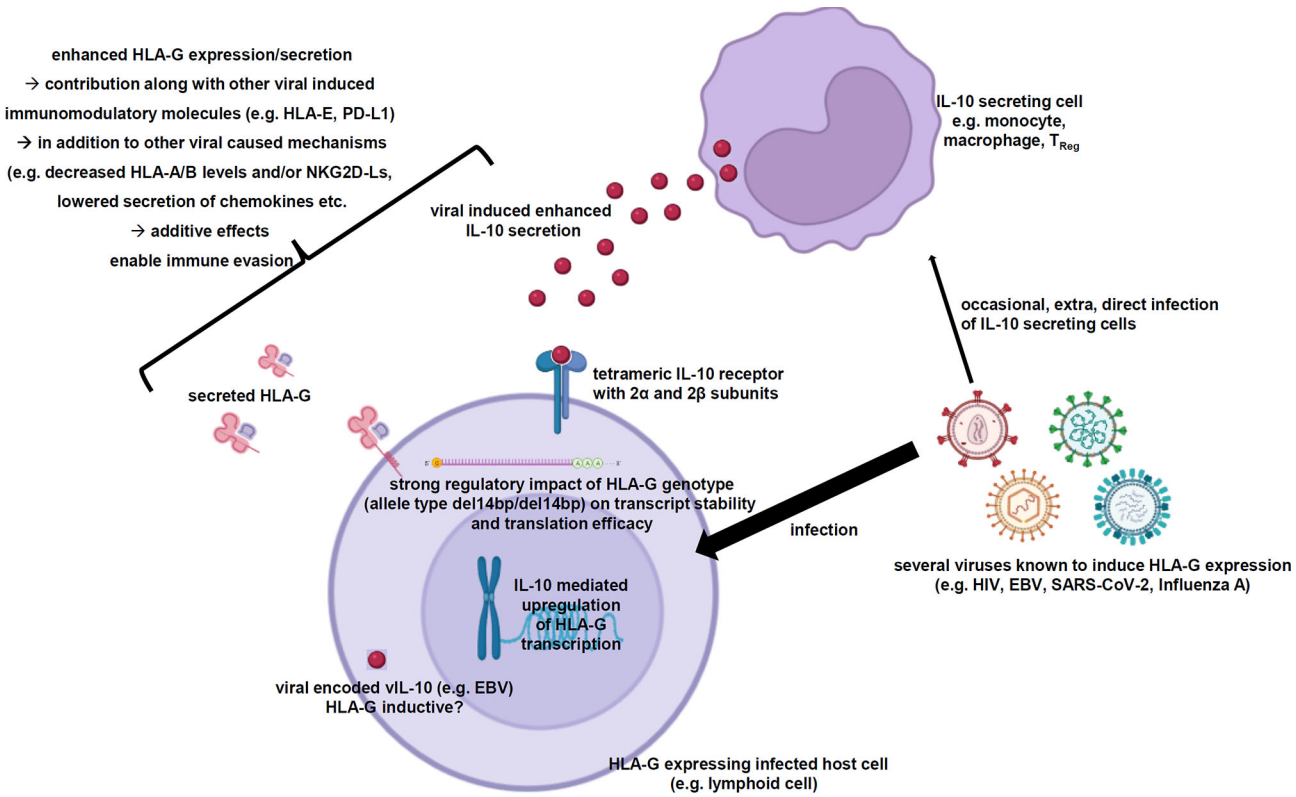


**FIGURE 2** | Scheme summarizing the virus-induced enhanced IL-10 secretion resulting in elevated HLA-G expression and secretion. The working hypothesis demonstrates the elevated IL-10 secretion of certain immune effector cells as consequence of viral infections, which is very likely the main driver of the increased HLA-G gene expression and secretion within the virally infected host cells strongly contributing to immune evasion. Created with BioRender.com.

autoimmune pathologies (144). Murine *in vivo* models inhibiting the IL-10R demonstrated that the viral infection with lymphocytic choriomeningitis virus resulted in a rapid resolution of the persistent infection. This blocked IL-10 signaling was also linked to an increased IFN-γ secretion by $CD8^+$ T cells (145). Another murine study with IL-10-deficient mice could also demonstrate a better anti-viral T cell response against the lymphocytic choriomeningitis virus (146).

The predominant IL-10-expressing cells are Th2 cells and Tregs, but also macrophages, dendritic cells, eosinophils and neutrophils are known to secrete IL-10 (144). Some of these immune cell populations were directly infected by HLA-G-inducing viruses and subsequently shifted towards increased IL-10 secretion. In other cases, indirect effects, like e.g. an increased PD-L1/PD-1 signaling, caused an enhanced IL-10 secretion of these immune effector cells.

Once the HLA-G gene transcription has been induced upon IL-10 stimulation, the homozygous genotype del14bp/del14bp has additional beneficial effects due to an enhanced HLA-G mRNA stability by avoiding miRNA-based downregulation and thereby resulting in increased HLA-G protein levels.

It is also known that IL-10 stimulation affects the RNA expression profile in IL-10-stimulated cells (147), but whether HLA-G-regulating miRs are downregulated upon IL-10 signaling is so far unknown. In addition, further direct or indirect effects of IL-10 on other HLA-G-regulating factors are rather limited with the exception of the transcriptional activator CREB1, which induces the transcription of HLA-G and IL-10 (78, 148). Thus, the regulation of HLA-G expression upon viral infection is complex and further studies are urgently needed to gain deeper insights into the molecular mechanisms of the viral infection-driven HLA-G neoexpression.

## AUTHOR CONTRIBUTIONS

SJ-B and BS designed the study. SJ-B, DS, OM, and BS wrote the manuscript. SJ-B and DS created the figures. All authors contributed to the article and approved the submitted version.

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# HLA-G and the MHC Cusp Theory

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Human leukocyte antigens (HLA) are significant genetic risk factors in a long list of diseases. However, the mechanisms underlying these associations remain elusive in many cases. The best-characterized function of classical major histocompatibility complex (MHC) antigens is to allow safe presentation of antigenic peptides *via* a self/non-self-discrimination process. Therefore, most hypotheses to date have posited that the observed associations between certain HLA molecules and human diseases involve antigen presentation (AP). However, these hypotheses often represent inconsistencies with current knowledge. To offer answers to the inconsistencies, a decade ago we have invoked the MHC Cusp theory, postulating that in addition to its main role in AP, the MHC codes for allele-specific molecules that act as ligands in a conformationally-conserved cusp-like fold, which upon interaction with cognate receptors can trigger MHC-associated diseases. In the ensuing years, we have provided empirical evidence that substantiates the theory in several HLA-Class II-associated autoimmune diseases. Notably, in a recent study we have demonstrated that *HLA-DRB1* alleles known to protect against several autoimmune diseases encode a protective epitope at the cusp region, which activates anti-inflammatory signaling leading to transcriptional and functional modulatory effects. Relevant to the topic of this session, cusp ligands demonstrate several similarities to the functional effects of HLA-G. The overall goal of this opinion article is to delineate the parallels and distinctive features of the MHC Cusp theory with structural and functional aspects of HLA-G molecules.

**Keywords: human leukocyte antigen (HLA), HLA-G, Cusp theory, antigen presentation (AP), HLA-disease association, immunity**

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## INTRODUCTION

The major histocompatibility complex (MHC), known in humans as HLA (human leukocyte antigen) is a cluster of genes nested in three regions, designated as Class I, Class II and Class III. Class I and II genes encode for molecules best known for their role in presentation of antigenic peptides through a self/non-self-discrimination process (1). Paradoxically, however, in spite of this meticulous self-protecting mechanism, particular HLA alleles and haplotypes are some of the most significant genetic risk factors in numerous human diseases (2). Over the decades, several hypotheses have been put forward to explain the mechanistic basis of the associations. Most of the salient hypotheses are based on the best-known function of HLA molecules: antigen presentation (AP). Among them: (*i*) Molecular mimicry with foreign antigens (3). (*ii*) Failures in T cell repertoire selection (4); (*iii*) Reactivity to altered self-antigens (5); (*iv*) Association through linkage disequilibrium (LD) (6). Although progress has been made (7), most hypotheses have not

been yet empirically validated, and AP flaws as a sole cause are incongruent with current knowledge, as we previously discussed (8, 9).

Given the limitations of AP-based hypotheses, a decade ago we have proposed an alternative theory (8, 9), postulating that in addition to their main role in AP, MHC molecules also express allelic epitopes, which activate immune modulatory pathways through interactions with cell surface receptors. Depending on permissive background genes and environmental influences, these otherwise physiologic pathways can excessively activate cellular events that lead to disease onset. We branded the new theory 'MHC Cusp' based on our prior finding that an epitope coded by a rheumatoid arthritis (RA)-associated *HLA-DR1* third allelic hypervariable region acts as a signaling ligand that can cause immune dysregulation and lead to autoimmune arthritis in experimental mouse models (10–23). The term 'cusp' was chosen based on the conformational properties of the epitope, which is located near a cusp-like prominence that is a common MHC fold feature shared by antigen presenting, as well as non-antigen presenting members of the MHC family. Since its postulation, we have provided empirical evidence in support of the MHC Cusp theory in RA, and more recently in two other diseases associated *DRB1* alleles. Directly relevant to the focus of this review, we have recently identified a new cusp ligand that is encoded by RA-protective *DRB1* alleles. That allelic cusp region, which we designated as protective epitope (PE), has the amino acid sequence DERAA in the cusp region (residues 70-74 of the DRβ chain), and activated anti-inflammatory transcriptomes as well as signaling events that culminate in immune modulatory effects (23). Being an HLA ligand that activates immune tolerance effect, the PE demonstrates resemblance to some of the widely documented HLA-G effects.

In the following segments, we review structural and functional aspects of classical versus the non-classical HLA molecules, discuss salient mechanistic theories of HLA-disease association, expend on the MHC Cusp theory and its parallels, as well as distinguishing features with functional and structural features of HLA-G ligands, and potential of harnessing these parallels for future exploration of new therapeutic strategies.

## HLA MOLECULES IN HEALTH AND DISEASE

### Structure and Canonical Functions of HLA Molecules

The HLA complex is a cluster of genes located on the short arm of chromosome 6 p21.3, that encode for MHC molecules in humans (24). The HLA is one of the most polymorphic segments in the human genome, having more than 45 genes accounting for over 25,000 currently known alleles (25). The HLA consists of three major regions, termed class I, containing classical (Ia) genes (*HLA-A, -B, -C*), as well as the nonclassical HLA (Ib) genes (*HLA-E, -F, -G, -H*), The HLA class II region contains the classical class II genes (*HLA-DRA, -DRB1, -DRB2, -DRB3, -DRB4, -DRB5, -DQA1, -DQA2, -DQB1, -DQB2, -DPA1, -DPB1*) and other, less variable, genes (*HLA-DMA, -DMB, -DOA, -DOB*). The class III region contains complement factor genes (*e.g. C2, C4*) and other genes related to inflammation, leukocyte maturation and immune response (*e.g. TNF, HSPA1*) (26).

HLA class I and II genes encode for transmembrane glycoproteins best known for their role in presentation of antigenic peptides (1). The MHC class I molecule is typically composed of one α heavy chain, containing two peptide-binding domains (α1, α2), one immunoglobulin-like domain (α3), a transmembrane region and a cytoplasmic tail, as well as a light chain, a non-HLA encoded low molecular protein, named Beta-2 microglobulin ($\beta_2$m) (27). The MHC class II molecule is composed of two heavy chains (α, β), each containing: a peptide-binding domain (α1 and β1, respectively), immunoglobulin-like domain (α2 and β2, respectively), a transmembrane region and a cytoplasmic tail (27). Three-dimensional structures of MHC Class I and II molecules determined by x-ray crystallography revealed a similar fold and peptide-binding groove between the two classes of molecules (28, 29).

MHC Class I molecules are expressed on the cell surface of human nucleated cells and typically present short endogenous peptides to cytotoxic CD8 T cells (30, 31). MHC class I molecules may also present exogenous peptides, in a process called "cross-presentation" (32). MHC class II molecules are expressed on the surface of professional antigen presenting cells (APCs), such as macrophages, dendritic cells, and B cells. They typically present exogenously synthesized antigens to CD4 T cells (33, 34).

HLA-G molecules are best known for their immunomodulatory properties and their involvement in maternal–foetal and organ transplantation immune tolerance (35). They act as ligands and have the ability to bind to inhibitory receptors expressed by a variety of immune cells (36). HLA-G molecules exert inhibitory effects on both innate and adaptative immune responses, such as inhibition of natural killer (NK) cells, as well modulation of B and T cell function (37). Their interaction with immunoglobulin-like receptors, such as receptor Ig-like transcripts 2 and 4 (ILT2, ILT4) and killer cell immunoglobulin-like receptor (KIR) KIR2DL4 expressed by peripheral NK cells, B- and T lymphocytes and monocytes, leading to inhibitory function has been well described (36, 38). HLA-G has also been shown to increase apoptosis of activated $CD8^+$ T cells and NK cells, as well as to reduce chemotaxis by downregulation of chemokine receptor expression in T cells (36). Moreover, its expression can be transactivated by the *AIRE* (Autoimmune Regulator) gene, the main regulator of negative selection in the thymus, which suggests a possible involvement of this molecule in autoimmunity (39). In physiologic conditions, HLA-G molecules display tissue-restricted expression. However, under certain pathological circumstances - such as viral infection, malignancy, transplantation, oxidative stress, hypoxia, inflammatory and autoimmune disorders - the expression of these molecules can be upregulated by interferons, interleukin 10, and other factors, such as microRNAs (36, 40, 41), and favour anti-inflammatory Th2-type responses, possibly in order to protect tissues against excessive inflammatory aggression (42). It is important to add that pro-inflammatory

responses have also been described for HLA-G molecules, and the HLA-G homodimer has been shown to induce secretion of the proinflammatory cytokines interleukins 6 and 8, as well as tumor necrosis factor alpha from both decidual macrophages and NK cells (43).

## Classical Versus Non-Classical MHC Molecules

Classical MHC genes are highly polymorphic, particularly in the peptide-binding domains. Polymorphisms are thought to be an adaptative evolution in response to pathogen exposures, driving selection on MHC, with resultant allele frequency changes in different populations (44). While classical class I genes (*HLA-A, -B, -C*) are highly polymorphic and mainly present antigenic peptides to CD8T cells, the nonclassical major HLA class Ib genes (*HLA-E, -F, -G, -H*) exhibit lower polymorphism and limited tissue distribution, and are typically recognized by alternative immune receptors or, are only expressed under certain stimuli (45), having important immunoregulatory and nonimmunological functions, such as stimulation or inhibition of natural killer (NK) cells (46). The nonclassical MHC class I genes are considered evolutionary conserved, and in addition to interaction with CD8 and NK cells, non-classical MHC molecules also interact with receptors such as CD94/NKG2 to modulate immunity (47).

HLA-G shows 86% amino acid similarity with classical class I genes, with the main difference being due to the occurrence of a stop codon in exon 6, resulting in a shorter protein, compared to classical class I molecules (48). HLA-G exhibits low polymorphism (49, 50), and a currently known polymorphic diversity of only 88 alleles, including 4 null and 2 questionable alleles (51). Seven isoforms of HLA-G produced by alternative splicing of the primary transcript (50) have been described, four of which are membrane-bound (HLA-G1, -G2, -G3, and -G4) and three of which are soluble molecules (HLA-G5, -G6, and -G7) (52). Additionally, HLA-G1 may form a soluble dimer. It was also proposed that novel HLA-G isoforms may be formed in specific conditions, such as carcinomas (36, 53). While HLA-G1 has the classical class I domains: ($\alpha$1, $\alpha$2, $\alpha$3) and is associated with $\beta_2$m; HLA-G2 is composed of $\alpha$1 and $\alpha$3 domains only, and HLA-G3 is composed of only $\alpha$1. HLA-G4 has domains $\alpha$1 and $\alpha$2; HLA-G5 and HLA-G6 are soluble isoforms and have the same extracellular globular domains of their membrane-bound counterparts: HLA-G1 ($\alpha$1, $\alpha$2, $\alpha$3, associated with $\beta_2$m) and HLA-G2 ($\alpha$1, $\alpha$3), respectively (54), **Figure 1**.

