HLA-G-MEDIATED IMMUNE TOLERANCE: PAST AND NEW OUTLOOKS

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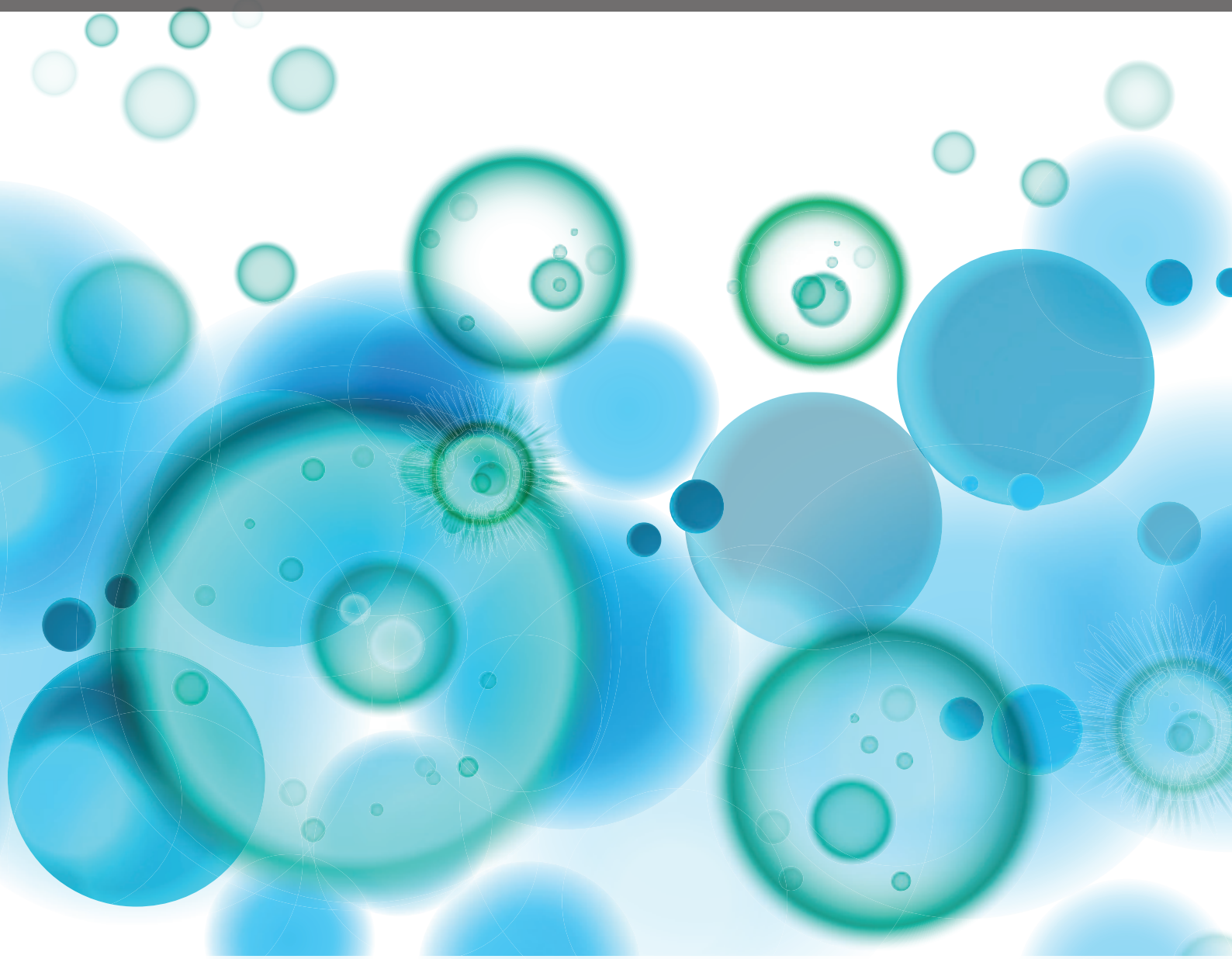

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HLA-G-MEDIATED IMMUNE TOLERANCE: PAST AND NEW OUTLOOKS

Topic Editors:
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The non-classical HLA class I molecule HLA-G is different from classical HLA class I molecules because of the low polymorphism in the coding region, the fact that HLA-G primary transcript is alternatively spliced in seven isoforms, and the inhibitory action on immune cells. Although HLA-G is low polymorphic, variants in both promoter and 3' un-translated region (UTR) of HLA-G locus regulate its expression.

In healthy conditions, a basal level of HLA-G gene transcription is observed in most cells and tissues; however, translation into HLA-G protein is restricted to trophoblasts in the placenta, where it participates in promoting tolerance at the fetal-maternal interface. HLA-G is also expressed by thymic epitelial, cornea, mesenchymal stem cells, nail matrix, pancreatic beta cells, erythroid, and endothelial precursors. HLA-G can be neo-expressed in adult tissues in pathological conditions, and its expression has been documented autoimmune disorders, viral infections, and cancer. In the latter setting de novo HLA-G expression is associated with the capability of tumor cells to evade the immune control.

In the last decade it has become evident that HLA-G expression on T cells and antigen-presenting cells confers to these cells tolerogenic properties. This Research Topic focused on i) summarizing updated clinical and immunological evidences that HLA-G expression is associate with beneficial or detrimental tolerance, ii) gathering new insights into the mechanisms governing the expression of HLA-G in healthy and pathological conditions, such as pre-eclampsia, and iii) examining the mechanisms underlying HLA-G mediated tolerance.

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EDITORIAL
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Editorial: HLA-G-Mediated Immune Tolerance: Past and New Outlooks

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Keywords: HLA antigens, tolerance, pregnancy, infection, autoimmune diseases, genetic variation

Editorial on the Research Topic

HLA-G-Mediated Immune Tolerance: Past and New Outlooks

This research topic gathered several researchers who actively base their research and investigations on the non-classical HLA-G molecule. HLA-G differs from classical HLA class I molecules since it has limited protein variability, can be expressed in several membrane-bound and soluble isoforms generated by alternative splicing generating, and modulates immune response. HLA-G expression is physiologically restricted to the maternal–fetal interface and to immune privileged adult tissues. *De novo* expression of HLA-G is deleterious when present in tumor cells and in chronically infected cells, whereas it is advantageous in autoimmune diseases and after transplantation.

In the present collection of manuscripts, different aspects of the HLA-G biology have been discussed including the genetic variability, the relationship between HLA-G and other non-classical HLA class I molecules, and the role of HLA-G in promoting tolerance in T-cell-mediated diseases and in pregnancy.

In the contest of HLA-G genetics, it is intriguing that the overall *HLA-G* gene structure was preserved during the evolution, and the HLA-G variability has been established before human dispersion from Africa. Castelli et al. illustrated that most of the variation sites found in the HLA-G coding region are either synonymous or intronic mutations and that the HLA-G promoter region presents numerous polymorphic sites.

Regarding the role of HLA-G in maintaining tolerance, Rizzo et al. and Dias et al. delivered a solid snap shot on the physiological expression of HLA-G and its role in inducing tolerance in autoimmunity. The Authors also discussed that the *de novo* expression of HLA-G, specifically in tumors and after chronic infections, has important implication in promoting immune escape. Special attention received the association between HLA-G polymorphisms, specifically those present at 3′UTR of the gene, protein expression, and functions in healthy and pathological conditions.

HLA-G belongs to the HLA class Ib molecules family that contains HLA-E and HLA-F. Morandi and Pistoia summarized, for the first time, the relationship between HLA-G and HLA-E in different settings. They concluded that, in physiological conditions, HLA-E expression is strongly associated with HLA-G and both molecules co-operate in promoting anergy in immune effector cells, specifically in NK cells. Conversely, HLA-G/HLA-E interaction in pathological conditions, i.e., in autoimmune and inflammatory diseases, may exert divergent or potentially opposite effects. The central role mediated by the non-classical HLA class I molecules, HLA-G, HLA-E, and HLA-F, in promoting tolerance during pregnancy and preeclampsia has been extensively discussed. Djurisic and Hviid indicated that in preeclampsia, HLA-F function is still unknown and that despite HLA-E is involved in immune suppression, increased soluble HLA-E levels has not been associated with preeclampsia. Conversely, the high expression of HLA-G compared to HLA-E and -F in the placenta, and the presence of HLA-G in semen, endometrium, in matured cumulus–oocyte complex, as well as the rise in soluble level after conception, imply an important role for HLA-G in early pregnancy.

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Furthermore, the role of HLA-G in immune regulation and spiral artery remodeling highlights its importance and multifaceted activities. In line with this view, Gregori et al. proposed that, at the fetal/maternal interface, the expression of HLA-G coordinates the cross-talk between fetal extravillous trophoblasts (EVTs) and maternal decidual and immune cells. Upon blastocyst is implantation into the uterine wall, trophoblasts indeed differentiate into EVTs that regulate their cell migration in the decidua, support the induction of the pro-angiogenic microenvironment necessary for vascular remodeling, inhibit effector innate and adaptive immune responses, and promote a tolerogenic loop in which resident cells become tolerogenic.

Finally, Rebmann et al. presented and discussed a new and interesting novel aspect in the biology of the HLA-G, the HLA-G-bearing extracellular vesicles (EVs). Several cell types involved in immune tolerance and tissue remodeling, including tumor cells, trophoblasts, and mesenchymal stromal cells, secrete HLA-G-bearing EVs. The mechanisms underlying the functional consequences of HLA-G-bearing EVs are, thus far, little investigated. Nevertheless, HLA-G-bearing EVs represent a novel mode of HLA-G delivery within target cells, thereby bypassing the interaction between HLA-G and its specific receptors. This new concept opens new perspectives in the modulatory activity of HLA-G.

Overall, we gathered a nice compilation of old and new findings on HLA-G, and this research topic highlights the importance of this immune-modulatory molecule in healthy and pathological conditions and proposes new investigation avenue to better define HLA-G biology and potentially identify new therapeutic strategies for promoting or dampening tolerance.

AUTHOR CONTRIBUTIONS

This work was supported by Telethon Italy grant number Tele 16-G1. The author confirms being the sole contributor of this work and approved it for publication.

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Insights into *HLA-G* genetics provided by worldwide haplotype diversity

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Human leukocyte antigen G (*HLA-G*) belongs to the family of non-classical HLA class I genes, located within the major histocompatibility complex (MHC). *HLA-G* has been the target of most recent research regarding the function of class I non-classical genes. The main features that distinguish *HLA-G* from classical class I genes are (a) limited protein variability, (b) alternative splicing generating several membrane bound and soluble isoforms, (c) short cytoplasmic tail, (d) modulation of immune response (immune tolerance), and (e) restricted expression to certain tissues. In the present work, we describe the *HLA-G* gene structure and address the *HLA-G* variability and haplotype diversity among several populations around the world, considering each of its major segments [promoter, coding, and 3′ untranslated region (UTR)]. For this purpose, we developed a pipeline to reevaluate the 1000Genomes data and recover miscalled or missing genotypes and haplotypes. It became clear that the overall structure of the HLA-G molecule has been maintained during the evolutionary process and that most of the variation sites found in the *HLA-G* coding region are either coding synonymous or intronic mutations. In addition, only a few frequent and divergent extended haplotypes are found when the promoter, coding, and 3′UTRs are evaluated together. The divergence is particularly evident for the regulatory regions. The population comparisons confirmed that most of the *HLA-G* variability has originated before human dispersion from Africa and that the allele and haplotype frequencies have probably been shaped by strong selective pressures.

Keywords: HLA-G, haplotypes, polymorphisms, variability, gene structure and diversity, non-classical HLA, 1000Genomes Project, selective pressure

INTRODUCTION

Human leukocyte antigen G (*HLA-G*) belongs to the family of non-classical HLA class I genes, located within the major histocompatibility complex (MHC) at chromosomal region 6p21.3. The MHC segment is considered to be the most polymorphic region in vertebrate genome (1). Although the *HLA-G* product presents the same class I classical molecule structure, its main function is not antigen presentation. HLA-G function in the immune response regulation has been extensively studied since its discovery by Geraghty and colleagues in 1987 (2).

The *HLA-G* gene has been the target of most recent research regarding the function of class I non-classical genes. The main features that distinguish *HLA-G* from classical class I genes are (a) limited protein variability, (b) alternative splicing generating several membrane bound and soluble isoforms, (c) short cytoplasmic tail, (d) modulation of immune response (immune tolerance), and (e) restricted expression to certain tissues (3).

The HLA-G molecule does not seem to stimulate immune responses, however, it exerts inhibitory functions against natural killer (NK) cells (4), T lymphocytes (4), and antigen-presenting cells (APC) (5) through direct interaction with multiple inhibitory receptors such as ILT2/CD85j/LILRB1 (ILT2), expressed by all monocytes, B cells, some lineages of T cells, and NK cells (6); ILT4/CD85d/LILRB2 (ILT4), only expressed by monocytes and dendritic cells (7); and KIR2DL4/CD158d (KIR2DL4) that has a restricted expression to CD56 NK cells (8).

HLA-G role in immune tolerance was first studied in trophoblast cells at the maternal–fetal interface (9). Several studies reported an aberrant or reduced HLA-G expression in both mRNA and protein levels. This phenomenon was observed in pathological conditions such as preeclampsia (10) and recurrent spontaneous abortion (11) in comparison with normal placentas.

Beyond trophoblast expression, HLA-G is related to a variety of physiological and pathological conditions. In physiological conditions, HLA-G expression has been documented in cornea (12), thymus (13), and erythroid and endothelial precursors (14). On the other hand, HLA-G variation sites and/or expression levels are associated with pathological conditions such as viral infections (15–20), cancer (21–27), recurrent miscarriage (28–37), pregnancy outcome and pregnancy complications (37–45),

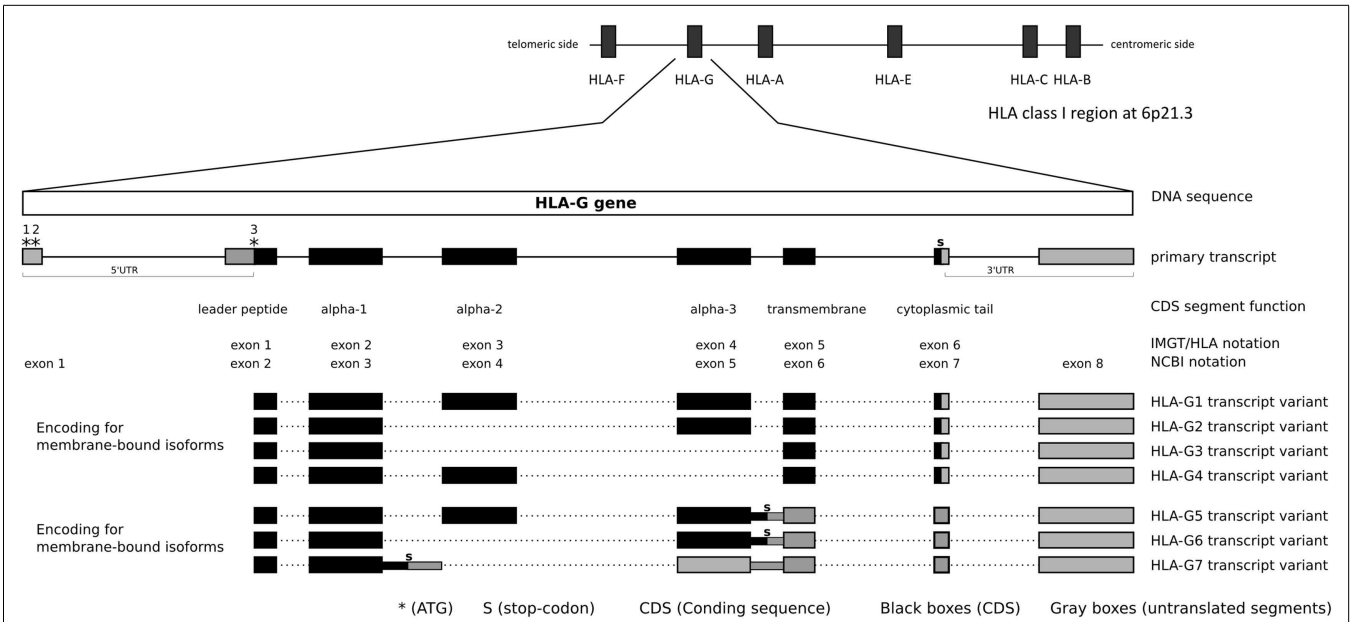

FIGURE 1 | *HLA-G* gene structure and transcripts.

autoimmune diseases (46–54), transplantation outcome (55–57), and inflammatory diseases (58–61), indicating that *HLA-G* encodes a critical molecule for the immune system.

HLA-G GENETIC STRUCTURE

The *HLA-G* gene presents a structure that resembles other classical class I genes such as *HLA-A*, *HLA-B*, and *HLA-C*. *HLA-G* encodes for a membrane-bound molecule with the same extracellular domains presented by other class I molecules, including the association with the β2-microglobulin. However, its main function is not antigen presentation.

The *HLA-G* gene exon/intron structure and splicing patterns are well defined, but there are inconsistencies between the National Center for Biotechnology Information (NCBI)[1], the International Immunogenetics Database (IMGT/HLA[2]), and the Ensembl database[3] annotations regarding its structure, mainly because the IMGT/HLA database only presents sequences within 300 bases upstream the coding sequence (CDS) and the database does not consider most of the 3′ untranslated region (UTR) segment. Therefore, in the present work, the structure defined by NCBI/Ensembl will be used throughout the text.

According to the NCBI reference sequence NC_000006.12 (GRCh38 or hg19) and transcripts such as NM_002127.5 (NCBI), ENST00000428701, and ENST00000376828 (Ensembl), the *HLA-G* gene (NCBI Gene ID: 3135) presents eight exons and seven introns, consistent with a classical class I gene structure, and encompasses a region of 4144 nucleotides between positions 29826979 and 29831122 at 6p21.3 (GRCh38). This gene is surrounded by some of the most polymorphic genes in the human genome (**Figure 1**), such as *HLA-A* (115 Kb downstream), *HLA-B* (1526 Kb downstream), and *HLA-C* (1441 Kb downstream), and other non-classical HLA loci such as *HLA-E* (662 Kb downstream) and *HLA-F* (103 Kb upstream). According to the NCBI annotation and hg19, the *HLA-G* DNA segment encodes a full-length mRNA of 1578 nucleotides and alternative smaller ones, as discussed later. Considering the full-length mRNA, 1017 nucleotides represent the CDS encoding for a full-length protein of 338 amino acids, 178 nucleotides represent the 5′UTR segment, and 383 nucleotides represent the 3′UTR segment.

There is no consensus regarding the exact location where the *HLA-G* transcription may start. Considering the NCBI and Ensembl annotations, and the transcripts NM_002127.5 from NCBI and ENST00000428701 from Ensembl, the *HLA-G* transcription starts 866 nucleotides upstream the initial translated ATG (third * at **Figure 1**). However, other transcripts tell us a different story: ENST00000376828 indicates that the *HLA-G* transcription might start even earlier, while ENST00000360323 indicates that the transcription starts 24 nucleotides upstream the initial translated ATG. Given these contradictory information, it is possible that the *HLA-G* gene presents multiple transcription start points depending on the presence of specific transcription factors or other expression inducing mechanisms, but it probably presents only one translation start point as described further. Since there is no consensus, in the present work, we opt to use the annotation presented by both NCBI and Ensembl, considering NM_002127.5 and ENST00000428701 as references. Considering the transcription start site indicated by NM_002127.5/ENST00000428701 or ENST00000360323, *HLA-G* presents a large 5′UTR segment. Within this segment, there is an intron (intron 1) of about 688 nucleotides that is spliced out, giving rise to 5′UTR of about 178 nucleotides composed of DNA segments of two adjacent exons. Considering this transcription start point, the *HLA-G* 5′

[1]http://www.ncbi.nlm.nih.gov
[2]http://www.ebi.ac.uk/ipd/imgt/hla/
[3]http://www.ensembl.org/index.html

sequence presents at least three potential translation start points, i.e., two in the 5′UTR and the third one defining the beginning of the CDS. In the present work, we will consider the Adenine of this third ATG, i.e., the first base of the CDS, as nucleotide +1. Although conventional nomenclature would suggest the first transcribed base as nucleotide +1, our decision will avoid unnecessary confusion regarding the position of various well-established *HLA-G* variation sites. All nucleotides before the CDS will be noted as negative numbers and nucleotides in the CDS segment will be noted as positive numbers, using as a reference sequence the one available at the official human genome hg19 or NC_000006.12.

The first ATG is found between nucleotides −154 and −152 (mRNA) or nucleotides −842 and −840 (DNA). The second one is found between nucleotides −118 and −116 (mRNA) or nucleotides −806 and −804 (DNA). Both of these translation start points are in the same frame and are included in a sequence that does not resemble the preferred translation initiation sequence (Kozak consensus sequence) and might not initiate translation (62). Even if the first ATG is used, it would produce a peptide of only eight residues due to a stop codon found downstream in the reading frame. Alternatively, if the second ATG is used, a protein of about 136 amino acid residues would be produced. Although in a different frame from the main translation start point (the third one), this 136 amino acid molecule is quite similar to other human and primate class I molecule alpha-1 domains. The third and main ATG is compatible with the preferred Kozac sequence (62) and it initiates the translation of the full-length 338 amino acid residues protein and defines the beginning of the CDS segment.

The *HLA-G* CDS is composed of joining segments of six exons, in which the first contains the translation start point and the last one contains the stop codon (**Table 1**, **Figure 1**). It should be noted that there is no consensus regarding exon and intron nomenclature between NCBI/Ensembl and the IMGT/HLA databases. IMGT/HLA considers as exon 1 the first mRNA segment that is translated, i.e., exon 2 for NCBI/Ensembl (**Figure 1**). The actual exon 2, which encodes the final portion of the 5′UTR, contains the main translation start point and in fact encodes the HLA-G leader peptide (**Figure 1**). In addition, exons 3, 4, and 5 encode the alpha-1, alpha-2, and alpha-3 domains, respectively, exon 6 encodes the transmembrane domain, and exon 7 the cytoplasmic tail. A premature stop codon at exon 7 leads to a shorter cytoplasmic tail when compared to other class I molecules (**Figure 1**, **Table 1**). The segment downstream the stop codon at exon 7 extending to exon 8 composes the *HLA-G* 3′UTR. The *HLA-G* mRNA 3′UTR is short when compared to other class I genes. This gene structure description highlights one of the widely spread misconceptions regarding *HLA-G* gene structure: in 1987, Geraghty and colleagues proposed the existence of an exon 7 based on homology with classical class I genes (2). This "exon 7" was in fact part of the intron 7 (NCBI) and it is usually absent in most of the *HLA-G* transcripts. Although this "exon 7" segment has been found in alternative transcripts (e.g., ENST00000478519), other intron segments are also sometimes kept in rare alternative transcripts (e.g., ENST00000478355), since alternative splicing is an important characteristic of the *HLA-G* gene as described further.

Table 1 | The *HLA-G* exons and introns, their size, function, and nomenclature.

According to NC_000006.12 (hg19)	According to IMGT/HLA	Size (nt)	Function considering the full-length mRNA
Exon 1	–	66	5′UTR
Intron 1	–	688	Spliced out
Exon 2	Exon 1	185	5′UTR/Leader peptide
Intron 2	Intron 1	129	Spliced out
Exon 3	Exon 2	270	Alpha-1 domain
Intron 3	Intron 2	226	Spliced out
Exon 4	Exon 3	276	Alpha-2 domain
Intron 4	Intron 3	599	Spliced out
Exon 5	Exon 4	276	Alpha-3 domain
Intron 5	Intron 4	122	Spliced out
Exon 6	Exon 5	117	Transmembrane domain/cytoplasmic tail
Intron 6	Intron 5	445	Spliced out
Exon 7	Exon 6	33	Cytoplasmic tail/stop codon/3′UTR
Intron 7	–	357	Spliced out
Exon 8	–	355	3′UTR

The *HLA-G* gene may produce at least seven protein isoforms generated by alternative splicing of the primary transcript (**Figure 1**). Four isoforms are membrane bound presenting the transmembrane domain and the short cytoplasmic tail. HLA-G1 is the full-length membrane-bound isoform with a structure that resembles classical class I molecules. HLA-G2 lacks alpha-2 domain, HLA-G3 lacks alpha-2 and alpha-3 domains, and HLA-G4 lacks alpha-3 domain. Three isoforms are soluble due to the lack of the transmembrane domain. The soluble HLA-G5 and HLA-G6 isoforms present the same extracellular domains of HLA-G1 and HLA-G2, respectively; however, both transcript variants retain intron 5 leading to a stop codon before the translation of the transmembrane domain, and a tail of 21 amino acids implicated in their solubility. HLA-G7 transcript variant retains intron 3 leading to a premature stop codon. Therefore, HLA-G7 isoform presents only the alpha-1 domain linked to two amino acids encoded by intron 2 (**Figure 1**) (63–65).

In the next sections, we will address the *HLA-G* variability and haplotype diversity among several populations around the world.

HLA-G VARIABILITY AS DESCRIBED IN THE 1000GENOMES PROJECT

The 1000Genomes Project is a large survey aiming to sequence the entire genome of thousands of individuals in several populations around the world (66). In the initial released data, the phased

genotypes of 1092 individuals from 14 populations were available. These data have driven several studies regarding *HLA-G* variability and evolutionary aspects (67–69).

The initial genotype published by the 1000Genomes Project was based on exome sequencing or whole genome low coverage sequencing and lacks several known *HLA-G* polymorphisms due to limitations in the genotype detection procedures at that moment. Among the missing polymorphic sites, we may highlight some known indels, such as the traditionally studied 14-bp presence or absence (insertion/deletion) in the *HLA-G* 3′UTR. In addition, the method used to infer genotypes and haplotypes failed to clearly distinguish triallelic SNPs, reporting them as biallelic ones (e.g., the *HLA-G* promoter SNP at position −725C/T/G, rs1233334).

Considering these technical limitations and considering the fact that most of the bioinformatics tools used in the initial survey are now more advanced and developed, we have reevaluated the 1000Genomes raw sequencing data regarding the *HLA-G* gene using a locally developed pipeline to get genotypes and haplotypes, to better understand the *HLA-G* variability around the world and to retrieve data regarding some *HLA-G* missed polymorphic sites.

First, by using Samtools (70) subroutine view, we downloaded the BAM files (binary alignment map) containing the 1000Genomes official alignment data for the *HLA-G* gene region (between positions 29793317 and 29799834 at chromosome 6) directly from the 1000Genomes server (ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/). The reads downloaded were already trimmed on both ends for primer sequences. The download was performed for each of the initial 1092 samples and included data from both low coverage whole genome and exome when available. It should be mentioned that we got the sequences (reads) from BAM files representing the *HLA-G* region, thus, the next step of our pipeline used only the reads that were previously mapped to the *HLA-G* region by the 1000Genomes Consortium. Each BAM file was converted into a Fastq format file retrieving all reads that were previously mapped to the *HLA-G* region. The BAM to Fastq conversion was made using Bamtools (https://github.com/pezmaster31/bamtools/) and Perl scripts (locally developed) to filter out duplicated reads and to classify the reads as paired or unpaired.

Both paired and unpaired Fastq files were mapped to a masked chromosome 6 (hg19), in which only the *HLA-G* region was available and the rest of the chromosome was masked with "N" to preserve nucleotide positions regarding hg19. To date, hg19 presents a *HLA-G* coding region sequence compatible with the widely spread *HLA-G* allele known as G*01:01:01:05. Mapping was performed using the application BWA, subroutine ALN (71), configured to allow the extension of a deletion up to 20 nucleotides, in order to evaluate the 14-bp polymorphism. The resulting BAM files from the newly mapped reads, from both paired-end and unpaired sequences, were joined using Picard-tools (http://picard.sourceforge.net/index.shtml). Regions containing indels were locally realigned by using the application GATK (72), routines RealignerTargetCreator and IndelRealigner. This local realignment used as reference a file containing known *HLA-G* indels. The Bamtools software was also used to remove reads mapped with low mapping quality (MQ) scores (MQ < 40). After the procedure described above, 16 samples were discarded because all mapped reads (or most of them) were withdrawn due to poor MQ scores. The GATK routine UnifiedGenotyper was used to infer genotypes and a VCF file (variant call format) was generated.

Given the low coverage nature of the 1000Genomes data, some genotypes called by GATK are far uncertain, mainly in situations in which a homozygous genotype is inferred when that position presents low depth coverage. In addition, given the polymorphic nature and the high level of sequence similarity of HLA genes, some level of miss-mapped reads is expected and might bias genotype inference. To circumvent this issue, the VCF file generated by GATK was treated with a locally developed Perl script that applied the rules described below. This script uses the number of different reads detected for each allele at a given position (provided by GATK when the VCF file was generated).

- Homozygosity was only inferred when a minimal coverage of seven reads was achieved; otherwise, a missing allele was introduced in this genotype. This procedure assures ($p > 0.99$) that a homozygous genotype is called because of lack of variance at that position and not because the second allele was not sampled.
- Genotypes, in which one allele was extremely underrepresented (proportion of reads under 5%), were considered as homozygous for the most represented allele. This procedure minimizes the influence of miss-mapped reads to the *HLA-G* region and the high level of sequencing errors that characterizes next-generation sequencing data, and such correction was applied only in situations characterized by high depth of coverage (20 or more reads available for the evaluated position).
- For genotypes in which one allele was mildly underrepresented (with a proportion of reads between 5 and 20%), a missing allele was introduced representing this underrepresented allele. This procedure is particularly helpful in situations characterized by low depth of coverage (less than 20 reads available for the evaluated position), in which a single read may indicate the existence of an alternative allele, such read may be a miss-mapped read (false positive variant) or may represent a true unbalanced heterozygous genotype (true positive variant). Therefore, the definitive status of this kind of genotype (homozygous or heterozygous) was inferred during a final imputation step.
- Genotypes in which the proportion of reads for the less represented allele was higher than 20% were considered to be heterozygous. This procedure assures that only high-quality heterozygous genotypes are passed forward to the imputation procedure.

After applying the rules described above, the *HLA-G* database presented 8.42% of missing alleles, i.e., alleles that were considered uncertain because of low coverage or bad proportions. Some single nucleotide variations (SNVs) previously detected (with low quality) were converted into monomorphic as the alternative allele was removed or coded as missing, thus, they were not considered for further analyses. By using the VCFtools package (73), we removed SNVs that were no longer variable or that were represented just once in the dataset (i.e., singletons). In addition, we predicted the functional effect of each SNV, i.e., they were classified as coding synonymous mutations, coding non-synonymous

mutations, splice site acceptors, stop-codon generation, and others, by using Snpeff (74). The missing alleles were imputed as well as *HLA-G* haplotypes were inferred by using the PHASE algorithm (75) as previously described (76, 77). For this purpose, a database containing high-quality genotype information for 133 SNVs for each of the 1076 remaining samples was used. The haplotyping procedure generated 200 haplotypes, with a mean haplotype pair probability of 0.7965 and with 524 samples (48.70%) presenting a haplotype pair with a probability higher than 0.9. The results of the procedure described above were presented separately for each *HLA-G* region (coding, 3′UTR and promoter) and, finally, as fully characterized extended haplotypes.