Despite the significance of HLA-G in immunomodulation, pregnancy maintenance and graft survival, surprisingly little is known about the molecular structure and dynamic behavior of the HLA-G isoforms (54). In fact, to date, the Protein Data Bank (PDB) has only made available a few crystal structures related to HLA-G1 (54, 55). Recently, with the use of structural modeling, Arns et al. (54) have provided insight into the structure and dynamic behavior of the membrane-bound HLA-G1, as well as for the soluble HLA-G1 dimer. The authors demonstrated a "tilting" movement for membrane-bound HLA-G and have argued that the location of major leukocyte receptor binding sites in the $\alpha$3 domain, close to the membrane, could restrict these interactions with ILT2 and ILT4 receptors. By contrast, full rotational freedom was demonstrated for soluble HLA-G1 dimer, which would allow a large conformational flexibility for this molecule in solution, resulting in larger exposure of the binding sites and additional binding possibilities, as compared to its membrane-bound counterpart, which could explain the observed higher affinity of soluble HLA-G1 dimer to ILT2 and ILT4 receptors as compared to the membrane-bound HLA-G1 (54). These findings illustrate the structural diversity of HLA-G molecules, with associated impact on their functional versatility.

In MHC Class I molecules, the $\alpha$1 and $\alpha$2 domains fold into an oval-shaped groove that can accommodate short peptides (usually 8-10 amino acid residues). Class II molecules are formed jointly by two different gene products, $\alpha$ and $\beta$ chain and have a peptide binding groove surrounded by two parallel helical structures ($\alpha$1 and $\beta$1), which can accommodate larger peptides (commonly 13-25 amino acid residues) (28).

**Figure 2** shows a comparison of the three-dimensional structures of HLA-G1 and HLA-DR molecules. Of note, is a similar cusp-shaped fold in the $\alpha$2 domain of HLA-G1, and the $\beta$1 domain of the HLA-DR molecule, despite their markedly different chain composition, and considerable evolutionary distance. This suggests that the respective conformationally homologous regions may have conserved their common shape through evolution to retain a fundamental functional advantage that this region provides. Below we discuss the possibility the cusp region has retained its shape through evolution to preserve important ligand functions.

The MHC system is not without flaws, and HLA-associated diseases have been described since the 1960s. Over the years it has been realized that the HLA region in the human genome has the greatest known number of associated diseases, such as type 1 diabetes (57), RA (58), Alzheimer's disease (59), schizophrenia (60), and many others (2). Recently, a study evaluated HLA type-I genotypes with outcomes in course of COVID-19, and demonstrated a high risk for death of adults with the presence of the *HLA-A*01:01* allele (61), highlighting the importance of the HLA phenotype also in acute illnesses. Although genes outside of the MHC locus also encode antigen-presenting molecules (62), this review will focus on HLA-related molecules.

# HLA-DISEASE ASSOCIATION - CANONICAL HYPOTHESES

The HLA complex is one of the most extensive and polymorphic gene groups in the human genome. It has undergone extensive refinements through evolution to assure effective protection against foreign organisms on the one hand, while preserving the health of the host, on the other. Paradoxically, however, it is the very same group of genes that is implicated as risk factors in a long list of diseases that affect every organ system and tissue in the body. How has it happened that a safeguard that has been so meticulously perfected to police and defend the body against disease turned into a culprit? This enigma, which has been at the focus of scientific debate over the last 5 decades, has generated

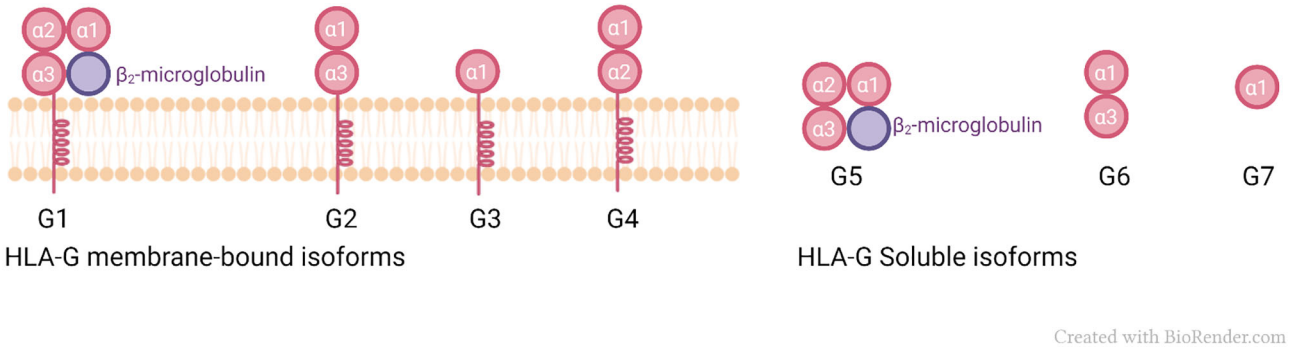


**FIGURE 1** | Membrane-bound and soluble isoforms of HLA-G.

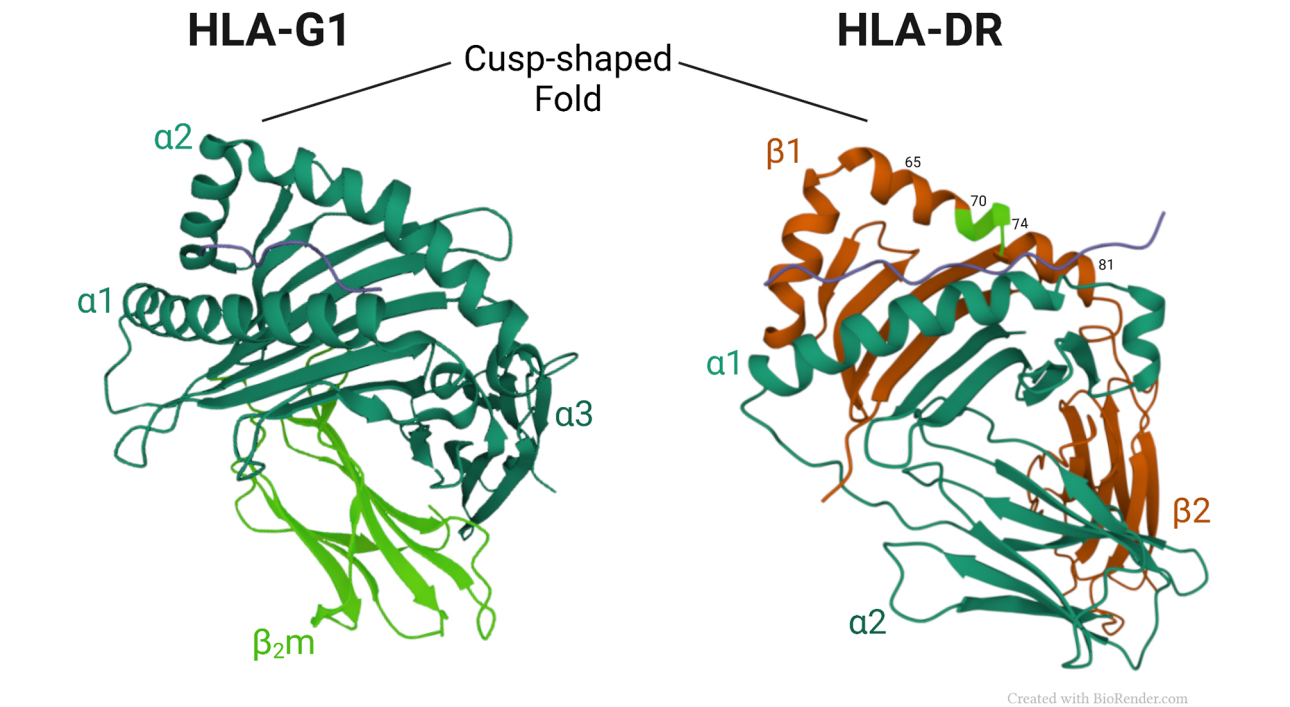


**FIGURE 2** | Crystal structures of HLA-*G1* (pdb, 3KYN) and *HLA-DR* (pdb, 1A6A) (56), focusing on their respective cusp folds. Amino-acids at positions 70–74 of the DRβ-chain are highlighted in light green.

multiple different hypotheses that offer a variety of answers to this perplexing question. Given that the best-known function of HLA molecules is to present peptide antigens to T cells, the "canonical" hypotheses are focused on various AP flaws.

Among the most widely discussed canonical hypotheses are reactivity to self-antigens due to molecular mimicry with foreign antigens; skewed T cell repertoire selection; reactivity to altered self-antigens; and association through LD. Additional theories concerning the mechanistic basis of HLA-disease association are discussed in a recent review article (63).

## Molecular Mimicry With Foreign Antigens

The term "molecular mimicry", also known as "immune cross-reactivity", refers to mistaken identification by the immune system of a self-antigen as foreign, due to their structural resemblance, as schematically represented in **Figure 3A**. The concept, invoked as far back as 60 years ago (65),was rationalized based on primary sequence homology between human and microbial antigens (66), as well as cases of test tube cross-reactivity of antiviral monoclonal antibodies with host tissues and cells (3, 67). Molecular mimicry has been implicated in many diseases and autoimmune disorders, such

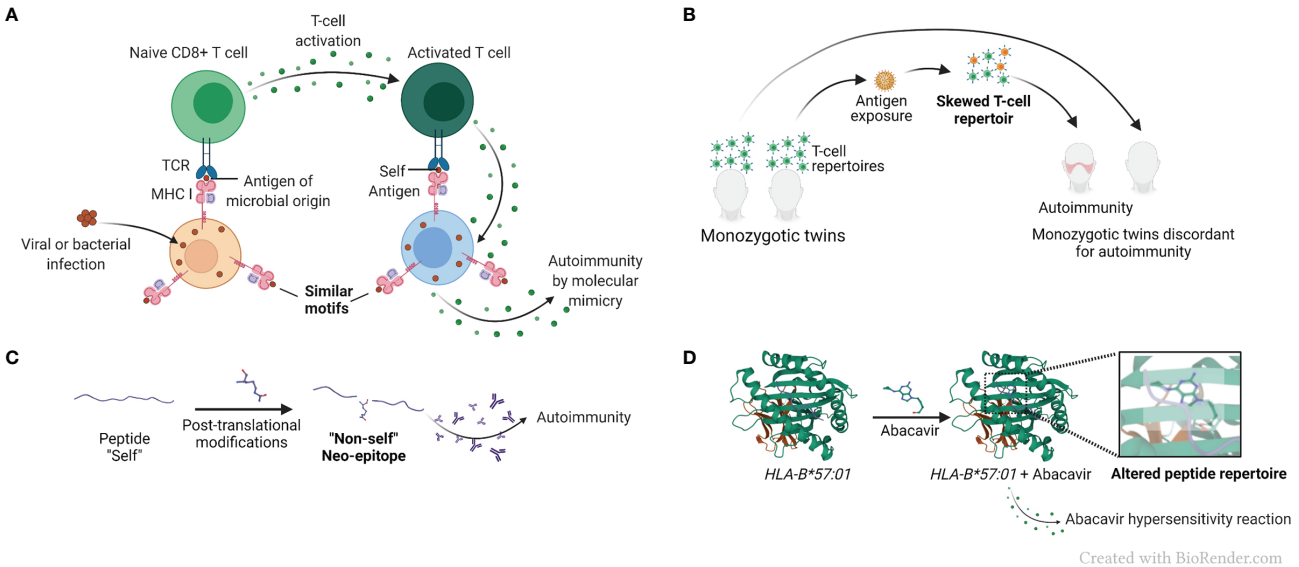


**FIGURE 3** | AP-based hypotheses for the mechanistic basis of HLA-disease association. **(A)** Simplified representation of the molecular mimicry hypothesis. Molecular mimicry occurs when the similarities between foreign peptides and self-peptides are such that auto-reactive T cells are activated, triggering autoimmunity. **(B)** Simplified representation of the skewed T cell repertoire hypothesis in monozygotic twins. Naïve T cells are activated after recognition of non-self-antigens presented by MHC molecules, and T cell repertoire is skewed towards new antigen-driven specificities. **(C)** Representation of the reactivity to altered self-antigens caused by protein citrullination. In the reactivity to altered self-antigens hypothesis, proteins that were previously tolerated by the immune system suffer changes and become neo-epitopes and recognized as "non-self", breaking immunological tolerance. **(D)** Representation of a drug-associated HLA modification caused by abacavir, pdb, 3VRJ (64).

as COVID-19 (68), uveitis (69) and multiple sclerosis (70), among other conditions. A recent study has implicated molecular mimicry as an underlying mechanism of protection against RA by certain *HLA-DRB1* alleles encoding for a DERAA sequence in the cusp region due to a tri-lateral sequence homology among a self-protein named vinculin, microbial proteins, and the products of the protective *DRB1* alleles (71). However, the premise of this complex hypothesis, which postulates antigenic mimicry among proteins derived from three different sources (microbial, vinculin and HLA-DR), as well as AP by an HLA-DQ that is in LD with the protective *HLA-DRB1* allele has raised some questions (23, 72).

Importantly, despite much effort in the past decades, a direct link between molecular mimicry and autoimmunity remains elusive, and the identity of putative antigens have not been convincingly and reproducibly demonstrated in HLA-associated diseases (8). It is noteworthy that a series of studies with viral and bacterial proteomes demonstrated extensive overlaps between human and microbial motifs, suggesting that this homology is such that it should have led to a frequency of autoimmunity much greater than the 5% currently reported (73). This analysis, and lack of direct cause-effect data to implicate molecular mimicry in human autoimmune diseases strongly suggest that molecular mimicry does exist; however, it is unlikely to be a significant pathogenic factor in autoimmunity (74–77).

## Skewed T Cell Repertoire Selection

T cell repertoire is shaped through a series of positive and negative thymic selection processes, leading to deletion of self-reactive T cell receptors (TCR) in the context of self-MHC (4), (**Figure 3B**). Studying monozygotic twins, Utz et al. demonstrated distinct T cell repertoires in multiple sclerosis-discordant twin pairs (78). The skewing was observed in peripheral blood lymphocytes stimulated both with a putative target self-antigen, myelin basic protein, as well as with an unrelated foreign antigen tetanus toxoid, demonstrating an antigen-nonspecific variations in T cell repertoires between the discordant twins (78). These findings were interpreted to suggest that factors other than genetics exert influence on an individual's T cell repertoire in multiple sclerosis, an HLA-associated disease. Additionally, one significant limitation of the T cell repertoire selection hypothesis is that despite much effort, clonality of antigen-specific T cells is yet to be convincingly demonstrated in HLA-associated diseases.

## Reactivity to Altered Self-Antigens

Alterations of self-antigens might render them recognizable as foreign by the immune system. A classic example of a post-translational modification is protein citrullination (5), which has been proposed as a triggering event in RA disease development (79). Protein citrullination is an important process necessary to maintain physiological function of several proteins. In patients carrying *HLA-DRB1* alleles encoding for the shared epitope (SE) (80), anti-citrullinated protein antibodies (ACPA) are useful diagnostic and prognostic marker (81). It has been hypothesized that exposure to environmental triggers, such as cigarette smoke (82) leads to accelerated protein citrullination,

which in turn become a target for auto-antibodies (5, 83), (**Figure 3C**). The increased risk of RA development in smokers has been established for years (84), especially amongst individuals carrying particular *HLA-DRB1* alleles encoding the SE (85, 86). Although it has been demonstrated that some citrullinated proteins have a higher binding affinity to the SE than their native counterparts (87), that observation is not universal and does not necessarily govern T cell response (88). Thus, it has been argued that presentation of citrullinated peptides is unlikely to be the sole link between the SE and generation of ACPA (88, 89). It has also been hypothesized that cigarette smoke facilitates protein citrullination in the lungs of susceptible individuals, resulting in generation of autoantibodies directed against these citrullinated proteins (90, 91). However, this hypothesis does not explain the increased risk of RA development in children of women who smoked during pregnancy (92).

### Association Through Linkage Disequilibrium

The HLA complex is characterized by extensive LD, meaning a non-random (either higher or lower) association of alleles at different loci (93). LD frequently renders the identification of the causative genes in disease associations more challenging (94). It should be mentioned, however, that associations through LD do not provide a mechanism, they just identify spurious associations while the mechanistic basis remains unknown. Indeed, erroneous HLA-disease associations have been established due to LD, such as in the case of narcolepsy, where predisposing factors had been mistakenly attributed to *HLA-DRB1*15*, and later were found to be associated to the *HLA-DQ* genes that are in LD with *DRB1*15* (6). These inaccurate associations are becoming less common, as genetic and epidemiological research technologies have evolved and are being steadily optimized (95).

### Drug-Induced HLA Modifications

Certain small-molecule drugs have been shown to bind to specific HLA proteins and cause modifications that result in altered binding repertoires and subsequent HLA-associated adverse drug reactions. A case in point is the abacavir hypersensitivity syndrome, developed by individuals carrying the *HLA-B*57:01* allele. It has been demonstrated that the drug used in HIV therapy, binds non-covalently to the peptide-binding cleft of *HLA-B*57:01*-coded protein, resulting in a modification of its peptide-binding repertoire, which may lead to presenting endogenous peptides (96), (**Figure 3D**). The knowledge of this HLA-associated hypersensitivity has prompted a change in HIV treatment guidelines (97), with resultant marked decline in hypersensitivity occurrence (98).

## HLA-DISEASE ASSOCIATION - NONCANONICAL HYPOTHESES

While most hypotheses concerning HLA-disease association are centred on AP, evidence in support of AP-independent mechanisms has been presented as well. For example, certain *HLA-B*27* alleles are found in more than 90% of patients with ankylosing spondylitis and explain 20.1% of the heritability of the disease (99). It has been found that amino acid Cys67 in such HLA-B27 molecules favors an open conformation of the molecule, which is a stronger ligand for leukocyte Ig-like receptor B2 (LILRB2) than other HLA class I molecules (100). It has also been demonstrated that HLA-B27 molecules are predisposed to misfolding (101, 102), generating disulfide-linked homodimers, which are recognized by KIR 3DL2 on $CD4^+$ $T_H17$ cells, and stimulate the production of IL-17 (103). Notably, misfolded proteins have been shown to accumulate in the endoplasmic reticulum and activate the unfolded protein response, an aberration which has been implicated in the pathogenesis of rheumatic diseases (102).

Another example of non-canonical hypothesis is the modulation of cell surface receptors by MHC molecules. An example is in the case of the human cytomegalovirus (HCMV) infection, which has been demonstrated to modulate HLA-C, favoring a potent KIR2DS1-mediated NK cell activation (104).

### The MHC Cusp Theory

In addition to the individual limitations discussed above, AP as a sole causative factor in HLA-associated diseases is difficult to reconcile with several fundamental epidemiologic and pathogenic characteristics of those diseases:

1. Many of the diseases that are known to associate with specific HLA alleles display neither autoimmune basis, nor evidence of AP. For example, HLA allele *DQB1*06:02* is found in almost all patients with narcolepsy type 1 (105), yet convincing, cause-effect relationships between the *DQB1*06:02* allele and autoantibodies or AP have not been shown in this disease, which is caused by an abnormal neurotransmission.
2. A single allele can associate with multiple diseases with distinct pathogeneses or target tissues. For example, *HLA-DRB1*03:01*, has been associated with systemic lupus erythematosus (106), Graves' disease (107), and type 1 diabetes (108), to name just few. Likewise, the aforementioned narcolepsy-associated *DQB1*06:02* allele is also a genetic risk factor for an unrelated disease, multiple sclerosis (109).
3. Particular alleles can offer disease-susceptibility across species, for example, the SE sequence has been found to associate with human RA (110), as well as canine arthritis (111) and murine models of the disease (112). It is hard to conceptualize the evolutionary advantage of such trans-species AP-based susceptibility factor among species that have vastly different adaptive immune system antigen recognition repertoires.
4. The impact of HLA allele-dose on disease severity by specific alleles does not fit well with mainstream AP-based concepts. For example, it has been demonstrated that in RA, allele-dose effects can determine disease incidence, severity, age of onset and concordance rates in RA-discordant monozygotic twins (113, 114). The effect of homozygosity is incongruent with AP-based clonal expansion. Moreover, HLA-DR heterozygosity has actually been shown to increase disease susceptibility in

several conditions, including systemic lupus erythematosus (115), and type 1 diabetes (116).

We put forward the MHC Cusp theory a decade ago (9) to provide an alternative explanation to the aforementioned inconsistencies that cannot be convincingly answered by the AP-based hypotheses. The theory postulates that: *"HLA molecules encode ligands in one of their hypervariable regions, designated a "cusp" based on its three-dimensional cusp-like conformation. Under certain environmental and background gene conditions, these cusp-ligands can interact with non-major histocompatibility complex (MHC) receptors thereby activating aberrant cell signaling events that cause disease development"* (9).

To empirically examine the MHC Cusp theory, our group has so far focused on two representative *HLA-DRB1* alleles known for their association with autoimmune diseases. The SE, a five amino acid motif (QKRAA, QRRAA or RRRAA) in the cusp 70-74 region of the DRβ chain coded by RA-associated *DRB1* alleles, and is strongly associated with RA susceptibility (110), was the first to be studied. Our findings to date have demonstrated that the SE acts as a signal transduction ligand that interacts with cell surface calreticulin (CRT) and activates signaling events, which facilitate pro-arthritogenic and bone erosive changes (10–23). We have demonstrated that human lymphoblastoid B cell lines carrying SE-coding HLA-DR alleles triggered spontaneous production of nitric oxide (NO) (10). We later demonstrated that the SE caused increased NO and reactive oxygen species (ROS) production, and facilitated osteoclast differentiation in RAW 264.7 cells, bone marrow cells (BMCs) derived from DBA/1 mice, and in healthy human peripheral blood mononuclear cells (PBMCs). The SE was also found to promote increased production of interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α), and enhanced the differentiation of receptor activator of NF-kB ligand (RANKL)-expressing IL-17 producing T cells, causing Th17 cell-mediated pro-osteoclastogenic effects independent of antigen presentation (15).

Our studies with this cusp ligand have also provided a mechanistic basis for the long-observed association between RA, cigarette smoking and carriage of SE-coding alleles (117), by demonstrating that in the presence of aryl hydrocarbon receptor (AhR) agonists - environmental pollutants found in cigarette smoke - the activation potency of the SE-mediated pathway is amplified synergistically with resultant augmented inflammatory response and bone erosive damage that lead to more severe experimental arthritis in mice (21). In a subsequent study, we demonstrated in RAW 264.7 cells, that lipopolysaccharide (LPS) facilitated cell surface translocation of CRT, which in turn enabled increased SE-activated calcium signaling and activation of peptidylarginine deiminase, increasing cellular abundance of citrullinated proteins. Intraperitoneal administration of LPS in transgenic mice carrying a human SE-coding *HLA-DRB1* allele caused increased serum levels of TNF-α and ACPA production, as well as terminal phalanx bone destruction (22), thereby suggesting a mechanistic basis for the long documented association between RA and protein citrullination. Additionally, we have demonstrated that the interaction between SE and its receptor, CRT, is facilitated by citrullination of the latter (16). Thus, the Cusp theory provides a multifaceted, AP-independent model of HLA-disease association and its interaction with environmental factors.

The second cusp motif that we have studied was the 70-DERAA-74 sequence, which is coded by *DRB1* alleles known to protect against RA and several other autoimmune conditions (118–122). An AP-based hypothesis to explain the contrasting effects of the SE and PE has been offered based on the charge of critical amino acid residues coded by these two functionally distinct alleles. It has been proposed that the positively charged residues lysine and arginine in the SE would favor the binding of citrullinated proteins, while the PE, which contain negatively charged residues, can bind to both arginine and citrulline residues (123). However, the observation that other HLA molecules (e.g. DQ), and peptide binding pockets outside of the 70-74 region in HLA-DR molecules could accommodate citrullinated peptides as well (123), suggests that the interactions between HLA molecules and citrullinated peptides associations are more complex.

Given the limitations of the canonical, AP-based hypotheses to provide convincing explanation for the effects of protective *DRB1* alleles, we have taken a cusp-based experimental approach. As discussed above, an amino acid sequence motif 70-DERAA-74 in the DRβ chain shows a protective effect (118, 123). Using short synthetic peptides corresponding to this cusp region, we have demonstrated that these AP-incompetent peptides activated transcriptomes that are consistent with anti-inflammatory (M2) macrophage polarization. A diametrically opposite effect was observed with synthetic peptides corresponding to the SE motif, which activated a pro-inflammatory macrophage (M1) transcriptome (23).

Thus, our findings provide empirical evidence in support of an AP-independent mechanism that contributes to HLA-disease association. It is important to clarify that the MHC Cusp theory does not aim to supplanting the role of AP, it rather demonstrates that in addition to their well-studied classical AP functions, MHC molecules express cusp-region epitopes that under certain environmental conditions can trigger activation of disease-facilitating pathways. In summary, the amino acid sequence in the cusp-region of these molecules would govern the binding specificity of these binding sites, while the three-dimensional cusp-like structure makes it more accessible for interaction with cognate receptors. A proposed scheme of the role of MHC cusp ligands in disease pathogenesis is shown in **Figure 4**.

## A Summary of the Experimental Evidence Supporting the Ligand Function of the SE

Our research to date, using a range of approaches and techniques has provided considerable evidence in support of the SE effects as a cusp ligand. Our early findings involved lymphoblastoid B cell lines carrying SE-positive or -negative *HLA-DRB1* alleles, as well as L cell transfectants that carry allele-distinct HLA-DR molecules, or fibroblast cell transfectants expressing HLA-DR molecules with single amino acid point mutations. In all cases, SE-positive cells showed increased NO production. Moreover, the signaling effects were found to be independent of any cellular context, since cell-free tetrameric HLA-DR molecules that

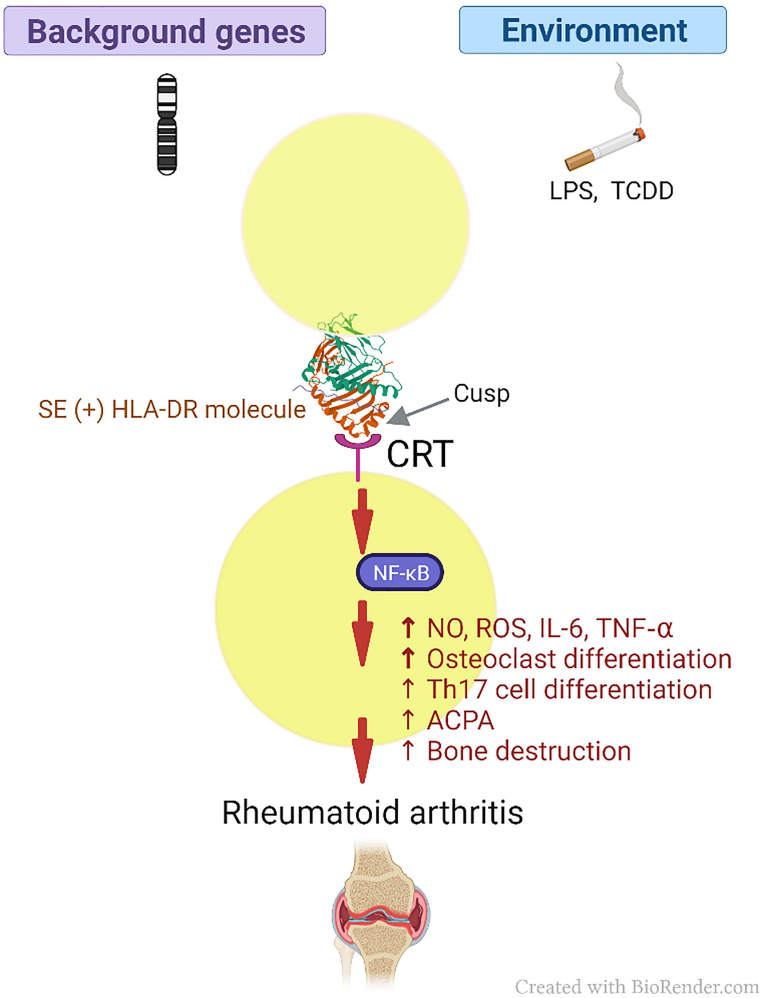


**FIGURE 4** | Schematic model of the MHC Cusp theory (as exemplified by the effect of the SE in RA). The cusp region of RA risk-conferring HLA-DR molecules contains an allele-specific physiologic signal transduction ligand (SE) that can help polarize the immune response through interaction with CRT, which acts as a cell surface receptor, with resultant activation of immune modulating pathways. Depending on permissive background genes and environmental influences such as pollutants, the otherwise physiologic pathways can activate cellular events that lead to the onset of RA (please see text for details). CRT, calreticulin; LPS, lipopolysaccharide; TCDD, 2,3,7,8 -Tetrachlorodibenzo-p-dioxin; SE, shared epitope; NF-κB, Nuclear factor-κB; RA, rheumatoid arthritis; NO, nitric oxide; ROS, reactive oxygen species; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; Th17, T helper 17; ACPA, anticitrullinated cyclic peptide antibodies.

possess a SE-positive DRβ molecules stimulated fibroblast cells to produce higher levels of NO compared with cells stimulated with a control HLA-DR tetramer. Likewise, multimeric hepatitis B core proteins engineered to express the SE cusp region, but not the same region encoded by a SE-negative allele, stimulated NO production in fibroblasts. Furthermore, cell-free soluble synthetic peptides corresponding to the cusp region of SE-positive HLA-DR molecules triggered increased NO levels in class II HLA-negative cells (10).

Consistent with the known association between the pro-oxidative effect of NO and the proposed role of oxidative stress in the pathogenesis of RA, we have demonstrated that cells exposed to cell-surface-expressed, as well as cell-free soluble SE-positive cusp regions showed increased ROS levels, and higher vulnerability to oxidative DNA damage in an NO-dependent fashion. Using single amino acid substitutions, we have identified a sequence motif of Q/R-K/R-X-X-A that is critical for the SE cusp region signaling effect (11).

In other studies, we identified allele-specific cusp ligand-triggered activation of cellular calcium increase, and activation of the NF-kB pathway (10, 11, 21, 22). We have also demonstrated synergistic cross talk between the SE and the AhR pathways (21) and allele-specific distinct cytokine and chemokine production profiles by different cusp region ligands (14, 15, 17, 23). Additionally, cell activation by cusp region ligands was found to trigger allele-specific signature transcriptomes (23).

Allele-specific signal transduction pathway modulation by the SE versus the PE has been mapped, demonstrating that under M1-polarizing conditions, bone marrow-derived macrophages

(BMDM) of SE-positive transgenic mice demonstrate a cascade of signaling events, involving pSHIP1 activation, inhibition of Pi3k and pAkt, and NF-κB activation, leading to enhanced M1 polarization. In contrast, in BMDM of PE-positive transgenic mice, a mirror image of signaling event is found, leading to decreased M1 polarization (23). Under M2-polarizing conditions, BMDM of PE-positive transgenic mice show a tandem upstream pathway effect through activation of pAkt and pStat6, which activate transcription of M2-polarization genes *Arg1* and *Yam1*, respectively, with resultant enhanced M2 polarization. In contrast, in SE-positive transgenic mice, effective M2 polarization is inhibited by sustained NF-κB activity (23).

Relevant to the role of SE in RA disease pathogenesis, we demonstrated allele-specific RA-associated cytokine and chemokine production profiles by the SE, as compared to other allelic cusp ligands (14, 15, 17, 23). In addition, the SE ligand was found to specifically facilitate Th17 and inhibit Treg differentiation (14). It also polarized macrophage differentiation toward a pro-inflammatory subset (M1), with a diametrically opposite effect by the PE cusp ligand, which was found to facilitate differentiation of anti-inflammatory subset of macrophages (M2) (23). Another RA-relevant finding is the pro-arthritogenic effect of synthetic peptide and peptidomimetic SE ligands *in vivo* and potent activation of osteoclast differentiation and bone erosive effects, both *in vitro* and *in vivo* (15, 18).

To identify the SE signal-transducing cell surface receptor, we used a combination of affinity chromatography, cell-binding assays, surface plasmon resonance, and time-resolved fluorescence resonance energy transfer techniques to demonstrate specific interactions between the SE ligand and CRT at a cell-free level. The interaction was confirmed at the cellular level by studying CRT-KO, as well as CD91-negative cells, along with antibody-mediated blocking experiments and reconstitution techniques. Thus, these experiments demonstrated that SE-triggered cell signaling effects depend on CRT and its known co-receptor, CD91 (12).