To characterize and explore global patterns of *HLA-G* diversity, a population genetics approach was performed using the ARLEQUIN 3.5.1.3 software (78, 79). The frequencies of each *HLA-G* haplotype were computed by the direct counting method and adherences of diplotype proportions to expectations under Hardy–Weinberg equilibrium were tested by the exact test of Guo and Thompson (80). Intrapopulational genetic diversity parameters were assessed in each population by computation of gene diversity (average expected heterozygosity across variation sites), haplotype diversity, nucleotide diversity, and the number of private haplotypes. Interpopulation genetic diversity was explored by means of pair-wise F_{ST} estimates (81), by the exact test of population differentiation (82), and by the analysis of molecular variance (AMOVA) (83), all based on haplotype frequencies. Since the pair-wise F_{ST} and the exact test of population differentiation between pairs of populations represent 91 statistical comparisons, the Bonferroni correction was used to adjust the significance level for multiple testing, resulting in a $\alpha = 0.0005$ (i.e., 0.05/91). Reynolds' genetics distance was also estimated for each pair of population samples by the ARLEQUIN 3.5.1.3 software (78, 79, 84). The resulting matrix was used to generate a multidimensional scaling (MDS) using the PASW Statistics (17.0.2) software (SPSS Inc.).

HLA-G CODING REGION VARIABILITY AND HAPLOTYPES

In contrast to classical *HLA* class I genes, *HLA-G* presents low variability in its coding region. To date, only 50 coding alleles or haplotypes are officially recognized by the IMGT/HLA database[2] (version 3.17.0.1). Most of the SNVs in the *HLA-G* coding region are either coding synonymous mutations or intronic variants. Therefore, these 50 officially recognized *HLA-G* alleles encode only 16 different full-length proteins and two truncated molecules (null alleles). This is a distinctive feature of the *HLA-G* gene and also of other non-classical class I genes: only 36% of the known *HLA-G* alleles are associated with different HLA-G molecules when compared to classical class I genes, in which 75.4% for *HLA-A*, 77.8% for *HLA-B*, and 73.5% for *HLA-C* alleles are associated with different molecules (IMGT/HLA). The limited *HLA-G* coding region polymorphism is distributed among the alpha-1, alpha-2, and alpha-3 domains, while for classical class I genes, polymorphisms are found mainly around the region encoding the peptide binding groove, i.e., alpha-1 and alpha-2 domains (1). This is particularly evident for *HLA-B*, in which there is at least one recognized allele carrying a mutation for each nucleotide of exons 2 or 3, with few exceptions.

Generally, a SNV is considered as a polymorphic site if the minor allele presents a frequency of at least 1%. In this matter, some *HLA-G* variable sites may not be considered as true polymorphisms because they are rarely observed. Considering the 50 *HLA-G* alleles that have been officially recognized by IMGT/HLA, and taking into account the several studies evaluating the *HLA-G* coding region polymorphisms in normal or pathological conditions, only 13 alleles encoding four different HLA-G full-length molecules and a truncated one are frequently observed in worldwide populations (3, 19, 23, 34, 36, 37, 68, 69, 76, 85–104).

Among the high-frequency *HLA-G* coding alleles, we may find the G*01:01:01:01, G*01:01:01:04, G*01:01:01:05 (present at hg19), G*01:01:02:01, G*01:01:03:01, G*01:01:05, and G*01:01:07 alleles; all carrying intronic or synonymous mutations and encoding for the same full-length HLA-G molecule known as G*01:01. *HLA-G*01:01:01:01 is the reference allele used by IMGT/HLA, it was the first one described (2) and usually the most common allele in all populations studied so far. Among the frequent ones, we also find the G*01:03:01:01 allele that is characterized by a non-synonymous mutation at position 292, codon 31, exchanging a Threonine by a Serine, encoding the full-length molecule known as G*01:03. Another group of alleles are represented by G*01:04:01, G*01:04:03, and G*01:04:04, all of them encoding the same molecule known as G*01:04. They are characterized by a non-synonymous mutation at position 755, codon 110, exchanging a Leucine by an Isoleucine, and by other synonymous mutations. The null allele, G*01:05N, which is associated with a truncated HLA-G molecule due to a deletion of a cytosine around codon 130 that changes the reading frame, is also very frequent in some African, Asian, and admixed populations. Finally, the last frequent allele is G*01:06, which is characterized by a non-synonymous mutation at position 1799, codon 258, exchanging a Threonine by a Methionine, encoding a molecule known as G*01:06. Other *HLA-G* alleles are sporadically found around the world, but only the ones presented above have been described at polymorphic frequencies.

However, the variability in the *HLA-G* coding region may be higher than the one presented by IMGT/HLA, because IMGT/HLA only presents alleles that were cloned, sequenced, and properly characterized by the researchers. In addition, most of the known alleles are not fully characterized, presenting only some exons sequenced. Therefore, the variability at the *HLA-G* coding region may be greater than the one reported so far.

The reevaluation of the *HLA-G* sequencing data from the 1000Genomes Project indicated that the *HLA-G* coding region is indeed much conserved and just a few new coding alleles are frequently found worldwide. The approach described earlier evidenced the presence of 81 SNVs in the *HLA-G* coding region, as described in **Table 2**. Some of these variation sites are truly polymorphic, while some might be considered as mutations. In addition, some of these new sites are not represented in the IMGT/HLA database and might represent new *HLA-G* alleles.

As observed in **Table 2**, most of the 81 variation sites occur in introns (54 sites) or in exons as synonymous changes (16 sites). Thus, 86.4% of all variants are associated with the same HLA-G full-length molecule, unless they somehow influence *HLA-G* splicing pattern. Among the ones that might be related to different

Table 2 | List of all variation sites found in the *HLA-G* coding region, their genomic positions on chromosome 6 relative to hg19 and the *HLA-G* gene, and their allele frequencies considering all populations of the 1000Genomes Project (Phase 1).

Genomic position (hg19)	SNPid	*HLA-G* position	IMGT recognized	Allele 1 (reference)	Allele 1 frequency	Allele 2	Allele 2 frequency	Annotation
29795636	rs1630223	15	*	G	0.4967	A	0.5033	Synonymous
29795657	rs1630185	36	*	G	0.4967	A	0.5033	Synonymous
29795667	.	46		G	0.9991	T	0.0009	Non-synonymous
29795720	rs56388903	99	*	A	0.1120	G	0.8880	Intronic
29795747	rs6932888	126	*	G	0.7156	C	0.2844	Intronic
29795751	rs6932596	130	*	C	0.7161	T	0.2839	Intronic
29795768	rs1629329	147	*	T	0.4396	C	0.5604	Intronic
29795809	rs1628628	188	*	C	0.5669	T	0.4331	Intronic
29795822	.	201		A	0.9963	G	0.0037	Splice site acceptor
29795840	.	219		G	0.9967	T	0.0033	Non-synonymous
29795913	rs41551813	292	*	A	0.9503	T	0.0497	Non-synonymous
29795914	rs72558173	293	*	C	0.9986	T	0.0014	Non-synonymous
29795918	rs80153902	297	*	G	0.9958	A	0.0042	Synonymous
29795927	rs72558174	306	*	G	0.9972	A	0.0028	Synonymous
29795945	rs9258495	324	*	G	0.9991	T	0.0009	Synonymous
29795987	rs78627024	366	*	G	0.9972	A	0.0028	Synonymous
29795993	rs1130355	372	*	G	0.4967	A	0.5033	Synonymous
29796103	rs1626038	482	*	T	0.4340	C	0.5660	Intronic
29796106	rs17875399	485	*	G	0.9526	T	0.0474	Intronic
29796114	.	493		G	0.9991	A	0.0009	Intronic
29796115	rs1736927	494	*	A	0.4336	C	0.5665	Intronic
29796119	rs201510147	498		G	0.9986	A	0.0014	Intronic
29796126	rs3215482	505	*	A	0.4828	AC	0.5172	Intronic
29796128	.	507	*	C	0.9517	A	0.0483	Intronic
29796149	.	528		A	0.9967	C	0.0033	Intronic
29796152	rs1625907	531	*	G	0.4819	C	0.5181	Intronic
29796228	.	607		G	0.9981	A	0.0019	Intronic
29796234	rs375939243	613	*	CA	0.4991	C	0.5009	Intronic
29796245	.	624	*	T	0.9991	C	0.0009	Intronic
29796257	rs1625035	636	*	C	0.4493	T	0.5507	Intronic
29796265	rs17875401	644	*	G	0.9493	T	0.0507	Intronic
29796273	.	652		C	0.9981	T	0.0019	Intronic
29796306	rs1624337	685	*	G	0.4986	A	0.5014	Intronic
29796327	rs1130356	706	*	C	0.7621	T	0.2379	Synonymous
29796348	rs79303923	727	*	C	0.9981	T	0.0019	Synonymous
29796362	.	741	*	C	0.9991	G	0.0009	Non-synonymous
29796369	rs3873252	748	*	A	0.9345	T	0.0655	Synonymous
29796376	rs12722477	755	*	C	0.8053	A	0.1947	Non-synonymous
29796434	rs41557518	813	*	AC	0.9642	A	0.0358	Frame Shift
29796492	rs17875402	871	*	G	0.9944	A	0.0056	Synonymous
29796637	rs17875403	1016	*	C	0.9949	T	0.0051	Intronic
29796640	rs1632942	1019	*	T	0.4475	C	0.5525	Intronic
29796675	rs17875404	1054	*	G	0.9503	T	0.0497	Intronic
29796685	rs1632941	1064	*	T	0.4972	C	0.5028	Intronic
29796700	rs148061958	1079		C	0.9972	T	0.0028	Intronic
29796725	rs370704534	1104		C	0.9981	G	0.0019	Intronic
29796749	rs62391965	1128	*	C	0.9345	A	0.0655	Intronic
29796752	.	1131		A	0.9991	T	0.0009	Intronic
29796768	rs1632940	1147	*	T	0.2040	C	0.7960	Intronic

(Continued)

Table 2 | Continued

Genomic position (hg19)	SNPid	*HLA-G* position	IMGT recognized	Allele 1 (reference)	Allele 1 frequency	Allele 2	Allele 2 frequency	Annotation
29796800	rs140935623	1179		A	0.9981	G	0.0019	Intronic
29796838	rs1736923	1217	*	A	0.4963	G	0.5037	Intronic
29796934	rs114041958	1313	*	G	0.9507	A	0.0493	Intronic
29796935	rs1632939	1314	*	G	0.4972	A	0.5028	Intronic
29796986	rs1632938	1365	*	G	0.4972	A	0.5028	Intronic
29797043	rs145023077	1422		C	0.9912	T	0.0088	Intronic
29797052	rs116139267	1431		C	0.9967	T	0.0033	Intronic
29797073	rs188836562	1452		G	0.9991	C	0.0009	Intronic
29797155	rs17875405	1534	*	G	0.9503	C	0.0497	Intronic
29797173	rs1736920	1552	*	A	0.4470	G	0.5530	Intronic
29797195	.	1574		A	0.9986	AC	0.0014	Frame Shift
29797211	rs41562616	1590	*	C	0.9503	T	0.0497	Synonymous
29797380	rs200931762	1759		G	0.9991	A	0.0009	Non-synonymous
29797420	rs12722482	1799	*	C	0.9698	T	0.0302	Non-synonymous
29797421	rs76951509	1800	*	G	0.9963	A	0.0037	Synonymous
29797448	rs17875406	1827	*	G	0.9554	A	0.0446	Synonymous
29797553	rs1632937	1932	*	G	0.4972	C	0.5028	Intronic
29797639	rs1049033	2018	*	C	0.7742	T	0.2258	Synonymous
29797696	rs1130363	2075	*	A	0.4470	G	0.5530	Synonymous
29797782	rs1611627	2161	*	T	0.5627	C	0.4373	Intronic
29797899	rs1632934	2278	*	T	0.4972	C	0.5028	Intronic
29797933	rs1632933	2312	*	C	0.4972	T	0.5028	Intronic
29797951	rs1736912	2330	*	A	0.4972	G	0.5028	Intronic
29798029	.	2408		T	0.9991	A	0.0009	Intronic
29798033	rs17179080	2412		G	0.9707	A	0.0293	Intronic
29798039	rs1632932	2418	*	G	0.4972	A	0.5028	Intronic
29798083	rs114038308	2462	*	C	0.9345	T	0.0655	Intronic
29798140	rs915667	2519	*	A	0.5084	G	0.4916	Intronic
29798248	rs186170315	2627		G	0.9991	A	0.0009	Intronic
29798419	rs915670	2798	*	G	0.7742	A	0.2258	Intronic
29798425	rs915669	2804	*	G	0.4480	T	0.5520	Intronic
29798459	rs915668	2838	*	C	0.4480	G	0.5520	Intronic

**Denotes a variation site that is recognized by the IMGT/HLA database.*

HLA-G full-length proteins, we may find two frameshift mutations: the first associated with the G*01:05N null allele and the second representing a low-frequency variation site not recognized by IMGT/HLA (genomic position 29797195); one variation site associated with a splicing acceptor site (genomic position 29795822, HLA-G position + 201) and eight non-synonymous modifications, most of them recognized by IMGT/HLA. Interestingly, one synonymous modification was found presenting a high frequency (2.93%) and is not associated with any known *HLA-G* allele described so far (*HLA-G* position + 2412, rs17179080, **Table 2**). Although a triallelic SNV is described at exon 2 (HLA-G position + 372), associated with the G*01:04:02 allele, we did not find the third allele in the present data.

As described earlier, haplotypes were inferred considering all variation sites found in the *HLA-G* region. When the coding region is isolated from these haplotypes, we found 93 different *HLA-G* coding haplotypes, a number far higher than the number of *HLA-G* alleles officially recognized. The complete table of haplotypes is available upon request. **Table 3** describes all coding haplotypes presenting a minimum global frequency of 1% and the closest known *HLA-G* allele in terms of sequence similarity. It should be mentioned that non-variable positions for the haplotypes presented in **Table 3** were removed. Although 93 different haplotypes were inferred, only 11 present a frequency higher than 1%. Of those, 10 were compatible with a specific allele described at the IMGT/HLA database and mentioned earlier as high-frequency alleles that usually occur in any population, and 1 is a new allele that is close to G*01:01:01:01 but presents the frequent nucleotide change at position + 2412, not recognized by IMGT/HLA. As previously observed in other studies, the most frequent *HLA-G* allele is G*01:01:01:01, followed by G*01:01:02:01 and G*01:04:01. These 11 haplotypes or coding

Table 3 | List of *HLA-G* coding haplotypes presenting a global frequency higher than 1%, considering all populations of the 1000Genomes Project (Phase 1).

HLA-G position	Genomic position on chromosome 6 (hg19)	SNPid	G*01:01:01:01	G*01:01:01:01new	G*01:01:01:04	G*01:01:01:05	G*01:01:02:01	G*01:01:03:03	G*01:03:01:02	G*01:04:01	G*01:04:04	G*01:05N	G*01:06
15	29795636	rs1630223	G	G	G	G	A	A	G	A	A	A	A
36	29795657	rs1630185	G	G	G	G	A	A	G	A	A	A	A
99	29795720	rs56388903	G	G	G	A	G	G	G	G	G	G	G
126	29795747	rs6932888	C	C	G	G	G	G	G	G	G	G	G
130	29795751	rs6932596	T	T	C	C	C	C	C	C	C	C	C
147	29795768	rs1629329	T	T	T	T	C	C	C	C	C	C	C
188	29795809	rs1628628	C	C	C	C	T	C	C	T	T	T	T
292	29795913	rs41551813	A	A	A	A	A	A	T	A	A	A	A
372	29795993	rs1130355	G	G	G	G	A	A	G	A	A	A	A
482	29796103	rs1626038	T	T	T	T	C	C	C	C	C	C	C
485	29796106	rs17875399	G	G	G	G	G	G	T	G	G	G	G
494	29796115	rs1736927	A	A	A	A	C	C	C	C	C	C	C
505	29796126	rs3215482	–	–	–	–	C	C	–	C	C	C	C
507	29796128		C	C	C	C	C	C	A	C	C	C	C
531	29796152	rs1625907	G	G	G	G	C	C	G	C	C	C	C
613	29796234	rs375939243	A	A	A	A	–	–	A	–	–	–	–
636	29796257	rs1625035	C	C	C	C	T	T	T	T	T	T	T
644	29796265	rs17875401	G	G	G	G	G	G	T	G	G	G	G
685	29796306	rs1624337	G	G	G	G	A	A	G	A	A	A	A
706	29796327	rs1130356	C	C	C	C	T	C	C	C	C	T	T
748	29796369	rs3873252	A	A	A	A	A	T	A	A	A	A	A
755	29796376	rs12722477	C	C	C	C	C	C	C	A	A	C	C
813	29796434	rs41557518	C	C	C	C	C	C	C	C	C	–	C
1019	29796640	rs1632942	T	T	T	T	C	C	C	C	C	C	C
1054	29796675	rs17875404	G	G	G	G	G	G	T	G	G	G	G
1064	29796685	rs1632941	T	T	T	T	C	C	T	C	C	C	C
1128	29796749	rs62391965	C	C	C	C	C	A	C	C	C	C	C
1147	29796768	rs1632940	C	C	T	T	C	C	T	C	C	C	C
1217	29796838	rs1736923	A	A	A	A	G	G	A	G	G	G	G
1313	29796934	rs114041958	G	G	G	G	G	G	A	G	G	G	G
1314	29796935	rs1632939	G	G	G	G	A	A	G	A	A	A	A
1365	29796986	rs1632938	G	G	G	G	A	A	G	A	A	A	A
1534	29797155	rs17875405	G	G	G	G	G	G	C	G	G	G	G
1552	29797173	rs1736920	A	A	A	A	G	G	G	G	G	G	G
1590	29797211	rs41562616	C	C	C	C	C	C	T	C	C	C	C
1799	29797420	rs12722482	C	C	C	C	C	C	C	C	C	C	T
1827	29797448	rs17875406	G	G	G	G	G	G	G	G	A	G	G
1932	29797553	rs1632937	G	G	G	G	C	C	G	C	C	C	C
2018	29797639	rs1049033	C	C	C	C	T	C	C	C	C	T	T
2075	29797696	rs1130363	A	A	A	A	G	G	G	G	G	G	G
2161	29797782	rs1611627	T	T	T	T	C	T	T	C	C	C	C
2278	29797899	rs1632934	T	T	T	T	C	C	T	C	C	C	C
2312	29797933	rs1632933	C	C	C	C	T	T	C	T	T	T	T
2330	29797951	rs1736912	A	A	A	A	G	G	A	G	G	G	G
2412	29798033	rs17179080	G	A	G	G	G	G	G	G	G	G	G
2418	29798039	rs1632932	G	G	G	G	A	A	G	A	A	A	A

(Continued)

Table 3 | Continued

HLA-G position	Genomic position on chromosome 6 (hg19)	SNPid	G*01:01:01:01	G*01:01:01:01new	G*01:01:01:04	G*01:01:01:05	G*01:01:02:01	G*01:01:03:03	G*01:03:01:02	G*01:04:01	G*01:04:04	G*01:05N	G*01:06
2462	29798083	rs114038308	C	C	C	C	C	T	C	C	C	C	C
2519	29798140	rs915667	A	A	A	A	G	G	A	G	G	G	G
2798	29798419	rs915670	G	G	G	G	A	G	G	G	G	A	A
2804	29798425	rs915669	G	G	G	G	T	T	T	T	T	T	T
2838	29798459	rs915668	C	C	C	C	G	G	G	G	G	G	G
Global haplotype frequency ($2n = 2152$)			0.2528	0.0200	0.0376	0.0911	0.1445	0.0627	0.0446	0.1329	0.0404	0.0330	0.0283

HLA-G coding haplotypes were converted into coding alleles based on the International Immunogenetics Database (IMGT/HLA). The new HLA-G allele presenting a frequency of about 1% is defined with the suffix "new."

alleles do represent 88.8% of all *HLA-G* coding haplotypes and are associated with only four different HLA-G full-length molecules and a truncated one. Moreover, taking into account these 11 haplotypes, at least 60.87% of all HLA-G full-length molecules would be the same (from G*01:01:01:01, G*01:01:02:01, G:01:01:03:03, G*01:01:01:04, and G*01:01:01:01new) and a higher proportion is expected if other rare haplotypes are considered.

The haplotypes listed in **Table 3** do present heterogeneous frequencies among the 1000Genomes populations (**Table 4**). The G*01:01:01:01 allele, for example, is very frequent among Europeans and Asians, presents intermediate frequencies among admixed populations and lower frequencies in African populations, while an opposite pattern is observed for the G*01:05N null allele. In addition, allele G*01:01:03:03 is absent or very rare in African populations, and the G*01:04:04, G*01:01:01:04, and G*01:01:01:01new alleles are absent in Asians.

HLA-G 3′ UNTRANSLATED REGION VARIABILITY AND HAPLOTYPES

The reevaluation of the *HLA-G* sequencing data indicated that its 3′UTR presents several high-frequency variation sites in a short segment. The approach described earlier evidenced as much as 17 variation sites in this short region, as described in **Table 5**. Some of these variation sites are polymorphic and have been previously described in several studies that evaluated the *HLA-G* 3′UTR (38, 69, 76, 88, 105–117), while some might be considered as mutations. In general, nine variation sites can be considered as true polymorphisms. It should be noted that the nomenclature used to designate *HLA-G* 3′UTR variation sites is based on our previous reports, being designated as UTR-1, UTR-2, and so forth (88). In this matter, the 14-bp insertion (rs371194629), although less frequent and not represented in the hg19 human genome, is considered to be the ancestral allele and should be counted for designate *HLA-G* 3′UTR positions.

When the 3′UTR segment is isolated from the 200 extended haplotypes found, we observe 41 different haplotypes for this region. **Table 6** presents all haplotypes that reached a global frequency higher than 1% and the complete table of haplotypes is available upon request. Monomorphic positions considering these high-frequency haplotypes are removed from **Table 6**. Considering the global frequency of each haplotype, it is noteworthy that only nine haplotypes account for more than 95% of all haplotypes found. These haplotypes were named according to the previous studies addressing the *HLA-G* 3′UTR variability (38, 69, 76, 88, 105–117).

The haplotypes found considering the reevaluation of the 1000Genomes data are consistent with the ones found in several other populations, and some haplotypes that were previously considered as rare ones (such as UTR-10 and UTR-18) are actually more frequent than previously thought considering all populations pooled together (global frequency). Some rare SNVs that were previously described using Sanger sequencing, such as the one at position +3001 (69, 110, 111), and others that were described in studies evaluating the 1000Genomes data, such as +3032, +3052, +3092, +3121, and +3227, were also detected in this reevaluation (**Table 5**). In addition, it should be pointed out that the 14-bp polymorphism, which is absent at the 1000Genomes initial released VCF files, was retrieved from the raw sequence data and its genotypes were inferred for most of the samples.

Similar to the *HLA-G* coding region, a heterogeneous distribution of these nine 3′UTR haplotypes is observed among the 1000Genomes populations (**Table 7**). The UTR-1 haplotype, for example, is very common in European populations, but presents lower frequencies in populations from Africa. The UTR-7 haplotype is absent or rare in populations of African ancestry, and haplotypes UTR-6 and UTR-18 are absent or rare in Asia. The 3′UTR haplotype frequencies in admixed populations are close to the ones reported for other admixed populations such as Brazilians (76, 88, 110, 111). In addition, the frequencies observed for the 1000Genomes African populations are close to the ones reported for other African populations described in isolated reports (108, 116, 117). Moreover, the frequencies reported here are close to the ones presented for the same data in another manuscript (69), with some minor differences since this latter manuscript only imputed the 14-bp polymorphism and used the original 1000Genomes VCF data.

Table 4 | The most frequent *HLA-G* coding haplotypes and their frequencies among the 1000Genomes Project (Phase 1) populations.

***HLA-G* coding alleles according to IMGT/HLA[a]**	**Europe**					**Asia**			**Africa**		**Admixed**			
	CEU $2n = 170$	**TSI** $2n = 196$	**GBR** $2n = 174$	**FIN** $2n = 184$	**IBS** $2n = 28$	**CHB** $2n = 192$	**CHS** $2n = 200$	**JPT** $2n = 178$	**YRI** $2n = 174$	**LWK** $2n = 188$	**ASW** $2n = 118$	**MXL** $2n = 124$	**PUR** $2n = 110$	**CLM** $2n = 116$
G*01:01:01:01	0.3824	0.2755	0.2989	0.3370	0.2857	0.2813	0.3900	0.2360	0.0690	0.1489	0.1271	0.2339	0.2182	0.1810
G*01:01:02:01	0.1824	0.1735	0.1954	0.1196	0.2500	0.0938	0.0350	0.1742	0.1379	0.1436	0.1780	0.2097	0.1000	0.1552
G*01:04:01	0.0647	0.1020	0.0517	0.0543	0.0714	0.2656	0.2400	0.3764	0.0402	0.0106	0.0339	0.1532	0.1364	0.1810
G*01:01:01:05	0.1529	0.1429	0.1092	0.2609	0.1071	0.0469	0.0150	0.0056	0.0632	0.0319	0.0339	0.0806	0.1182	0.1293
G*01:01:03:03	0.0529	0.0408	0.0920	0.0435	0.0357	0.1719	0.2050	0.0337	0.0000	0.0000	0.0085	0.0484	0.0455	0.0086
G*01:03:01:02	0.0353	0.0306	0.0230	0.0163	0.0000	0.0260	0.0000	0.0169	0.0690	0.0798	0.1186	0.0968	0.0818	0.0603
G*01:04:04	0.0235	0.0306	0.0115	0.0054	0.0000	0.0000	0.0000	0.0000	0.2299	0.0745	0.1102	0.0081	0.0273	0.0259
G*01:01:01:04	0.0118	0.0153	0.0632	0.0109	0.0714	0.0000	0.0000	0.0000	0.0747	0.1011	0.0763	0.0403	0.0727	0.0603
G*01:05N	0.0059	0.0408	0.0000	0.0109	0.0000	0.0417	0.0150	0.0056	0.1207	0.0638	0.0847	0.0242	0.0000	0.0172
G*01:06	0.0412	0.0714	0.0632	0.0272	0.1071	0.0260	0.0100	0.0056	0.0000	0.0053	0.0085	0.0242	0.0273	0.0431
G*01:01:01:01new	0.0059	0.0153	0.0115	0.0000	0.0000	0.0000	0.0000	0.0000	0.0460	0.0585	0.0593	0.0242	0.0364	0.0345

[a] *HLA-G coding haplotypes were converted into coding alleles based on the International Immunogenetics Database (IMGT/HLA). The new HLA-G allele presenting high frequencies is defined with the suffix "new."*

CEU, Utah residents with Northern and Western European ancestry; TSI, Toscani from Italy; GBR, British from England and Scotland; FIN, Finnish from Finland; IBS, Iberian populations from Spain; CHB, Han Chinese from Beijing; CHS, Han Chinese from South China; JPT, Japanese from Tokyo, Japan; YRI, Yoruba from Ibadan, Nigeria; LWK, Luhya from Webuye, Kenya; ASW, people of African ancestry from the southwestern United States; MXL, people of Mexican ancestry from Los Angeles, California; PUR, Puerto Ricans from Puerto Rico; CLM, Colombians from Medellin, Colombia.

Haplotypes are ordered according to their global frequency.

Table 5 | List of all variation sites found in the *HLA-G* 3′ untranslated region, their positions regarding hg19 and the *HLA-G* gene, and their allele frequencies considering all populations of the 1000Genomes Project (Phase 1).

Genomic position hg19 (Chr6)	SNPid	HLA-G position	Allele 1 (reference)	Allele 1 frequency	Allele 2	Allele 2 frequency
29798563		2942	T	0.9986	C	0.0014
29798581	rs371194629	2960	G	0.7068	GATTTGTTCATGCCT	0.2932
29798608		3001	C	0.9986	T	0.0014
29798610	rs1707	3003	C	0.1152	T	0.8848
29798617	rs1710	3010	G	0.4610	C	0.5390
29798634	rs17179101	3027	C	0.9359	A	0.0641
29798639	rs146339774	3032	G	0.9967	C	0.0033
29798642	rs17179108	3035	C	0.8829	T	0.1171
29798659		3052	C	0.9991	T	0.0009
29798699	rs180827037	3092	G	0.9986	T	0.0014
29798728	rs138249160	3121	T	0.9967	C	0.0033
29798749	rs1063320	3142	C	0.4484	G	0.5516
29798784		3177	G	0.9991	T	0.0009
29798790	rs187320344	3183	G	0.9991	A	0.0009
29798794	rs9380142	3187	A	0.7045	G	0.2955
29798803	rs1610696	3196	C	0.7625	G	0.2375
29798834	rs1233331	3227	G	0.9707	A	0.0293

Table 6 | The most frequent *HLA-G* 3′ untranslated region haplotypes presenting frequencies higher than 1% considering all populations of the 1000Genomes Project (Phase 1).

dbSNP	rs371194629	rs1707	rs1710	rs17179101	rs17179108	rs1063320	rs9380142	rs1610696	rs1233331	Global
HLA-G position	2960 (14 bp)	3003	3010	3027	3035	3142	3187	3196	3227	frequency,
HG19 (Chr6)	29798581	29798610	29798617	29798634	29798642	29798749	29798794	29798803	29798834	$2n = 2152$
UTR-1	Del	T	G	C	C	C	G	C	G	0.2904
UTR-2	Ins	T	C	C	C	G	A	G	G	0.1938
UTR-3	Del	T	C	C	C	G	A	C	G	0.1938
UTR-4	Del	C	G	C	C	C	A	C	G	0.1083
UTR-7	Ins	T	C	A	T	G	A	C	G	0.0558
UTR-10	Del	T	C	C	C	G	A	G	G	0.0367
UTR-5	Ins	T	C	C	T	G	A	C	G	0.0358
UTR-18	Del	T	G	C	C	C	A	C	A	0.0283
UTR-6	Del	T	G	C	C	C	A	C	G	0.0125
Major allele	Del	T	C	C	C	G	A	C	G	
Frequency	0.7068	0.8848	0.5390	0.9359	0.8829	0.5516	0.7045	0.7625	0.9707	

HLA-G 3′ untranslated region haplotypes were named following the same nomenclature used in the previous studies (69, 76, 88, 110).
Haplotypes are ordered according to their global frequency.