We have mapped the SE binding site on CRT to a discrete region on CRT P-domain. This was accomplished by using surface plasmon resonance-based experiments with domain deletion mutants, and a photoactive cross-linking method. Based on in silico-based docking interactions between the SE in its natural conformation and the CRT P-domain, followed by site-directed mutagenesis we identified the amino acid residues in the binding site that are critical for binding to the SE ligand and effectively transducing signaling (13).

Based on the molecular details of SE interaction at the specific binding site on CRT, we designed synthetic SE agonists that mimic the SE effects, as well as antagonists that block SE-CRT interaction. Based on those data, we have successfully designed different synthetic molecules that specifically target the interaction site and facilitate arthritis or inhibit it in an experimental model of arthritis (15, 18, 112, 124).

Thus, we have deciphered key molecular mechanisms associated with the cusp ligand SE effects *in vitro* and *in vivo* and have embarked on reducing this knowledge to practice.

# PARALLELS BETWEEN HLA-G AND THE MHC CUSP THEORY

HLA-G and the MHC Cusp theory demonstrate functional and conceptual parallels. Reminiscent of the Cusp theory that has identified several *HLA-DRB1* alleles which code for ligands that activate signaling events that result in transcriptional and functional immune modulation, HLA-G activity has been shown to involve ligands that exert profound immune modulatory effects. Like the Cusp theory, it has been proposed that in at least some HLA-G isoforms, AP is not required for their functional activities (125), and both Cusp epitopes and certain HLA-G molecules involve activation of specific signaling events. The following are four areas illustrating salient functional and structural characteristics, that illustrate the parallels between the MHC Cusp theory and HLA-G.

## Immune Modulatory and Autoimmune Disease Effects

As discussed above, the MHC Cusp theory offers a non-AP mechanistic basis for HLA-associated diseases and health traits. The cusp region in class II HLA molecules encompasses the third allelic hypervariable region of their β chain, with which numerous epidemiologic studies have identified allele-specific associations in many autoimmune diseases, such as RA, systemic lupus erythematosus, type 1 diabetes, and many other conditions. While AP might explain some of the associations, it is important to note that in most HLA-associated diseases, the identities of the self or foreign antigens and conclusive evidence to substantiate AP-based mechanism is limited despite decades of research effort. Based partly on this reality, as well as the promiscuous associations of many alleles with diseases and conditions that have no common pathogenetic, mechanistic, or tissue distributions, we have previously proposed that notwithstanding a putative role of AP, the Cusp theory may provide a mechanistic framework for at least some allele-specific disease association. Using two emblematic *HLA-DRB1* alleles known to associate with autoimmune disease susceptibility or protection, *DRB1*04:01; DRB1:04:02*, we have shown that AP-incompetent ligands - peptide sequences unable to perform antigen presentation - corresponding to the cusp regions of these alleles trigger disease-relevant transcriptional, signaling, cell activation and disease phenomes (14, 15, 19, 21–23).

Like class II HLA alleles, HLA-G products have been shown to associate with diverse and mechanistically unrelated autoimmune and neoplastic diseases, and to play a crucial role in preserving fetal-maternal tolerance, independent of AP. The best characterized roles of HLA-G are in implantation and maintenance of pregnancy (126, 127), and tolerance to solid organ allografts (35). In the context of the Cusp theory, however, it is important to mention that HLA-G immune modulatory effect has been documented in several autoimmune conditions as well. For example, polymorphisms in the HLA-G gene have been found to be a significant risk factor for type 1 diabetes (128), systemic lupus erythematosus (129, 130), RA (131), experimental autoimmune uveitis (132), systemic sclerosis (130), as well as

multiple sclerosis and several autoimmune skin conditions (133), asthma (134) and Graves' disease (135), however, it is important to mention that results for associations between HLA-G and diseases are often times contradictory or inconclusive, and warrant further investigation.

The promiscuous immune modulatory effects of HLA-G in multiple diseases that lack common tissue distribution or pathogenesis suggest a fundamental, antigen-nonspecific effects, as discussed below.

## Ligand Effect Versus AP

Like classical class I HLA molecules, certain HLA-G isoforms are folded in a three-domain conformation, associate with $\beta_2$m, possess a peptide-binding groove, and interacts with CD8+ T cells (136). Further, HLA-G has been reported to carry natural peptides with characteristic groove-binding motifs (137), and has been implicated in HLA-restricted antigen-specific cytolytic CD8+ repertoire selection (138).

However, different from class Ia molecules, HLA-G has a limited tissue distribution and a short intracytoplasmic domain, it shows a low level of polymorphism, and its mRNA is spliced into multiple isoforms. Further, HLA-G cleft peptides are positioned in a constrained mode of binding, which is distinct from the mode that characterizes peptide groove binding in class Ia HLA molecules (139).

Based on these considerations and others, it has been proposed as far back as the mid-1990's that AP might not be the only - or even the primary - function of HLA-G molecules (125, 140). As discussed above, various HLA-G isoforms have been shown to interact with immune receptors such as KIR and activate in trans several functional effects, including modulation of innate and adaptive immunity, anti-viral, anti-tumor, as well as non-immune functions. Those trans-activation events involve defined signaling pathways, which are detailed elsewhere (127).

Potentially relevant to HLA-G-activated pathways, the PE, coded by *HLA-DRB1* alleles known to associate with protection against several autoimmune diseases (118, 141), can activate signaling and transcriptional events that characterize immune tolerance (23). Although the identity of the PE receptor is not yet known, our findings indicate that the PE ligand activates S473Akt phosphorylation with downstream effects on the NF-κB pathway, and M2 macrophage polarization, reminiscent of signaling events observed with HLA-G (142, 143). Thus HLA-DR cusp region ligands in the form of short linear synthetic peptides, which lack AP capacity, can activate cascades of signaling and cell differentiation events with potential effects on health and disease processes.

## Topologies of Receptor-Ligand Interactions

The MHC Cusp theory has been prompted by growing evidence linking a specific region on the MHC molecule, which despite substantial polymorphism and evolutionary distance, is conformationally conserved in the entire MHC family of molecules and other MHC fold proteins (144). As previously discussed (8, 9), that region, which was named by us "cusp" due to its conformationally conserved cusp-like structure, appears to be a hub of epitopes that interact with various non-MHC receptors and activate functional effects. Those receptor binding epitopes have been identified across the MHC family regardless of the antigen presentation capabilities of the parent molecules, as follows.

In class II HLA molecules, the cusp region which shows immune modulating ligand properties has been mapped to the amino acid residues 65-79 in the β1 domain, which forms an α helix with an 'upward kink' (**Figure 2**). The conformationally analogous region to the MHC class II cusp region in class I molecules is in the α2 domain, where a similar 'kink' is found. That region in Class I is enriched in ligands as well. For example, in class Ia molecules, such as HLA-A (145) and HLA-C (146) the cusp region has been shown to bind natural killer cell inhibitory receptors, such as KIRDL1, in a tandem binding geometry, where the KIR domain D1 binds to the α1 domain of the HLA molecule, while domain D2 interacts with the α2 domain, at the cusp region. Equivalent cusp-region binding topologies were found between HLA-C molecules and KIRDL2 and KIRDL3 (147). Cusp-region interaction involvement has been found in the class Ib molecules as well. Reminiscent to the above-mentioned binding topology of class Ia HLA molecules, a tandem of CD94-NKG2A binding sites has been mapped, respectively, to the α1 and α2 domains of the class Ib HLA-E molecule (148–150). Noteworthy, cusp region binding sites have been found also in non-antigen presenting members of the extended MHC family, such as the hereditary haemochromatosis protein HFE, which interacts in its cusp region with transferrin receptors (151). Another example is mouse M10, a pheromone receptor-associated MHC molecule with an open and empty groove whose cusp region interacts with odorant receptors of the vomeronasal organ (152).

Given the common structural and functional properties between HLA-G and other HLA molecules (144), and since evidence exists for an immune receptor-binding site on the α2 domain of the latter, it is reasonable to predict that the HLA-G could contain an equivalent receptor binding site in the cusp domain as well. Evidence to support this scenario, however, is inconclusive. On the one hand, specific polymorphic residues in the HLA-G1 α2 domain were found to impact HLA-G NK cell recognition and immune regulation (153), however, a recent study has concluded, based on co-crystallization experiments, that there was no evidence to support a role for interactions between immune receptors and the HLA-G1 α2 domain (154). It should be added that confirmatory structural analyses, or functional studies are yet to be published, particularly considering the interplay between soluble and membrane-bound isoforms. Given the exquisite sensitivity of crystallography studies to even minor physicochemical alterations, it is still possible that the final word on whether the HLA-G α2 cusp region plays a role in ligand-receptor immune modulating interactions is yet to be said.

## Therapeutic Implications

One of the practical outcomes provided by the MHC Cusp theory is the prospect of identification of specific ligand-receptor targets for therapeutic intervention. A practical example of the therapeutic potentials was realized upon the identification of the SE binding site on CRT by our group (13).

That finding has opened the door to the development of an antagonistic ligand, which showed potent therapeutic effect in mice with experimental erosive arthritis (112). Likewise, the identification of the anti-inflammatory pathways activated by the PE might offer the mechanistic insights for the development of PE-mimicking ligands with anti-inflammatory properties thereby offering therapeutic protection against HLA-associated diseases, such as RA.

A similar rationale may be used for the development of HLA-G related peptides, which might hold therapeutic promise in a number of immune-mediated diseases (133) and transplantation. Additionally, a recent work has evaluated HLA-G expression in biopsy renal tissue of patients with lupus nephritis (155). That study demonstrated that the percentage of patients who did not respond to treatment was significantly lower in the HLA-G expressing group compared to the group that did not express HLA-G, suggesting a possible role of HLA-G molecules in response to treatment (155). Synthetic, HLA-G-derived molecules have already been developed for potential use to improve tolerance to organ transplants (156).

On the other hand, HLA-G expression has been shown to play a role in escape of cancerous tumours (157) and virally infected cells from immune control (52), suggesting deleterious actions of HLA-G (38), which should be carefully considered when developing HLA-G synthetic molecules as a potential therapeutic agents. Because of the involvement of HLA-G in immune evasion and consequently tumour growth, this molecule has been considered to exert an immune checkpoint function in cancer (48). Although monoclonal antibodies have shown therapeutic success in blocking other checkpoint molecules, the structural and functional diversity of the various HLA-G isoforms have posed limitations to the development of specific antibodies for immunotherapy in cancer, and although HLA-G antibodies have been developed, they commonly do not interact with all HLA-G isoforms (56). Using specific HLA-G based ligands would surpass antibody related limitations. Thus, efforts to improve understanding of HLA-G specific receptor interactions, signaling pathways and potential targets may open the door to developing inhibitory ligands for the use in cancer therapy, which holds a great potential for tumor-specificity, since HLA-G has restricted expression in healthy tissues (48).

## A Clarification

All theories, including the MHC Cusp require independent corroboration. To date, we have presented published evidence that supports AP-independent allele-specific immune dysregulation by cusp-region sequences coded by two *HLA-DRB1* alleles*: 04:01* and *04:02*. Although our Cusp theory-based work has been very well cited, other groups have not yet publicly validated the theory. For contextual purposes, it is important to note that although the MHC Cusp theory does not supplant AP-based hypotheses of HLA-disease association, it is nevertheless often incorrectly interpreted as challenging the more prevalent, AP theory. As we clarified in previous publications, and reiterated it here as well, based on literature evidence, and our own experimental data, we propose that as plausible as they are, AP-based mechanisms alone cannot explain many of the epidemiologic and immunogenetic findings in HLA-associated diseases. We submit that the MHC Cusp theory could effectively answer several of these inconsistencies. We also wish to point out that while 10 years have passed since we first invoked the MHC Cusp theory, such time delay is not uncommon. Many other unorthodox theories awaited even a longer time before they were accepted by the mainstream scientific community.

# CONCLUDING REMARKS

Decades of research into the structure-function characteristics of HLA-G, and paralleled research effort to decipher the mechanistic basis of HLA-disease association have led to better appreciation of the versatility and functional plasticity of this family of molecules. The MHC Cusp theory and HLA-G structure-function characteristics - seemingly unrelated entities - appear to display multiple parallels. Obviously, many questions are yet to be answered; however, research data gathered over the years has already uncovered insights about possible evolutionary inter-relatedness and specialization within the vast MHC group of genes. The lessons that have been learned thus far, and those that are awaiting discovery, could help to better understand the evolution and structure-function aspects of the MHC, which in turn might generate new ideas how to harness this knowledge to promote health, and cure diseases.

# AUTHOR CONTRIBUTIONS

BMS searched the literature and wrote the manuscript. VD participated in editing the manuscript. VD also critically reviewed the article. JH conceptualized the Cusp theory and the parallels with HLA-G and wrote sections of the manuscript. All authors read and approved the final manuscript.

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# Primary Trophoblast Cultures: Characterization of HLA Profiles and Immune Cell Interactions

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**Introduction:** Trophoblasts are essential in fetal-maternal interaction during pregnancy. The goal was to study HLA profiles of primary trophoblasts derived from placentas, and to investigate their usefulness in studying interaction with immune cells.

**Methods:** After enzymatic digestion of first-trimester placental tissue from seven donors (6-9 weeks gestation) and trophoblast enrichment we cultured cytotrophoblasts (CTB) in stem cell medium. CTB were differentiated into EVT in a Matrigel-containing medium. A subset of CTB/EVT was profiled for microRNA levels. Expression of classical HLA molecules and of HLA-G was studied by flow cytometry, qPCR, and ELISA. Secondary trophoblast cell lines JAR and JEG-3 were studied as controls. Lymphocytes were investigated during co-culturing with EVT.

**Results:** The trophoblasts could be easily maintained for several passages, upregulated classical trophoblast markers (GATA3, TFAP2C, chromosome-19 microRNAs), and upon differentiation to EVT they were selective in expressing HLA-C. EVT showed increasing expression of total HLA-G, an increasing proportion of HLA-G1 over G2- and G3 isoforms, and elevated excretion of soluble HLA-G. These features were distinct from those of the secondary trophoblast cell lines. TNF-α and IL-8 represented the most abundantly secreted cytokines by CTB, but their levels were minimal in EVT cultures. As proof of principle, we showed that EVT affect lymphocytes in three-day co-cultures (n=4) by decreasing activation marker HLA-DR.

**Conclusion:** We verified the possibility culturing trophoblasts from first-term placentas, and their capability of differentiating to HLA-G expressing EVT. This culture model better represents the *in-vivo* situation than previously studied secondary trophoblast cell lines and enables mechanistic studies of fetal-maternal interactions.

**Keywords: trophoblast, HLA-G, placenta, pregnancy, culturing, stem cell, differentiation, immune cell**

## INTRODUCTION

Trophoblasts play a significant role during pregnancy in fetal-maternal interaction. These cells are essential in the placentation process, and while invading in the uterine wall and the decidua of the mother they need to be tolerated by maternal immune cells. To study trophoblast characteristics and their interactions with other cell types, it is useful to establish a culture system after isolation of the trophoblasts. Following enzymatic digestion of placental tissue, trophoblasts may be directly enriched and cultured thereafter, but such primary cultures are relatively short-term due to limited proliferation capacity of the cells. Immortalized trophoblast cell lines including JEG-3, JAR, and HTR-8/SVneo are frequently used for *in vitro* research, but because of differences with primary trophoblasts (1–5) it is questionable whether such secondary cell lines are reliably modelling trophoblasts that are encountered *in vivo* (6). Placental explants in culture represent a more natural homologue to a placenta *in vivo*, but these explants show stress, cell death, and impaired nucleic acid content under certain conditions (7–11), which may confound results obtained in consecutive co-cultures.

The availability of long-term trophoblast cultures would circumvent the issues outlined above. Therefore, organoid cultures of trophoblasts from placentas at six to nine weeks of gestation have been generated, which were differentiated into EVT (12, 13). However, the drawback may be that three-dimensional organoids constitute multiple cell types, including villous trophoblasts and syncytiotrophoblast, and that these cell types display an inside-out orientation in comparison to villous tissue from the placenta. A possibly more appropriate model to study trophoblast-immune cell interactions may be a two-dimensional culture of one trophoblast cell type. A protocol was described to maintain cultured cytotrophoblasts (CTB) cultures, generated from trophoblast stem cells from first trimester placentas (14). The cell lines have the capacity to give rise to different trophoblast lineages, they show transcriptomes similar to those of primary trophoblast cells from the placenta, and they grow for at least one year, remaining genetically stable during that course (14).

Our objective was to establish 2D cultures of primary trophoblasts derived from first-term placentas, to study their profile of classical HLA molecules and HLA-G, and to examine if they could be used to study the interaction with immune cells.

**Abbreviations:** ACTB, Beta-actin; BSA, Bovine Serum Albumin; CDH1, Cadherin-1/E-cadherin; CGA, Chorionic Gonadotropin Subunit Alpha; CTB, Cytotrophoblasts; DMEM, Dulbecco's Modified Eagle Medium; EDTA, Ethylenediaminetetraacetic Acid; EGFR, Epidermal Growth Factor Receptor; ELISA, Enzyme-linked Immunosorbent Assay; EPCAM, Epithelial Cell Adhesion Molecule; EVT, Extravillous Trophoblasts; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; HLA, Human Leukocyte Antigen; ITGA, Integrin Subunit Alpha; ITGB, Integrin Subunit Beta; IL, Interleukin; ITS, Insulin-Transferrin-Selenium; KSR, KnockOut Serum Replacement; KRT, Cytokeratins; MSC, Mesenchymal Stromal Cells; NRG1, Neuregulin-1; PBL, Peripheral Blood Lymphocytes; PBS, Phosphate-Buffered Saline; p/s, Penicillin/Streptomycin; SD, Standard Deviation; SEM, Standard Error of the Mean; TEAD4, TEA Domain Transcription Factor 4; TGF-β, Transforming Growth Factor, Beta; TNF-α, Tumor Necrosis Factor, Alpha; TS, Trophoblast Stem Cell; VIM, Vimentin.

## MATERIALS AND METHODS

### Tissue Collection and Processing

Placental tissue was collected from donors (n=7) who had undergone termination of pregnancy due to social reasons between six and nine weeks of gestation. All samples were obtained after informed consent, and the study was carried out in accordance with the guidelines issued by the Medical Ethics Committee of the LUMC (P08.087) and in accordance with the Declaration of Helsinki.

Villous tissue was separated from the chorionic plate, minced, and washed three times with PBS (pH 7.4, Thermo Fisher Scientific, #AM9624). The tissue pellet was digested in 20-30 mL of 1:1 Accutase/TrypLE for 30 min at 37°C and filtered through a 70 μm-filter. The flow-through was taken up in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). After centrifugation for 5 min at 1,400 rpm, the pellet was taken up in 5 mL of DMEM/10% FBS and kept at 4°C. The lysate on the filter was digested again, followed by filtering and washing steps. This second pellet was pooled with the first pellet and centrifuged for 5 min at 1,500 rpm. The total pellet was resuspended in 1-2 mL of DMEM/10% FBS. A Percoll gradient was composed, containing 15 mL of 70% Percoll (Percoll SIP (Sigma-Aldrich/Merck/GE Healthcare, #17-0891-01) in PBS) and 20 mL of 15% Percoll (diluted in RPMI). The cell suspension was layered on this Percoll gradient and centrifuged for 30 min at 2,000 rpm with no brake. The trophoblast-containing interphase layer was separated, taken up in DMEM/10% FBS, and centrifuged for 5 min at 1,500 rpm. The pellet was washed in PBS/2% FBS/1 nM EDTA, taken up in 1 mL of the same buffer, and the number of cells were counted.

### Trophoblast Enrichment

Further enrichment of trophoblasts was established by magnetic bead retraction. For this, the EasySep human PE positive selection kit II was used (Stem Cell Technologies, #17684). All incubations were performed at room temperature in the dark: 100 μL of FcR blocker and 20 μL of PE-labeled ITGA6 (CD49f) antibody were added to the cells for 15 min. Further steps included addition of 30 μL of Selection Cocktail and 30 μL of RapidSpheres, both followed by 15 min of incubation. The step of bead retraction was repeated three times by replacing the flow-through each time again on the column. After the final step, cells were washed in PBS/2% FBS/1 nM EDTA and resuspended in TS-basal medium. This medium has been described previously (14) and contains DMEM/F-12 with 0.05 mM 2-mercapto-ethanol, 0.2% FBS, 1% penicillin/streptomycin (p/s), 0.3% bovine serum albumin (BSA), 0.5% KnockOut Serum Replacement (KSR), and 1% Insulin-Transferrin-Selenium (ITS)-X. A summary of reagents and growth factors is displayed in **Supplementary Table 1**.

### Culturing of CTB and Differentiation to EVT

Cells were cultured on collagen IV-coated plates in TS medium, which represents TS-basal medium (see above) supplemented with 1.5 μg/mL of L-ascorbic acid, 50 ng/mL of epithelial growth

factor (EGF), 2 μM CHIR-99021, 0.5 μM A 83-01, 1 μM SB431542, 0.8 mM Valproic acid, and 5 μM Y-27632 (14). For coating of the plates, 5 μg/mL of collagen IV in PBS was incubated for 90 min at 37°C. After washing with PBS, 0.5 x $10^6$ CTB were incubated per 2 mL of TS medium at 37°C. During culturing the medium was refreshed every 2-3 days. Cells were transferred to a new, coated dish when they had reached a confluence of around 80%.

For phenotypic characterization of the basal CTB cultures, the cells were kept in culture for three days and subjected to flow cytometry, qPCR analysis, and Luminex analysis (see below). To harvest the cells, TS medium was removed and cells were washed by PBS. After addition of 1 mL of TrypLE reagent (to dissociate cells), the plate was incubated 15 min at 37°C and 1 mL of TS medium was added.

To study EVT, CTB cultures were harvested and 0.75 x $10^5$ cells were transferred to a fresh, collagen IV-coated (1 μg/mL) dish and incubated in 2 mL of EVT medium. This medium contains DMEM/F-12 supplemented with 0.1 mM 2-mercapto-ethanol, 0.5% p/s, 0.3% BSA, 1% ITS-X, 7.5 μM A 83-01, 2.5 μM Y-27632, 100 ng/ml of NRG1, and 4% KSR (14). At the end of resuspension, Matrigel (final concentration of 2%) was added. After three days, the medium was replaced with the same content but minus NRG1 and with 0.5% Matrigel. At six days, at approximately 80% confluence, the cells were either harvested for phenotypic characterization or transferred to a new, coated dish and incubated further for several days in EVT medium minus NRG1 and KSR and with 0.5% Matrigel. After this, cells and medium were subjected to characterization.

## Culturing of Immortalized Trophoblast Cell Lines and Mesenchymal Stromal Cells

As a control to the primary trophoblast cultures, the trophoblast cell lines JEG-3 and JAR were studied. These were cultured in IMDM with 10% FBS and p/s. In addition, fetal mesenchymal stromal cells (MSC) were studied, which were cultured on collagen-IV-coated plates in DMEM/F-12 supplemented with 10% FBS and p/s.

## Karyotyping

Three cell lines (QH1, QG1, RC2) were checked for DNA stability at later passages (17-18) by karyotyping, along with JAR and JEG-3. For this, 25 μL (250 ng) colcemid per mL of medium was added and incubated for 1 hour. Cells were then removed from the plate by trypsin/EDTA, fixed with methanol/acetic acid (3:1), incubated overnight at 60°C, and treated with Giemsa/Leishman stain to examine GTG banding on metaphase chromosomes from five cells per sample. Metaphases were analyzed on a GSL-120 platform (Leica) using CytoVision software.

## Genotyping

Typing of fetal tissues and maternal blood for the classical HLA molecules was performed at the National Reference Laboratory for Histocompatibility Testing at the LUMC, The Netherlands. Assessment of the fully phased *HLA-G* sequence was performed as described in a previous paper (15).

## Quantitative PCR for mRNA Analysis

RNA was extracted according to a previously described protocol (16). Quantitative PCR analysis was performed on a ViA7 in 15 μL-reactions containing 3 μL of 1:25 diluted cDNA, 0.3 μM primers, and 7.5 μL SybrGreen mix (Bio-Rad). Primer sequences are summarized in **Table 1**. The PCR program for *HLA-A*, *-B*, *-C*, and *DRB1* started with 95°C for 10 min, followed by 40 cycles of 95° C for 15 seconds, 68°C (decreasing one degree every cycle to 64°C) for 45 seconds, and 72°C for 30 seconds. All other markers were tested by the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. Each PCR run was ended with a melting curve whereby the temperature was gradually increased from 55°C to 95°C at a rate of 0.05°C per second. Signals for the transcripts of interest were corrected for the average signal of two reference genes (*GAPDH* and *ACTB*) using the ΔCq calculation. For HLA-G-isoform-specific PCR assays, plasmid constructs containing either the G1, G2 or G3 isoform were tested as controls. These constructs were custom-made through GeneArt Gene Synthesis (Thermofisher Scientific).

## MicroRNA Profiling

Thirteen samples (CTB/EVT from QG1, QH1, RF1; JAR; JEG-3; MSC from fetus and mother) were screened for approximately 750 microRNAs by real-time PCR using human miRNOME panels I +II (Exiqon, Vedbaek, Denmark). For each sample, 40 ng of RNA was reverse transcribed into cDNA using the Universal cDNA synthesis kit II (Exiqon) and dispersed over the wells. To correct for inter-assay variation, the samples were standardized to each other using the interplate controls (UniSp3) present on each plate. Next, input variations between samples were corrected using the geometric mean of four reference small-RNAs (423-3p, 423-5p, 103, U6), as indicated in the Exiqon guidelines. Then, detectable microRNAs were selected by including only the ones showing Cq < 37 in more than half of all samples.

## Flow Cytometry

To detect surface markers, cells were incubated with antibodies for 30 min on ice, washed with PBS/1% FBS, and incubated for 60 min in 1 mL of Fixation/Permeabilization diluent (ThermoFisher Scientific, 00-5123-43 and 00-5223) at room temperature. For intracellular stainings (VIM, KRT) cells were subsequently incubated in 2 mL of Permeabilization Buffer (ThermoFisher Scientific, 00-8333), followed by addition of the antibody, washing, and resuspension in PBS/1% FCS. An overview of antibodies for flow cytometry is summarized in **Table 2**.

## Assessment of Proteins in the Supernatant

Culture medium of three CTB/EVT cultures (QG1, QH1, RC2) and control cell lines was subjected to assessment of soluble (s) HLA-G levels using an ELISA kit (MyBioSource, San Diego CA, #MBS2516229). Positive control in this assay was the HLA-G standard that was included in the kit, while MSC that are expected to not secrete sHLA-G functioned as negative control. Cytokines using a 17-plex Luminex assay were measured in four cultures (QG1, QG2, QH1, RG3). The procedure was carried as described previously (16). The following proteins were assessed: G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8,

**TABLE 1** | Overview of primers used for quantitative PCR assays in the study.

| Target | Forward sequence | Reverse sequence |
|---|---|---|
| ACTB | ACCACACCTTCTACAATGAG | TAGCACAGCCTGGATAGC |
| CGA | AACCCATTCTTCTCCCAGCC | GTGGACTCTGAGGTGACGTT |
| ELF3 | GGCCGATGACTTGGTACTGA | CAGCTCCTCAAGGGCACAAT |
| ELF5 | TAAATCGGAAGCCCTGGCAA | ACTAACCTTCGGTCAACCCG |
| EPCAM | GAATGGCAAAGTATGAGAAGGCTGA | TCCCACGCACACACATTTGTAA |
| GAPDH | ACCCACTCCTCCACCTTTGAC | TCCACCACCCTGTTGCTGTAG |
| GATA3 | CTCATTAAGCCCAAGCGAAG | GCATTCCTCCTCCAGAGTGT |
| HLA-A generic | TGGAGGAGGAAYAGCTCAGATA | CAAGGCAGCTGTCTCACAC |
| HLA-B generic | CCTAGCAGTTGTGGTCATC | AGCCCTGGGCACGGTCG |
| HLA-C generic | TGTCCTGGYTGTCCTAGCT | TGTCTCAGGCTTTACAAGYGA |
| HLA-C*01 | CACAGACTGACCGAGTGAG | CCCCAGGTCGCAGCCAC |
| HLA-C*02/12 | CCGCGGGTATGACCAGTC | CTCCAGGTAGGCTCTCCA |
| HLA-C*02/17 | CGAGTGAACCTGCGGAAA | GAGCCACTCCACGCACTC |
| HLA-C*03 | CACAGACTGACCGAGTGAG | AGCGTCTCCTTCCCATTCTT |
| HLA-C*04/18 | CGAGTGAACCTGCGGAAA | GCCCCAGGTCGCAGCCAA |
| HLA-C*05 | CGAGTGAACCTGCGGAAA | CGCGCGCTGCAGCGTCTT |
| HLA-C*06 | TACTACAACCAGAGCGAGGA | GGTCGCAGCCATACATCCA |
| HLA-C*07 | TACTACAACCAGAGCGAGGA | ACGGGCCGCCTCCA |
| HLA-C*07:03 | TACTACAACCAGAGCGAGGA | GAGCCACTCCACGCACAG |
| HLA-C*08 | ACGACACGCAGTTCGTGCA | GCGCAGGTTCCGCAGGC |
| HLA-C*14 | CCACTCCATGAGGTATTTCTC | GGTCGCAGCCAAACATCCA |
| HLA-C*16 | CCGCGGGTATGACCAGTC | CCCTCCAGGTAGGCTCTCT |
| HLA-C*18 | AGTCCGAGAGGGGAGCCC | GCCCCAGGTCGCAGCCAA |
| HLA-DRB1 generic | ACAGTGGAATGGAGAGCACGG | CCAGAGTGTCCTTTCTGATTCCT |
| HLA-G generic | GACAGCGACTCGGCGT | GTGTTCCGTGTCTCCTCT |
| HLA-G1 | ATGCTGCAGCGCGCGGACC | TGGGCAGGGAAGACTGCTTCCA |
| HLA-G2 | ACCAGAGCGAGGCCAACCC | GGCAGGGAAGACTGCTTCCA |
| HLA-G3 | AACCAGAGCGAGGCCAAGCAG | AGCTCCCTCCTTTTCAATCTGAG |
| ITGA1 | TCAATGACTTTCAGCGGCCC | ACCTCTCCCAACTGGACACT |
| ITGA5 | GGCTTCAACTTAGACGCGGA | GGCCGGTAAAACTCCACTGA |
| ITGA6 | GAGCTTTTGTGATGGGCGATT | CTCTCCACCAACTTCATAAGGC |
| TEAD4 | TGGACAAGCCCATCGACAAT | TGCCTGGTCCTTTAGCTTGG |
| TFAP2C | TGGACGAGGTGCAGAATGTC | TCAGTGGGGTTCATTACGGC |

IL-10, IL-12-p70, IL-13, IL-17, MCP-1 (CCL2), MIP-1β (CCL4), and TNF-α. In addition, active TGF-β1, TGF-β2, and TGF-β3 were analyzed by single-assay Luminex.

## Co-culture of EVT and Immune Cells

CTB from QH1 were differentiated for six days to EVT. On the sixth day, 300,000 peripheral blood lymphocytes (PBL) of four different donors, obtained during pregnancy, were added to 150,000 EVT. This co-culture was maintained for three days in a 1:1 mixture of RPMI/HS/p/s/glut and EVT medium (see above). HLA-C genotypes of the PBL were C*03/C*07, C*04/C*07, C*07/C*07, and C*04/C*06. As control, PBL were cultured in the absence of EVT in either RPMI/HS/p/s/glut medium or in the 1:1 mixture with EVT medium. EVT remained attached to the plate, whereas immune cells remained floating. Hence, at the end of the co-culture immune cells were removed from the culture medium, gated for CD3 together with CD4 or CD8, and analyzed by flow cytometry for expression of CD69 and HLA-DR. These represent early and late T cell activation markers, respectively (18).

## Statistics

Difference in markers on T cells in absence or presence of EVT was calculated with paired samples t-tests. $P<0.05$ was considered statistically significant.

# RESULTS

## Establishment of CTB and EVT Cultures and Success Rate

After digestion of the placental tissue and enrichment steps, trophoblasts were transferred to collagen-IV-coated culture plates in TS-basal medium. At a certain point, cultures were differentiated to EVT in specific medium containing Matrigel. Whereas cells at the CTB stage were forming a dense cluster, were rounded in structure and adhered to the plate, cells at the EVT stage had a more spindle-shaped appearance. Macroscopic and microscopic pictures of a representative successful experiment are shown in **Figure 1**.