HLA-G 5′ PROMOTER REGION VARIABILITY AND HAPLOTYPES

As previously discussed, there is no consensus regarding where the *HLA-G* transcription starts. Considering NCBI and NM_002127.5, the *HLA-G* transcription starts 866 nucleotides upstream the initiation codon ATG. However, most of the studies performed so far regarding the *HLA-G* promoter structure did consider 1500 nucleotides upstream the main initiation codon ATG as the *HLA-G* promoter region. In this scenario, only SNVs above −866 should be considered as promoter SNVs (or SNVs from the upstream regulatory region) and the ones between −866 and −1 should be considered as 5′UTR SNVs. Nevertheless, despite of this inconsistency and considering the fact that there is no consensus yet regarding the *HLA-G* initial transcription starting point, in the present work we considered all SNVs upstream the main translation start point as promoter (5′ upstream regulatory region) SNVs.

The approach described earlier evidenced the presence of 35 SNVs in the *HLA-G* promoter region, as described in **Table 8**. Among them, 26 of all variable sites (74.3%) can be considered

Table 7 | The most frequent *HLA-G* 3′ untranslated region haplotypes and their frequencies among the 1000Genomes Project (Phase 1) populations.

***HLA-G* 3′UTR haplotypes**[a]	**Europe**					**Asia**			**Africa**		**Admixed**			
	CEU $2n = 170$	**TSI** $2n = 196$	**GBR** $2n = 174$	**FIN** $2n = 184$	**IBS** $2n = 28$	**CHB** $2n = 192$	**CHS** $2n = 200$	**JPT** $2n = 178$	**YRI** $2n = 174$	**LWK** $2n = 188$	**ASW** $2n = 118$	**MXL** $2n = 124$	**PUR** $2n = 110$	**CLM** $2n = 116$
UTR-1	0.3882	0.2959	0.3333	0.3533	0.3214	0.2865	0.4200	0.2472	0.1322	0.2287	0.2288	0.2823	0.2909	0.2241
UTR-3	0.0882	0.1276	0.0575	0.0652	0.0714	0.2813	0.2600	0.4944	0.2989	0.1170	0.1610	0.1532	0.1818	0.2328
UTR-2	0.2471	0.2398	0.2644	0.1739	0.3929	0.1510	0.0500	0.1685	0.1667	0.2340	0.2627	0.2419	0.1000	0.2155
UTR-4	0.1529	0.1378	0.1092	0.2826	0.1071	0.0469	0.0200	0.0056	0.1322	0.1117	0.0508	0.0887	0.1273	0.1466
UTR-7	0.0471	0.0408	0.0747	0.0435	0.0357	0.1563	0.1800	0.0281	0.0000	0.0000	0.0085	0.0403	0.0455	0.0000
UTR-10	0.0000	0.0714	0.0230	0.0380	0.0000	0.0313	0.0100	0.0225	0.0977	0.0585	0.0339	0.0161	0.0364	0.0345
UTR-5	0.0353	0.0255	0.0172	0.0163	0.0000	0.0156	0.0000	0.0169	0.0460	0.0479	0.1017	0.0806	0.0909	0.0431
UTR-18	0.0118	0.0153	0.0517	0.0109	0.0714	0.0000	0.0000	0.0000	0.0172	0.0798	0.0508	0.0323	0.0727	0.0603
UTR-6	0.0059	0.0153	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0747	0.0266	0.0254	0.0081	0.0091	0.0000
others	0.0235	0.0306	0.0690	0.0163	0.0000	0.0313	0.0600	0.0169	0.0345	0.0957	0.0763	0.0565	0.0455	0.0431

[a] *HLA-G 3′ untranslated haplotypes were named following the same nomenclature used in the previous studies (69, 76, 88, 110).*

CEU, Utah residents with Northern and Western European ancestry; TSI, Toscani from Italy; GBR, British from England and Scotland; FIN, Finnish from Finland; IBS, Iberian populations from Spain; CHB, Han Chinese from Beijing; CHS, Han Chinese from South China; JPT, Japanese from Tokyo, Japan; YRI, Yoruba from Ibadan, Nigeria; LWK, Luhya from Webuye, Kenya; ASW, people of African ancestry from the southwestern United States; MXL, people of Mexican ancestry from Los Angeles, California; PUR, Puerto Ricans from Puerto Rico; CLM, Colombians from Medellin, Colombia.

Haplotypes are ordered according to their global frequency.

Table 8 | List of all variation sites found at the *HLA-G* 5′ promoter region, their positions regarding hg19 and the *HLA-G* gene, and their allele frequencies considering all populations of the 1000Genomes Project (Phase 1).

Genomic position hg19 (Chr6)	SNPid	HLA-G position	Allele 1 (reference)	Allele 1 frequency	Allele 2	Allele 2 frequency	Allele 3	Allele 3 frequency
29794317	rs1736936	−1305	G	0.4995	A	0.5005		
29794443	rs1736935	−1179	A	0.4466	G	0.5534		
29794467	rs3823321	−1155	G	0.8020	A	0.1980		
29794482	rs1736934	−1140	A	0.6952	T	0.3048		
29794484	rs17875389	−1138	A	0.9493	G	0.0507		
29794501	rs3115630	−1121	T	0.0428	C	0.9572		
29794524	rs146374870	−1098	G	0.9972	A	0.0028		
29794658	rs1632947	−964	G	0.4986	A	0.5014		
29794700	rs370338057	−922	C	0.9981	A	0.0019		
29794812	rs182801644	−810	C	0.9986	T	0.0014		
29794860	rs1632946	−762	C	0.4972	T	0.5028		
29794897	rs1233334	−725	G	0.0953	C	0.8550	T	0.0497
29794906	rs2249863	−716	T	0.4963	G	0.5037		
29794933	rs2735022	−689	A	0.4963	G	0.5037		
29794956	rs35674592	−666	G	0.4981	T	0.5019		
29794976	rs17875391	−646	A	0.9749	G	0.0251		
29794989	rs1632944	−633	G	0.4995	A	0.5005		
29795076	rs201221694	−546/−540	A	0.9744	AG	0.0256		
29795081	rs368205133	−541/−533	GA	0.9545	G	0.0455		
29795083	rs112940953	−539	A	0.9967	G	0.0033		
29795101	rs138987412	−521	C	0.9986	A	0.0014		
29795113	rs17875393	−509	C	0.9559	G	0.0441		
29795136	rs1736933	−486	A	0.4991	C	0.5009		
29795139	rs149890776	−483	A	0.9717	G	0.0283		
29795145	rs1736932	−477	C	0.4461	G	0.5539		
29795179	rs17875394	−443	G	0.9638	A	0.0362		
29795222	rs17875395	−400	G	0.9559	A	0.0441		
29795231	rs17875396	−391	G	0.9559	A	0.0441		
29795253	rs1632943	−369	C	0.4480	A	0.5520		
29795267	rs191630481	−355	G	0.9967	A	0.0033		
29795338	.	−284	G	0.9991	A	0.0009		
29795366	.	−256	TC	0.9958	T	0.0042		
29795421	rs1233333	−201	G	0.4967	A	0.5033		
29795472	.	−150	C	0.9977	T	0.0023		
29795566	rs17875397	−56	C	0.9503	T	0.0497		

as true polymorphisms (minor allele frequency above 1%), and at least 11 present frequencies around 50%. In addition, the tri-alleic SNP at position −725, as well as other known indels at the promoter region, was properly recovered.

When the promoter region is isolated from the 200 extended haplotypes found, we observe 64 haplotypes for this region. **Table 9** presents all haplotypes that reached a frequency higher than 1% and the complete table of haplotypes is available upon request. Monomorphic positions considering these frequent haplotypes were removed from **Table 9**. Considering the global frequency of each haplotype, it is worth mentioning that only nine haplotypes account for more than 95% of all haplotypes found. These haplotypes were named according to previously published works addressing the *HLA-G* promoter region variability (76, 118–120). As previously observed for both the coding and 3′UTR regions, promoter haplotype frequencies greatly vary among populations (**Table 10**).

HLA-G EXTENDED HAPLOTYPES

As described earlier, 200 extended haplotypes were inferred considering the whole *HLA-G* sequence encompassing the promoter, coding, and 3′UTR segments. Since there is no official nomenclature for the entire MHC genes, the *HLA-G* extended haplotypes were named according to the nomenclature adopted for each *HLA-G* segment. As already observed for some populations (76, 88, 118–120), the promoter haplotypes are usually associated with the same coding and 3′UTR haplotypes (**Table 11**). For example, promoter haplotype 010101a is usually associated with the coding

Table 9 | The most frequent *HLA-G* 5′ promoter region haplotypes presenting frequencies higher than 1% considering all populations of the 1000Genomes Project (Phase 1).

SNV Identification			HLA-G Promoter Haplotypes									
HG19 (Chr6)	**SNPid**	**HLA-G position**	**010102a**	**010101a**	**010104a**	**010101b**	**010101f**	**010101c**	**010104b**	**010101d**	**0103a**	**0103e**
29794317	rs1736936	−1305	A	G	A	G	G	G	A	G	G	G
29794443	rs1736935	−1179	G	A	G	A	A	A	G	A	G	G
29794467	rs3823321	−1155	G	G	A	G	G	G	A	G	G	G
29794482	rs1736934	−1140	T	A	A	A	A	A	A	A	A	A
29794484	rs17875389	−1138	A	A	A	A	A	A	A	A	G	G
29794501	rs3115630	−1121	C	C	C	C	C	T	C	C	C	C
29794658	rs1632947	−964	A	G	A	G	G	G	A	G	G	G
29794860	rs1632946	−762	T	C	T	C	C	C	T	C	C	C
29794897	rs1233334	−725	C	C	C	G	C	G	C	C	T	T
29794906	rs2249863	−716	G	T	G	T	T	T	G	T	T	T
29794933	rs2735022	−689	G	A	G	A	A	A	G	A	A	A
29794956	rs35674592	−666	T	G	T	G	G	G	T	G	G	G
29794976	rs17875391	−646	A	A	A	A	A	A	A	A	A	G
29794989	rs1632944	−633	A	G	A	G	G	G	A	G	G	G
29795076	rs201221694	−546	–	–	–	–	–	–	–	–	G	–
29795081	rs368205133	−541	A	A	A	A	–	A	A	A	A	A
29795113	rs17875393	−509	C	C	C	C	C	C	C	C	G	G
29795136	rs1736933	−486	C	A	C	A	A	A	C	A	A	A
29795139	rs149890776	−483	A	A	A	A	A	A	A	G	A	A
29795145	rs1736932	−477	G	C	G	C	C	C	G	C	G	G
29795179	rs17875394	−443	G	G	G	G	G	G	A	G	G	G
29795222	rs17875395	−400	G	G	G	G	G	G	G	G	A	A
29795231	rs17875396	−391	G	G	G	G	G	G	G	G	A	A
29795253	rs1632943	−369	A	C	A	C	C	C	A	C	A	A
29795421	rs1233333	−201	A	G	A	G	G	G	A	G	G	G
29795566	rs17875397	−56	C	C	C	C	C	C	C	C	T	T
29795636	rs1630223	15	A	G	A	G	G	G	A	G	G	G
Global Frequency ($2n = 2152$)			0.2825	0.2728	0.1501	0.0520	0.0446	0.0418	0.0353	0.0260	0.0191	0.0149

HLA-G promoter haplotypes were named following the same nomenclature used in the previous studies (76, 118).
Haplotypes are ordered according to their global frequency.

allele G*01:01:01:01 and the 3′UTR haplotype named UTR-1. The same phenomenon is observed for each of the main *HLA-G* promoter, coding, or 3′UTR haplotypes. In this matter, only 24 extended *HLA-G* haplotypes were found presenting a minimum frequency of 0.5% and representing more than 85% of all haplotypes, and only 15 present frequencies higher than 1%.

The extended haplotypes shown in **Table 11** were classified according to previously defined *HLA-G* lineages (76, 118). It becomes clear that most of the extended haplotypes are associated with the same encoded full-length molecule and functional polymorphisms are mainly present at the regulatory regions. In fact, many polymorphisms in the regulatory regions do present high frequencies (around 50%), what is compatible with the evidence of balancing selection acting on the *HLA-G* regulatory regions (3, 69, 76, 88, 115, 118, 121). For example, lineages HG010101 (a, b or c) and HG010102 are associated with *HLA-G* coding alleles that usually encode the same HLA-G molecules (exception made to the G*01:06 and G*01:05N alleles), but the promoter and 3′UTR haplotypes are the most divergent ones compared to each other.

Recently, the Neanderthal genome sequence corresponding to a sample dating 40,000 years was published (122). The same pipeline described above was applied to this Neanderthal genome and we found that this unique sample does present a *HLA-G* haplotype found among modern humans with a frequency of 0.00604 (G010101f/G*01:01:01:04/UTR-6) and another haplotype that was not found in the present series and is composed of a recombined promoter, an unknown *HLA-G* coding allele close to G*01:01:02:01 and UTR-2.

HLA-G WORLDWIDE DIVERSITY

Human leukocyte antigen G worldwide intrapopulational genetic diversity was evaluated by means of different population genetics parameters (**Table 12**). Except for the number of private

Table 10 | The most frequent *HLA-G* 5′ promoter region haplotypes and their frequencies among the 1000Genomes Project (Phase 1) populations.

Promoter haplotypes[a]	Europe					Asia			Africa		Admixed			
	CEU $2n = 170$	TSI $2n = 196$	GBR $2n = 174$	FIN $2n = 184$	IBS $2n = 28$	CHB $2n = 192$	CHS $2n = 200$	JPT $2n = 178$	YRI $2n = 174$	LWK $2n = 188$	ASW $2n = 118$	MXL $2n = 124$	PUR $2n = 110$	CLM $2n = 116$
010102a	0.2824	0.3418	0.3908	0.2283	0.4286	0.3385	0.2750	0.2360	0.2586	0.2713	0.2881	0.2742	0.1636	0.2328
010101a	0.3941	0.2704	0.3103	0.3370	0.3214	0.2813	0.4150	0.2303	0.1379	0.2394	0.1695	0.2419	0.2182	0.1810
010104a	0.0882	0.1327	0.0575	0.0652	0.0714	0.1979	0.1800	0.3820	0.2701	0.0904	0.1356	0.0806	0.1455	0.0862
010101b	0.0471	0.0510	0.0230	0.1902	0.0000	0.0417	0.0100	0.0056	0.0805	0.0266	0.0339	0.0645	0.0455	0.0690
010101f	0.0118	0.0255	0.0747	0.0109	0.0714	0.0000	0.0050	0.0056	0.0747	0.1277	0.0847	0.0484	0.0909	0.0603
010101c	0.1059	0.0867	0.0862	0.0870	0.1071	0.0052	0.0050	0.0000	0.0000	0.0053	0.0085	0.0161	0.0727	0.0603
010104b	0.0000	0.0000	0.0000	0.0000	0.0000	0.0781	0.0800	0.0899	0.0000	0.0000	0.0085	0.0726	0.0273	0.1379
010101d	0.0059	0.0153	0.0115	0.0000	0.0000	0.0000	0.0000	0.0000	0.0632	0.0691	0.0763	0.0403	0.0636	0.0431
0103a	0.0235	0.0153	0.0115	0.0163	0.0000	0.0156	0.0000	0.0169	0.0000	0.0000	0.0339	0.0887	0.0364	0.0345
0103e	0.0059	0.0051	0.0057	0.0000	0.0000	0.0104	0.0000	0.0000	0.0402	0.0479	0.0339	0.0081	0.0273	0.0259

[a] *HLA-G promoter lineages were named according to the previous studies (76, 118).*

CEU, Utah residents with Northern and Western European ancestry; TSI, Toscani from Italy; GBR, British from England and Scotland; FIN, Finnish from Finland; IBS, Iberian populations from Spain; CHB, Han Chinese from Beijing; CHS, Han Chinese from South China; JPT, Japanese from Tokyo, Japan; YRI, Yoruba from Ibadan, Nigeria; LWK, Luhya from Webuye, Kenya; ASW, people of African ancestry from the southwestern United States; MXL, people of Mexican ancestry from Los Angeles, California; PUR, Puerto Ricans from Puerto Rico; CLM, Colombians from Medellin, Colombia.

Haplotypes are ordered according to their global frequency.

Table 11 | The most frequent *HLA-G* extended haplotypes presenting frequencies higher than 0.5% considering all populations of the 1000Genomes Project (Phase 1).

Promoter haplotype[a]	Coding allele[b]	3′UTR haplotype[c]	*HLA-G* lineage[d]	Global frequency	Extended haplotype[e]
010101a	G*01:01:01:01	UTR-1	HG010101a	0.24257	G010101a/G*01:01:01:01/UTR-1
010102a	G*01:01:02:01	UTR-2	HG010102	0.11803	G010102a/G*01:01:02:01/UTR-2
0104a	G*01:04:01	UTR-3	HG0104	0.09108	G0104a/G*01:04:01/UTR-3
010102a	G*01:01:03:03	UTR-7	HG010103	0.05112	G010102a/G*01:01:03:03/UTR-7
010101b	G*01:01:01:05	UTR-4	HG010101c	0.04786	G010101b/G*01:01:01:05/UTR-4
010101c	G*01:01:01:05	UTR-4	HG010101c	0.04136	G010101c/G*01:01:01:05/UTR-4
0104a	G*01:04:04	UTR-3	HG0104	0.03810	G0104a/G*01:04:04/UTR-3
0104b	G*01:04:01	UTR-3	HG0104	0.03392	G0104b/G*01:04:01/UTR-3
010101f	G*01:01:01:04	UTR-18	HG010101b	0.02835	G010101f/G*01:01:01:04/UTR-18
010102a	G*01:06	UTR-2	HG010102	0.02556	G010102a/G*01:06/UTR-2
010101d	G*01:01:01:01new	UTR-1	HG010101a	0.01859	G010101d/G*01:01:01:01new/UTR-1
010102a	G*01:05N	UTR-10	HG010102	0.01812	G010102a/G*01:05N/UTR-10
0103a	G*01:03:01:02	UTR-5	HG0103	0.01766	G0103a/G*01:03:01:02/UTR-5
010102a	G*01:05N	UTR-2	HG010102	0.01255	G010102a/G*01:05N/UTR-2
010102a	G*01:01:02:01	UTR-10	HG010102	0.01115	G010102a/G*01:01:02:01/UTR-10
0104a	G*01:04:01-Like	UTR-3	HG0104	0.00883	G0104a/G*01:04:01-Like/UTR-3
010101d	G*01:01:01:04-Like	UTR-1	HG010101a	0.00651	G010101d/G*01:01:01:04-Like/UTR-1
0103c	G*01:03:01:02	UTR-5	HG0103	0.00651	G0103c/G*01:03:01:02/UTR-5
010101f	G*01:01:01:04	UTR-6	HG010101b	0.00604	G010101f/G*01:01:01:04/UTR-6
010101a	G*01:01:01:06	UTR-4	HG010101*	0.00604	G010101a/G*01:01:01:06/UTR-4
010102a	G*01:01:03:03	UTR-7-Like	HG010103	0.00604	G010102a/G*01:01:03:03/UTR-7-Like
0103e	G*01:03:01:02	UTR-13	HG0103	0.00558	G0103e/G*01:03:01:02/UTR-13
010102a	Unknown/new	UTR-2	HG010102	0.00558	G010102a/unknown/UTR-2
010101a	G*01:01:09	UTR-4	HG010101*	0.00558	G010101a/G*01:01:09/UTR-4

[a] *HLA-G promoter haplotypes were named according to the previous studies (76, 118).*

[b] *HLA-G coding haplotypes were converted into coding alleles based on the International Immunogenetics Database (IMGT/HLA). When a haplotype is close to one known haplotype, except for a single nucleotide modification, suffix "-Like" was added. The new HLA-G allele is defined with the suffix "new."*

[c] *HLA-G 3′ untranslated haplotypes were named according to the previous studies (69, 76, 88, 110).*

[d] *HLA-G lineages were named according to a previous study (76).*

[e] *Names proposed for the HLA-G extended haplotypes.*

**Denotes possible crossing overs among known lineages*

Haplotypes are ordered according to their global frequency.

alleles, which is greatly influenced by sample sizes and the number of different samples from a same geographic area (group), African populations exhibited higher levels of genetic diversity in comparison with Europeans and Asians. Admixed populations sampled in America also revealed high levels of diversity. These findings are consistent with the current knowledge that older and admixed populations are prone to exhibit larger diversity than younger and non-admixed populations. Similar observations are made when the promoter (**Table 13**) and coding (**Table 14**) regions are considered separately. Since these differences between Africans and non-Africans are not as substantial as those observed for neutral markers (123), such similar levels of diversity may be reflecting both demographic events and the action of balancing selection. However, when the 3′UTR is considered (**Table 15**), a different pattern arises, regarding gene and nucleotide diversity. For instance, Europeans present the highest levels while Africans presents the lowest levels. This finding does not present a straightforward explanation, although one may suppose that a stronger signature of balancing selection over *HLA-G* 3′UTR may have distorted demographic signatures, resulting in a higher diversity in Eurasia. It should be emphasized that, as previously reported for a Brazilian population sample (76) and also for the populations of the 1000Genomes Project (69), both the promoter and 3′UTR diversity have been shaped by a strong balancing pressure.

The comparison of the three different *HLA-G* regions (**Tables 13–15**) also reveals interesting aspects. The average expected heterozygosity (gene diversity) for variation sites at the 3′UTR is ∼20% higher (0.2730) than the estimated ones for the promoter (0.2323) and coding (0.2244) regions. As a consequence, nucleotide diversity is 4.5 times higher for the 3′UTR (2.8640%) than for the promoter (0.6331%) and coding (0.6432%) regions. Nucleotide diversity at *HLA-G* 3′UTR is almost 40 times higher than the human genome average (0.075%) (118, 124), resulting in an astonishing average of 8.19 differences when two randomly chosen 3′UTR (286-bp long) haplotypes are compared. Balancing selection favors the maintenance of different alleles in

Table 12 | Genetic diversity parameters and probability of adherence of diplotype frequencies to Hardy–Weinberg equilibrium expectations (*p*HWE), considering whole *HLA-G* haplotypes.

Population sample	Gene diversity	Private haplotypes	Haplotype diversity	Nucleotide diversity (%)	*p*HWE
Africa ($2n = 362$)	0.2913 ± 0.1949	36	0.9417 ± 0.0054	0.7643 ± 0.3690	0.6582 ± 0.0137
LWK ($2n = 188$)	0.3108 ± 0.1888	24	0.9497 ± 0.0075	0.7815 ± 0.3781	0.7200 ± 0.0130
YRI ($2n = 174$)	0.3175 ± 0.1722	10	0.9118 ± 0.0121	0.7283 ± 0.3531	0.5892 ± 0.0134
Europe ($2n = 752$)	0.2663 ± 0.2162	33	0.8622 ± 0.0088	0.7399 ± 0.3570	0.8219 ± 0.0113
CEU ($2n = 170$)	0.3315 ± 0.1902	6	0.8210 ± 0.0231	0.7384 ± 0.3579	0.5821 ± 0.0133
FIN ($2n = 184$)	0.2940 ± 0.1828	17	0.8501 ± 0.0187	0.6679 ± 0.3243	0.4973 ± 0.0142
GBR ($2n = 174$)	0.3234 ± 0.2036	8	0.8679 ± 0.0168	0.7632 ± 0.3696	0.3129 ± 0.0126
IBS ($2n = 28$)	0.4330 ± 0.1566	0	0.8492 ± 0.0412	0.7737 ± 0.3867	0.6021 ± 0.0065
TSI ($2n = 196$)	0.3055 ± 0.2078	9	0.8883 ± 0.0141	0.7546 ± 0.3653	0.7044 ± 0.0125
Asia ($2n = 570$)	0.2675 ± 0.2013	41	0.8503 ± 0.0090	0.6782 ± 0.3280	0.6628 ± 0.0137
CHB ($2n = 192$)	0.3185 ± 0.1816	5	0.8560 ± 0.0141	0.7093 ± 0.3439	0.3700 ± 0.0131
CHS ($2n = 200$)	0.3362 ± 0.1953	19	0.8141 ± 0.0204	0.6898 ± 0.3345	0.6625 ± 0.0134
JPT ($2n = 178$)	0.2710 ± 0.1617	4	0.8468 ± 0.0141	0.5857 ± 0.2854	0.5297 ± 0.0136
Admixed ($2n = 468$)	0.2908 ± 0.1999	26	0.9332 ± 0.0059	0.7890 ± 0.3805	0.6699 ± 0.0136
ASW ($2n = 118$)	0.3253 ± 0.1908	6	0.9483 ± 0.0092	0.8108 ± 0.3933	0.7233 ± 0.0130
CLM ($2n = 116$)	0.3337 ± 0.1786	8	0.9237 ± 0.0113	0.7655 ± 0.3718	0.3765 ± 0.0131
MXL ($2n = 124$)	0.3508 ± 0.1774	3	0.9110 ± 0.0146	0.8045 ± 0.3902	0.6571 ± 0.0129
PUR ($2n = 110$)	0.3220 ± 0.1687	7	0.9296 ± 0.0140	0.7599 ± 0.3693	0.3774 ± 0.0134
Total ($2n = 2152$)	0.2345 ± 0.2149	-	0.9068 ± 0.0040	0.7548 ± 0.3637	0.9025 ± 0.0089

CEU, Utah residents with Northern and Western European ancestry; TSI, Toscani from Italy; GBR, British from England and Scotland; FIN, Finnish from Finland; IBS, Iberian populations from Spain; CHB, Han Chinese from Beijing; CHS, Han Chinese from South China; JPT, Japanese from Tokyo, Japan; YRI, Yoruba from Ibadan, Nigeria; LWK, Luhya from Webuye, Kenya; ASW, people of African ancestry from the southwestern United States; MXL, people of Mexican ancestry from Los Angeles, California; PUR, Puerto Ricans from Puerto Rico; CLM, Colombians from Medellin, Colombia.

Table 13 | Genetic diversity parameters and probability of adherence of diplotype frequencies to Hardy–Weinberg equilibrium expectations (*p*HWE), considering *HLA-G* promoter haplotypes.

Population sample	Gene diversity	Private haplotypes	Haplotype diversity	Nucleotide diversity (%)	*p*HWE
Africa ($2n = 362$)	0.2908 ± 0.2034	7	0.8438 ± 0.0092	0.6604 ± 0.3380	0.4466 ± 0.0127
LWK ($2n = 188$)	0.3000 ± 0.1941	5	0.8397 ± 0.0147	0.6590 ± 0.3382	0.7370 ± 0.0110
YRI ($2n = 174$)	0.3154 ± 0.1907	1	0.8269 ± 0.0149	0.6447 ± 0.3315	0.0849 ± 0.0051
Europe ($2n = 752$)	0.2401 ± 0.2252	14	0.7725 ± 0.0091	0.5998 ± 0.3088	0.5186 ± 0.0138
CEU ($2n = 170$)	0.2818 ± 0.2120	1	0.7471 ± 0.0217	0.5972 ± 0.3090	0.9768 ± 0.0026
FIN ($2n = 184$)	0.2584 ± 0.2054	7	0.7899 ± 0.0164	0.5476 ± 0.2852	0.2223 ± 0.0107
GBR ($2n = 174$)	0.2970 ± 0.2193	1	0.7379 ± 0.0216	0.6069 ± 0.3135	0.0324 ± 0.0036
IBS ($2n = 28$)	0.4400 ± 0.1504	0	0.7169 ± 0.0559	0.6000 ± 0.3202	0.6445 ± 0.0027
TSI ($2n = 196$)	0.2723 ± 0.2249	4	0.7848 ± 0.0176	0.6183 ± 0.3188	0.3980 ± 0.0125
Asia ($2n = 570$)	0.2517 ± 0.2189	8	0.7536 ± 0.0076	0.5524 ± 0.2864	0.5938 ± 0.0129
CHB ($2n = 192$)	0.2878 ± 0.2038	1	0.7627 ± 0.0155	0.5664 ± 0.2941	0.6127 ± 0.0108
CHS ($2n = 200$)	0.3403 ± 0.2187	3	0.7166 ± 0.0183	0.5672 ± 0.2944	0.5743 ± 0.0112
JPT ($2n = 178$)	0.2574 ± 0.1806	1	0.7409 ± 0.0171	0.4871 ± 0.2564	0.3093 ± 0.0104
Admixed ($2n = 468$)	0.2927 ± 0.1958	9	0.8700 ± 0.0081	0.6868 ± 0.3502	0.3354 ± 0.0122
ASW ($2n = 118$)	0.3128 ± 0.1907	1	0.8573 ± 0.0189	0.6867 ± 0.3525	0.3945 ± 0.0122
CLM ($2n = 116$)	0.3136 ± 0.1923	2	0.8777 ± 0.0147	0.6884 ± 0.3533	0.3855 ± 0.0108
MXL ($2n = 124$)	0.3241 ± 0.1851	0	0.8432 ± 0.0185	0.6870 ± 0.3525	0.5318 ± 0.0100
PUR ($2n = 110$)	0.3097 ± 0.1790	4	0.8881 ± 0.0142	0.6798 ± 0.3494	0.7863 ± 0.0092
Total ($2n = 2152$)	0.2323 ± 0.2208	–	0.8145 ± 0.0047	0.6331 ± 0.3243	0.4803 ± 0.0142

CEU, Utah residents with Northern and Western European ancestry; TSI, Toscani from Italy; GBR, British from England and Scotland; FIN, Finnish from Finland; IBS, Iberian populations from Spain; CHB, Han Chinese from Beijing; CHS, Han Chinese from South China; JPT, Japanese from Tokyo, Japan; YRI, Yoruba from Ibadan, Nigeria; LWK, Luhya from Webuye, Kenya; ASW, people of African ancestry from the southwestern United States; MXL, people of Mexican ancestry from Los Angeles, California; PUR, Puerto Ricans from Puerto Rico; CLM, Colombians from Medellin, Colombia.

Table 14 | Genetic diversity parameters and probability of adherence of diplotype frequencies to Hardy–Weinberg equilibrium expectations (*p*HWE), considering *HLA-G* coding region haplotypes.