Cells from a total of 12 donors were processed for culturing. Cultures from five donors were excluded from further study due to various reasons. Within the first weeks of culturing, cells from donor QI2 got infected and cells from donor PW1 were growing very slowly. Characterization of RC1 showed a considerable number of VIM-positive cells indicating contamination by mesenchymal stromal cells (MSC). Cells from PX1 still showed a circular morphology after six days of incubation in EVT differentiation medium. Cells from donor RF2 did not give interpretable results by genotyping. Hence, these five culture were excluded from further studies.

**TABLE 2** | Overview of antibodies used for flow cytometry in the study[1].

| Protein | Fluorochrome | Company | Clone | Catalogue number |
|---|---|---|---|---|
| CD14 | FITC | BD Bioscience | MΦP9 | 345784 |
| CD31 | FITC | BioLegend | WM59 | 303104 |
| CD45 | APC | BioLegend | HI30 | 304012 |
| CD56 | Pacific Blue | BioLegend | HCD56 | 318325 |
| CD73 | BV421 | Biolegend | AD2 | 344008 |
| CD90/THY1 | PE | Miltenyi Biotec | REA897 | 130-114-902 |
| CDH1/CD324 | Alexa-488 | BioLegend | 67A4 | 324110 |
| EGFR/ERBB1 | Brilliant Violet 421 | BioLegend | AY13 | 352911 |
| ERBB2 | FITC | BioLegend | 24D2 | 324404 |
| ERBB3 | PE | BioLegend | 1B4C3 | 324706 |
| HLA-A, B | Biotin | in-house developed HuMoAbs [2] | | |
| HLA-A/B/C | Pacific Blue | BioLegend | W6/32 | 311417 |
| HLA-C | PE | BD Pharmingen | DT-9 | 566372 |
| HLA-G | Alexa-488/PE | BioLegend | 87G | 335917 |
| HLA-G | PE | Abcam | MEMG9 | ab24384 |
| HLA-G | FITC | Abcam | G233 | ab7904-100 |
| IgG1 | Brilliant Violet 422 | BioLegend | MCOP-21 | 400157 |
| IgG1 | Alexa-488 | eBioscience | P3.6.2.8.1 | 53-4724-81 |
| IgG1 | PE | eBioscience | P3.6.2.8.1 | 12-4717-42 |
| IgG2a (mouse) | Alexa-488 | BioLegend | MOCP-173 | 400238 |
| IgG2a (mouse) | Alexa-647 | Biolegend | MOCP-173 | 400239 |
| IgG2a (Rt) | PE | Biolegend | RTK2758 | 400508 |
| IgG2a (mouse) | FITC | ThermoFisher | eBM2a | 11-4724-81 |
| IgG2b (mouse) | APC | BioLegend | 27-35 | 402206 |
| IgG2b (mouse) | PE | Biolegend | 27-35 | 402204 |
| ITGA1/CD49a | APC | BioLegend | TS2/7 | 328314 |
| ITGA2/CD49b | APC | BioLegend | P1E6-C5 | 359309 |
| ITGA2/CD49b | PE | BioLegend | P1E6-C5 | 359307 |
| ITGA4/CD49d | PE | BioLegend | 9F10 | 304303 |
| ITGA5/CD49e | APC | BioLegend | NKI-SAM-1 | 328011 |
| ITGA6/CD49f | PE | BioLegend | GoH3 | 313612 |
| ITGB1/CD29 | APC | BioLegend | TS2/16 | 303007 |
| KRT | FITC | Miltenyi Biotec | CK-6H5 | 130-118-964 |
| NOTCH1 | APC | BioLegend | MHN1-519 | 352107 |
| VIM | Alexa-647 | BioLegend | O91D3 | 677807 |

[1]*CDH1, cadherin-1; EGFR, epithelial growth factor receptor; KRT, cytokeratins; VIM, vimentin.*
[2]*HuMoAbs used were GV5D1 (A1/A9), SN607D8 (A2/A28), SN230G6 (A2/B57/58), BRO11F6 (A3/A11/A24), VTM1F11 (B7/B27/B60), BVK1F9 (B8), DK7C11 (B12), and HDG8D9 (B35/51), as described previously (17).*

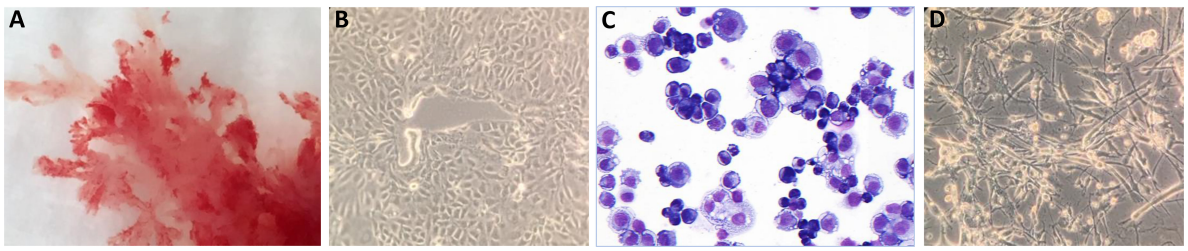


**FIGURE 1** | Macroscopic and microscopic pictures of trophoblast cells. **(A)** Cultured trophoblasts from a representative experiment (donor QH1) are shown. CTB were evaluated by **(B)** light microscopy or **(C)** after May Grunwald-Giemsa staining. The cells form a dense cluster, are rounded in structure and adhere to the plate. **(D)** Cells at the EVT stage have a more spindle-shaped appearance.

Seven cultures were characterized for cell surface markers by flow cytometry. First, gating strategies were set by forward-sideward scatter, 7-AAD live-dead staining, and CD45 staining to select singlet, viable, and non-hematopoietic cells (**Figure 2A**). Whereas immediately after isolation most cells (>95%) were positive for ITGA6 and KRT with around 50% of the cells showing positivity of HLA-G and CDH1/CD324 and only the minority (<9%) being positive for ITGA5 and HLA-C, upon culturing more cells gained expression of HLA-C (51%), HLA-G (>88%), and CDH1/CD324 (>90%) (**Figures 2B, C**). The relatively high proportion of HLA-$G^+$ cells after isolation probably may be explained because they mostly represented

distal cell column trophoblasts, as detected in first-trimester placenta tissue (**Supplementary Figure 1A**). Secondly, HLA-G expression may have been driven by the high collagen IV content in the tissue surrounding the cells (**Supplementary Figure 1B**).

In general, we experienced the CTB to be easily maintained for many passages. Maintenance of chromosomal stability of these cells at later passages has been shown before (14), and here we confirmed this by karyotyping on three CTB lines at passage 17-18 (**Supplementary Figure 2**). QG1 was normal except for a slight addition on chromosome 17 in two out of five cells. We genotyped the cultures for the classical HLA molecules and for *HLA-G*. **Table 3** shows the overview of the *HLA-C* allele genotypes and the coding allele sequence and 3'-UTR haplotypes of *HLA-G*.

## Classical Trophoblast Markers

GATA3, TFAP2c, ELF3, and ELF5 have previously been identified as trophoblast markers (19, 20). Indeed, expression of these markers was high in primary CTB and EVT compared to negative controls (MSC), while expression in secondary trophoblast cell lines JAR and JEG-3 followed the same pattern (**Figure 3A**). We next screened for microRNAs in a subset of samples: 140 microRNAs could be detected, of which 116 showing the biggest differences between groups were included in hierarchical clustering (**Figure 3B**). Expression of microRNAs on chromosome (C)19 is a typical marker of trophoblasts (19, 21, 22), and indeed we found 25 C19 microRNAs to be elevated in primary CTB and EVT compared to controls (**Figure 3B**, green boxes; **Figure 3C**).

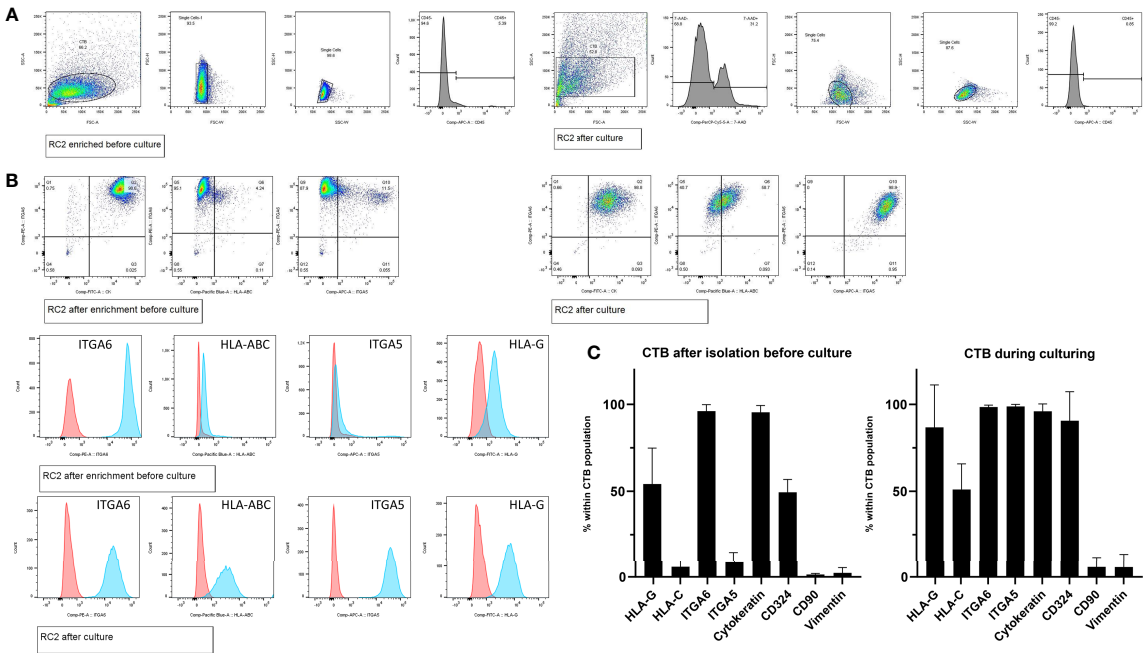


**FIGURE 2** | Cell frequencies by flow cytometry after enrichment and during CTB culturing. **(A)** Representative gating strategy from one donor (RC2), to select singlet cells by forward-sideward scatter that were viable (7-AAD$^{neg}$) and non-hematopoietic (CD45$^{neg}$). **(B)** Representative flow cytometry dot blots and histogram plots of trophoblasts from one donor (RC2), after cell enrichment but before culturing (upper panel) and during culturing in CTB stem cell medium (lower panel). **(C)** Mean cell frequencies from seven donors showing the positivity for each of the markers of interest, both after cell enrichment but before culturing (left graph) and during culturing in CTB stem cell medium (right graph).

**TABLE 3** | Overview of the trophoblast cultures characterized in the study and their HLA-C and HLA-G genotypes[1, 2].

| Donor code | GA (weeks) | HLA-C genotype | HLA-G coding sequence | HLA-G 3'-UTR |
|---|---|---|---|---|
| QG1 | 6 | C*07:01; C*07:04 | G*01:01:02:01 | UTR-2 |
| QG2 | 9 | C*07:01; C*07:02 | G*01:01:01:01/G*01:01:01:05 | UTR-1/UTR-4 |
| QH1 | 9 | C*07:01/02; C*07:02/10 | G*01:01:01:01 | UTR-1 |
| RC2 | 6 | C*01:02; C*05:01 | G*01:01:01:05 | UTR-4 |
| RF1 | 6 | C*03:04; C*16:01 | G*01:01:01:01/G*01:04:01:01 | UTR-1/UTR-3 |
| RG2 | 6 | C*04:01; C*07:01 | G*01:01:03:03/G*01:03:01:02 | UTR-7/UTR-17 |
| RG3 | 8 | C*02:10:02; C*03:03 | G*01:01:01:01 | UTR-1 |

[1]*GA, gestational age; CDS, coding sequence; 3'-UTR, 3 prime untranslated region.*
[2]*Five out of 12 cultures were excluded from further study and not genotyped. QI2 and PW1 due to infection and poor growth, respectively, at the start of the culture; RC1 due to contamination by VIM-positive MSC; PX1 due to their circular morphology after EVT differentiation; and RF2 due to non-interpretable genotype results.*

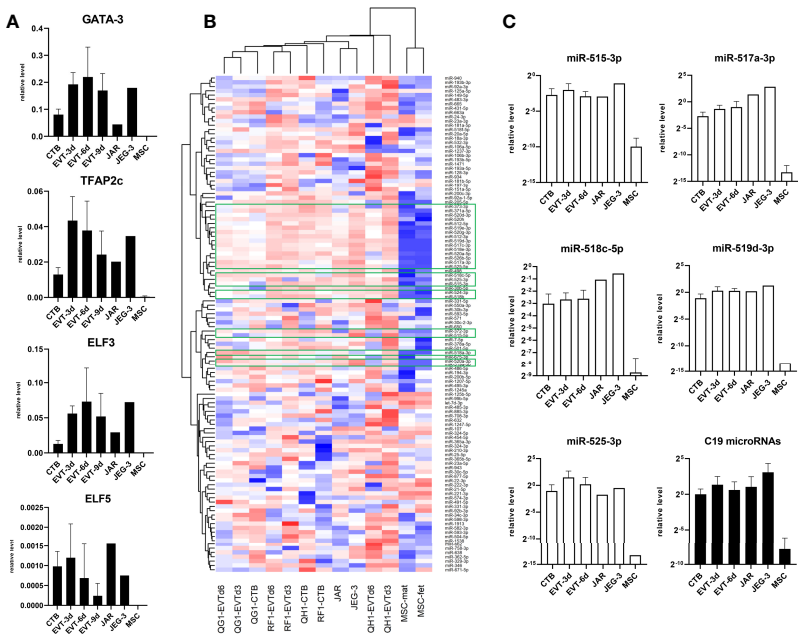


**FIGURE 3** | Expression of classical trophoblast markers in primary trophoblast cultures. **(A)** Messenger RNA expression of GATA3, TFAP2c, ELF3, and ELF5 (n = 6 primary cultures; n = 2 MSC cultures). **(B)** Approximately 750 microRNA were screened in primary CTB/EVT (n = 3), along with JAR, JEG-3, and two MSC lines. Out of these, 116 microRNAs were included for hierarchical clustering. Twenty-five C19 microRNA (green boxes) were typically expressed in trophoblasts. **(C)** Expression level of five typical C19 microRNAs (open bars) and average level of all C19 microRNAs per group (black bars). All graphs are represented by means with SD.

## CTB and EVT Express a Distinct Pattern of Cell Adhesion Markers

As expression of integrins and epithelial/mesenchymal markers may help in clarifying the source, identity, and differentiation of trophoblasts (23–26), we next characterized the trophoblast cultures by qPCR analysis for different cell adhesion markers. Messenger RNA expression of integrins *ITGA1* and *ITGA5* increased when CTB were differentiated to EVT and reached a plateau from six days of differentiation onwards (**Figure 4A**). In contrast, *ITGA6* expression was relatively high in CTB and it decreased in EVT. TEAD4 and EPCAM have been described as CTB markers (12, 14, 27), and we found their mRNA expression indeed to be high in CTB cultures but decreased in EVT cultures. CGA is specific for syncytiotrophoblast (14, 27, 28), but we found its RNA expression also to be high in CTB, the level of which further increased in EVT (**Figure 4A**).

The results by qPCR for integrin markers were verified by flow cytometry on four cultures (**Figures 4B, C**), after establishing gating strategies by forward-sideward scatter and 7-AAD live-dead staining to select viable singlet cells (**Figure 4B**). ITGA1 and ITGA5 showed low and medium signals, respectively, in all CTB cultures and increased upon differentiation to EVT. ITGA6 signals were relatively high in all CTB cultures. Other integrins including ITGA2 and ITGB1 displayed homogeneous expression among the different trophoblast cultures, showing relatively high signals in CTB which progressively decreased during EVT differentiation. Similarly, cell adhesion molecule E-cadherin displayed relatively high expression on CTB, which decreased between three and nine days of EVT differentiation. Three members of the ErbB family, namely epidermal growth factor receptor (EGFR/ERBB1), ERBB2, and ERBB3, all showed relatively low signals by flow cytometry in CTB. EGFR signals peaked in day-3 EVT cultures and thereafter decreased. Both ERBB2 and ERBB3 showed increasing signals in EVT between three days and nine days of differentiation.