Population sample	Gene diversity	Private haplotypes	Haplotype diversity	Nucleotide diversity (%)	*p*HWE
Africa ($2n = 362$)	0.2983 ± 0.2036	14	0.9177 ± 0.0053	0.6649 ± 0.3266	0.6983 ± 0.0122
LWK ($2n = 188$)	0.3100 ± 0.1981	9	0.9255 ± 0.0077	0.6691 ± 0.3295	0.6843 ± 0.0121
YRI ($2n = 174$)	0.3306 ± 0.1808	4	0.8934 ± 0.0116	0.6436 ± 0.3175	0.6841 ± 0.0110
Europe ($2n = 752$)	0.2588 ± 0.2233	15	0.8292 ± 0.0085	0.6229 ± 0.3063	0.6674 ± 0.0132
CEU ($2n = 170$)	0.3348 ± 0.1930	2	0.7908 ± 0.0221	0.6162 ± 0.3045	0.5567 ± 0.0117
FIN ($2n = 184$)	0.3019 ± 0.1893	8	0.8011 ± 0.0192	0.5665 ± 0.2808	0.5260 ± 0.0133
GBR ($2n = 174$)	0.3151 ± 0.2112	4	0.8449 ± 0.0163	0.6358 ± 0.3138	0.1818 ± 0.0096
IBS ($2n = 28$)	0.4308 ± 0.1625	0	0.8492 ± 0.0412	0.6405 ± 0.3262	0.5893 ± 0.0067
TSI ($2n = 196$)	0.3070 ± 0.2151	0	0.8563 ± 0.0136	0.6411 ± 0.3161	0.9138 ± 0.0062
Asia ($2n = 570$)	0.2631 ± 0.2097	13	0.7914 ± 0.0095	0.5772 ± 0.2848	0.4079 ± 0.0135
CHB ($2n = 192$)	0.3089 ± 0.1866	2	0.8106 ± 0.0144	0.5903 ± 0.2920	0.3012 ± 0.0107
CHS ($2n = 200$)	0.3567 ± 0.2013	8	0.7495 ± 0.0187	0.5934 ± 0.2934	0.4342 ± 0.0131
JPT ($2n = 178$)	0.2649 ± 0.1712	1	0.7645 ± 0.0188	0.4969 ± 0.2478	0.3456 ± 0.0110
Admixed ($2n = 468$)	0.2834 ± 0.2095	14	0.8970 ± 0.0060	0.6621 ± 0.3251	0.4418 ± 0.0136
ASW ($2n = 118$)	0.3200 ± 0.1953	3	0.9126 ± 0.0107	0.6796 ± 0.3355	0.4556 ± 0.0131
CLM ($2n = 116$)	0.3335 ± 0.1958	5	0.8888 ± 0.0127	0.6494 ± 0.3212	0.2857 ± 0.0113
MXL ($2n = 124$)	0.3482 ± 0.1815	2	0.8624 ± 0.0149	0.6655 ± 0.3287	0.9311 ± 0.0048
PUR ($2n = 110$)	0.3264 ± 0.1823	3	0.8992 ± 0.0138	0.6471 ± 0.3202	0.5820 ± 0.0123
Total ($2n = 2152$)	0.2244 ± 0.2219	–	0.8780 ± 0.0038	0.6432 ± 0.3156	0.5692 ± 0.0143

CEU, Utah residents with Northern and Western European ancestry; TSI, Toscani from Italy; GBR, British from England and Scotland; FIN, Finnish from Finland; IBS, Iberian populations from Spain; CHB, Han Chinese from Beijing; CHS, Han Chinese from South China; JPT, Japanese from Tokyo, Japan; YRI, Yoruba from Ibadan, Nigeria; LWK, Luhya from Webuye, Kenya; ASW, people of African ancestry from the southwestern United States; MXL, people of Mexican ancestry from Los Angeles, California; PUR, Puerto Ricans from Puerto Rico; CLM, Colombians from Medellin, Colombia.

Table 15 | Genetic diversity parameters and probability of adherence of diplotype frequencies to Hardy–Weinberg equilibrium expectations (*p*HWE), considering *HLA-G* 3′UTR haplotypes.

Population sample	Gene diversity	Private haplotypes	Haplotype diversity	Nucleotide diversity (%)	*p*HWE
Africa ($2n = 362$)	0.2833 ± 0.1700	8	0.8583 ± 0.0073	2.6744 ± 1.3827	0.1986 ± 0.0098
LWK ($2n = 188$)	0.3326 ± 0.1626	5	0.8573 ± 0.0124	2.9077 ± 1.4972	0.5067 ± 0.0116
YRI ($2n = 174$)	0.2965 ± 0.1268	3	0.8350 ± 0.0143	2.3841 ± 1.2486	0.6058 ± 0.0091
Europe ($2n = 752$)	0.3276 ± 0.1795	5	0.7885 ± 0.0084	2.9784 ± 1.5247	0.5801 ± 0.0127
CEU ($2n = 170$)	0.3938 ± 0.1332	0	0.7577 ± 0.0203	3.0292 ± 1.5558	0.8857 ± 0.0057
FIN ($2n = 184$)	0.3258 ± 0.1294	1	0.7612 ± 0.0173	2.6197 ± 1.3603	0.9146 ± 0.0043
GBR ($2n = 174$)	0.3802 ± 0.1585	1	0.7986 ± 0.0189	3.1900 ± 1.6321	0.0704 ± 0.0059
IBS ($2n = 28$)	0.4352 ± 0.1545	0	0.7460 ± 0.0537	3.3476 ± 1.7617	0.8526 ± 0.0025
TSI ($2n = 196$)	0.3515 ± 0.1613	1	0.8158 ± 0.0141	2.9499 ± 1.5169	0.5941 ± 0.0105
Asia ($2n = 570$)	0.3045 ± 0.1569	5	0.7507 ± 0.0098	2.6613 ± 1.3750	0.1824 ± 0.0093
CHB ($2n = 192$)	0.3849 ± 0.1194	0	0.7920 ± 0.0133	2.9605 ± 1.5222	0.3045 ± 0.0084
CHS ($2n = 200$)	0.3006 ± 0.1598	5	0.7234 ± 0.0198	2.6274 ± 1.3634	0.3031 ± 0.0104
JPT ($2n = 178$)	0.3086 ± 0.1024	0	0.6681 ± 0.0253	2.2658 ± 1.1920	0.6259 ± 0.0076
Admixed ($2n = 468$)	0.3147 ± 0.1855	1	0.8385 ± 0.0077	2.9705 ± 1.5222	0.3325 ± 0.0117
ASW ($2n = 118$)	0.3598 ± 0.1835	0	0.8415 ± 0.0172	3.1446 ± 1.6150	0.2936 ± 0.0101
CLM ($2n = 116$)	0.3702 ± 0.0917	0	0.8273 ± 0.0139	2.7185 ± 1.4119	0.9862 ± 0.0011
MXL ($2n = 124$)	0.3958 ± 0.1545	1	0.8270 ± 0.0178	3.1832 ± 1.6327	0.9469 ± 0.0039
PUR ($2n = 110$)	0.3338 ± 0.1180	0	0.8459 ± 0.0184	2.6841 ± 1.3962	0.0933 ± 0.0045
Total ($2n = 2152$)	0.2730 ± 0.1921	–	0.8223 ± 0.0041	2.8640 ± 1.4692	0.2546 ± 0.0118

CEU, Utah residents with Northern and Western European ancestry; TSI, Toscani from Italy; GBR, British from England and Scotland; FIN, Finnish from Finland; IBS, Iberian populations from Spain; CHB, Han Chinese from Beijing; CHS, Han Chinese from South China; JPT, Japanese from Tokyo, Japan; YRI, Yoruba from Ibadan, Nigeria; LWK, Luhya from Webuye, Kenya; ASW, people of African ancestry from the southwestern United States; MXL, people of Mexican ancestry from Los Angeles, California; PUR, Puerto Ricans from Puerto Rico; CLM, Colombians from Medellin, Colombia.

Table 16 | Matrix of pair-wise F_{ST} values based on whole *HLA-G* haplotype frequencies (below the diagonal) and probabilities associated with pair-wise F_{ST} values (above the diagonal) for the 14 populations analyzed in the present study.

	CEU	TSI	GBR	FIN	IBS	CHB	JPT	CHS	YRI	LWK	ASW	MXL	PUR	CLM
CEU		**0.0360**	0.3423	0.1081	0.3604	***0.0000****	***0.0000****	***0.0090***	***0.0000****	0.0901	**0.0180**	0.0541	0.1892	**0.0451**
TSI	**0.0086**		0.3694	***0.0000****	0.6396	**0.0180**	***0.0000****	**0.0180**	**0.0180**	0.2342	0.1532	0.3063	**0.0451**	0.4775
GBR	0.0005	−0.0012		***0.0090***	0.8288	***0.0000****	***0.0000****	***0.0090***	***0.0000****	0.1441	**0.0360**	0.2342	0.0541	0.1171
FIN	0.0083	***0.0391****	***0.0288***		**0.0270**	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	**0.0180**	***0.0000****
IBS	−0.0018	−0.0123	−0.0150	**0.0411**		0.1261	***0.0090***	0.0991	0.0721	0.3514	0.5135	0.6577	0.1441	0.3694
CHB	***0.0679****	**0.0251**	***0.0385****	***0.1219****	0.0246		**0.0270**	**0.0180**	***0.0090***	***0.0000****	**0.0270**	***0.0000****	***0.0000****	**0.0270**
JPT	***0.1434****	***0.0772****	***0.1067****	***0.2037****	***0.0981***	**0.0203**		***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****
CHS	***0.0366***	**0.0179**	***0.0233***	***0.0707****	0.0249	**0.0152**	***0.0610****		***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	**0.0180**
YRI	***0.0562****	**0.0174**	***0.0365****	***0.0940****	0.0270	***0.0182***	***0.0362****	***0.0317****		***0.0000****	**0.0360**	***0.0090***	***0.0000****	0.1712
LWK	0.0070	0.0028	0.0037	***0.0294****	−0.0020	***0.0469****	***0.1041****	***0.0331****	***0.0221****		0.1532	0.1622	0.2883	0.3153
ASW	**0.0237**	0.0044	**0.0087**	***0.0659****	−0.0056	**0.0252**	***0.0767****	***0.0344****	**0.0130**	0.0035		0.7748	**0.0270**	0.2883
MXL	0.0142	0.0006	0.0021	***0.0535****	−0.0101	***0.0236****	***0.0810****	***0.0256****	***0.0191***	0.0029	−0.0057		0.0541	0.3423
PUR	0.0053	**0.0128**	0.0111	**0.0151**	0.0178	***0.0625****	***0.1287****	***0.0311****	***0.0369****	0.0027	**0.0183**	0.0128		0.1982
CLM	**0.0164**	−0.0011	0.0074	***0.0450****	0.0005	**0.0235**	***0.0671****	**0.0180**	0.0054	0.0009	0.0015	0.0000	0.0055	

CEU, Utah residents with Northern and Western European ancestry; TSI, Toscani from Italy; GBR, British from England and Scotland; FIN, Finnish from Finland; IBS, Iberian populations from Spain; CHB, Han Chinese from Beijing; CHS, Han Chinese from South China; JPT, Japanese from Tokyo, Japan; YRI, Yoruba from Ibadan, Nigeria; LWK, Luhya from Webuye, Kenya; ASW, people of African ancestry from the southwestern United States; MXL, people of Mexican ancestry from Los Angeles, California; PUR, Puerto Ricans from Puerto Rico; CLM, Colombians from Medellin, Colombia.

Statistically significant F_{ST} values are in boldface ($p < 0.05$) or italicized boldface ($p < 0.01$). Statistically significant values at a 5% significance level after Bonferroni correction are marked with an asterisk ($p < 0.0005$).

a population, resulting in a proportionally higher average pairwise difference as compared with the measure of diversity based on the number of polymorphic sites. The worldwide nucleotide diversity at the whole *HLA-G* locus (0.7548%) is as expected slightly higher than that observed for the Brazilian population sample (0.00643%) (76). The direct comparison of haplotype diversity between the three regions could not be performed, since the very different lengths and number of variation sites of the three regions (**Tables 2**, **5**, and **8**) may bias any retrieved conclusions.

Two independent approaches were used to evaluate the extent of differentiation between pairs of populations (interpopulation diversity): F_{ST} and the exact test of population differentiation based on haplotype frequencies. Although these analyses have the same purpose and may provide similar results, both were performed to provide more reliable and robust conclusions. The analysis of the pair-wise F_{ST} matrix revealed a large range of variation of F_{ST} values: from −0.0150, between British from England and Scotland (GBR) and Iberian populations from Spain (IBS), to 0.2037, between Finnish (FIN) and Japanese (JPT) (**Table 16**). While only 1 out of 6 (16.7%) pairs of admixed populations and 4 out of 10 (40%) European populations differed significantly at the 5% unadjusted significance level; it is noteworthy that the two African populations, as well as the three Asian populations, differed. IBS presented the lowest number of significant comparisons (2 out of 13), a fact that is clearly related to the lack of statistical power due to the small sample size. On the other hand, JPT (all comparisons), CHB (12 out of 13), CHS (12 out of 13), FIN (12 out of 13), and YRI (11 out of 13) presented the largest number of significant comparisons. An overall stronger differentiation was observed by the matrix composed of non-differentiation probability values obtained through the exact test of population differentiation (**Table 17**). While only 3 out of 10 (30%) European populations differed significantly at the 5% significance level, it is noteworthy that the two African populations, as well as the three Asian populations and four admixed populations, differed. IBS presented the lowest number of significant comparisons (4 out of 13), while JPT, CHB, CHS and YRI differed in all pairwise comparisons including them. To sum up, both the exact test of population differentiation based on haplotype frequencies and the F_{ST} estimate revealed the existence of highly significant difference between the 14 populations. Since the more frequent *HLA-G* haplotypes are shared between most of the populations, these pairwise population differences may be due to the existence of many low-frequency haplotypes that are restricted to two or three populations (22.5% of the 200 identified haplotypes) or are private to a single population (63% of the 200 haplotypes).

To further explore the genetic relationships between populations, an AMOVA was performed assuming a hierarchical structure in which the 14 populations were divided into four groups: African, Asian, European, and admixed populations (**Table 18**). Considering the whole *HLA-G* gene, differences between the four groups account for only 2.45% of the variance, whereas 1.64% of the variance occurs as a consequence of differences between populations that belong to a same group. Almost all the variance (95.91%) is observed within populations. This same pattern is observed when each *HLA-G* region, i.e., promoter, coding, and 3′UTR, is considered separately, with the exception of the 3′UTR where the variance among groups (0.65%) gets even lower than the variance among populations that belong to a same group (1.32%), and is statistically non-significant.

Since the group composed of admixed populations represent an assembly of populations whose individuals present varying levels

Table 17 | Matrix of non-differentiation probabilities obtained by means of exact tests of population differentiation based on haplotype frequencies for the 14 populations analyzed in the present study.

	CEU	TSI	GBR	FIN	IBS	CHB	JPT	CHS	YRI	LWK	ASW	MXL	PUR	CLM
CEU														
TSI	0.2109													
GBR	0.1051	0.0765												
FIN	***0.0062***	***0.0004****	***0.0000****											
IBS	0.6345	0.9226	0.9772	0.2932										
CHB	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0057***									
JPT	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0002****	***0.0000****								
CHS	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0001****	**0.0105**	***0.0000****							
YRI	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****						
LWK	***0.0000****	***0.0000****	***0.0000****	***0.0000****	0.3488	***0.0000****	***0.0000****	***0.0000****	***0.0000****					
ASW	***0.0000****	***0.0000****	***0.0000****	***0.0000****	0.3020	***0.0000****	***0.0000****	***0.0000****	***0.0000****	0.1072				
MXL	***0.0000****	***0.0004****	***0.0000****	***0.0000****	0.4085	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0004****			
PUR	***0.0001****	**0.0048**	***0.0006***	***0.0000****	0.7816	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	0.0677		
CLM	***0.0000****	***0.0000****	***0.0000****	***0.0000****	0.5290	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0000****	***0.0001****	**0.0437**	**0.0117**	

CEU, Utah residents with Northern and Western European ancestry; TSI, Toscani from Italy; GBR, British from England and Scotland; FIN, Finnish from Finland; IBS, Iberian populations from Spain; CHB, Han Chinese from Beijing; CHS, Han Chinese from South China; JPT, Japanese from Tokyo, Japan; YRI, Yoruba from Ibadan, Nigeria; LWK, Luhya from Webuye, Kenya; ASW, people of African ancestry from the southwestern United States; MXL, people of Mexican ancestry from Los Angeles, California; PUR, Puerto Ricans from Puerto Rico; CLM, Colombians from Medellin, Colombia.

Statistically significant F_{ST} values are in boldface ($p < 0.05$) or italicized boldface ($p < 0.01$). Statistically significant values at a 5% significance level after Bonferroni correction are marked with an asterisk ($p < 0.0005$).

Table 18 | Analysis of molecular variance (AMOVA) for *HLA-G* haplotype frequencies, according to two different hierarchical structures and four different *HLA-G* datasets.

Groups composing the hierarchical structure [a]	HLA-G data type	Variance		
		Among groups (F_{CT})	Among populations within groups (F_{SC})	Within populations (F_{ST})
Africa: LWK, YRI;	Promoter	3.09% ($p = 0.0098 \pm 0.0033$)	1.57% ($p = 0.0000 \pm 0.0000$)	95.34% ($p = 0.0000 \pm 0.0000$)
Asia: CHB, CHS, JPT;	Coding region	2.99% ($p = 0.0049 \pm 0.0020$)	1.81% ($p = 0.0000 \pm 0.0000$)	95.20% ($p = 0.0000 \pm 0.0000$)
Europe: CEU, FIN, GBR, IBS, TSI;	3′UTR	0.65% ($p = 0.0665 \pm 0.0000$)	1.32% ($p = 0.0000 \pm 0.0000$)	98.02% ($p = 0.0000 \pm 0.0000$)
Admixed: ASW, CLM, MXL, PUR	Whole gene	2.45% ($p = 0.0029 \pm 0.0016$)	1.64% ($p = 0.0000 \pm 0.0000$)	95.91% ($p = 0.0000 \pm 0.0000$)
Africa: LWK, YRI;	Promoter	4.28% ($p = 0.0156 \pm 0.0039$)	2.01% ($p = 0.0000 \pm 0.0000$)	93.71% ($p = 0.0000 \pm 0.0000$)
Asia: CHB, CHS, JPT;	Coding region	4.14% ($p = 0.0147 \pm 0.0042$)	2.28% ($p = 0.0000 \pm 0.0000$)	93.58% ($p = 0.0000 \pm 0.0000$)
Europe: CEU, FIN, GBR, IBS, TSI	3′UTR	1.00% ($p = 0.0332 \pm 0.0065$)	1.32% ($p = 0.0010 \pm 0.0010$)	97.68% ($p = 0.0000 \pm 0.0000$)
	Whole gene	3.42% ($p = 0.0166 \pm 0.0000$)	1.99% ($p = 0.0000 \pm 0.0000$)	94.59% ($p = 0.0000 \pm 0.0000$)

of ancestry that can be assigned to Africans, Amerindians/Asians, and Europeans, this group was removed from a second round of analysis (**Table 18**). As a result, levels of variance between groups increased, although still lower than the expected ones for neutrally evolving sequences (123). Therefore, one may conclude that this analysis reflects the fact that most of the *HLA-G* diversity, particularly that from the 3′UTR, (a) originated from Africa before *Homo sapiens* dispersion to other continents and (b) has been maintained in worldwide populations by non-neutral evolutionary forces, particularly balancing selection. These conclusions are corroborated by previous data on *HLA-G* (68, 69, 76, 89, 121). Moreover, many different low-frequency haplotypes are being generated within populations by mutation and recombination. These features are responsible for the relatively poor resolution of the MDS plot (**Figure 2**) obtained with the matrix of Reynolds' genetic distance based on the whole *HLA-G* gene. Unexpectedly, (a) populations from a same geographic group, for example Asians (CHB, CHS and JPT), are distributed across large distances in the plot and (b) admixed populations (CLM, MXL, and PUR) that present major European, intermediate Amerindian, and minor African ancestry contributions (66), as revealed by the analysis of Ancestry Informative Markers (data not shown), are clustered together with African populations. These unexpected findings support the hypothesis that a strong signature of balancing selection over *HLA-G* may have distorted the expected demographic signatures.

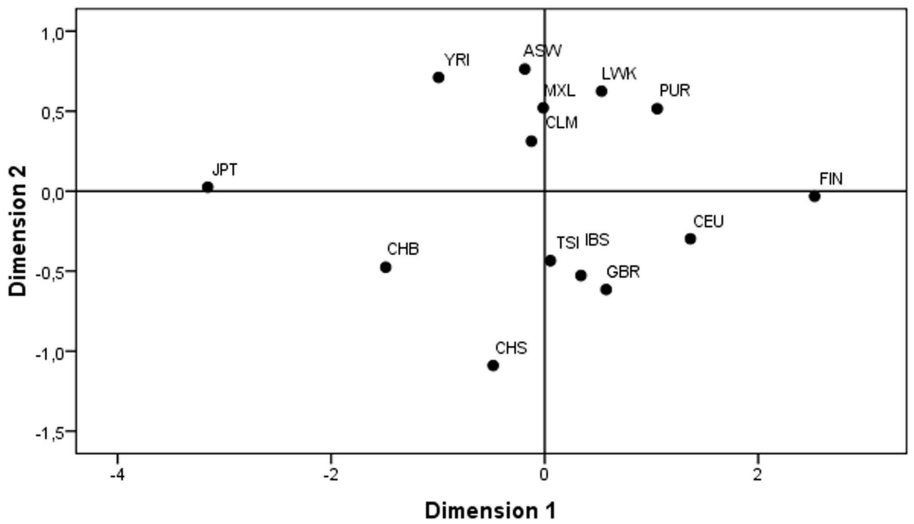

FIGURE 2 | Multidimensional scaling (MDS) plot revealing the genetics relationships between the 14 populations of the 1000Genomes Project (Phase 1).

HLA-G EVOLUTION ASPECTS

The MHC class I molecules evolved by a series of events that include chromosomal duplication, gene recombination, and selection probably driven by pathogens (125–127). Apparently, *MHC-G*, the *HLA-G* homologous sequence in non-human primates, is the oldest class I gene and it would be responsible for the origin of the whole class I loci (127). In fact, MHC class I genes from the New World primates, such as the cotton-top tamarin (*Saguinus oedipus*), are much closer to the human *HLA-G* than other human classical class I genes (127). This primate lineage separated from the one that gave rise to the Old World monkeys (or anthropoids) about 38 million years ago. It is noteworthy that the HLA-G and MHC-G molecules are functionally different despite the high identity among exonic sequences (128). New World primates' MHC-G plays a role in antigen presentation that is uncommon for human HLA-G, and this fact suggests that they are not orthologous as theorized in the past (129, 130). In contrast, the cotton-top tamarin presents two MHC-C molecules with inhibitory properties that interact with KIR receptors (131). The regulation of MHC levels (in this case, MHC-C) in these non-human primates seems to be one of the responsible mechanisms for fetal acceptance as well as for the shorter pregnancy period (132).

Old World primates have a peculiar MHC-G molecule. It presents just the α1 domain due to a stop codon at codon 164 (133), which may not hinder fetal protection against maternal NK cells, unless there is a mechanism in which the stop codon is ignored, allowing translation to continue (which is not discarded). In addition, gorillas and chimpanzees present a conserved *MHC-G* coding segment with few variations (3, 128, 129). Even the pregnancy period being shorter than in human beings, these species are polygamous, which would expose the female to different allogeneic fetuses during the fertile age. Orangutans on the other hand have long-lasting relationships and five *MHC-G* variants have been found so far – the polymorphism levels are low but more similar to human beings (3). Orangutans and humans are separated by about 15 million years of evolution. Possibly, the differences between maternal-fetal relationships among different species are responsible for each *MHC-G* peculiarities and for its function and variation levels.

In addition to alignments between human and other primates coding *MHC-G* sequences, analyses of *HLA-G* non-coding regions have proved to be highly informative about the evolutionary history of this gene. For example, the polymorphism of 14-pb located on *HLA-G* exon 8 (3′UTR) is exclusively found in the human lineage, suggesting that UTR haplotypes bearing the deletion such as UTR-1 are more recent than the ones that present the 14-bp fragment (134).

An interesting finding confirmed recently is that one of the most frequent *HLA-G* coding allele (global frequency of 0.24257), *G*01:01:01:01*, which is usually associated with UTR-1 and the promoter haplotype G010101a [described in Ref. (76) and **Table 11**], is probably the most recent haplotype. These data were established by the association between *G*01:01:01:01*/UTR-1 with an *Alu* insertion (*AluyHG*) that occurred before human dispersion from Africa, in a location 20 Kb downstream *HLA-G* 3′UTR. The frequency of this Alu element increases with distance from Africa (68).

Given the HLA-G immunomodulatory properties and the unique tissue expression patterns, *HLA-G* expression levels must be maintained under a fine regulatory control. In addition, the lack of variability found in its coding region and limited number of proteins coded by this gene lead us to believe that this region

is under tight evolutionary forces that limit variation. The differences on mammalian pregnancy and species-specific pathogens must be considered when studying the evolution of the immune system molecules.

HLA-G TRANSCRIPTION REGULATION

Most of the studies already performed to understand *HLA-G* regulation considered as the *HLA-G* promoter 200 nucleotides upstream the first translated ATG and within 1.5 Kb upstream the CDS. The *HLA-G* regulation is unique among all class I genes [reviewed at Ref. (67)]. Generally, HLA class I genes present two main regulatory modules in the proximal promoter region (within 200 bases upstream the CDS) that includes [reviewed at Ref. (67)] (a) the Enhancer-A (EnhA) that interacts with NF-κB family of transcription factors, which are important elements to induce HLA class I genes expression (135); (b) the interferon-stimulated response element (ISRE) that consists of a target site for interferon regulatory factors (IRF), which might act as class I activators (IRF-1) or inhibitors (IRF-2 and IRF-8) (135). The ISRE module is located adjacent to the EnhA element, and both work cooperatively controlling HLA class I genes expression; (c) the SXY module in which the transcription apparatus is mounted.

However, the *HLA-G* gene presents regulation peculiarities that differ from other class I genes [reviewed at Ref. (67)]. First, the *HLA-G* EnhA is the most divergent one among the class I genes and is unresponsive to NF-κB (136) and might only interact with p50 homodimers, which are not potent HLA class I gene transactivators (137). In addition, the *HLA-G* ISRE is also unresponsive to IFN-γ (138) due to modified ISRE. In fact, the *HLA-G* locus presents the most divergent ISRE sequence among the class I genes (135, 136), what could explain the absence of IFN-γ induced transactivation. The ISRE is also a target for other protein complexes that may mediate HLA class I transactivation. However, both *HLA-G* EnhA and ISRE seem to bind only the expressed factor Sp1, which apparently does not modulate the constitutive or IFN-induced transactivation of *HLA-G* (136). Some polymorphisms in promoter region, such as −725 C > G/T, are close to known regulatory elements. In this matter, the −725 G allele was related with higher *HLA-G* expression levels (120).

The SXY module comprises the S, X1, X2, and Y boxes and is an important target for regulatory binding elements and HLA class I genes transactivation. Box X1 is a target for the multiprotein complex regulatory factor X (RFX), including RFX5, RFX-associated protein, and RFXANK (137, 139–141). The RFX members use to interact with an important element for HLA class II transactivation (CIITA), also important to HLA class I gene transactivation (139). The X2 box is a binding target for activating transcription factor/cAMP response element binding protein (ATF/CREB) transcription factor family (142) and Y box is a binding target for nuclear factor Y (NFY), which includes subunits alpha, beta, and gamma (NFYA, BFYB, and NFYC) (67, 139). For *HLA-G*, the SXY module presents sequences compatible only with S and X1 elements, but divergent from X2 and Y. Because CIITA is dependent of a functional SXY module, which includes X2 and Y elements, the SXY module does not transactivate *HLA-G* gene (139, 143–146).

Other regulatory elements within the *HLA-G* promoter have been described, such as heat shock element, located at −469/−454 position, that bind with heat shock factor-1 (HSF-1), important elements involved in immune responses modulation (147), and progesterone, which is a steroid hormone secreted from corpus luteum and placenta, involved with endometrium maintenance and embryo implantation [reviewed at Ref. (67)]. The mechanism involved in HLA-G expression induced by progesterone is primarily mediated by the activation of progesterone receptor and a subsequent binding to a progesterone response element, found in the promoter region (148). The transactivation of *HLA-G* transcription has also been demonstrated by leukemia inhibitory factor (LIF) (149) and methotrexate cell exposure (150). In addition, it was demonstrated an increased *HLA-G* transcription level in choriocarcinoma cell JEG3 line after the treatment with LIF. Furthermore, LIF induces HLA-G expression in the presence of endoplasmic reticulum aminopeptidase-1 (ERAP1), expressed in the endoplasmic reticulum, and repression of ERAP1 culminates in *HLA-G* downregulation, indicating that ERAP1 has an important role in HLA-G regulation (151). Finally, it is necessary to highlight the importance of methylation status of the *HLA-G* promoter, since it appears to be very important for *HLA-G* transcription (152, 153).

Although some *HLA-G* regulatory elements are known, it is not clear why balancing selection is maintaining divergent lineages since most of the polymorphisms would not theoretically influence *HLA-G* transcription by the known mechanisms, mainly because they do not coincide with known regulatory elements [reviewed at Ref. (67)]. It should be noted that the same SNVs described for the *HLA-G* promoter in other manuscripts are also found in the present analysis.

HLA-G POST-TRANSCRIPTIONAL REGULATION

HLA-G might also be regulated by post-transcriptional mechanisms such as alternative splicing and microRNAs. Several studies have reported polymorphisms influencing splicing, mRNA stability, and also the ability of some microRNAs to bind to the *HLA-G* mRNA. The *HLA-G* 3′UTR segment is a key feature for its regulation mainly by the binding of microRNAs and influencing mRNA stability. *HLA-G* 3′UTR presents several polymorphic sites that influence gene expression [reviewed at Ref. (67)].

The 14-bp presence or absence (insertion or deletion) polymorphism was implicated in the *HLA-G* transcriptional levels and mRNA stability. The presence of the 14 bases segment in trophoblast samples has been associated with lower mRNA production for most membrane-bound and soluble isoforms (98, 154), and the absence of this segment seems to stabilize mRNA with a consequent higher HLA-G expression (98, 155, 156). In addition, *HLA-G* transcripts presenting the 14 bases segment can be further processed with the removal of 92 bases from the complete mRNA (98), giving rise to a shorter *HLA-G* transcript reported to be more stable than the complete isoform (157). The alternative splicing associated with the presence of the 14 bases segment is probably driven by other polymorphic sites in Linkage Disequilibrium with this polymorphic site (3).

The SNP located at position +3142 has been associated with differential HLA-G expression, because it might influence microRNA binding (158). The presence of a Guanine at the + 3142 is associated with a stronger binding of specific microRNAs,

such as miR-148a, miR-148b, and miR-152, decreasing HLA-G expression by mRNA degradation and translation suppression (3, 158, 159). In addition, the 14-bp region might also be a target for specific microRNAs and other 3′UTR polymorphisms might also influence microRNA binding (159). Another polymorphic site that would influence HLA-G expression is located at +3187. The allele +3187A is associated with decreased HLA-G expression because it extends an AU-rich motif that mediates mRNA degradation (106).

UTR-1 (**Table 6**) is the only frequent 3′UTR haplotype that do not carry the 14-bp sequence, and both the high expression alleles +3142G and +3187A. Therefore, it was postulated that this haplotype would be associated with high HLA-G expression; this was confirmed by another study evaluating soluble HLA-G levels and 3′UTR haplotypes (109). In addition, as already introduced, this haplotype (together with the coding allele *G*01:01:01:01*) is probably the most recent one (109) and its frequency might be increased worldwide due to its high-expressing feature.

CONCLUDING REMARKS

Due to the key features of HLA-G on the regulation of immune response and immune modulation, particularly during pregnancy, the overall structure of the HLA-G molecule has been maintained during the evolution process. This is evident when the variability of more than a thousand individuals is taking into account, and only few encoded different molecules are frequently found. Most of the variation sites found in the *HLA-G* coding region are either synonymous or intronic mutations. The *HLA-G* promoter region presents numerous polymorphic sites, with several examples of variation sites in which both alleles are equally represented. Although the mechanisms underlying why some divergent promoter haplotypes are preferentially selected are still unclear, just a few divergent and frequent promoter haplotypes are found worldwide. The *HLA-G* 3′UTR variability is quite expressive considering the fact that most of the SNVs are true polymorphisms, they are equally represented, and this segment is of short size. These observations, for both promoter and 3′UTR, are compatible with the evidences of balancing selection acting on these regions. Finally, the population comparisons confirmed that most of the *HLA-G* variability has arisen before human dispersion from Africa and that the allele and haplotype frequencies might have been shaped by strong selective pressures.

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HLA-G molecules in autoimmune diseases and infections

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Human leukocyte antigen (HLA)-G molecule, a non-classical HLA-Ib molecule, is less polymorphic when compared to classical HLA class I molecules. Human leukocyte antigen-G (HLA-G) was first detected on cytotrophoblast cells at the feto-maternal interface but its expression is prevalent during viral infections and several autoimmune diseases. *HLA-G* gene is characterized by polymorphisms at the 3′ un-translated region and 5′ upstream regulatory region that regulate its expression and are associated with autoimmune diseases and viral infection susceptibility, creating an unbalanced and pathologic environment. This review focuses on the role of HLA-G genetic polymorphisms, mRNA, and protein expression in autoimmune conditions and viral infections.

Keywords: HLA-G, inflammation, autoimmunity, infection, regulation

INTRODUCTION

Human Leukocyte Antigen-G (HLA-G) is a functional molecule belonging to class Ib human leukocyte antigens (HLA) characterized by a non-covalent link between β_2-microglobulin (β2m) and glycoprotein heavy chain. The gene is located within Major Histocompatibility Complex (MHC) locus on chromosome 6 (1, 2). HLA-G products show some peculiar features for which they are considered as non-classical HLA-I antigens: (1) the limitation of their allelic polymorphism (3); (2) the expression of seven isoforms represented by four membrane-bound (G1, G2, G3, and G4) and three soluble (G5, G6, and G7) proteins (4); and (3) the restriction of their tissue distribution (5). Polymorphisms at the 5′ upstream regulatory region and at the 3′ UTR of the *HLA-G* gene play an important role in the regulation of HLA-G production (6). Mainly, two polymorphisms at the 3′ UTR: a deletion/insertion (*DEL/INS*) of 14 base pairs (*14bp*) polymorphism (rs371194629) and a $C > G$ single-nucleotide polymorphism (SNP) at the *+3142bp* position (rs1063320) (7) (**Figure 1**) are able to affect mRNA stability *in vivo* and protein production and implicated in pathological conditions: *14bpINS* allele is associated with mRNA instability (8, 9); *+3142G* allele creates a binding site for three microRNAs (miRNAs) (*miR-148a*, *miR-148b*, and *miR-152*) reducing soluble protein production (10). These observations suggest that *14bpINS/INS* and *+3142G/G* genotypes are associated with a lower HLA-G production than *14bpDEL/INS* and *DEL/DEL*, *+3142C/G*, and *C/C* genotypes (8, 10).

Membrane-bound HLA-G1 and soluble HLA-G5 (HLA-G5) represent the mainly expressed and investigated HLA-G isoforms (1) and are currently supposed to be the most important and functional isoforms (11). However, while HLA-G5 molecules are actively secreted as soluble isoforms, HLA-G1 proteins could be released by proteolytic shedding from cell surface (sHLA-G1) via matrix metalloproteinase-2 (MMP-2) (12–16). HLA-G can exist as β2m-associated and -free monomers (17, 18) and as disulfide-linked dimers or multimers (17, 19, 20). HLA-G disulfide-linked dimers are linked by disulfide bonds between two cysteine residues at position 42 of the HLA-G alpha-1 domain (19–21) and present higher affinity for ILT-2 and ILT-4 receptors compared to monomers (22, 23). Placental trophoblast cells (24), thymus (25), cornea (26), nail matrix (27), pancreas (28), erythroid, and endothelial precursors (29) present a physiological expression of HLA-G molecules. However, HLA-G can be ectopically expressed also on monocytes (30), in transplantation, tumors, viral infections, and autoimmune diseases (1, 2). HLA-G antigens are currently considered as immune-modulatory molecules due to their role in preserving immune tolerance at the feto-maternal interface (31), promoting graft tolerance (32), reducing inflammatory and immune responses (33), favoring tumors (34), and virus infection via immune escape (35). Both membrane-bound and soluble HLA-G antigens exert their immune-suppressive properties: (a) inhibiting the activity and inducing apoptosis of cytotoxic $CD8^+$ T cells and NK cells (36–38); (b) inhibiting the proliferation of $CD4^+$ T cells that are shifted to an immune-suppressive profile (39, 40); (c) inhibiting antigen-presenting cells and B cell differentiation (41, 42); (d) inducing a Th2 polarization (43); and (e) inducing regulatory T cells (44) and Interleukin (IL)-10 secreting dendritic cells (DC10) (45) (**Figure 2**). The interactions between HLA-G proteins and their specific inhibitory receptors ILT-2 (LILRB1/CD85j), ILT-4 (LILRB2/CD85d), and KIR2DL4 (CD158d) expressed by immune cells (46) account for the effects of these molecules on immune cells.

Moreover, HLA-G expression is up-regulated by the secretion of anti-inflammatory cytokines such as IL-10 which, in its turn, is enhanced by HLA-G (30). For these reasons, the implication of HLA-G molecules in inflammatory, immune-mediated, and infective conditions has been investigated (47, 48). The knowledge of

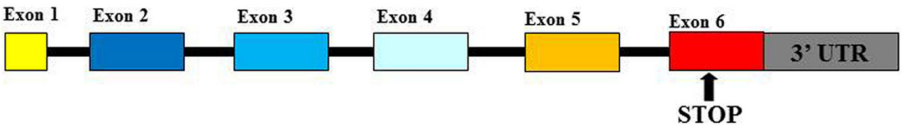

FIGURE 1 | Human leukocyte antigen-G gene. UTR, un-translated region.

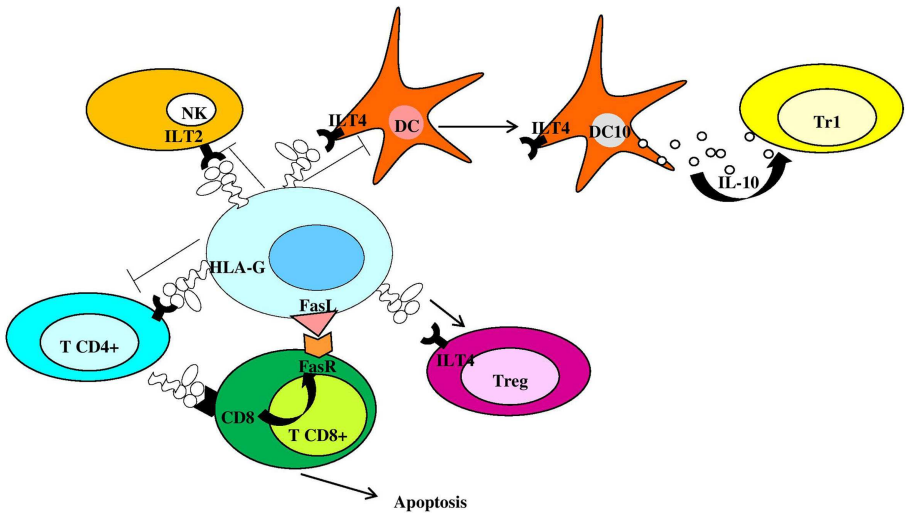

FIGURE 2 | Human leukocyte antigen-G is an anti-inflammatory molecule inhibiting and controlling immune cell activation. NK, natural killer cells; Tr1, type 1 regulatory T cells; DC, dendritic cell; Treg, regulatory T cell; FasR, Fas receptor; DC10, IL-10-differentiated dendritic cells.

the interactions between HLA-G molecules and immune mechanisms and their implication in pathological conditions may assist in improving our knowledge on the mechanisms at the basis of several autoimmune diseases and viral infections.

HLA-G AND GASTROINTESTINAL DISEASES

Celiac disease is a gluten sensitivity, which induces an inflammation that damages the villi in the small intestine of genetically predisposed subjects. Both genetic and environmental factors contribute to the development of celiac disease (CD). Torres and coauthors (49) have shown the presence of HLA-G in biopsies from celiac patients and have observed higher sHLA-G amounts in comparison with control subjects. The evaluation of the *14bp INS/DEL* polymorphism in a group of 522 celiac patients (50), subdivided accordingly with the presence of HLA-DQ2 molecule, encoded by *DQA1*05/DQB1*02* genes, has demonstrated an increased frequency of the *14bp INS/INS* genotype in comparison with controls. These data suggest that the *14bp INS* allele may increase the risk of gut inflammation, most likely leading to chronicity. Ulcerative colitis (UC) and Crohn's disease are characterized by a different sHLA-G expression pattern (51) by peripheral blood mononuclear cells. Non-activated peripheral blood mononuclear cells from Crohn's disease patients secrete spontaneously sHLA-G while those from UC patients and healthy donors do not. Furthermore, after stimulation with LPS, both cells from Crohn's disease and healthy subjects show sHLA-G production, while this does not happen in UC patients. The different HLA-G expression profiles in UC and Crohn's disease patients sustain the different aethiopathogenesis at the origin of these two diseases. In particular, the responses to therapies in UC and Crohn's disease correspond to different sHLA-G secretion levels (52). The immunosuppressant therapy normalizes the production of HLA-G molecules in Crohn's disease while it starts the release of HLA-G in UC patients. These data confirm the diversity in the behavior of these two pathologies and propose the analysis of sHLA-G levels with the final goal of distinguishing between UC and Crohn's disease patients and to monitor therapy.

HLA-G AND RHEUMATOLOGIC DISEASES

Rheumatic diseases are inflammatory and autoimmune diseases, which are the second most common cause of disability after musculoskeletal injuries. Rheumatoid arthritis (RA) is an autoimmune disease caused by the immune system attacking synovial cells. A combination of genetic and environmental factors may increase the risk of RA. Gene expression profiles (GEPs) in bone marrow-derived RA mononuclear cells (53) have shown 1,910 down-regulated and 764 up-regulated gene, which include the *HLA-G* gene. Several studies have evaluated the role of *HLA-G* polymorphisms in RA susceptibility without reaching a final common result. The evaluation on 256 RA patients and 356 healthy controls genotyped for the *HLA-G 14bp INS/DEL* polymorphism has reported no differences in allelic and genotypic

frequencies and no correlation with disease characteristics (54). The analysis of two SNPs (rs1736936, −1305G/A and rs2735022, −689A/G) in *HLA-G* promoter in the Korean population has not presented any connection to the development of RA (55). The evaluation in a Brazilian cohort documented the implication of 3′ UTR polymorphisms in RA follow-up (56). The authors have observed a significant association of the *−762C > T*, *−716T > G*, *−689A > G*, *−666G > T*, *−633G > A*, *−486A > C*, and *−201G > A* (*rs1632946*; *rs2249863*; *rs2735022*; *rs35674592*; *rs1632944*; *rs1736933*; and *rs1233333*) SNPs with the disease. The analysis of 106 patients with juvenile idiopathic arthritis (JIA) has shown an association between JIA female susceptibility and the 14 bp DEL allele. These different associations support the presence of different pathogenic elements between RA and JIA (54). RA (57) and JIA patients present lower serum sHLA-G concentration than in controls (58), with a possible contribution to the chronicity of the inflammation. On the contrary, JIA synovial fluids showed higher sHLA-G levels than controls (SF) (56). Since we have observed that HLA-G molecules are enhanced in synovial fibroblasts from inflamed joints (59) and that high sHLA-G levels correlate with disease activity (57), we may suggest an impaired control of immune reaction at joint, which characterizes JIA disease. The *HLA-G 14bp INS/DEL* polymorphism has also been evaluated as a marker for RA therapy. Methotrexate (MTX), a disease-modifying anti-rheumatic drug (DMARD), induces an increased production of IL-10 in RA patients with a better therapeutic response (60) and is able to enhance HLA-G secretion by peripheral blood mononuclear cells (61). Interestingly, the *14bp DEL/DEL* genotype is increased in RA patients with a good response to MTX therapy (62), with a possible implication in the control of immune activation. It must be underlined, however, that contrasting results have been obtained (63, 64), possibly due to a different dosage of MTX, a different cut-off value for RA therapy response assessment. Scleroderma (SSc) is an autoimmune rheumatic disease of the connective tissue (65). Only SSc patients with a longer survival, lower frequency of vascular cutaneous ulcers, telangiectasias, and inflammatory polyarthralgia present HLA-G molecule expression in skin biopsies (66) suggesting an implication of this molecule on the control of immune response at the skin level.

Systemic lupus erythematosus is a systemic autoimmune disease of the connective tissue that can affect any part of the body. The immune response is mainly characterized by Th2-cell predominance. Rosado and coauthors (67) and Chen and coauthors (68) have shown higher sHLA-G and IL-10 levels in systemic lupus erythematosus (SLE) patients in comparison with healthy controls, while Rizzo and coauthors (69) have observed lower sHLA-G concentrations in SLE patients (70). Interesting, the analysis of monocytes and mature CD83 positive dendritic cells from SLE patients has evidenced a diminished expression of HLA-G in comparison with healthy controls (71), a lower HLA-G expression in response to IL-10 and a lower HLA-G trogocytosis from autologous monocytes compared with controls. Using the SNPs mapping approach, *HLA-G* gene is recognized as a novel independent locus for SLE (72). In particular, *HLA-G 14bp INS/DEL* polymorphism and *HLA-G +3142C > G* SNP have been analyzed in a SLE population. SLE patients showed a higher frequency of *14bp INS* allele and *14bp INS/INS* genotype (69) and the heterozygote group showed lower systemic lupus erythematosus disease activity index (SLEDAI) indexes than homozygous groups (73). On the contrary, the evaluation of *HLA-G 14bp INS/DEL* polymorphism in a SLE Brazilian population did not present an association (74), while the *+3142G* allele and the *+3142 GG* genotype frequencies were increased among SLE patients as compared with controls (75, 76). These data sustain a possible role of HLA-G expression in modifying SLE condition. Behçet (BD) and Kawasaki diseases are autoimmune vasculitis. The *HLA-G*01:01:01* allele is associated with a reduced risk of BD while *HLA-G*01:01:02* and *G*01:05N* alleles are associated with an increased risk of BD (77, 78). Non-synonymous SNP *(+755A/C)* of the *HLA-G* gene (rs12722477, *G*01:04*) is significantly associated with Kawasaki disease (79). These data suggest an influence of *HLA-G* polymorphisms in determining disease risk, possibly affecting HLA-G production and consequently inflammation status.

HLA-G AND CUTANEOUS DISEASES

The skin is characterized by a "skin immune system (SIS)," where immune cells and humoral components support cutaneous inflammation. The deregulation of skin defense mechanisms is evident in a large variety of inflammatory disorders of the skin, such as psoriasis, atopic dermatitis, pemfigo, vitiligo, and systemic sclerosis (80). HLA-G protein is not expressed in the skin from healthy controls (81, 82). Ectopic HLA-G expression has been described in skin pathologies (83–86).

Psoriasis is a chronic inflammatory skin disease with an autoimmune component. Both membrane-bound and soluble HLA-G proteins have been detected in psoriatic skin lesions with the main compound characterized by macrophage lining at the dermo-epidermal junctions (82). The up-regulation of HLA-G molecules by macrophages could represent an attempt to control auto-reactive T cells, induced by activated keratinocytes-derived cytokines/chemokines. HLA-G may prevent keratinocyte destruction by modulating the activity of cytotoxic lymphocytes and promoting the development of Treg cells (87). Interestingly, significantly lower plasma sHLA-G levels have been found in psoriatic patients compared with controls (88), suggesting a difference in systemic HLA-G expression that could be associated with the IL-10 deficiency typical of psoriasis. Psoriasis management can be divided into three main types: topical drugs, light therapy, and systemic medications. Evaluation of therapeutic effects on sHLA-G expression has shown an increase in plasmatic levels of systemic treated patients (efalizumab, cyclosporin A, and acitretin) (88) and a significant association between *HLA-G 14bp DEL* allele and *14bp DEL/DEL* genotype with acitretin clinical outcome (89). We can suppose a possible direct effect of HLA-G in antagonizing systemic T helper 1 activation and with a potential role as a marker of response to acitretin in psoriatic patients.

Pemphigus vulgaris is a blistering disease caused by autoantibodies to desmoglein skin adhesion proteins. Skin tissue sections from pemphigus vulgaris (PV) patients express detectable HLA-G molecules at both transcriptional and translational levels, while control sections present only HLA-G transcription (90). Moreover, the HLA-G *14bp DEL* allele has been observed with higher frequency in PV patients in comparison with controls in a Jewish

population (91). These data suggest that HLA-G expression could be a detrimental factor for the development of PV.

HLA-G AND DIABETES

Type 1 and type 2 diabetes present immunologic defects that enhance insulin resistance as a result of genetics sedentary lifestyle, obesity, and other conditions, such as chronic inflammation or infection. It has been shown that higher levels of sHLA-G are frequent in subjects with an impaired glucose metabolism (92). These data suggest a possible implication of HLA-G antigens in the diabetic condition. In fact, SNPs *rs4122198*, *rs2394186*, *rs1619379*, and *rs1611133* near the *HLA-G* gene have been associated with type 1 diabetes (93); dendritic cells from type 1 diabetic patients produce lower HLA-G molecules in response to IFN-beta (94) in comparison with control subjects and the *HLA-G 14bp INS-INS* genotype might contribute to the development of high blood pressure in type 2 diabetes (95).

Interestingly, HLA-G has been found in some secretory granules and on the cell surface of primary islet cells induced to secrete insulin (28). On the basis of these data, it could be hypothesized that an impaired HLA-G expression at pancreatic islets could sustain T cell activation and onset of diabetes.

HLA-G IN MULTIPLE SCLEROSIS

Multiple sclerosis is the prototypic autoimmune disease of the central nervous system (CNS) characterized by chronic inflammatory demyelination and neurodegeneration of unidentified origin (96). Multiple sclerosis (MS) typically occurs in young adults and manifests in women twice as frequently as in men with neurological symptoms and signs, called relapses, which are usually disseminated in space and time (97). About the 80% of MS patients present a disease onset with a relapsing–remitting (RR) form followed by a secondary progressive (SP) course that arises after years, whereas MS starts with a primary progressive (PP) form in approximately the 20% of subjects (98). However, the recent proposed criteria (99) suggest that the coexistence of multi-focal lesions in the periventricular white matter on T2-weighted Magnetic Resonance Imaging (MRI) scans with or without Gadolinium (Gd) enhancement on T1-weighted MRI scans are needed for the diagnosis of MS. Based on epidemiological studies, exposure to an environmental factor, e.g., an infectious agent, in genetically predisposed individuals is currently thought to be crucial for MS pathogenesis (100) in which the traffic into the CNS of activated auto-reactive $CD4^+$ T helper 1 (Th1) cells plays a central role (96, 101, 102). The initiation of brain inflammation is due to the activation of microglia by infiltrating $CD4^+$ T cells leading to the generation of Th1-mediated immune responses (IL-12/IFN-γ and IL-23/IL-17), while the resolution of neuroinflammation is triggered by astrocytes, which promote anti-inflammatory Th2-polarized responses (IL-10 and TGF-β) and the elimination of infiltrating immune cells through Fas/FasL-dependent apoptosis (96, 101) (**Figure 3**).

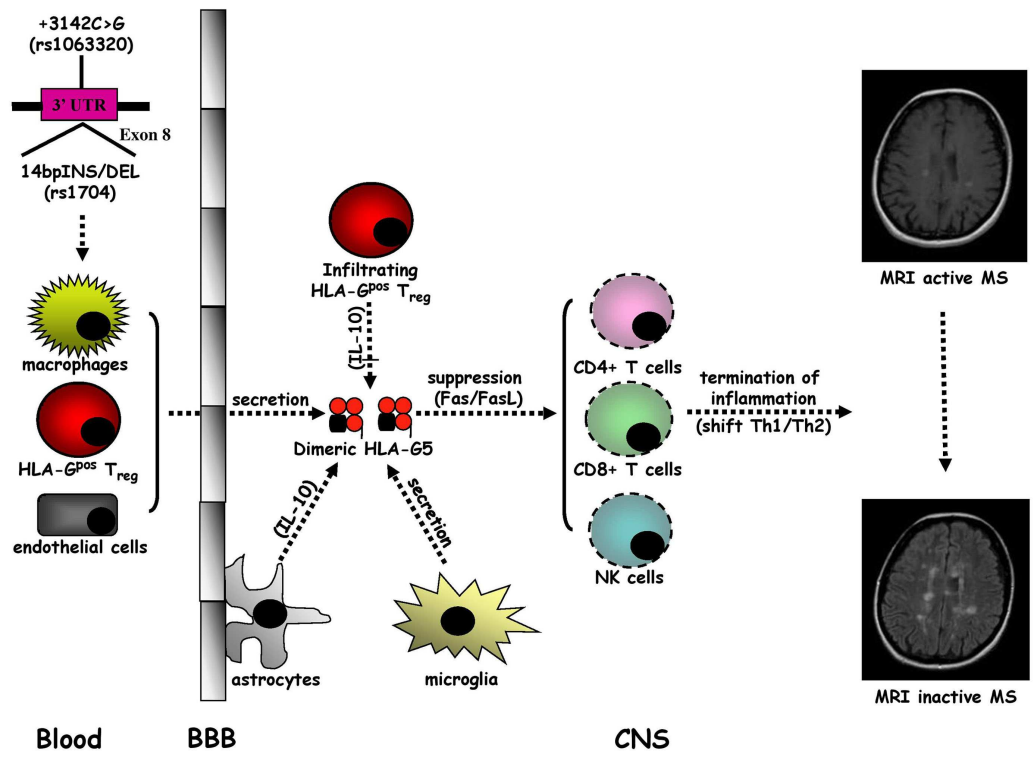

FIGURE 3 | Intrathecal immune milieu in MS. The secretion of HLA-G5 in dimeric form by macrophages and HLA-G^{pos} T_{reg} infiltrating the central nervous system (CNS) across the blood–brain barrier (BBB), endothelial cells, and microglia, sustained by a IL-10 release by astrocytes, may promote the suppression of $CD4^+$ Th1 cell activity and the apoptotic removal of $CD8^+$ T cells and NK cells that favor the formation of an anti-inflammatory intrathecal microenvironment leading to the termination of MS inflammation.

A growing body of evidence indicates that sHLA-G antigens may have a tolerogenic role in MS (102, 103). Cerebrospinal fluid (CSF) detectable sHLA-G has been detected in RRMS patients with higher levels in comparison with other inflammatory neurological disorders (OIND), non-inflammatory neurological disorders (NIND), and controls (104). Furthermore, higher CSF sHLA-G levels have been detected in RRMS without MRI evidence compared to those with MRI active disease. Notably, a positive correlation between CSF concentrations of sHLA-G and IL-10 has been found in MS patients without MRI evidence of active disease. Therefore, CSF levels of sHLA-G may act, together with IL-10, as anti-inflammatory molecules to regulate MS disease activity. The association between elevated CSF sHLA-G levels and clinical and MRI appearance of MS stable disease is supported by the intrathecal synthesis of sHLA-G in MS clinically and MRI inactive patients (105). We have found higher CSF levels of HLA-G5 and not of sHLA-G1 isoforms compared with controls and in presence rather than in absence of MRI Gd enhancing lesions (106) and an as well as inverse correlation between CSF levels of sHLA-G and anti-apoptotic sFas molecules in MS patients without MRI disease activity (107). Collectively, these results suggest a strong correlation between high CSF levels of sHLA-G antigens and the resolution of MS autoimmunity probably related to the anti-inflammatory properties of these molecules. The impact of HLA-G in MS pathogenesis was recently confirmed by other studies, which demonstrated that: (a) Th1 and Th2 cytokine production and $CD4^+$ T cell proliferation are suppressed by HLA-G from MS patient peripheral blood monocytes during the first month of treatment with IFN-β (108); (b) MS disease activity during pregnancy may be modulated by tolerogenic properties of sHLA-G since post-partum serum sHLA-G levels are higher in MS patients without clinical attacks (109); and (c) microglia, macrophages, and endothelial cells located within and around MS lesions present a strong immunohistochemical expression of HLA-G and its inhibitory receptors (ILT-2 and ILT-4), with an elevated protein HLA-G expression on cultured human microglial cells after activation with Th1 pro-inflammatory cytokines (110). Meanwhile, a novel subpopulation of naturally occurring $CD4^+$ and $CD8^+$ regulatory T cells of thymic origin expressing HLA-G ($HLA\text{-}G^{pos}$ T_{reg}), has been characterized in MS patients with a suppressive activity through the secretion of HLA-G5 and the shedding of sHLA-G1 (111–113). Overall, these data sustain anti-inflammatory properties of sHLA-G molecules, and in particular HLA-G-5 isoform, which could lead to the remission of MS autoimmunity. Although it has been demonstrated that SNP rs4959039, a SNP in the downstream un-translated region of HLA-G gene is independently associated with MS susceptibility (114), the possible link between *HLA-G* genetic polymorphisms and MS has not been intensively explored (102, 103). Conflicting results have been obtained. Although no association between *HLA-G* gene polymorphism and MS or severity of the disease has been initially found (115), *14bpINS* and *−725G* (rs1233334) alleles have been shown to be related to MS (116). However, a recent study, evaluating the influence of *14bpDEL/INS* and *+3142C > G* HLA-G polymorphisms on CSF and serum sHLA-G production, has documented a correlation between HLA-G genetic polymorphisms and sHLA-G concentrations in both CSF and serum (117). These findings indicate that CSF and serum sHLA-G levels in MS could be affected by two main HLA-G polymorphisms. Moreover, preliminary results from our laboratory have demonstrated that, MS patients present dimeric sHLA-G form more frequently than control, in particular in MRI inactive MS patients (unpublished data), suggesting that large amounts of biologically active dimeric sHLA-G form could be released in CSF of MS patients, possibly induced by pharmacological treatment (118). Nevertheless, in a recent study no association was found between serum sHLA-G levels, disability progression, disease MRI activity, and time to conversion from clinically isolated syndrome (CIS) to clinically definite MS (119). These findings suggest that the use of sHLA-G levels in CSF should be taken into consideration as a prognostic marker for monitoring disease conversion, activity, progression, and response to therapy.

HLA-G IMPACT IN VIRAL INFECTIONS

Even if host immune system present several mechanisms to control viral infections, the viruses have developed several strategies to counteract host immune defenses (120). HLA-G seems to be implicated in viral immune-escape from Natural Killer cells (121).

Human immunodeficiency virus type 1 (HIV-1) up-regulates HLA-G molecules and down-regulates classical HLA-A and -B. Studies have focused on the expression of HLA-G in monocytes, which are relevant as reservoirs of HIV-1, and in lymphocytes, which are more susceptible to infection by HIV-1. Monocytes from HIV-1 seropositive patients express HLA-G (122) with a possible association with antiretroviral therapy (HAART), since patients undergoing HAART present higher levels of HLA-G expression on monocytes in comparison with untreated and healthy subjects (122, 123). T cells obtained from HIV-1 seropositive individuals have been found to express HLA-G at a higher proportion (124) and behave like HLA-G+ Treg. Furthermore, on the basis of *HLA-G* genetics, it would seem that the *HLA-G 14bpINS* and *+3142G* polymorphisms affect the susceptibility to HIV (125) but not mother–child transmission (126) in African population.

Human cytomegalovirus is a herpes virus that persists in the host (127) by means of several strategies to evade the immune system. HLA-G expression is evidenced during viral reactivation in macrophages and astrocytoma cells (35) and the levels of expression on monocytes an in serum is higher during active human cytomegalovirus (HCMV) infection (128). This up-regulation is proposed to be associated with virus-encoded homologs of humanIL-10 (cmvIL-10) (129), which prevents NK cell recognition of infected cells.

There is also evidence to support also a role of HLA-G molecules in susceptibility and outcome of human papilloma virus (HPV) infections. The alleles HLA-G *14bp INS*, *+1537C* (rs12722477), *G*01:01*, *G*01:04*, and *G*01:06* have been associated with both high-grade squamous intraepithelial lesions and cervical cancer, while *HLA-G 14bp DEL* and *+3142C* alleles have been identified as protective (130–135). These results are in agreement with the low levels of HLA-G5 expression in cervical cancer (136). On the other hand, two researches recognized *HLA-G 14bp DEL* allele and *+3142C* as associated with increased risk of cervical cancer (137, 138), in agreement with increased expression

of HLA-G in cervical cancer tissues (139) and with the spontaneous de-methylation of *HLA-G* promoter that allows immune-evasion and the development of precancerous cervical lesions (140). HLA-G has been also implicated in nasal polyposis development in the presence of HPV infection (141). Nasal polyps with HPV11 infection have shown HLA-G expression on epithelial cells, while no HLA-G expression has been observed in HPV negative polyps.

Neurotropic viruses such as herpes simplex virus-1 (HSV-1) and Rabdovirus (RABV) (142) induce the expression and up-regulation of membrane and soluble HLA-G molecules in actively infected neurons with a consequent protection toward host NK cells.

Hepatitis C virus (HCV) and Hepatitis B virus (HBV) seems to induce HLA-G expression to control host immune response (125, 143–148).