## Primary Trophoblasts Are Distinct From Immortalized Trophoblast Cell Lines and MSC in Cell Surface Markers

We further studied the trophoblast cell lines JEG-3 and JAR and compared them to primary trophoblast cultures with respect to cell surface markers. A control of fetal MSC was additionally included. The expression patterns of the different cell types have been summarized in **Table 4**. The two secondary cell lines were more similar in their expression pattern to primary CTB than to EVT. Only ITGA2 was dissimilar between the cell lines and the CTB. In contrast to primary trophoblasts, JEG-3 cells had low expression of HLA-C and HLA-G and JAR cells were devoid of these molecules. MSC typically expressed VIM and CD73, which were absent on trophoblasts. Furthermore, MSC moderately expressed ITGA1, ITGA6, and HLA class I.

## EVT Express HLA-C but No Other Classical HLA Molecules

We investigated expression of the classical HLA molecules in the trophoblast cultures (**Figure 5**). As the cells had been subjected to *HLA-C* genotyping (**Table 3**), we verified mRNA expression of

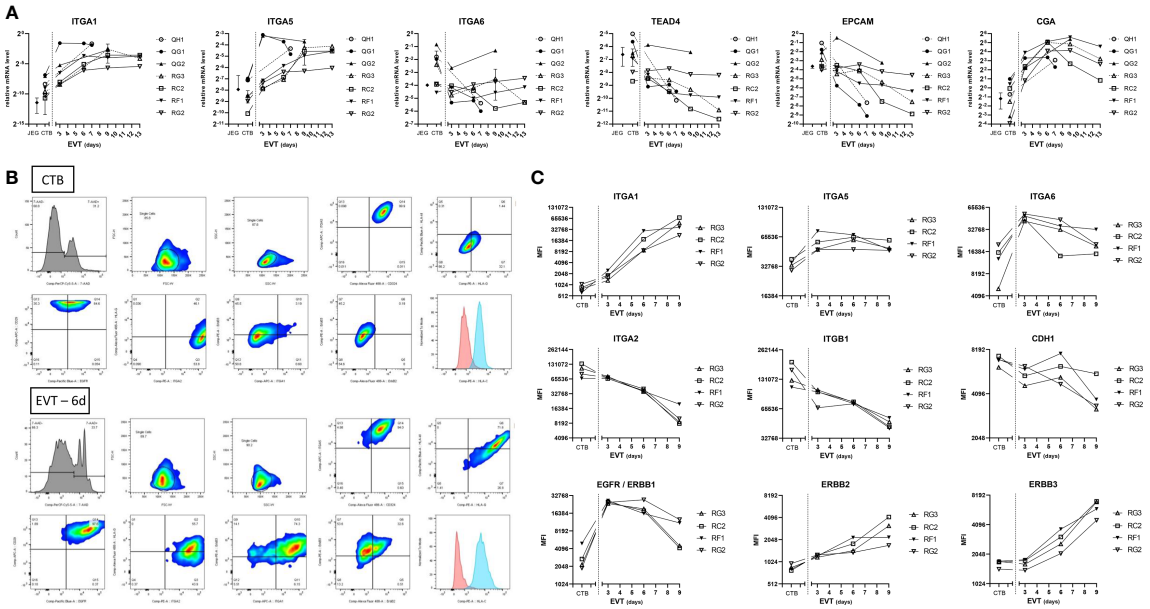


**FIGURE 4** | Expression of cell adhesion and epithelial markers in primary trophoblast cultures. **(A)** Expression of cell adhesion and trophoblast markers at the RNA level by culture days. Cultures (n = 7) were analyzed at the CTB and EVT phase for mRNA expression of *ITGA1*, *ITGA5*, *ITGA6*, *TEAD4*, *EPCAM*, and *CGA*. **(B)** Representative gating strategy from one donor (RC2), to select singlet cells by forward-sideward scatter that were viable (7-AAD$^{neg}$). The figure shows dot blots of the markers studied in CTB (upper two panels) and EVT after 6 days of differentiation (lower two panels). **(C)** Expression of cell adhesion and epithelial markers at the protein level by culture days. Cultures (n = 4) were analyzed by flow cytometry at the CTB and EVT phase for surface expression of integrins, CDH1, and three members of the ErbB family.

**TABLE 4** | Surface markers on various cell cultures[1].

| Protein | CTB | EVT-d3 | EVT-d6 | EVT-d9 | JEG-3 | JAR | MSC |
|---|---|---|---|---|---|---|---|
| ITGA1 | - | ++ | ++ | +++ | - | - | ++ |
| ITGA2 | +++ | ++ | + | + | ± | - | +++ |
| ITGA5 | ++ | +++ | ++ | +++ | +++ | ++ | ++ |
| ITGA6 | +++ | ++++ | ++ | ++ | ++++ | ++++ | ++ |
| ITGB1 | ++++ | +++ | ++ | ++ | +++ | ++ | ++++ |
| CDH1 | +++ | ++ | ++ | + | ++ | +++ | ± |
| KRT | +++ | +++ | ++ | + | ++ | ++++ | - |
| EGFR/ERBB1 | ++ | +++ | ++ | + | +++ | +++ | ++ |
| ERBB2 | ± | + | ++ | +++ | + | + | + |
| ERBB3 | + | + | ++ | +++ | + | + | - |
| HLA-ABC | ± | + | ++ | +++ | + | - | ++ |
| HLA-G | ± | + | ++ | +++ | ± | - | – |
| VIM | - | - | - | - | - | - | ++++ |
| CD73 | - | - | - | ± | - | - | ++++ |

[1]*The intensity of antibody staining was scored – (negative), ± (minimal), + (low), ++ (medium), +++ (high) or ++++ (very high). MSC, mesenchymal stromal cells; CDH1, cadherin-1; KRT, cytokeratins; EGFR, epithelial growth factor receptor; VIM, vimentin.*

*HLA-C* using allele-specific PCR primers. In each culture, positivity of mRNA expression was indeed only found for those alleles that had been found positive by DNA typing (**Figure 5A**). Upon differentiation to EVT, both *HLA-C* mRNA expression and surface expression of pan-HLA class I increased (**Figure 5B**). At the RNA level, out of all classical HLA loci *HLA-C* by far showed the most abundant expression both in CTB and EVT, the level being approximately eight, five, and 14 powers higher compared to that of *HLA-A*, *HLA-B*, and *HLA-DRB1*, respectively, on a log-2 scale (**Figure 5C**, left panel).

We next verified surface expression of individual HLA-A and -B alleles on two trophoblast cultures (QG1: HLA-A1/A68(28)/B8/B44 (12); QH1: HLA-A2/A2/B7/B7) by flow cytometry using human monoclonal antibodies that were developed in-house, as previously described (29). Four different genotyped maternal blood samples were incorporated as controls to verify HLA antibody specificity (**Figure 5C**, right panel; **Supplementary Figure 3A**). JAR and JEG-3 cell lines were negative for HLA-A and -B (**Supplementary Figure 3B**). In EVT after seven days of differentiation, the surface index for HLA-A1, -A28, -B8, and -B12 in QG1 was around zero

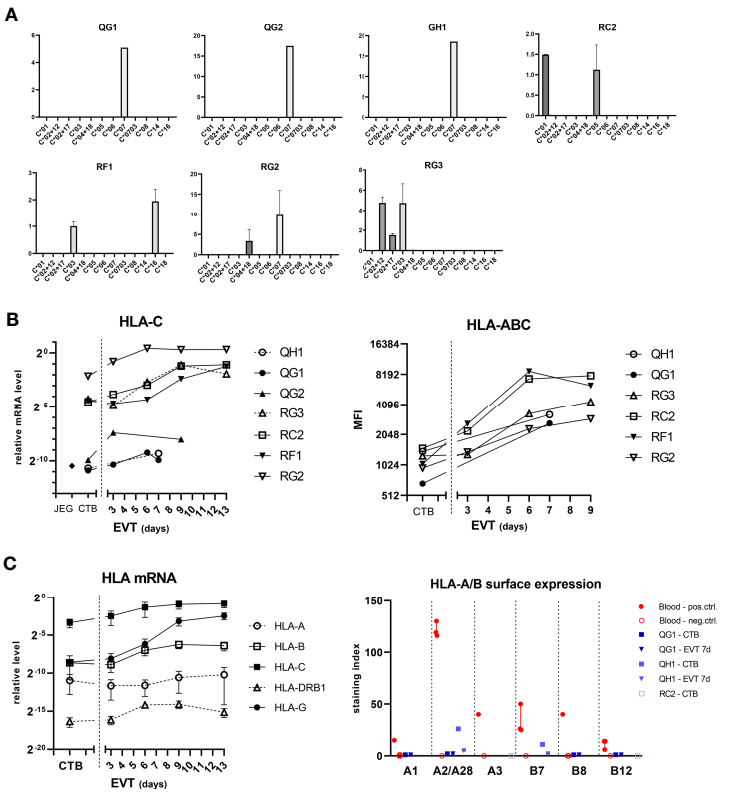


**FIGURE 5** | Expression of HLA-C in trophoblasts. **(A)** Messenger RNA expression of *HLA-C* according to the *HLA-C* alleles. RNA from CTB cultures (n = 7) was analyzed by quantitative PCR using primer sets that are specific for different *HLA-C* alleles. The vertical axes represent relative mRNA expression of *HLA-C* alleles, corrected for the average signal of two reference genes (*GAPDH*, *ACTB*). The graph shows means with SEM of two independent experiments per culture. **(B)** *HLA-C* mRNA expression was determined by quantitative PCR using generic primers that targeted all alleles (left panel). HLA-C was also studied by flow cytometry using antibodies against pan-HLA class I (right panel). **(C)** Messenger RNA expression of *HLA-A*, *-B*, *-C*, *-DRB1*, and *-G* (left panel) was studied in CTB and EVT cultures. RNA was analyzed by quantitative PCR using locus-specific primer sets. The graph shows means with SEM. Surface expression of individual HLA-A and HLA-B alleles was investigated by flow cytometry using human monoclonal antibodies on four blood cell samples and two trophoblast cultures (right panel). The graph displays medians with interquartile ranges.

compared to blood samples (0-2 vs. 15, 119, 40, and 6-14, respectively) and expression of HLA-A2 and -B7 was minimal in QH1 (2-5 vs. 116-130 and 25-50, respectively; **Figure 5C**, right panel). We did observe in one CTB culture (QH1) some positivity for HLA-A2 (26.5) and -B7 (11.1), whereas the other CTB culture (QG1) was negative (A1: 0; A28: 1.7; B8: 0; B12: 0) (**Figure 5C** and **Supplementary Figure 3B**). Therefore, we tested CTB from a third donor, RC2 (HLA-A3/B44(12)/B56(22)). Both HLA-A3 and -B44 were negative by specific HLA antibodies in flow cytometry (**Supplementary Figure 3B**).

## EVT Upregulate HLA-G Expression and Secrete Soluble HLA-G

Next, we studied HLA-G expression in the trophoblasts (**Figure 6**). *HLA-G* mRNA expression progressively increased upon differentiation to EVT from three days to 13 days, and this observation was verified by flow cytometry using two different anti-HLA-G antibodies (MEM-G9 and 87G) (**Figure 6A**). For *HLA-G* mRNA analysis we developed primer sets that distinguished a single isoform, either *HLA-G1*, *G2* or *G3* (**Figure 6B**, left panel). Most of the *HLA-G* detected in CTB and the JEG-3 cell line consisted of the *G1* isoform; remarkably, with increasing time of differentiation to EVT the *G1* proportion further increased while the proportion of *G2* and *G3* decreased (**Figure 6B**, right panel). Furthermore, we analyzed relationship in the CTB cultures between the 3'-UTR haplotype of HLA-G and its expression and found that UTR-1 and UTR-4 haplotypes were related to relatively high expression and UTR-2 and UTR-7 with low expression (**Figure 6C**). Corresponding to the upregulated HLA-G expression by EVT, the supernatants of EVT, but not those from CTB, JEG-3/JAR cell lines, and MSC, displayed elevated levels of sHLA-G (20-40 ng/mL; **Figure 6D**).

## CTB and EVT Secrete Several Cytokines

In addition to the sHLA-G levels, we analyzed 20 different cytokines and growth factors in the supernatants of CTB and EVT cultures, as these could also have a paracrine effect on immune cells upon interaction with trophoblasts. IL-2, IL-5, IL-6, IL-8, and TNF-α could be detected, of which the latter showed the highest levels. Generally, CTB showed the highest cytokine

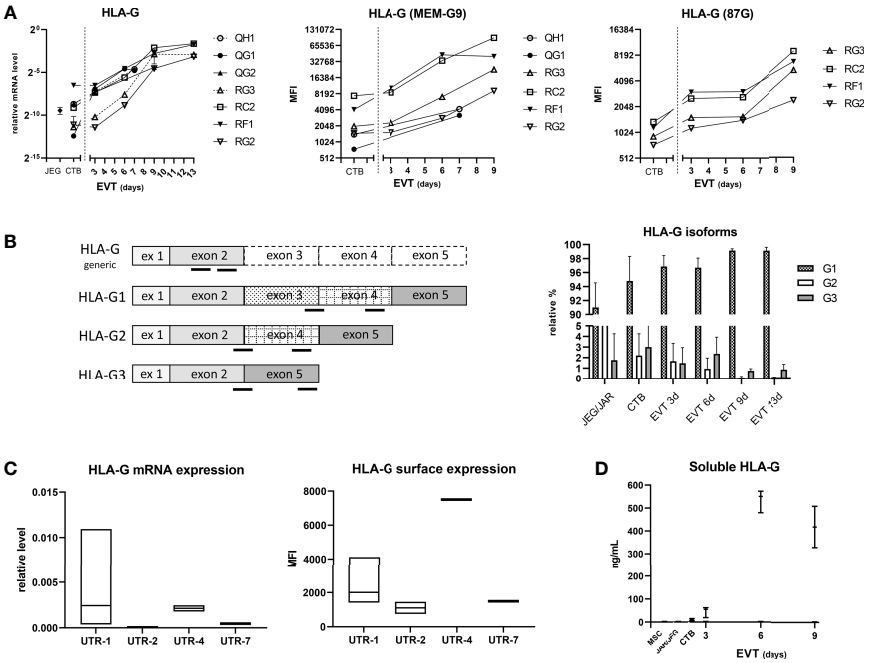


**FIGURE 6** | Expression of HLA-G in trophoblasts. **(A)** *HLA-G* mRNA expression was determined by quantitative PCR using generic primers that targeted all alleles (left panel). HLA-G was also studied by flow cytometry using antibodies against HLA class I and two epitopes on HLA-G1 and -G5 isoforms (MEM-G9 and 87G), respectively (middle and right panel). **(B)** *HLA-G1*, *G2*, and *G3* isoforms were separately studied by using isoform-specific primer sets (annealing locations indicated by black bars in the left panel). The results in the right panel are displayed as means with SD of n = 4 primary cultures. **(C)** HLA-G expression according to *HLA-G* 3'-UTR haplotype. Messenger RNA expression of *HLA-G* from seven CTB cultures was analyzed by quantitative PCR (corrected for the average signal of reference genes *GAPDH* and *ACTB*; left panel), whereas information of HLA-G surface expression by flow cytometry against MEMG9 was available for six CTB cultures (right panel). Expression data were grouped according to haplotype: UTR-1 (n = 4), UTR-2 (n = 2), UTR-4 (n = 2) or UTR-7 (n = 1). The graphs show floating bars (min to max with line at median). **(D)** Soluble HLA-G levels were determined by ELISA in supernatant of CTB (n = 3), EVT (n = 3), JEG-3 and JAR cell lines, and MSC (n = 2). The graph shows medians with the whiskers representing minimum and maximum values.

concentrations in the culture medium, and concentrations progressively decreased once the cells were differentiated to EVT (**Figure 7**).

## Co-Culturing of EVT and Immune Cells Leads to Decreased HLA-DR Expression on CD4$^+$ T Cells

Given our interest to use the trophoblast cultures as a model to study fetal-maternal interactions, as a proof of principle we investigated the effect of trophoblasts on immune cells. For this, we co-cultured four different sources of PBL together with EVT for three days. As control, PBL were cultured alone, either in their usual medium (RPMI plus supplements) or in a 1:1 mixture of EVT medium and RPMI medium. We evaluated two markers of T cell activation status, namely CD69 and HLA-DR. The composition of the medium did not affect expression of these markers on the T cells (**Figure 8**, first two bars of each graph). When cultured in the presence of EVT, CD4$^+$ T cells showed a significant decrease ($P<0.05$) in HLA-DR expression on their surface (**Figure 8**, upper right graph). No differences were seen when lymphocytes were fully matched, mismatched at one allele or fully mismatched for HLA-C in comparison to the trophoblasts. The activation marker CD69 did not show a difference in CD4$^+$ lymphocytes upon co-culturing. HLA-DR and CD69 expression on CD8$^+$ T cells was not affected (**Figure 8**, lower panel).