On the basis of these results, HLA-G proteins are expressed by virally infected cells as a mechanism to evade host immune control, preventing T cell and NK cell activation. The main challenge would be to block HLA-G up-regulation by viral infection, in order to allow the recognition by immune cells.

INTERACTION OF HLA-G MOLECULES WITH OTHER HLA-Ib MOLECULES

Other HLA-Ib molecules have been identified: HLA-E and HLA-F (149, 150) characterized by a low genetic diversity as well as by a particular expression pattern, structural organization and functional profile.

Similar to HLA-G, HLA-E forms a complex with β2-microglobulin. HLA-E is known to play an important role as immune-modulator during pregnancy and transplantation (151), inhibiting immune responses by its interaction with $CD8^+$ T cell receptors (TCRs) (152) and with the CD94/NKG2A inhibitory receptors of NK cells (153). Meanwhile, this molecule may present non-self antigens activating immune response (154).

Similar to other HLA molecules, HLA-F can form a complex with beta2 microglobuli and three splicing variants have been described. While the presence of HLA-G and HLA-E has been recently correlated with physiological and pathological conditions, the clinic-pathological significance of HLA-F is limited. HLA-F is expressed by peripheral blood B cells upon activation (155) and is detected in embryonic tissues, including the extravillous trophoblasts invading maternal deciduas, and in spermatozoids (156, 157) and in the serum of patients affected by tumors (158).

Only few data are available on the interaction of HLA-G molecules with the other HLA-Ib antigens. In physiological conditions, HLA-G molecules interact with HLA-E and co-operate to inhibit NK cells, mainly at feto-maternal interface, via interaction with ILT-2 and CD94/NKG2A, respectively (159). In pathological condition, the interaction between these two molecules facilitates the escape of tumor cells from NK cell recognition (160). In MS, HLA-G and HLA-E molecules are expressed by resident CNS cells and interact with NK cell and cytotoxic lymphocytes (161). HLA-G, -E, and -F expression by trophoblasts correlates with the protection of the fetus from destruction by the maternal immune system, suggesting a co-operation for fetal tissue preservation.

CONCLUSION

This review aims to focus on the key role of HLA-G molecules in autoimmune diseases and viral infections. The data herein summarized suggest that HLA-G may have a crucial role in the creation of an impaired immune response that characterizes these pathological conditions.

In fact, it appears even more evident that HLA-G proteins are involved in the regulation of the immune system during autoimmunity, such as gastrointestinal, skin, rheumatic and neurological diseases and in the immune-escape mechanisms during viral infections.

Here, we have reviewed a series of experimental and epidemiological studies that support the direct influence of HLA-G proteins on the balance of immune settings. On this basis, understanding the function of HLA-G in these disorders could help in the identification of new approaches to control HLA-G production.

For example, it is interesting to note that inflammatory cutaneous diseases present a disproportional expression of HLA-G molecules with respect to controls and that this could generate autoimmunity. Thus it appears that down/over-expression of HLA-G may not only act as an immunosuppressive and beneficial molecule but may also sustain an unbalanced immune stimulation and autoimmunity. With reference to bowel diseases especially, it appears clear that the different HLA-G expression levels could help in the differential diagnosis and consequently in the choice of appropriate treatment.

Furthermore, several studies have evidenced the possible role of sHLA-G antigens as a tolerogenic molecules in MS since their intrathecal production is associated with disease remission. It is of extreme importance to evaluate the role of HLA-G antigens in MS pathogenesis, in particular if they are implicated in disease progression or if they represent an indirect manifestation of MS inflammation of CNS. Still to be clarified are the functional differences between HLA-G5 and sHLA-G1, and whether dimers and monomers exert a different function in MS inflammatory disease activity. As far as viral infections are concerned, HLA-G could be considered a target for anti-viral treatment, so increased knowledge in this field could contribute to identifying different therapeutic strategies.

Collectively, the results emerging from the literature confirm the importance of the HLA-G molecule in the pathogenesis and progression of immune-based diseases and infections, underlining the relevance of its investigation with the aim to developing new therapeutic strategies and clinical markers. Meanwhile, the analysis of the interactions between HLA-G and other HLA-Ib molecules may be useful to understand the mechanisms for the creation of immune-suppressive microenvironments.

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The role of HLA-G molecule and *HLA-G* gene polymorphisms in tumors, viral hepatitis, and parasitic diseases

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Considering that the non-classical HLA-G molecule has well-recognized tolerogenic properties, HLA-G expression is expected to be deleterious when present in tumor cells and in cells chronically infected by viruses, whereas HLA-G expression is expected to be advantageous in autoimmune disorders. The expression of HLA-G on tissue or peripheral blood cells, the levels of soluble HLA-G and polymorphic sites along the gene have been studied in several disorders. In this study, we revised the role of the molecule and polymorphic sites along the *HLA-G* gene in tumors, viral hepatitis, and parasitic disorders. Overall, several lines of evidence clearly show that the induction of HLA-G expression in tumors has been associated with worse disease outcome and disease spread. In addition, the few studies conducted on hepatitis and parasitic disorders indicate that HLA-G may contribute to disease pathogenesis. Few isolated polymorphic sites, primarily located at the coding or 3′ untranslated *HLA-G* region, have been evaluated in these disorders, and a complete *HLA-G* typing together with the study of gene regulatory elements may further help on the understanding of the influence of the genetic background on disease susceptibility.

Keywords: HLA-G, tumors, viral hepatitis, parasitic disorders, polymorphism

INTRODUCTION

HLA-G is a non-classical class I gene of the human Major Histocompatility Complex (NCBI gene ID: 3135), presenting a restricted tissue expression pattern and encoding molecules with immune modulatory properties. This gene, firstly described by Geraghty and colleagues in 1987 (1), presents a genetic structure that resembles other classical HLA class I genes. However, contrary to that observed for classical class I genes (*HLA-A*, *-B*, and *-C*), the *HLA-G* gene is quite conserved among different populations and within the same population, presenting only a few non-synonymous mutations and several variation sites characterized as synonymous modifications, intronic variations, or variable sites at the regulatory regions [reviewed at Ref. (2)].

HLA-G does not seem to initiate immune responses as its classical counterparts. Instead, the HLA-G molecule is associated with the induction of inhibitory stimuli for T and B lymphocytes (3, 4), Natural Killer (NK) cells (3), and antigen-presenting cells (APC) (5). The HLA-G molecule may directly interact with multiple inhibitory receptors, including ILT2/CD85j/LILRB1 (ILT2), ILT4/CD85d/LILRB2 (ILT4), and KIR2DL4/CD158d (KIR2DL4).

The HLA-G molecule was firstly detected at the trophoblast in the maternal fetal interface, probably modulating the maternal immune system during pregnancy. Beyond trophoblast expression, HLA-G has been detected in few normal tissues, including cornea (6), thymus (7), and erythroid and endothelial precursors (8), and its upregulation has been detected in several pathological conditions as described in the present review.

Alternative splicing is also an important characteristic of the *HLA-G* gene. It may produce at least seven protein isoforms generated by alternative splicing of the primary transcript [reviewed at Ref. (2)], in which four isoforms are membrane-bound and three isoforms are soluble due to the lack of a transmembrane domain.

Much effort has been made to evaluate *HLA-G* worldwide variability. The *HLA-G* gene seems to present functional polymorphisms mainly in the regulatory regions, probably influencing its expression. Considering data from at least 18 different populations (9–12) the *HLA-G* locus presents few frequent extended haplotypes. These haplotypes are a combination among a small number of very divergent promoter and 3′ untranslated region (3′UTR) haplotypes (**Figures 1** and **2**), and a coding allele usually encodes the same HLA-G molecule (**Figure 3**). The regulatory segments are characterized by the occurrence of several polymorphic sites presenting high heterozygosis. Although there is no consensus regarding where the *HLA-G* transcription starts (13), the polymorphisms at the 5′ upstream regulatory region (5′URR) have been considered to influence HLA-G expression, mainly because of the fact that polymorphic sites coincides with, or are close to, known transcription factor binding sites (**Figure 1**) [Reviewed at Ref. (13)]. Likewise, haplotypes at the *HLA-G* 3′UTR segment have been considered influencing HLA-G expression, mainly because

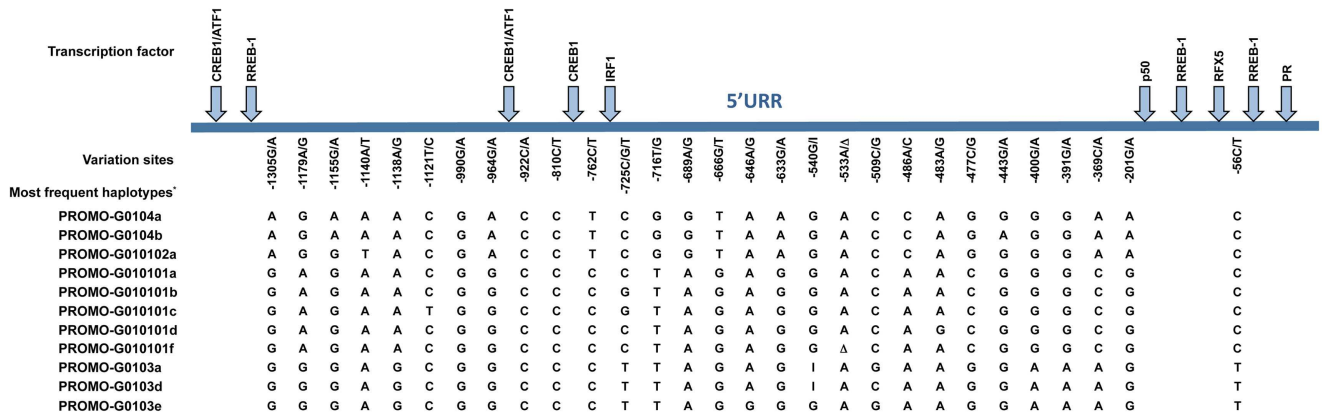

FIGURE 1 | Variation sites at the 5′ upstream regulatory region (5′URR) of the *HLA-G* gene (1.4 kb upstream of ATG), as well as the target binding sites of the described transcriptional factors. The position of the variation sites is determined in relation to Adenine of the initiation codon ATG. *Since there is no official nomenclature for 5′URR haplotypes, they were designed as previously reported (10). Transcription factors: CREB1, CAMP responsive element binding protein 1; ATF1, cyclic AMP-dependent transcription factor ATF-1; RREB1, Ras responsive element binding protein 1; IRF1, interferon regulatory factor 1; p50, nuclear factor NF-κ-B p105 subunit; RFX5, DNA-binding protein RFX5 (RFX family); PR, progesterone receptor. I, insertion of a guanine at position −540; Δ, deletion of an adenine at position −533.

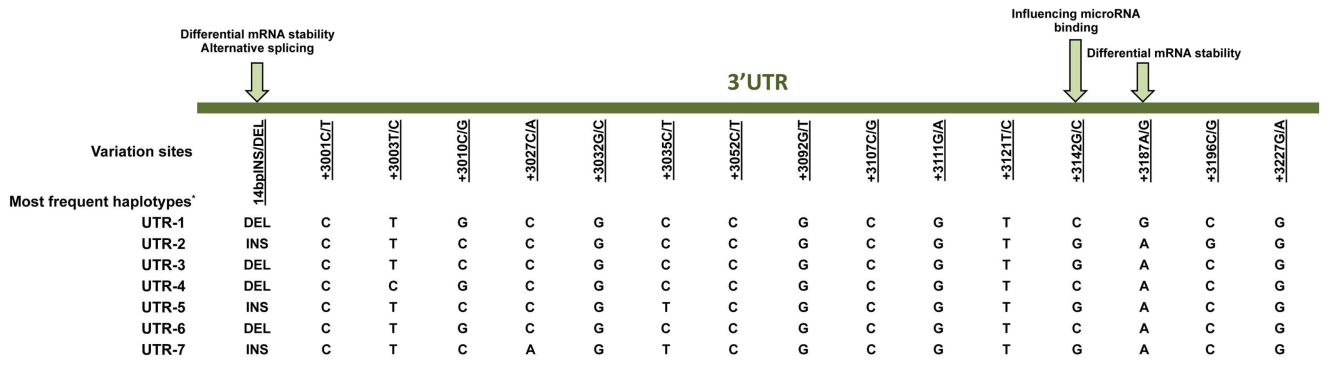

FIGURE 2 | Variation sites at the 3′ untranslated region (3′UTR) of the *HLA-G* gene that may influence HLA-G expression. Polymorphic sites associated with diseases presented in this review are underlined. Arrows indicate polymorphic sites that have been functionally studied. *Since there is no official nomenclature for 3′UTR haplotypes, they were designed as previously reported (11). DEL, deletion; INS, insertion.

the fact that some polymorphic sites (such as the one at position +3142) may influence the binding of specific microRNAs (14–17) or may influence mRNA stability (such as the one at position +3187) and alternative splicing (such as the 14-bp polymorphism) (**Figure 2**).

The *HLA-G* coding region presents mainly synonymous or intronic variation sites. Considering the most frequent *HLA-G* coding haplotypes found worldwide [reviewed at Ref. (2, 18)], only five different HLA-G full-length molecules are frequently found, in which four are complete molecules encoded by the *HLA-G*-*01:01, *01:03, *01:04, and *01:06 allele groups, and one is a truncated molecule encoded by the *HLA-G**01:05N null allele. Although some different HLA-G molecules were detected worldwide, they are usually quite rare and the same *HLA-G* coding alleles are usually detected in every population studied so far. Apparently, all these frequently found molecules (exception made to the G*01:05N) present the same modulatory effects described earlier (2). Considering that only a few extended haplotypes are usually found, and considering that most of the *HLA-G* coding alleles are associated with only one promoter or 3′UTR haplotype, it is possible that most of the associations described so far regarding *HLA-G* coding polymorphism and pathological conditions are reflecting the presence of specific promoter and 3′UTR sequences and specific HLA-G production capabilities.

In the present review, we report some diseases that have been associated with the modulation of the HLA-G expression, with the presence of specific *HLA-G* gene variation sites or both, and whenever known, the mechanisms underlying such associations are discussed.

TUMORS

The arisen of transformed cells and the spread of cancer cell clones are usually controlled by the immune system cells, particularly by the action of cytototoxic T and NK cells; however, cancer

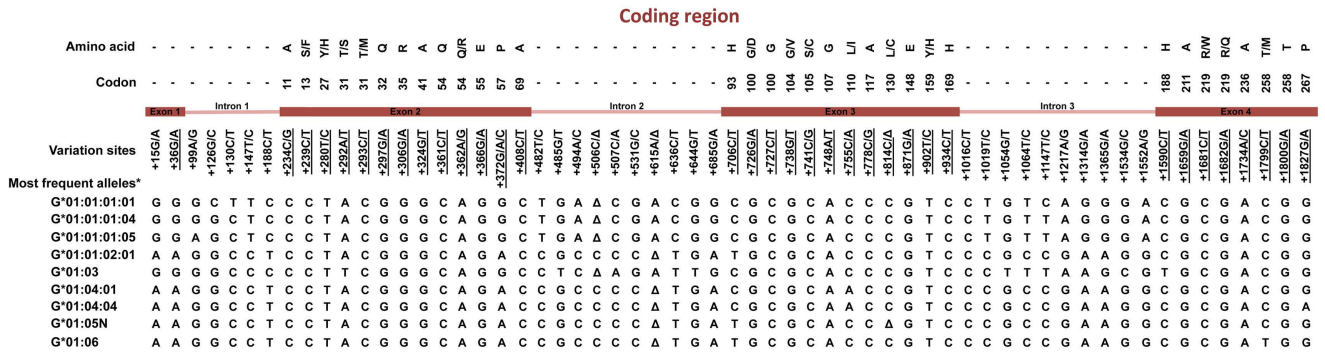

FIGURE 3 | Variation sites at the coding region of the *HLA-G* gene from exon 1–4. Polymorphic sites associated with diseases presented in this review are underlined. *Haplotypes presenting a global frequency higher than 1% in worldwide populations. Amino acids: A, alanine; S, serine; F, phenylalanine; Y, tyrosine; T, threonine; M, methionine; Q, glutamine; R, arginine; E, glutamic acid; P, proline; H, histidine; G, glycine; D, aspartic acid; V, valine; C, cysteine; L, leucine; I, isoleucine; W, tryptophan. Δ, deletion.

cells have developed several strategies to evade host immune surveillance. Since classical histocompatibility (HLA-A, -B, and -C) molecules present tumor antigens to cytotoxic T cells, tumor cells have developed strategies to escape the cytotoxic effect of T cells by interfering with the expression of these molecules on tumor cell surface. On the other hand, the absence of HLA classical molecules on the surface of tumor cells triggers NK cell activity to eliminate neoplastic cells. If tumor cell expresses HLA-G, the cytotoxic activity of both T and NK cells are inhibited, facilitating tumor cell spread. When the decreased expression of classical HLA molecules is accompanied by an increased expression of immunomodulatory molecules such as HLA-G, the effective cytototoxic immune response against tumor cells is much impoverished [reviewed at Ref. (2)].

Although the study of HLA-G expression in tumor cells has been widely explored [reviewed at Ref. (19–21)], the evaluation of the *HLA-G* gene polymorphic sites has not been studied at the same extent, and even rarer are the studies evaluating the relationship between HLA-G tumor expression and *HLA-G* polymorphic sites. Next, we highlight some peculiarities of tumors, for which HLA-G expression (tissue or soluble levels), gene polymorphisms, or both have been evaluated.

HLA-G EXPRESSION IN TUMORS

Increased HLA-G expression has been observed in different tumor types, including breast cancer (22–29), hepatocellular carcinoma (30–33), papillary thyroid carcinoma (34, 35), follicular thyroid carcinoma (35), follicular adenoma (35), nasopharyngeal carcinoma (36), neuroblastoma (37), bladder transitional cell carcinoma (TCC) (38), melanoma (39–42), colorectal cancer (43–45), gastric cancer (46–48), esophageal carcinoma (49–53), lung cancer (49, 54–57), renal cell carcinoma (58–62), glioblastoma (63–66), acute myeloid leukemia (67, 68), and B-cell chronic lymphocytic leukemia (69–73). **Table 1** summarizes the HLA-G expression in many types of tumors described in this review.

In most tumors, the increased HLA-G expression has been associated with advanced disease stages, shorter survival time, presence of metastasis, higher tumor grade, weak host immune response, greater tumor size, tumor recurrence, tumor invasion, poor histological grade, lower classical HLA antigen expression, presence of infiltrating T regulatory cells, cancer progression, increased inflammatory cell lesion infiltration, and tumor differentiation (23, 24, 26, 29–32, 34–36, 40–42, 44, 46–48, 50–54, 56, 57, 66, 69, 72, 73, 79). In other tumors, no association between increased HLA-G expression and clinicopathological features has been observed, including bladder TCC (38) and acute myeloid leukemia (67, 68).

Furthermore, increased sHLA-G levels have been reported for breast cancer (23–25, 75), hepatocellular carcinoma (31–33), papillary thyroid carcinoma (76), neuroblastoma (37), melanoma (39), colorectal cancer (49, 77), gastric cancer (47, 49), esophageal carcinoma (49–51), lung cancer (49, 54, 55), renal cell carcinoma (62), and acute myeloid leukemia (78). Higher sHLA-G levels have been associated with: (i) increased number of $CD4^+$ regulatory T (Treg) cells in breast cancer (23), (ii) more aggressive tumor behavior in papillary thyroid carcinoma (76), (iii) local or disseminated relapse in neuroblastoma (37), (iv) advanced stages of disease and tumor load in melanoma (39), (v) higher IL-10 production in esophageal carcinoma (51), (vi) absence of anterior myelodysplasia along with higher leukocytosis in acute myeloid leukemia (78), and (vii) shorter survival time, high-grade tumors, higher IL-10 production, and loss of HLA classical class I molecules in patients with lung cancer (54–56).

Interestingly, sHLA-G levels were significantly decreased in breast cancer patients at 6 and 12 months after surgery (25). In addition, no association between higher sHLA-G levels and clinicopathological features has been observed in hepatocellular carcinoma (33), colorectal cancer (77), gastric cancer (47), esophageal carcinoma (50, 51), and renal cell carcinoma (62). On the other hand, plasma sHLA-G levels were closely similar when bladder TCC patients and healthy controls were compared (38).

Overall, several laboratory (increased HLA-G tumor expression, increased sHLA-G levels, increased levels of IL-10, and a cytokine that induces HLA-G expression) and clinical (advanced disease stages, worse prognosis, and presence of metastasis) findings do corroborate the malefic role of HLA-G in cancer disorders.

Table 1 | Association between HLA-G expression and tumors.

Tumor	HLA-G molecule				Reference
	n	Expression (%)	Metastasis[a]	sHLA-G (*n*)	
Breast cancer	36	36^{IHC}	nd	nd	(22)
	46/39	$26^{(E)IHC}/41^{(S)IHC}$	No	nd	(74)
	58	70.7^{IHC}	nd	$\uparrow(92)^{ELISA}$	(23)
	235	66^{IHC}	Yes	$\uparrow(44)^{ELISA}$	(24)
	677	60^{IHC}	No	nd	(27)
	nd	nd	nd	$\uparrow(45)^{ELISA}$	(25)
	38	58^{IHC}	nd	nd	(28)
	nd	nd	nd	$\uparrow(120)^{ELISA}$	(75)
	52	59.6^{IHC}	No	nd	(29)
	45	62^{IHC}	Yes	nd	(26)
Hepatocellular carcinoma	173	57^{IHC}	nd	nd	(30)
	219	50.2^{IHC}	nd	$\uparrow(19)^{ELISA}$	(31)
	36	66.7^{WB}	nd	$\uparrow(36)^{ELISA}$	(32)
	nd	nd	nd	$\uparrow(80)^{ELISA}$	(33)
Thyroid cancer	nd	nd	nd	$\uparrow(183)^{ELISA}$	(76)
	70	44.3^{IHC}	Yes	nd	(34)
	72	77.5^{IHC}	No	nd	(35)
Nasopharyngeal carcinoma	552	79.2^{IHC}	Yes	nd	(36)
Neuroblastoma	12	0^{IHC}	nd	$\uparrow(53)^{ELISA}$	(37)
Bladder transitional cell carcinoma	75	68^{IHC}	nd	$\emptyset(15)^{ELISA}$	(38)
Melanoma	nd	nd	nd	$\uparrow(190)^{ELISA}$	(39)
	79	28^{IHC}	nd	nd	(40)
	35	34.2^{IHC}	nd	nd	(42)
Colorectal cancer	39	$87^{RT\text{-}PCR}$	nd	nd	(43)
	201	64.6^{IHC}	Yes	nd	(44)
	nd	nd	nd	$\uparrow(144)^{ELISA}$	(77)
	nd	nd	nd	$\uparrow(37)^{ELISA}$	(49)
	251	20.3^{IHC}	nd	nd	(45)
Gastric cancer	160	71^{IHC}	Yes	nd	(46)
	179	49.7^{IHC}	Yes	$\uparrow(179)^{ELISA}$	(47)
	nd	nd	nd	$\uparrow(28)^{ELISA}$	(49)
	52	31^{IHC}	Yes	nd	(48)
Esophageal carcinoma	121	90.9^{IHC}	Yes	nd	(52)
	79	65.8^{IHC}	nd	$\uparrow(41)^{ELISA}$	(50)
	nd	nd	nd	$\uparrow(58)^{ELISA}$	(49)
	60	75^{IHC}	No	nd	(53)
	60	70^{IHC}	Yes	$\uparrow(60)^{ELISA}$	(51)
Lung cancer	39	26^{IHC}	nd	nd	(56)
	106	75^{IHC}	Yes	nd	(57)
	101	41.6^{IHC}	nd	$\uparrow(91)^{ELISA}$	(54)
	nd	nd	nd	$\uparrow(137)^{ELISA}$	(55)
	nd	nd	nd	$\uparrow(43)^{ELISA}$	(49)
Renal cell carcinoma	18	61^{IHC}	nd	nd	(59)
	38	76^{qPCR}	nd	nd	(61)

(Continued)

Table 1 | Continued

Tumor	HLA-G molecule				Reference
	n	Expression (%)	Metastasis[a]	sHLA-G (*n*)	
Clear cell renal carcinoma	12	58^{IHC}	nd	nd	(60)
	95	46.8^{IHC}	nd	$\uparrow(16)^{ELISA}$	(62)
Glioblastoma	5	80^{IHC}	nd	nd	(63)
	26	$\geq 58^{IHC}$	nd	nd	(64)
	39	64^{IHC}	nd	nd	(65)
	108	60.2^{IHC}	nd	nd	(66)
Acute myeloid leukemia	nd	nd	nd	$\uparrow(75)^{ELISA}$	(78)
	77	45^{FC}	nd	nd	(67)
	22	68.2^{FC}	nd	nd	(68)
B-cell chronic lymphocytic leukemia	47	$1–54^{FC}$	nd	nd	(69)
	20	$1–34^{FC}$	nd	nd	(72)
	30	35.31^{FC}	nd	nd	(73)

[a]*Association between HLA-G expression and metastasis.*

sHLA-G, soluble HLA-G; IHC, imunohistochemistry; nd, not determined; (E), breast carcinoma effusions; (S), breast carcinoma solid lesions; ↑, increased sHLA-G levels in patients; ELISA, enzyme-linked immunosorbent assay; WB, western blotting; Ø, similar sHLA-G levels between patients and controls; RT-PCR, reverse transcriptase-PCR; qPCR, quantitative PCR; FC, flow cytometry.

POLYMORPHIC SITES AT *HLA-G* GENE AND TUMORS

Several isolated segments of the *HLA-G* gene have been studied in tumors, highlighting the 3′ untranslated and coding regions. Certainly, the 14-bpINS/DEL polymorphism is the most studied. In breast cancer patients, the 14-bpDEL allele and 14-bpDEL/DEL genotype were associated with susceptibility to breast cancer in Southeastern Iranian (80) and Korean patients (81); however, no association has been reported for Brazilians (26). In addition, Korean patients exhibiting the 14-bpINS/INS genotype exhibited no HLA-G expression in breast cancer lesions (81). A meta-analysis evaluating the role of the 14-bpINS/DEL polymorphism in breast cancer reports an overall cancer risk in Asian populations (82).

The 14-bpDEL allele was associated with susceptibility to hepatocellular carcinoma in Brazilian (83) and Chinese (84) patients, but not in Korean patients (84). In addition, Chinese patients exhibiting the 14-bpDEL/DEL genotype presented increased HLA-G expression in hepatocellular carcinoma specimens (84). The 14-bpINS/DEL genotype was associated with decreased risk for childhood neuroblastoma development in Australian and New Zealand patients (85). The *HLA-G* 3′UTR haplotype known as UTR-3 (86) was associated with susceptibility to acute myeloid leukemia development in Italian patients (68).

Considering the *HLA-G* coding segment, the +755C/A (non-synonymous Leu/Ile substitution at codon 110, which defines the *HLA-G**01:04 protein group) was associated with protection against more severe nasopharyngeal carcinoma tumor stages (87).

Regarding the bladder TCC, the *HLA-G**01:04:04 allele, and the *HLA-G**01:04 allelic group were associated with susceptibility to bladder TCC in smoking patients and the *HLA-G**01:03 allele and the *HLA-G**01:04 allelic group was associated with protection against bladder TCC development in non-smoking Brazilian patients. In addition, the *HLA-G**01:01 allelic group and *HLA-G**01:01/*G**01:01 genotype were associated with susceptibility to bladder TCC development in non-smokers. Considering the bladder TCC progression, the following associations were observed: (i) the *HLA-G**01:03 allele was associated with high-grade tumors among smokers; (ii) the *HLA-G**01:01:01/*G**01:01:02 genotype was associated with protection against high-grade tumors in the whole group of patients, whereas the same association was observed with the *HLA-G**01:01 group, but only among smokers; and (iii) the *HLA-G**01:04 allele group was associated with high-grade tumor development in smoker and in the whole group of patients (88).

No association has been observed for: (i) *HLA-G* coding region alleles in South Korean and Brazilian breast cancer patients (81, 89); (ii) 14-bpINS/DEL polymorphism in Italian patients presenting thyroid cancer (76); (iii) *HLA-G**01:03 allele and *HLA-G**01:05N null allele in Tunisian patients with nasopharyngeal carcinoma (87); (iv) *HLA-G**01:05N null allele with susceptibility to esophagus carcinoma development in Chinese patients (90); (v) 14-bp INS/DEL polymorphic site in Brazilian bladder TCC patients (88); and (vi) +292A/T, +755C/A, and +1799C/T in Australian and New Zealand childhood neuroblastoma patients (85).

To date, *HLA-G* polymorphisms have not been investigated in the context of melanoma, glioblastoma, colorectal cancer, gastric cancer, lung cancer, and renal cell carcinoma.

Although some polymorphic sites (14-bpDEL allele) and coding region allele groups (*HLA-G**01:04) have been previously associated with increased sHLA-G levels, few convincing associations have been reported, exception made to breast cancer for which an extensive meta-analysis has evidenced the role of this polymorphic site in Asiatic patients. Since several polymorphic sites have

been described at the *HLA-G* regulatory regions, exhibiting putative roles on HLA-G expression, the typing of the complete gene and the study of the regulatory elements (transcription factors and microRNAs) produced in the tumor environment may the helpful to understand the mechanisms of tumor evasion mechanisms.

VIRAL HEPATITIS

Similar to tumor cells, viruses have also developed several strategies to evade the cytotoxic effect of immune effector cells, including downregulation of HLA classical class I molecules and the upregulation of non-classical molecules, or both. As a corollary, the increased HLA-G expression, induced by the virus itself or by the presence of an inflammatory milieu containing transcription and post-transcription factors that positively modulate *HLA-G* expression, may exacerbate virus morbidity and/or patient mortality. The influence of HLA-G has been studied in several chronic viral infections; some of them associated with neoplastic transformation, including human immunodeficiency virus (HIV), human papillomavirus (HPV), human cytomegalovirus (hCMV), and hepatitis viruses [reviewed at Ref. (2)].

Increased HLA-G hepatocyte expression in HCV-infected liver specimens has been associated with milder stages of fibrosis and hemosiderin deposit (91). Besides hepatocytes, HLA-G expression was observed on mast cells present in areas of liver fibrosis (92). Increased plasma sHLA-G levels were associated with chronic HCV infection and with increased IL-10 and IFN-γ levels (93). Since the treatment of mast cells with IL-10 and class I interferons induces HLA-G expression (92), infiltrating cells may play an important role on the maintenance of chronic infection and induction of chronic complications.