# DISCUSSION

For adequate study of the biology of trophoblasts and of their interaction with immune cells, stable trophoblast cultures are preferred. Here, we adopted a previously described protocol (14) for culturing CTB from first-term placental tissue and differentiating these to EVT. Isolation of trophoblasts from seven out of 12 donors (58.3%) led to successful results. Over the course of differentiation, trophoblasts upregulated HLA-C, HLA-G, ITGA1, and members of the ErbB family, and downregulated CDH1, ITGA2, and ITGB1. We verified that, like the *in-vivo* situation in the placenta, cultured EVT specifically express HLA-G and HLA-C but no other types of HLA molecules. In this way, the primary trophoblasts are clearly distinct from secondary, immortalized trophoblasts cell lines. Co-culturing of lymphocytes for three days with EVT led to decreased expression of HLA-DR on CD4$^+$ T cells.

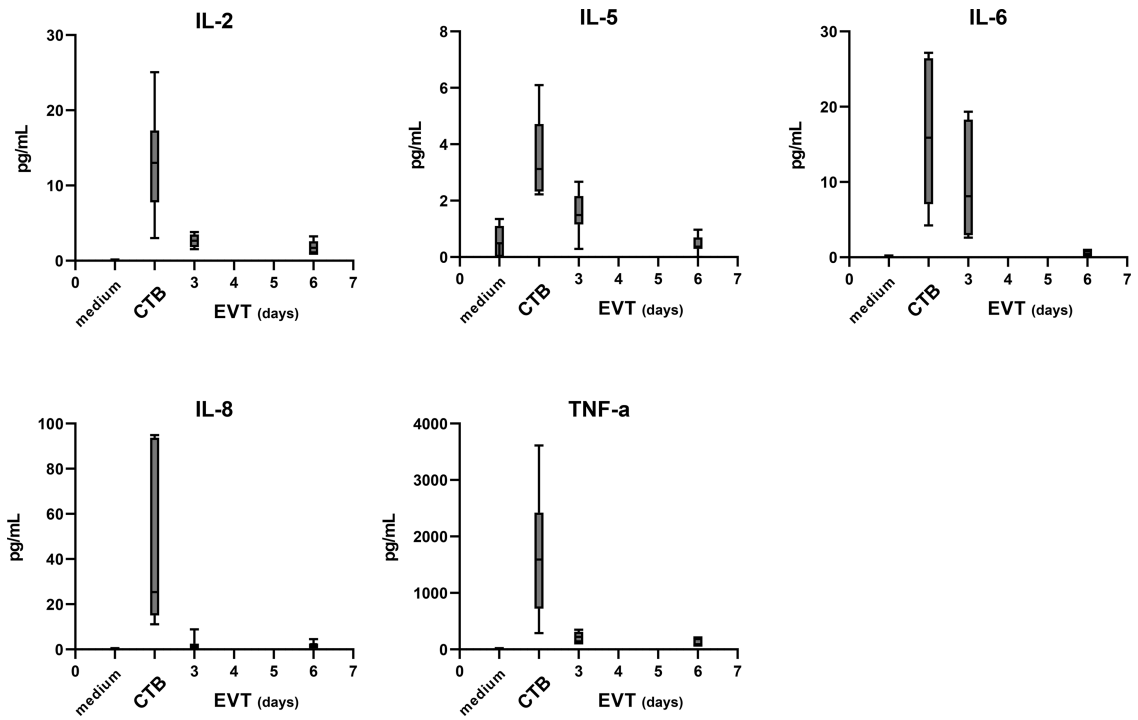


**FIGURE 7** | Cytokine secretion by trophoblasts in the culture medium. The level of 20 different cytokines and growth factors was analyzed by Luminex analysis in the medium from four cultures (QG1, QG2, RG3, and QH1 in triplicate). IL-2, IL-5, IL-6, IL-8, and TNF-α were detected above the minimum threshold value and are shown in this figure. The graph shows medians and the whiskers representing minimum and maximum values.

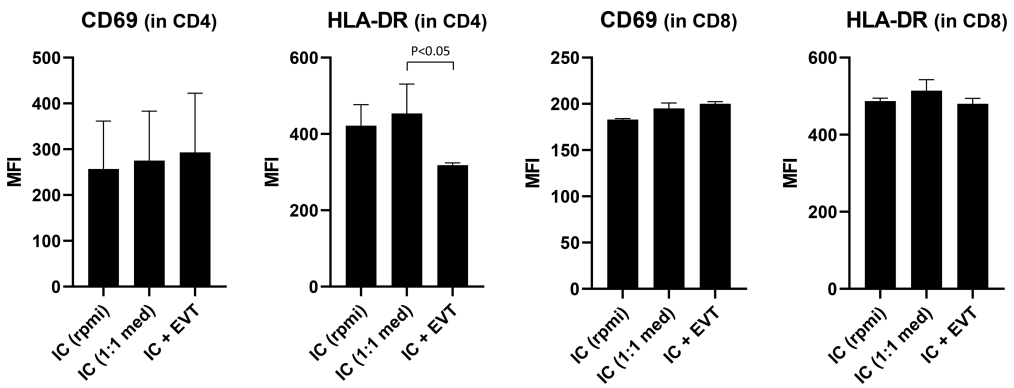


**FIGURE 8** | Expression of activation markers on $CD4^+$ and $CD8^+$ T cells following co-culturing of lymphocytes with EVT. First, EVT (culture QH1) were differentiated for six days. Then, lymphocytes from four different donors were added to the EVT for three days. HLA-DR and CD69 were studied by flow cytometry in both the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell population, both in the absence of EVT (first two columns) or in the presence of EVT (third column). The graph shows means with SEM. *P < 0.05.

We did verify three out of four features typical of trophoblasts, as reviewed before (19, 30–32), in our cultures: expression of classical trophoblast genes (TFAP2C, GATA3), expression of chromosome 19 microRNAs, and specific upregulation of HLA-G and HLA-C during differentiation to EVT. ELF5 methylation was not tested, but despite of this we did find ELF5 expression to be specifically elevated in the trophoblasts. Expression of other markers that we studied in CTB and EVT corresponds with that of earlier findings. CTB typically express epithelial cell markers (CDH1, EPCAM, EGFR) (12, 14, 27, 33–35) and ITGA6 (33, 36), express the specific marker TEAD4 (14, 37), and also produce a considerable amount of CGA (37–41). Indeed, early studies have shown that not only syncytiotrophoblast but also EVT express and produce human chorionic gonadotropin, which promotes trophoblast invasion (42–44). The relatively high proportion of $HLA\text{-}G^+$ cells directly

after cell isolation from first-trimester placentas may be explained because they mostly represent distal cell column trophoblasts. We verified that these cells at the base of the cell column indeed were HLA-G positive by immunohistochemistry, and they have been shown to be ITGA6$^+$ as well (45), similar to what we found in isolated cell fractions. The primary CTB cultures typically express ITGA2 and EPCAM, which suggests that the cells were derived from the cytotrophoblast column niche of the placenta, as put forward in previous studies (24, 46, 47). Of note, ITGA2$^+$ cytotrophoblasts at the base of the cell column express medium levels of HLA-G and EGFR and relatively high levels of CDH1 (24), similar to what we found in CTB after isolation. Upon differentiation to EVT, the cells showed a spindle-shaped morphology, reminiscent of MSC and in line with the earlier described process of epithelial-to-mesenchymal transition (23). EVT show upregulation of HLA-G (14, 19, 27, 28, 38, 48–51), HLA-C (36, 52), ITGA1 (14, 25, 49, 51), and ITGA5 (14, 25, 33, 36, 49, 51, 53), whereas CDH1 is downregulated (33, 34). EVT express ERBB2 and ERBB3, which have been shown to be activated by NRG1 (54), a compound that is part of the EVT differentiation medium. In our study, a fair number of CTB during culture expressed ITGA5 and HLA-G, which showed further increase to only moderate extent at the EVT stage. These observations may be related to a certain degree of differentiation, as cells were cultured from the start on collagen-IV coated plates. Indeed, differentiation of CTB upon attachment to matrix molecules has been demonstrated before (36, 55, 56). With respect to HLA-A and HLA-B, interestingly a recent study showed detectable surface expression on CTB and EVT in the 2D trophoblast stem cell model by flow cytometry (47). Here, we did find moderate surface expression of these molecules on one of the CTB cultures studied. Nonetheless, we did not detect HLA-A and HLA-B expression whatsoever at the RNA and protein level in EVT, which represented the principal cell type that was used further in co-culture experiments.

Immortalized trophoblast cell lines including HTR-8/SVneo, JEG-3, and JAR are frequently used for *in vitro* research as a model of human placental physiology. The HTR-8/SVneo cell line was shown to represent not a homogenous collection of cells, but rather to contain two populations of cells, namely cytokeratin-7 positive trophoblasts and vimentin-positive mesenchymal cells (3). JEG-3 and JAR are human trophoblast cell lines derived from a choriocarcinoma tumor. Their transcriptomic profile is considerably divergent from that of primary trophoblasts (1, 2), and due to their tumorigenic nature the three cell lines display cytogenomic differences (4), suggesting that any results obtained from immortalized cell lines should be confirmed in primary trophoblasts. In the current study, JEG-3 and JAR were similar to primary CTB cultures for most of the cell adhesion markers studied, but in contrast to primary EVT cultures these cell lines expressed no or minimal amounts of HLA-C and HLA-G and did not secrete sHLA-G. Similar findings have been described before (5). Hence, these secondary cell lines are less suitable to be employed in co-culture studies.

Of the cytokines assessed in the supernatant of the trophoblast cultures, five could be detected above background levels, of which TNF-α and IL-8 showed the highest level for CTB. TNF-α positivity was found in EVT of placental bed biopsies at 10 weeks of gestation (57). IL-8 was shown to be produced by placental explants, and immunohistochemistry confirmed that trophoblasts are positive for IL-8 (58). One study determined 12 cytokines in culture medium of CTB and EVT derived from first-term placental tissue, whereby IL-8 showed the highest levels but TNF-α and IL-6 were not tested (59). Interestingly, while in their study cytokine levels were higher in EVT compared to villous CTB, we found the opposite. Another study profiled 92 proteins in conditioned medium from chorionic villous samples, and found IL-6 and IL-8 among the highest secreted but TNF-α one of the lowest ones (60).

In the current study, we presented results of a 2D trophoblast culture model. Previous studies have described establishment of a 3D organoid culture (12, 13, 47). The advantage of organoid cultures over 2D trophoblast stem cells may be that they represent more the organization of villous structures, as seen *in vivo*. The drawback is that they are more cumbersome to grow and expand, and that due to their higher structural complexity and inside-out orientation of CTB and SCT the effect following any interactions with immune cells is harder to dissect. This latter issue is expected to be more easily studyable in the 2D culture model. As described earlier (14), we experienced the trophoblast cultures to be easily frozen, stored, and thawed, in case they were needed for later culturing. Collection and processing of multiple placentas for this purpose generates a panel of trophoblast cell lines, which encompasses a diversity of HLA-C and HLA-G constitutions. Establishing a repository of trophoblast cultures with heterogeneous HLA surface molecules gives unique opportunity to study the effect of genetic variants in these molecules on expression and on outcome of cell-cell interactions. This became apparent for HLA-G where we found in the relatively small group of trophoblast cultures that UTR-1 and UTR-4 haplotypes are related to high expression and UTR-2 and UTR-7 are related to low expression. Similar haplotype-expression relationships have been previously found by us and others (61–64). Various allelic variants of HLA-G on EVT may interact differently with receptors on NK cells (65) and possibly with the ILT2 (LILRB1) receptor on T cells. Seven isoforms of HLA-G have been identified, which include four membrane-bound proteins (HLA-G1 to HLA-G4). Particularly HLA-G1 may be efficient in inhibiting proliferation and cytolysis of NK cells and cytotoxic T lymphocytes (66, 67). In this respect it is interesting that in the current study differentiating EVT showed increasing prevalence of the HLA-G1 isoform and decreasing percentage of HLA-G2 and -G3. HLA-G2 and G3 isoforms inhibit lysis by T cells to slightly lesser extent than the HLA-G1 isoform (68), whereas the effect on NK cell cytotoxicity is not consistent between studies (66, 68, 69). HLA-C represents the only classical class I molecule that is present on the EVT, and it may display a recognition and triggering signal for T cells. As a proof of principle, we tested the effect of the presence of EVT on blood lymphocytes by co-culturing for three days. This co-culture led to decreased expression of HLA-DR on the surface of CD4$^+$ T cells. Here, we preferred cells from blood over those

from first-trimester placentas, since the latter often represent small pieces of tissues containing relatively few lymphocytes and the T cells may already have been directed to a more memory/activation phenotype. Usage of blood PBL also more easily enabled to pick specific HLA genotypes in relation to the primary trophoblasts. Next studies would need to incorporate lymphocytes from the decidua to investigate cell-cell interactions. Further research is also needed to find out if the effect on T cells by trophoblasts is mediated by cell-cell contact or by the action of one or more soluble components, and by which mechanism HLA-DR expression is affected. Nevertheless, since a homogeneous response was observed among different lymphocyte cultures, irrespective of HLA genotype, we think the effect on T cells may have been caused by soluble factors secreted by EVT in the medium. In fact, a similar observation of decreased HLA-DR on $CD4^+$ T cells was made by Svensson-Arvelund and colleagues after incubating blood $CD4^+$ T cells for several days with conditioned medium from first-term placental explants (70).

One remark concerning the study setup needs to be made. With trophoblast cultures established from placentas of electively ended pregnancies it is not possible to know if the placenta would have developed properly with advancing gestation. One way to compensate for this lack of knowledge and to know if results represent a physiological behaviour would be to have sufficiently high sample numbers when studying trophoblast actions.

In summary, we verified the possibility of establishing trophoblast cultures from first-term placentas and their capability of differentiating to EVT. These primary trophoblast cultures specifically upregulate HLA-C and HLA-G on their surface upon differentiation and are clearly dissimilar from immortalized trophoblast cell lines. The primary trophoblast cultures described represent a suitable model system to further perform mechanistic studies of their interaction with immune cells.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the LUMC (P08.087). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

ME analyzed and interpreted results and wrote down the manuscript. CK set up the cultures, performed flow cytometric analyses, and executed co-culture experiments. JA executed RNA isolations, cDNA analysis, and qPCR experiments. JD was involved in planning the genotyping experiments and interpreting their outcome. EB performed the Luminex assays. SC participated in coordinating the material transfer necessary for this study and in reviewing the content of the manuscript. M-LH participated in reviewing the clinical aspects and the content of the manuscript. All authors have read and approved the final 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.2022.814019/full#supplementary-material

**Supplementary Figure 1 |** Identification of distal cell column trophoblasts in placenta from electively ended pregnancy. **(A)** Tissue was obtained at first trimester and stained by hematoxylin-eosin (left picture) and for HLA-G (right picture). Proximal- and distal cell column trophoblast have been indicated in the figure. **(B)** First-trimester placental tissue was stained for HLA-G (left picture) and collagen IV (right picture).

**Supplementary Figure 2 |** Karyotyping of trophoblast cell lines. Three primary lines (QG1, QH1, RC2) were analyzed at passage 16-17, along with secondary trophoblast lines JAR and JEG-3 as positive controls. No deviations were observed for QH1 (46,XX [5]) and RC2 (46,XY[5]). QG1 was also normal except for a small addition to chromosome 17 (inlay at lower right corner) in two out of five cells (46,XX[3]/46,XX,add (17)(q25)[2]). As comparison, JAR and JEG-3 (not shown) are broadly deviating.

**Supplementary Figure 3 |** FACS histogram plots of human monoclonal HLA antibody stainings. **(A)** Four maternal blood samples were included as controls. **(B)** Expression of classical HLA class I antigens and HLA-G in three primary EVT cultures, in secondary trophoblast cell lines JEG-3 and JAR, and in maternal decidual stromal cells.

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