One study has associated increased HLA-G expression in hepatocytes with the HBV viral load (94). Different studies associated the increased serum/plasma sHLA-G levels with hepatitis B virus infection (33, 95, 96), which were associated with increased percentage of $CD4^+CD25^+FoxP3^+$ T regulatory and $HLA\text{-}G^+CD14^+$ monocytes cells in patients exhibiting acute or chronic hepatitis (95), active hepatitis B virus infection (33) and HBeAg negative hepatitis, hepatocellular carcinoma, and increased alanine aminotransferase levels (96).

Regarding the typing of *HLA-G* 3′UTR polymorphic sites in HCV- and HBV-infected patients, the +3142C allele and 14-bpDEL/+3142C haplotype were underrepresented in Brazilian HCV-infected patients presenting sickle cells disease compared with HCV-negative group (97). On the other hand, the 14-bpINS/INS genotype was overrepresented in African-Brazilian HIV^+ patients co-infected with HCV (HIV^+/HCV^+) compared with HIV^+/HCV^- patients. Regarding the *HLA-G*+3142 C/G and 14-bp INS/DEL variants, no significant association has been reported for HIV^+/HCV^+- (98) and HBV-infected patients (99), respectively, when compared with their respective controls.

Considering that many viruses have developed evasion strategies that are similar to cancer cells and considering that many chronic viral disorders have been associated with cell transformation and malignancy, the expression of HLA-G in these disorders may predict a worse outcome and greater susceptibility to cell transformation.

PROTOZOAN PARASITE INFECTIONS

HUMAN MALARIA INFECTION

Plasmodium spp. is the etiologic agent of the human malaria and little is known about the role of HLA-G during malaria infection, and all studies have been performed to understand the mother to child transmission. One study reported a decreased HLA-G expression in extravillous trophoblast of *Plasmodium falciparum*-infected placentas compared to uninfected placentas. If by one hand, HLA-G molecule is almost exclusively expressed in extravillous trophoblast of healthy placenta specimens, on the other hand, HLA-G is detected in intervillous space macrophages of *Plasmodium*-infected placentas. In addition, NK cells are increased in infected compared to uninfected placentas (100). Furthermore, increased cord plasma levels of sHLA-G have been associated with low birth weight and increased risk of *P. falciparum* infection in infancy (101).

A family based association study performed on individuals from Niakhar, Senegal, reported that the +3187G allele was associated with higher transmission to children and lower level of parasite density during asymptomatic *P. falciparum* infection. The *HLA-G* 3′UTR haplotype known as UTR-1 was associated with a decreased level of parasite density during asymptomatic infection under a dominant model, whereas the *HLA-G* UTR-3 haplotype was associated with an increased level of parasite density during the follow-up and increased intensity of asymptomatic infection under a recessive model (102).

A second family based association study also conducted on Senegalese population has tested the association of *HLA-G* 3′UTR variants with acquired anti-malarial humoral immunity. The +3010G and +3142C alleles were overtransmitted to children with increased total IgG and IgG1 antibodies levels against glutamate-rich protein (GLURP) of *P. falciparum*, and the +3196G allele had a preferential transmission to children with a lower IgG3 response against merozoite surface protein 2 (MSP2). The *HLA-G* 3′UTR-2 haplotype was associated with a decreased IgG3 response against MSP2, suggesting a role of HLA-G on the regulation of immune humoral response during *P. falciparum* infection (103).

HUMAN AFRICAN TRYPANOSOMIASIS

Human African trypanosomiasis, also known as sleeping sickness, is caused by protozoan parasites of the *Trypanosoma brucei* species. Although no studies are available regarding HLA-G expression, genetic studies report associations of *HLA-G* gene single nucleotide variation sites with the disease. A family based association study reported that the *HLA-G* 3′UTR-14-bpINS and +3196G alleles had a preferential transmission from heterozygote parents to children and were associated with susceptibility to human African trypanosomiasis (HAT) development. In contrast, the *HLA-G* 3′UTR +3003C, +3010G, and +3187G alleles showed lower transmission from parents to children and were associated with decreased risk of developing the disease. Regarding *HLA-G* 3′UTR haplotypes, UTR-2 and UTR-5 haplotypes were associated with higher susceptibility to HAT development, whereas the *HLA-G* UTR-4 haplotype was associated with decreased risk for HAT development (104).

AMERICAN TRYPANOSOMIASIS

The parasite *Trypanosoma cruzi* is the etiologic agent of American trypanosomiasis, also known as Chagas disease (105). In the chronic phase, four major clinical forms are observed: (i) cardiac that presents progressive congestive heart failure, various cardiac arrhythmias, thromboembolic events, and sudden death; (ii) digestive that is characterized by clinical signs of megaesophagus, megacolon, or both; (iii) cardiodigestive that comprises clinical and pathological signs of cardiac and digestive involvement; and (iv) indeterminate that develops without evident clinical and pathological signs (106). Recently, our group reported a decreased HLA-G expression on cardiac muscle and colonic cells in patients presenting cardiac or digestive clinical variants, respectively. On the other hand, no significant differences were observed regarding HLA-G expression in the esophagus of patients with digestive form when compared to non-chagasic patients.

Furthermore, we evaluated the polymorphic sites at the *HLA-G* 3′UTR region in Brazilian chagasic patients. The +3003T allele and +3003TT and +3187GG genotypes were overrepresented, whereas the +3003C allele and +3003CT, +3010GC, and +3042GC genotypes were underrepresented in symptomatic patients. In addition, the +3027CC and +3035CC genotypes, and the +3027C and +3035C alleles were associated with the digestive form of Chagas disease. Regarding *HLA-G* 3′UTR haplotypes, decreased UTR-4 and UTR-7 frequencies were associated with symptomatic patients and with the digestive form, respectively. On the other hand, UTR-13 was associated with the indeterminate variant and UTR-14 with the cardiac form (107).

Overall, studies on the association between HLA-G and parasitic disorders are still scarce and only the *HLA-G* 3′UTR has been evaluated.

CONCLUSION

Considering the tolerogenic properties of HLA-G and considering the aphorism that the induced expression of HLA-G may be detrimental in tumors and chronic viral infection, the overall findings reported is this revision corroborates this idea. Noteworthy, is the induced expression of HLA-G on the surface of tumor cells, which has been associated with greater tumor morbidity, tumor progression, and spreading. In addition, in chronic viral infections associated with pre-neoplastic and neoplastic transformation. On the other hand, the repression of HLA-G expression is less well studied; i.e., the decreased expression of HLA-G in organs or conditions in which a constitutive expression of the molecule is expected. For instance, the decreased expression of HLA-G (placentas of *P. falciparum*-infected mothers or heart and colonic specimens of Chagas disease) has been associated with morbidity of the chronic parasitic infection.

Studies on the association of the *HLA-G* gene with diseases of diverse etiology have underestimated the myriad of polymorphic sites present at the various gene segments and have primarily focused on the evaluation of one or few polymorphic sites, particularly at the 3′UTR. Considering that many polymorphic sites along the *HLA-G* gene can be readily performed and analyzed, and considering the relevant role of isolated polymorphic sites or *HLA-G* haplotypes on HLA-G expression, HLA-G typing on diseases should add an additional tool on the understanding of the role of HLA-G on disease associations.

Theoretically, polymorphic sites observed along the coding region may modify the encoded protein and consequently the interaction with HLA-G receptors and the formation of HLA-G dimers that may more efficiently bind to HLA-G receptors. Thus, a particular allele and a particular molecule could provide susceptibility or protection against a disease development; however, such associations have not been strong enough to be considered a disease marker, as has been observed for the classical association between HLA-B27 and ankylosing spondylitis. On the other hand, polymorphic sites observed along the *HLA-G* promoter and 3′UTR gene segments may modify gene expression, accounting for disease morbidity. Unfortunately, few polymorphic sites along regulatory regions have extensively been evaluated regarding their function, and probably a combination of regulatory transcriptional and posttranscriptional elements may account for the final HLA-G production. Therefore, a complete gene evaluation together with the availability of transcription and protein profiles may provide light to the understanding of the mechanisms of HLA-G induction or repression in a specific disorder.

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Interactions between HLA-G and HLA-E in physiological and pathological conditions

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HLA-G and HLA-E are immunoregulatory molecules that belong to HLA-Ib family. The role of these molecules in the control of the immune response has been extensively analyzed, both in physiological and pathological conditions. We have here summarized data present in the literature regarding the interaction of these molecules in different settings. These data suggested that HLA-G and -E co-operate in physiological conditions (i.e., establishment of an immune tolerance at maternal/fetal interface during pregnancy), whereas their role in the course of tumors or autoimmune/inflammatory diseases may be different or even opposite. Future studies aimed at investigating the interaction between HLA-G and HLA-E will help to clarify mechanism(s) underlying the regulation of immune effector cells in health and disease.

Keywords: HLA-G, HLA-E, tumor, autoimmune disease, viral infections

INTRODUCTION

HLA-G and HLA-E belong to non-classical HLA-class Ib family that also includes HLA-F and HLA-H. In contrast with classical HLA-Ia molecules (HLA-A, -B, and -C), these molecules display a limited polymorphism, with a small number of proteins encoded by few alleles (http://hla.alleles.org/nomenclature/stats.html, data are summarized in Table S1 in Supplementary Material). Moreover, the function of HLA-class Ia and Ib molecules is different. In fact, HLA-class Ia molecules bind peptides generated from cytoplasmic proteins (in general represented by viral or tumor-associated antigens) and interact with antigen-specific T-cell receptor expressed on cytotoxic $CD8^+$ T-cells, leading to the recognition of virus infected or transformed cells (1). In addition, HLA-class Ia molecules can interact with killer-inhibitory receptors expressed on NK cells, thus modulating NK cell functions (2).

HLA-class Ib molecules are also able to bind peptides generated from intracellular antigens, but the main function of these molecules is to modulate the immune response by interacting with specific inhibitory receptors expressed on different immune effector cells (3).

HLA-G is the best characterized among HLA-class Ib molecules. Seven different isoforms are encoded by the same primary mRNA through alternative splicing. Four isoforms (HLA-G1, -G2, -G3, and -G4) retain the transmembrane domain and therefore are membrane-bound, whereas the other three isoforms (HLA-G5, -G6, and -G7) retain the intron-4 and lose the transmembrane domain, and are therefore released as soluble molecules. In addition, soluble(s) HLA-G can be generated from membrane-bound molecules, through the cleavage operated by metalloproteases (4). In this respect, Rizzo et al. have recently reported that metalloprotease 2, but not 9, is involved in this process (5).

HLA-G expression is extremely restricted, being detected in physiological conditions in placental trophoblast cells at maternal–fetal interface during pregnancy (6), in thymus (7), cornea (8), nail matrix (9), pancreas (10), monocytes (11), erythroid (12), and endothelial precursors (13). However, HLA-G expression can be also detected in different immune cell populations, such as T-cells (14, 15), antigen-presenting cells (15–17), and in immunoregulatory cell populations, such as mesenchymal stem cells (18, 19). Nevertheless, HLA-G is up-regulated in different pathological conditions, such as transplantation, tumors, viral infections, and inflammatory diseases (20, 21).

The role of this molecule is to regulate the immune response, both in physiological and pathological conditions. This feature is important at maternal–fetal interface, to avoid the lysis of semi-allogeneic fetal tissue by maternal NK cells (22–25). Similarly, in transplanted patients, an increased expression of surface HLA-G (26) and an augmented concentration of serum sHLA-G (27) may protect the transplanted organs from the rejection by the host's immune system. Conversely, HLA-G expression on transformed cells (tumor cells and virus-infected cells) provides them with an immune escape mechanism, avoiding the recognition and lysis by cytotoxic immune effectors, such as NK cells and cytotoxic T lymphocytes (28).

The immunoregulatory properties of this molecule are related to the inhibition of the function of different immune cell populations, such as T- and B-lymphocytes, NK cells, and antigen-presenting cells. Such inhibition is mediated by the interaction of HLA-G molecules with at least four inhibitory receptors expressed on immune effector cells: immunoglobulin-like transcript (ILT)2 on NK cells, T- and B-lymphocytes; ILT4 on myeloid cells; KIR2DL4 on NK cells and T-lymphocytes; and CD160 on NK cells and T-lymphocytes (4).

The expression of HLA-E mRNA can be virtually detected in all nucleated cells. However, the surface expression of HLA-E, that requires the presence of peptides derived from other HLA-class I molecules and β2-microglobulin, is extremely restricted and it has been related to cell activation (29). In fact, the function of HLA-E is to bind peptides derived from the leader sequence of HLA-class I molecules (HLA-A, -B, -C, and -G) and to present them to NK cells through the interaction with the inhibitory receptor CD94/NKG2A, thus inhibiting NK cell lysis against cells that express normal levels of HLA-class I molecules. Conversely, cells with low levels of HLA-class I expression generate low levels of HLA-class I derived peptides and consequently display a low level of HLA-E, thus allowing NK cell lysis (30). HLA-E can also interact with CD94/NKG2C activating receptor on NK cells, in particular when it binds peptides generated from HLA-G. This feature is employed to activate NK cell lysis against HLA-G$^+$ trophoblast cells during placental invasion, leading to tissue remodeling (31). However, it has been demonstrated that HLA-E affinity to the inhibitory NKG2A/CD94 receptor is sixfold higher than its affinity to the activating NKG2C/CD94 receptor (32). Finally, HLA-E can present different peptides to HLA-E restricted effector cells. Romagnani et al. have identified a CD8$^+$ T-cell subset that recognized different peptides associated to HLA-E on allogeneic cells, thus highlighting their importance in transplantation and anti-tumor immune responses (33). Moreover, it has been reported that HLA-E present CMV-derived peptides to a subset of HLA-E restricted CMV-specific CD8 + T-cells (34). This feature may be relevant in the control of viral infections, since cytomegalovirus is able to avoid the control of conventional CTL or NK cells. On the other hand, Jiang et al. have demonstrated that peptides derived from the signal peptide of Hsp60 and loaded on HLA-E are recognized by a subset of CD8$^+$ regulatory T-cells that are able to control self-reactive T-cells. The loss of this recognition may lead to the development of autoimmune diseases (35).

In this review, we summarize for the first time data present in literature regarding the interaction between HLA-G and HLA-E, focusing on the role of this interaction in the control of the immune response both in physiological and pathological conditions.

HLA-G AND HLA-E CO-OPERATE IN PHYSIOLOGICAL CONDITIONS

Several authors have demonstrated that HLA-G can influence and modulate HLA-E expression. In particular, the expression of different isoforms of HLA-G may affect surface HLA-E expression, which depends on the availability of peptides derived from HLA-G molecules and other HLA-class I molecules. In this view, it has been demonstrated that HLA-E surface expression was higher in cells transfected with HLA-G1 or -G3 than in untransfected cells. Moreover, HLA-E expression was higher in cells transfected with HLA-G1 than in cells transfected with HLA-G3 (36). Similarly, Ulbrecht et al. have demonstrated that the truncated isoforms of HLA-G (HLA-G2, -G3, and -G4) are less efficient to provide peptides to HLA-E molecules. Consequently, HLA-E expression is lower in cells that express high levels of HLA-G truncated isoforms than in cells expressing HLA-G1 (37). This effect was likely related to the ability of full-length transmembrane isoforms to act as chaperone for HLA-E molecules, since the leader sequence, that generates HLA-E binding peptides, is identical across different HLA-G isoforms. However, data obtained by Sala et al. are partially in contrast with this conclusion. They transfected JAR cell line with *HLA-G*0105N* allele, which encodes a truncated isoform containing the leader peptide, the complete α1 domain, and the first half of the α2 domain. Although this truncated HLA-G1 protein is rapidly degraded, its leader sequence after cleavage might still be available for binding to the HLA-E molecule. In fact, transfected cells do not express HLA-G1 molecule on the surface, but express a functional HLA-E molecule that is capable to inhibit NK cell lysis by interacting with CD94/NKG2A receptor (38).

HLA-G and HLA-E are physiologically co-expressed on different cell populations and can interact to modulate the immune response. In this regard, Ishitani et al. have demonstrated that HLA-E expression in trophoblast cells was strongly related to HLA-G expression. In fact, surface expression of HLA-G was found in extravillous trophoblasts, whereas sHLA-G production was found in all placental trophoblasts, including villous cytotrophoblasts and syncytiotrophoblasts. HLA-E expression was detected in all cells that expressed either form of HLA-G, suggesting that HLA-E requires peptides derived from all isoforms of HLA-G to be expressed (39). Similarly, Shaikly et al. have demonstrated that HLA-G and HLA-E co-localize on the surface of trophoectodermal cells, and may regulate implantation through the regulation of the effector functions of uterine leukocytes, by interacting with different receptors expressed by different cell populations, leading to an addictive effect (40). Moreover, it has been recently demonstrated that mesenchymal stromal cells derived from gestational tissue (in particular derived from the cord blood) are poorly immunogenic, and this feature is related to the co-expression of HLA-G and HLA-E on their cell surface (41). Similarly, induced pluripotent stem cells (iPSCs) express low levels of classical HLA-class I molecules, but express high levels of HLA-G and HLA-E and are able to avoid the recognition of HLA-restricted cytotoxic T-cells, which become anergic when co-cultured with iPSCs (42).

HLA-G AND HLA-E INTERACTION MAY BE RELEVANT DURING CANCER AND VIRAL INFECTIONS

HLA-G and HLA-E can co-operate to establish an immunosuppressive microenvironment in human tumors and viral infections, facilitating the escape of transformed cells from the recognition by the immune system.

In this view, de Kruijf et al. have demonstrated that in patients with breast cancer either HLA-G or HLA-E expression correlated with worse overall and event-free survival. This was observed only in patients with tumors that display a loss of classical HLA-I molecules, thus suggesting that it may occur only when activated NK cells are present. Notably, patients with tumors co-expressing HLA-G and -E display the worst clinical outcome, thus suggesting that the two molecules may co-operate shutting down NK cell-mediated anti-tumor immune response (43). Similarly, it has been demonstrated that HLA-G and -E co-expression correlated with the presence of metastasis and with a worse event-free and overall survival in patients with colon cancer, irrespective of the expression of HLA-class Ia molecules (44). Nevertheless, Malmberg et al. have demonstrated that short-term ovarian carcinoma cell lines

treated with IFN-γ become resistant to CTL-mediated lysis. Such effect was mediated by increased HLA-G expression, which in turn leads to up-regulation of HLA-E on tumor cells. Surface HLA-E inhibits CTL activity by interacting with the inhibitory receptor CD94/NKG2A (45).

In contrast with these studies, several groups have demonstrated that HLA-G and -E may have different or even opposite roles in tumor progression. In this respect, da Silva et al. have demonstrated that HLA-G was overexpressed in the majority of biopsies derived from patients with breast cancer, whereas HLA-E expression was detected at low level in a small number of biopsies, thus suggesting that, at least in this cohort of breast cancer patients, HLA-G and -E interaction does not likely take place (46). Similarly, HLA-G is specifically expressed in renal cell carcinoma and not in normal renal parenchyma, whereas HLA-E is expressed in both normal and pathological tissues. Moreover, a better relapse-free survival was associated with a low HLA-G expression and with a high HLA-E expression, thus suggesting a divergent role of these molecules in the progression of this type of tumor (47). On the contrary, Silva et al. have demonstrated that, in patients with laryngeal lesions, HLA-G expression was detected in benign and premalignant lesions and not in invasive carcinomas, whereas HLA-E expression correlated with lesion grade, with a high expression in the draining lymph nodes of malignant lesions. Also, in this case, however, an opposite role of HLA-G and -E in tumor progression was demonstrated (48). Similarly, in patients with cervical carcinoma, HLA-G expression was detected in atypical glandular cells of undetermined significance and disappeared in cervical intraepithelial neoplasia (CIN) and invasive cancer, whereas HLA-E expression increased from CIN1 to CIN3 grade and the highest HLA-E expression was detected in invasive cancer, thus suggesting that HLA-E, rather than HLA-G, has a role in immune escape of transformed cells (49). Finally, HLA-G and HLA-E expression was detected in about 70% of biopsies from glioblastoma cells, and co-expression was detected in 36% of cases. A high HLA-E expression was related to a better overall survival, whereas no correlation was found between HLA-G expression and clinical outcome of patients (50).

HLA-G and -E can also co-operate in the tumor microenvironment to induce local anergy (51). It has been demonstrated that tumor-associated macrophages (TAM) express HLA-G on their surface (52). HLA-G expressed and/or released by TAM may interact with inhibitory receptors on NK cells stimulating the release of pro-angiogenic cytokines, as reported (53). Kren et al. have demonstrated that TAM may also express HLA-E (54), which interacting with the inhibitory receptor CD94/NKG2A on NK cells may further stimulate the release of immunosuppressive cytokines from NK cells (55). Thus, HLA-E may collaborate with HLA-G in the protection of TAM from NK cell lysis (30, 56) and in the establishment of a tolerogenic tumor microenvironment.

HLA-G and HLA-E interaction may also take place during viral infections. In this view, it has been demonstrated that rabies virus is able to up-regulate both HLA-G and -E expression in infected human neuronal precursors, and both molecules facilitate the immune escape of infected cells (57). Similarly, Vasireddi and Hilliard have demonstrated that, in contrast with other herpesviruses, herpes B virus does not downregulate the expression of HLA-Ia molecules. In contrast, HLA-G and -E expression is significantly up-regulated in infected cells, thus again suggesting a role of both molecules in the escape of infected cells from the recognition of the immune system (58).

HLA-G AND HLA-E MAY HAVE OPPOSITE ROLES IN INFLAMMATORY/AUTOIMMUNE DISEASES

Only few data are present in the literature regarding the role of both HLA-G and -E in inflammatory/autoimmune diseases. However, data obtained from our group in patients with juvenile idiopathic arthritis (JIA) and multiple sclerosis suggest that HLA-G and -E may have either an opposite or a synergistic role in the course of these pathological conditions.

In fact, we have demonstrated that in JIA patients, HLA-G may be more relevant as soluble molecule in the biological fluids, since serum levels of sHLA-G are decreased in patients as compared to controls. This may lead to an uncontrolled activation of immune effector cells, which eventually migrate to the synovium, causing tissue damage. In contrast, HLA-E appears to be more important as surface molecule, since its expression is higher on infiltrating synovial cells (mostly on B cells and monocytes) than in peripheral blood counterparts. This feature may be relevant to protect autoreactive cells from NK cell-mediated lysis, thus exacerbating local inflammation. Nevertheless, sHLA-E concentration in synovial fluid correlated with disease severity, thus suggesting that this molecule may represent a marker of cell activation (59).

In contrast with these observations, data obtained in multiple sclerosis patients suggested that HLA-G and HLA-E can co-operate in the resolution of inflammation. In fact, concentration of both sHLA-G and sHLA-E was higher in sera from patients than controls (represented by patients with other neurological disorders). More importantly, intrathecal synthesis of HLA-G and -E was detected, and concentration of both molecules was increased in cerebrospinal fluid (CSF) from MS patients as compared to controls. Moreover, sHLA-E concentration was higher in clinically stable patients than in those with clinically active disease. Finally, CSF samples inhibited *in vitro* NK- and CTL-mediated lysis. Such inhibition was higher using samples containing both HLA-G and -E than samples containing HLA-G or HLA-E, or devoid of both molecules. Taken together, these data suggested that HLA-G and HLA-E co-operate in the inhibition of immune effector cell function, and may have a role in the resolution of neuroinflammation (60).

CONCLUDING REMARKS

We have here summarized for the first time the interaction between HLA-G and -E in different settings (data are summarized in **Figure 1**). We can conclude that, in physiological conditions, HLA-E expression is strongly related to HLA-G, and normally both molecules are involved in inducing anergy of activated immune effector cells (mostly NK cells). Conversely, the interaction of these molecules in pathological conditions may be variable, ranging from a strong correlation and co-operation to an opposite function and role in the progression of the disease (see **Table 1**). Future studies aimed at a better knowledge of these interactions may explain the mechanisms underlying the establishment of an immunosuppressive microenvironment.

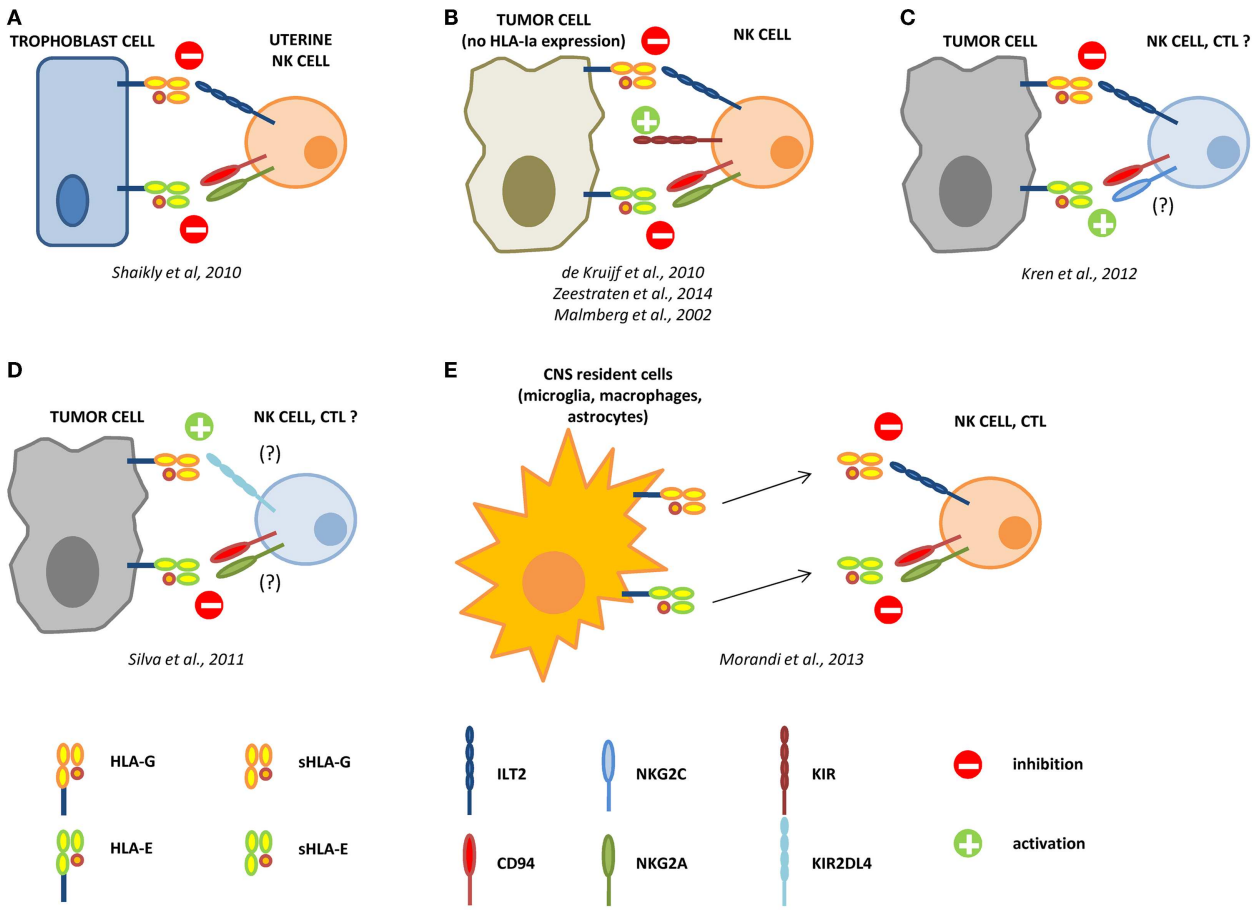

FIGURE 1 | Interactions between HLA-G and HLA-E in the control of the immune response. During pregnancy, HLA-G and -E are both expressed by trophoblast cells and co-operate in the inhibition of NK cell functions, by interacting with ILT2 and CD94/NKG2A receptors, respectively **(A)**. In different tumors, the loss of HLA-class Ia molecules activate NK cells through KIR ligand mismatch. HLA-G and -E co-operate in the inhibition of activated NK cells in the tumor microenvironment, facilitating the escape of tumor cells from NK cell recognition **(B)**. In renal cell carcinoma, HLA-G expression correlates with worse prognosis, whereas HLA-E expression represents a favorable prognostic marker. We can speculate that in this case HLA-G preferentially interacts with inhibitory receptors on NK cells and CTL, whereas HLA-E possibly interacts with CD94/NKG2C activating receptor on immune effector cells **(C)**. On the contrary, in laryngeal carcinoma, HLA-G predicts a good prognosis, whereas HLA-E is associated with worse prognosis. In this case, we speculate that HLA-G may predominantly interact with KIR2DL4 activating receptor, whereas HLA-E interacts with CD94/NKG2A inhibitory receptor on NK cells and CTL **(D)**. In multiple sclerosis patients, HLA-G and HLA-E are expressed and released by resident cells in the central nervous system (CNS), and both soluble molecules co-operate in the inhibition of NK cells and CTL function, by interacting with inhibitory receptors **(E)**.

Table 1 | Summary of HLA-G and HLA-E interactions in pathological conditions.

	Disease	Co-operation	Correlation	No correlation	Opposite role
Tumors	Breast cancer (43)	x			
	Colon cancer (44)	x			
	Ovarian carcinoma (45)	x			
	Breast cancer (46)			x	
	Renal cell carcinoma (47)				x
	Laryngeal carcinoma (48)				x
	Cervical carcinoma (49)			x	
	Glioblastoma (50)			x	
Viral infections	Rabies virus (57)		x		
	Herpes B virus (58)		x		
Autoimmune disease	Juvenile idiopathic arthritis (59)			x	
	Multiple sclerosis (60)	x			

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SUPPLEMENTARY MATERIAL

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HLA class Ib molecules and immune cells in pregnancy and preeclampsia

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Despite decades of research, the highly prevalent pregnancy complication preeclampsia, "the disease of theories," has remained an enigma. Indeed, the etiology of preeclampsia is largely unknown. A compiling amount of studies indicates that the pathological basis involves a complex array of genetic predisposition and immunological maladaptation, and that a contribution from the mother, the father, and the fetus is likely to be important. The Human Leukocyte Antigen (HLA)-G is an increasing focus of research in relation to preeclampsia. The HLA-G molecule is primarily expressed by the extravillous trophoblast cells lining the placenta together with the two other HLA class Ib molecules, HLA-E and HLA-F. Soluble isoforms of HLA-G have been detected in the early endometrium, the matured cumulus–oocyte complex, maternal blood of pregnant women, in umbilical cord blood, and lately, in seminal plasma. HLA-G is believed to be involved in modulating immune responses in the context of vascular remodeling during pregnancy as well as in dampening potential harmful immune attacks raised against the semi-allogeneic fetus. In addition, HLA-G genetic variants are associated with both membrane-bound and soluble forms of HLA-G, and, in some studies, with preeclampsia. In this review, a genetic contribution from the mother, the father, and the fetus, together with the presence and function of various immune cells of relevance in pregnancy are reviewed in relation to HLA-G and preeclampsia.

Keywords: HLA class Ib, HLA-E, HLA-F, HLA-G, preeclampsia, immune cells

INTRODUCTION

Preeclampsia is believed to develop in two stages: a pre-clinical stage without symptoms typically characterized by poor placentation, and a clinical stage occurring some point after 20 weeks of gestation with symptoms of increased blood pressure accompanied by proteinuria. Subclinical changes include placental oxidative stress and endothelial activation.

A unique subset of cytokine-producing decidual NK (dNK) cells is identified in the placenta during pregnancy. In contrast to the conventional NK cells of the periphery (pNK), which make up 5% of the peripheral leukocyte population, dNK cells are enriched in the placental compartment constituting up to 75% of the placental leukocyte population (1, 2). dNK cells are known to produce angiogenetic factors, and the poor trophoblastic vascular remodeling of the spiral arteries in preeclampsia has been attributed a decrease in dNK cell numbers and/or abrogated functions. Moreover, T and NK cells of the periphery are known to be activated in preeclampsia (3).

The human Major Histocompatibility Complex (MHC) is a large gene family located on chromosome 6. It includes the classical Human Leukocyte Antigen (HLA) class Ia and II genes (HLA-A, -B, -C, -DR, -DQ, and -DP). These genes and molecules are well known for their importance in antigen-peptide presentation and in organ transplantation, and for their association with a range of diseases, especially autoimmune diseases (4, 5). However, the MHC region also includes the so-called non-classical HLA class Ib genes: HLA-E, -F, and -G (6–9). The role of these genes and molecules in pregnancy and in preeclampsia is a main focus of this review.

There are two anatomical contact-points between the maternal immune cells and the fetus: the systemic immune response between maternal circulating immune cells and the syncytiotrophoblasts, and the local immune response between decidual immune cells and the extravillous trophoblast cells (**Figure 1**) (10). The syncytiotrophoblast cells are devoid of HLA I molecules (11), and it is unlikely that T cell responses are directed against these. Protection from NK lysis is provided by the non-classical HLA class Ib molecules, HLA-E and HLA-G, which are highly expressed in extravillous trophoblast cells lining the placenta, and possibly also expressed by syncytiotrophoblast cells (12, 13). However, in addition to expressing the HLA class Ib molecules, extravillous trophoblast cells express low amounts of the polymorphic HLA-C, which could serve as a source of allorecognition by maternal immune cells.

HLA CLASS Ib IN PREGNANCY

Human trophoblast cells express one HLA class Ia molecule (HLA-C) and all HLA class Ib molecules (HLA-E, -F, and -G) (6, 12, 14). Considering the unique co-expression of HLA-E, -F, and -G in the placenta and their mutual involvement in immune modulation, a combined effect or interaction of all three class Ib molecules would not seem far stretched to hypothesize (12). HLA-G has

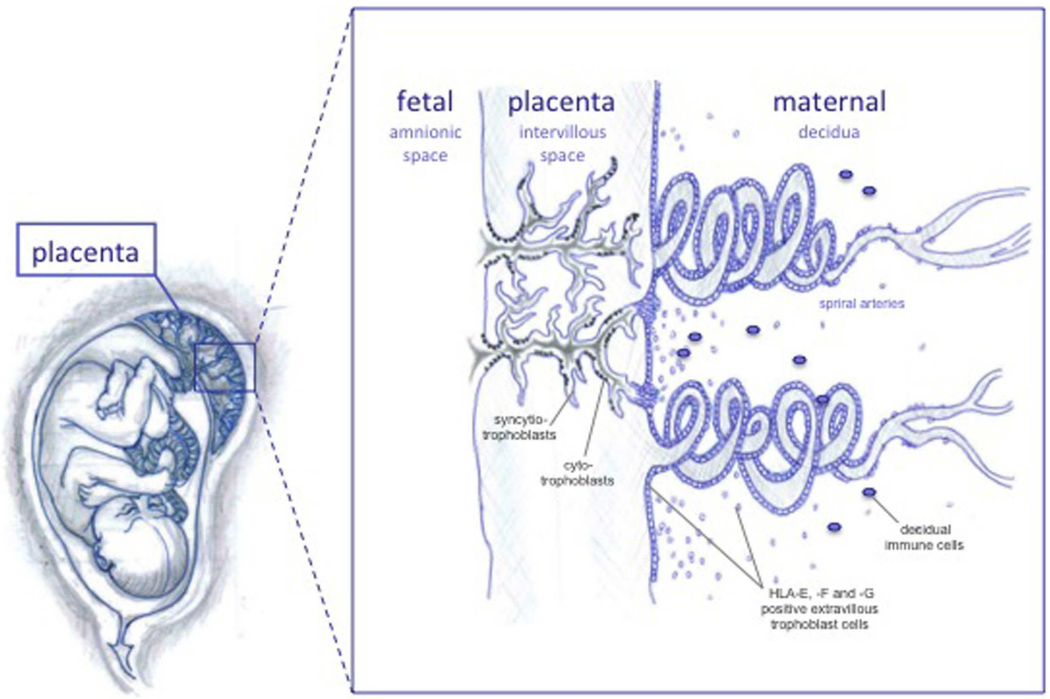

FIGURE 1 | The feto-maternal interface. The extravillous trophoblast cells invades the maternal decidua and the spiral arteries, possibly remodeling these in order to increase blood flow to the fetus as pregnancy progresses. HLA-G and HLA-E protect invading trophoblast cells from lysis by NK cells throughout pregnancy, while HLA-F is expressed on the surface of extravillous trophoblast cells at later stages.

been intensively studied, HLA-E moderately studied, while little is known about HLA-F. Nonetheless, some studies on the expression and function exist, and can be related to their possible role in pregnancy.

Human leukocyte antigen-G is strongly expressed throughout pregnancy, both in the cytoplasm of extravillous trophoblast cells and on the cell surface (15, 16). HLA-F is weakly expressed in the extravillous trophoblast during the first trimester of pregnancy (16). From second trimester and on, the expression increases continuously and HLA-F translocates to the cell surface. HLA-E expression is similar to HLA-F, but HLA-E is additionally found on the cell surface in the first trimester. The increase in HLA-E and HLA-F expression coincides with fetal growth (16), and implies a role, at least for HLA-F, in this context.

Unlike classical HLA Ia molecules, the primary role of HLA-G is not antigen presentation, but rather immune regulation through the receptors ILT2, ILT4, and KIR2DL4 (**Figure 2**) (17–19). HLA-E mRNA has been detected in all cells and tissues examined and its function is likely to extend that of pregnancy (20). In contrast to HLA-G, HLA-E has been demonstrated to present antigens to a restricted subset of T cells (21), and in addition, to act as a ligand for the NK-specific CD94/NKG2 lectin receptors that regulate the activity of these cells (**Figure 2**) (22, 23). In the placenta, ligands for HLA-E are restricted to leader peptides from HLA-G and HLA-C, partly because of its hydrophobic properties, which limit the selection of peptides it can bind (24).

The functional role of HLA-F is the least defined. HLA-F is not believed to act in antigen presentation as it is expressed on the surface of proliferating viral-transformed lymphoid and monocyte cells without bound peptide (25, 26), and sometimes found associated with other HLA class I molecules also devoid of peptide as open conformers (27). The functional relevance of open HLA class I conformers is unclear, but it is possibly related to their unusual ability to cis-associate with themselves and other receptors (28). At least some studies indicate that these forms enable them to act as regulators of ligand–receptor interactions (28). Interestingly, similarly to HLA-G, HLA-F tetramers are able to bind ILT2 and ILT4 (**Figure 2**) (29).

HLA-G GENE AND HLA-G mRNA AND PROTEIN ISOFORMS

Eighteen HLA-G alleles have been described at the protein level according to the WHO Nomenclature Committee for Factors of the HLA System and the International Immunogenetics Information System (IMGT)/HLA Database. HLA-G exhibits low nucleotide variability in the coding regions. Most HLA-G polymorphisms do not alter the amino acid sequence, and are not expected to affect secondary structures of the heavy chains. HLA-G is alternatively spliced to produce seven mRNA isoforms, four of which encode membrane-bound protein isoforms (HLA-G1, -G2, -G3, and -G4) and three that encode soluble protein isoforms (HLA-G5, -G6, and -G7) (30–34). HLA-G1 represents the full-length isoform. HLA-G2 results from the removal of exon 3. HLA-G3 results from the removal of exon 3 and 4, and HLA-G4 from out-splicing of exon 4. HLA-G5 and -6 are soluble isoforms due to inclusion of intron 4 in the mature mRNA, which leads to secreted proteins with additional 21 amino acids

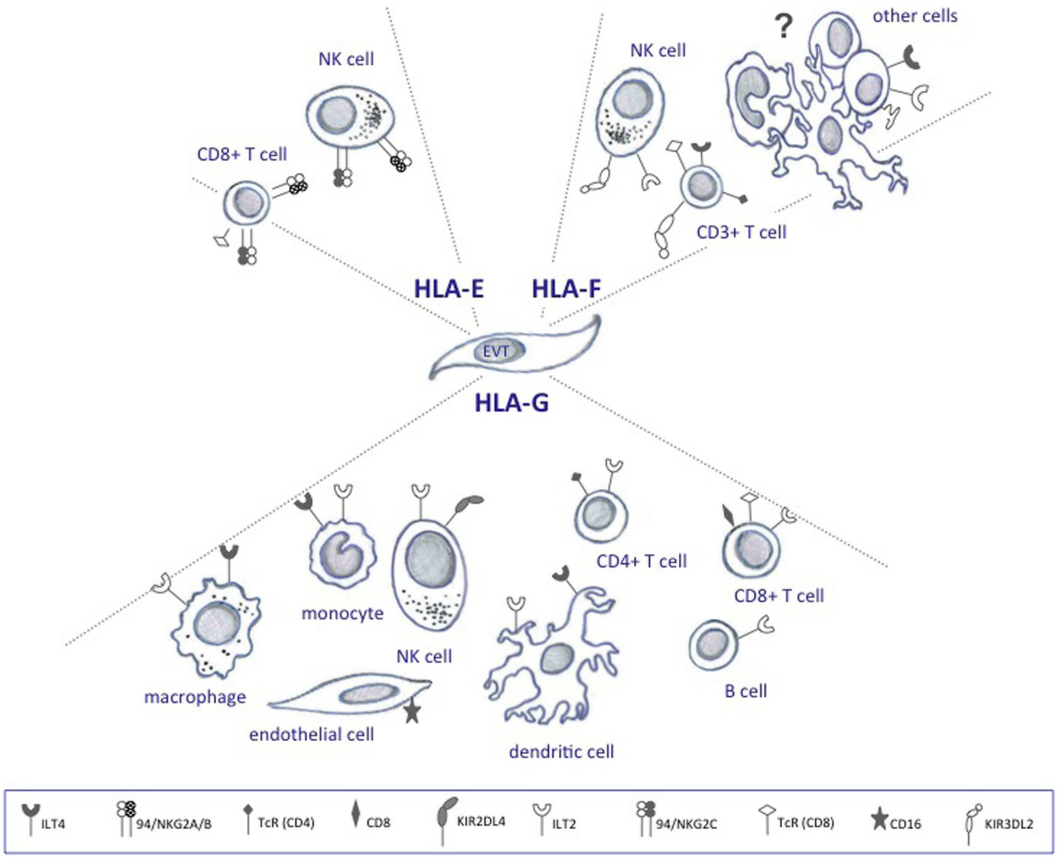

FIGURE 2 | HLA class Ib and cognate receptors expressed on decidual immune cells.

encoded by the intron 4 sequence (31). HLA-G7 includes exon 2 and part of intron 2, and is predicted to encode a small soluble isoform, however, more studies are needed to demonstrate the presence of this isoform *in vivo* (34). With relevance for pregnancy, HLA-G4 and -7 mRNAs are not abundant in placentas (35).

Human leukocyte antigen-F and HLA-E have, like their counterpart, a low degree of polymorphism (36, 37). Compared to HLA-G and -E, HLA-F is distinguished in the literature by lacking exon 7, which produces a protein with a shortened cytoplasmic domain. However, HLA-G also lacks exon 7, and a newer interpretation of the intron and exon nomenclature of the HLA-G gene is currently receiving attention.

SOURCE OF HLA CLASS Ib AND CELLULAR LOCALIZATION IN THE PLACENTA

Soluble HLA-G in the maternal circulation is predominantly produced and shed from trophoblast cells during pregnancy, but a quantity of sHLA-G is possibly produced by regulatory T cells and antigen-presenting cells like monocytes and dendritic cells (DCs) derived hereof (14, 38, 39). In non-pregnant individuals, sHLA-G likely reflects expression from monocytes (40, 41). Other tissues or biological fluids where HLA-G has been detected include the matured cumulus–oocyte complex, thymus, follicular fluid, and seminal plasma; furthermore at immune privileged sites, HLA-G expression has been confirmed to the eye, brain, testis, the epididymis, and the prostate gland (42–46). Also, HLA-G is secreted by erythroblasts (47), which is interesting as increased fetal erythroblastosis is detected in women who subsequently develop preeclampsia (48).

Human leukocyte antigen-E mRNA expression has been detected in virtually all cells and tissues examined and is expressed on the surface of a wide variety of cells (20).

Cellular localization of HLA-F is verified in the placenta (12), the tonsils, spleen, bladder, skin, thymus tissue, and liver cell lines (25, 49). While surface expression is absent in most tissues (25), surface expression has been demonstrated on trophoblast cells during later stages of gestation (12).

Human leukocyte antigen-G mRNA transcripts have been detected in first trimester and at term in extravillous (12, 15) and in syncytiotrophoblast cells (12), in the latter case, only mRNA transcript encoding the non-membrane forms have been confirmed (12). Because HLA-G is highly homologous to other HLA class I molecules, specific antibodies have been difficult to develop (50), and the protein expression of soluble HLA-G isoforms by syncytiotrophoblast cells cannot be ruled out, as sporadic patches with HLA-E expression have been detected in this trophoblast cell fraction (12, 13), which probably requires availability of leader

peptides from HLA-G. Thus, the exact HLA-G expression profile in the syncytiotrophoblast cells is still a controversial issue.

In the placental choriocarcinoma cell line JEG-3, a physical co-localization of HLA class Ib was evidenced, showing HLA-E, -G, and -C on the cell surface, while HLA-F expression was confined to the cytoplasm (51). Also, using cell bio-imaging, a recent study revealed that HLA-G and HLA-E are co-localized in preimplanted embryos (52), indicating a prerequisite for co-expression of HLA class Ib molecules, which also could apply in the uterine compartment.

HLA-G CONFORMATIONAL VARIANTS AND HIGH MOLECULAR WEIGHT COMPLEXES

A recombinant HLA-G protein consisting of the α1 and α2 domains was synthesized to mimic the extracellular part of HLA-G2 and HLA-G6 in one study (53). It showed that this HLA-G protein bound ILT4, but not ILT2, and was the first to report a binding of a HLA-G receptor with truncated HLA-G isoforms. In continuation of these findings, it was demonstrated that the same structure is able to induce tolerance and prolong the endurance of skin allografts in B6-mice and in an ILT4-transgenic mouse model (53).

In one study, HLA-G5 was hypothesized to indirectly regulate trophoblast invasion by binding to decidual leukocytes and inducing cytokine production, and as a consequence positively affect placentation (54). More specifically, recombinant HLA-G5 (rHLA-G5) was demonstrated to stimulate trophoblast invasion upon binding to KIR2DL4 and ILT2, which led to activation of the ERK pathway via phosphorylation of ERKs (54). Accordingly, trophoblast invasion was reversed with blocking antibodies for ILT2 and KIR2DL4 (54). Since insufficient trophoblast invasion is a characteristic of preeclampsia, it would be interesting if further studies of the effects of HLA-G5 on placentation were performed.

Recently, high molecular weight HLA-G complexes circulating in exosomes were identified (55). Trophoblast-derived exosomes are endocytic nanoparticles (<100 nm) shed from the placenta into the circulation, where they may stimulate or inhibit peripheral immune cells, while simultaneously expose paternal antigens systemically (56). Interestingly, the HLA-G complexes reported in exosomes were heterogeneous in nature, some proteins corresponding to ubiquitinated HLA-G, while other structures exhibited unclassified protein modifications (55). HLA-G protein alterations may affect quantification in biological fluids. Indeed, soluble HLA-G is readily detected in EDTA-stabilized blood plasma using a specific ELISA and the MEM-G/9 antibody, while the detection level is decreased in heparin-stabilized blood plasma and in serum samples (own unpublished observations). This may have important implications for detection of sHLA-G and possibly sHLA-E in the circulation of preeclamptic women, specifically when assessing their potential as biomarkers, and could explain some of the discrepancies in soluble levels previously described between studies.

Human leukocyte antigen-G exists in different forms, commonly as a monomer associated with or without the β_2m-subunits or as hetero- or homodimers, but unique trimeric and oligomeric forms have also been acknowledged (57–59). The physiological significance of different forms remains unclear. Recent reports have demonstrated that β_2m-associated HLA-G monomers comprise the majority of all HLA-G forms expressed by trophoblast cells (53), but a significant fraction exists in the form of HLA-G homodimers by forming an intermolecular disulfide bridge between two cysteine residues of the α1 domains of two HLA-G molecules (60). So far, the homodimer form has shown to be the most active arrangement with a higher affinity for ILT2 and ILT4 compared with the monomer (18). Furthermore, the homodimer enhances the ILT2-mediated signaling at the cellular level (18). Interestingly, in trophoblast cell lines, cell bio-imaging showed that app. 40% of HLA-E and HLA-G are co-localized in the form of tetramers or higher-order homodimer clusters (51, 52) and that HLA-E and -G form heterotypic associations with HLA-C (51), indicating a physical association on the cell surface in higher-order complexes. If these findings reflect a co-dependency of HLA-E and -G surface expression and co-localization, then a possibly reduced level of HLA-G in preeclampsia – in addition to reducing availability of leader peptide necessary for stable HLA-E surface expression – could also affect the functionality of HLA-E by other means.

Similar to HLA-G, HLA-F exists with and without association with β_2m, and can form homodimers as well as associate with other HLA class I (25, 26). The possibility that HLA-F heavy chains have hidden functions that are determined by the amino acid sequence of the α domains is plausible (28) and should be investigated in relation to receptor–ligand interactions in pregnancy and preeclampsia.

HLA-G IN PREGNANCY AND PREECLAMPSIA

Elevated levels of sHLA-G have been observed in the maternal circulation during pregnancy (61–64). An association between HLA-G and preeclampsia is supported by several findings. First, a direct association between reduced HLA-G expression in term placentas and preeclampsia has been demonstrated with *in situ* hybridization, immunohistochemistry on frozen sections, and with a ribonuclease protection assay (65–67). Second, circulating sHLA-G levels are decreased in preeclampsia, and in some cases this is observed as an early event in pregnancy in women who subsequently develop preeclampsia compared with women with uncomplicated pregnancy (62, 64, 68–70). Third, HLA-G polymorphisms have been associated with sHLA-G levels in peripheral blood from blood donors and with HLA-G protein expression in the placenta during pregnancy (71, 72), and fourth, HLA-G polymorphisms, some of which are associated with circulating levels, are further associated with increased risk of preeclampsia in some studies (73–76) but not in all (77–80). While the beneficial role of HLA-G is recognized in relation to pregnancy, a precise relationship between HLA-G and preeclampsia needs further appraisal.

FUNCTIONAL SIGNIFICANCE OF HLA-G ISOFORMS IN RELATION TO PREGNANCY AND PREECLAMPSIA

To emphasize the function of HLA-G in relation to pregnancy and preeclampsia, several questions need to be addressed. First, which cells express cognate receptors and what is their function, second, does HLA-G exhibit isoform-specific functions, and third,

what molecular structures can HLA-G form, and could it have functional relevance?

ILT2 and ILT4 are the major receptors for HLA-G. Since ILT2 and ILT4 are expressed by leukocytes – the former by most leukocytes, and the latter primarily by monocytes, macrophages, and DCs – most attention has been drawn to the interaction between HLA-G and immune cells (81). However, novel functions of HLA-G have been suggested, possibly in the context of vascular events during placentation. Indeed, both ILT2 and ILT4 have been identified in the mesenchyme of term placentas, but with different localization. ILT2 was abundant in stromal cells, while ILT4 was prominent in perivascular smooth muscles. Interestingly, trophoblast cells express neither receptor (82). This is consistent with recent findings showing that HLA-G5 dimers engage with ILT4 in airway smooth muscle (83). Although ILT2 may be the major binding protein for leukocytes, ILT4 has been suggested as the main receptor for HLA-G. Additionally supporting an alternative role of HLA-G is the observation that CD160, an sHLA-G1 receptor found on endothelial cells but not reported on trophoblast cells, inhibits angiogenesis by an apoptotic pathway (84).

Arguments for existence of HLA-G-isoform-specific functions include the observation that HLA-G2 and -G6 isoforms are expressed exclusively in the extravillous trophoblast cells distal to the villous, while HLA-G5 is ubiquitously expressed in syncytiotrophoblast cells (85, 86) and maternal blood (62). The major isoform-specific distinction supported by experimental studies is based on a functional concentration-dependency, which implicates HLA-G5 as a potentially more effective stimulator according to some studies (59, 87). HLA-G5 expression in the placenta seems to be sparse, at least at the mRNA level (50, 88, 89). Moreover, an isoform-specific role for HLA-G5 in relation to pregnancy was indicated in a recent study where HLA-G5 – while low or completely absent in maternal blood at term in normal pregnancies – was significantly increased in preeclampsia (62).

On the other hand, an argument for similar functions between different HLA-G isoforms is given by studies that describe women who are homozygous for the HLA-G*01:05N null allele (597DeltaC) and thereby lack expression of HLA-G1 and -G5. However, they have demonstrable HLA-G levels in the placenta and produce viable offspring, which is consistent with the idea that other isoforms – or other HLA class Ib molecules – provide functional compensation (90).

Most studies correlating circulating sHLA-G levels with preeclampsia have focused on the HLA-G1 and -G5 isoforms, which are nearly identical. Soluble HLA-G1 is derived from the full-length membrane-bound isoform containing a transmembrane cytoplasmic region, which may be cleaved by metalloproteases and shed from the cell surface (91, 92).

The soluble isoform HLA-G5 is generated due to a stop codon in intron 4 that prevents translation of the transmembrane cytoplasmic domain. Due to technical challenges, HLA-G5 has long been difficult to identify with specific monoclonal antibodies, but this issue seems lately to have been overcome (62). One argument for focusing on HLA-G1 is that it represents the most abundant isoform in the placenta. However, a functional distinction among HLA-G isoforms is plausible.

Human leukocyte antigen-G1 is by far the most abundant HLA-G mRNA isoform, both in preeclamptic placental biopsies and control placental biopsies, followed by G3, G5, G2, and G6 (35, 88). HLA-G4 and -G7 mRNA transcripts are not abundant in placentas (35). An *in vitro* functional study showed that the truncated isoforms G2, G3, and G4 are expressed on the surface of transfected cells and protect against NK and T cell-mediated cytotoxicity (93), and more recently a transfection study showed that HLA-G1 and HLA-G3 differentially increased HLA-E surface expression (94), indicating that the less abundant HLA-G isoforms are able to functionally compensate for HLA-G1 but with different effectiveness. However, low transcript abundance and/or protein expression in the placenta has prompted researchers to assume that these transcripts are less relevant, and *in vivo* relevance is typically only supported for G1 and G5. Interestingly, a study found that the HLA-G mRNA profile in term placental biopsies is shifted toward a higher frequency of HLA-G5 in preeclampsia (35), which is supported by higher HLA-G5 protein levels in maternal blood in preeclampsia compared to controls according to another, independent study (62).

HLA-G POLYMORPHISMS LINKED TO PREECLAMPSIA

A 14 bp insertion/deletion (ins/del) HLA-G polymorphism in the 3′ untranslated region (3′UTR) first described by Harrison et al. (95), is the best studied HLA-G polymorphism and has shown to influence HLA-G mRNA transcript size and stability (31, 88, 96–98).

Preeclampsia is a pregnancy condition unique to humans (99). The HLA-G 14 bp deletion allele is also unique to humans (100), and interestingly, this allele is more prevalent than the insertion allele (101, 102), raising the question whether the 14 bp deletion variant evolved evolutionary as a compensatory mechanism to counter pathological conditions only seen in humans. It is an intriguing thought that this theory could apply to preeclampsia.

Several studies have been undertaken in effort to clarify, whether the fetal HLA-G 14 bp ins/del genotype predisposes to preeclampsia in the mother (**Table 1**). One study found an association between the 14 bp insertion allele in offspring from primiparous preeclamptic women and controls (76, 103), which was supported by another study that further demonstrated a reduced level of the G3 isoform in placentas homozygous for the insertion in mild preeclampsia (73). Conversely, other studies found no association in offspring cases of preeclampsia, but noteworthy, included women with different degrees of preeclampsia (78, 104, 105). The discrepant results from different studies leave the influence of the fetal 14 bp ins/del genotype on the risk of developing preeclampsia controversial. However, published studies are characterized by small sample sizes, and larger scale studies are necessary. Furthermore, assessing combined mother–child HLA-G genotypes may be a better approach. The above mentioned case-control study of 155 family triads of mother, father, and offspring performed by Hylenius et al. showed an association of homozygosity for the 14 bp ins allele in offspring from primiparous women with severe preeclampsia (103), also supported by others (104, 106). Furthermore, the results suggested that a 14 bp ins/del contribution from the father influenced the risk of developing preeclampsia (103).

Table 1 | Summary of previous studies investigating possible associations between HLA-G polymorphisms/alleles and preeclampsia.

Study	Study size (case/control)	Parity subjects (case/control)	Subject	Association with preeclampsia
14 bp ins/del polymorphism				
Bermingham et al. (105)	68/74	Primiparous: all	Parents and offspring	No
O'Brien et al. (73)	7/11	ND	Offspring	Yes
Hylenius et al. (103)	57/98	Primiparous: 40/70 Multiparous: 17/28	Parents and offspring	Yes. Association in offspring and in mother/offspring pairs. Association with paternal inheritance (only significant in primiparous cases)
Vianna et al. (77)	157/162	ND	Mothers	No. A trend showing higher allele frequency of 14 bp del in mothers with preeclampsia
Moreau et al. (74)	36/60	ND	Offspring	Yes
Iversen et al. (78)	31/43	ND	Mothers and offspring	No
Zhang et al. (106)	120/158; 82/87; 67/75	ND	Mothers and offspring; parents; fathers and offspring	Yes. Association in offspring, in mother/offspring pairs and father/offspring pairs
+3187 polymphism				
Yie et al. (75)	29/15	Nulliparous	Offspring	Yes
G*01:04:xx				
Carreiras et al. (207)	104/29	ND	Mothers and offspring	Partly, when the allele was maternally inherited
Hylenius et al. (103)	57/98	Primiparous: 40/70 Multiparous: 17/28	Parents and offspring	No
G*01:05N				
Aldrich et al. (79)	57/36	ND	Offspring	No
Hylenius et al. (103)	57/98	Primiparous: 40/70 Multiparous: 17/28	Parents and offspring	No
Loisel et al. (111)	58/314	ND	Mothers	Yes
G*01:06				
Moreau et al. (74)	36/60	ND	Offspring	Yes
Tan et al. (104)	83/240	Primigravidas: 20/92 Multigravidas: 63/148	Mothers and offspring	Yes. Also when paternally inherited (multiparous women)

ND, not determined/not described.

A puzzling thing about the 14 bp ins/del polymorphism is the controversy about the abundance, and possibly, stability of the two alleles. In fact, as stated earlier, the mRNA deletion transcript has been shown to be more abundant than the mRNA insertion transcript. This fits well with studies showing higher sHLA-G levels when homozygous for the deletion, and importantly, with studies that support an association between the insertion allele, reduced HLA-G levels and preeclampsia (72, 88). A mechanism that might be compensatory to the lower HLA-G protein expression associated with the insertion allele exists: the presence of an alternative splice transcript produced from, and secondary to, the 14 bp insertion mRNA transcript. An *in vitro* study inducing a transcriptional stop with Actinomycin D treatment in JEG-3 and M8 cell lines, showed that the alternate transcript, characterized by removal of 92 bases from the insertion transcript, is more stable than the 14 bp insertion transcript (96). However, the −92 bp variant does not represent the majority of transcripts (88, 96), and its physiological relevance *in vivo* remains to be investigated. Complicating the matter of linking differential HLA-G protein expression to either the insertion or deletion mRNA transcripts, a recent study using a K562 cell line transfected with the insertion and deletion sequences separately, reported that membrane-bound HLA-G was higher in insertion transfectants, while sHLA-G was lower (98). Although these findings need verification, the study by Svendsen et al. indicates that the 14 bp ins/del genotype could have an impact on the soluble/membrane-bound HLA-G ratio, and could help clarify some of the conflicting results from preeclampsia studies. As a highly debatable explanation to the findings by Svendsen et al., ins/del HLA-G mRNA transcripts could have different structural features of the untranslated regions and coding sequences – a major and overlooked part in the control of mRNA translation. Relaxed secondary structures in UTRs are common for many mRNAs and characterize transcripts that are translated at a high rate (107). Conversely, more stable mRNA secondary structures containing e.g., hairpin loops, although exhibiting low turnover of mRNA, may be translated at a slower rate (107). The

secondary structures of the 14 bp ins/del mRNA transcripts have not been elucidated, but potential differences could explain why the insertion allele, albeit less abundant, is associated with high membrane-bound HLA-G. It does not, however, explain the lower sHLA-G levels associated with the insertion allele, which could be related to differences in the dynamics of HLA-G translation and post-translational mechanisms, e.g., shedding of HLA-G1 from the cell surface.

Several HLA-G SNPs are shown to be in strong linkage disequilibrium with the 14 bp ins/del polymorphism. These include a −725 SNP located in the promoter region previously shown to affect the transcriptional rates of HLA-G (108), and an array of SNPs in the 3′UTR downstream from the 14 bp ins/del that may act as microRNA sites and influence mRNA size and stability (109, 110). These include SNPs at +3142, +3187, and +3196 (109). Yie et al. reported that the +3187 SNP was associated with differences in mRNA stability, and that homozygous offspring were strongly correlated with severe preeclampsia (75). An association between HLA-G haplotypes and preeclampsia has been reported in some studies (76) but not in all (111). In the study by Larsen et al., a fetal HLA-G 3′UTR haplotype consisting of the 14 bp insertion sequence, a C at the +3010 SNP, a G at the +3142 SNP, an A at the +3187 SNP, and a G at the +3196 SNP was associated with the risk of developing severe preeclampsia in primipara (76). Interestingly, another fetal HLA-G 3′UTR haplotype with the 14 bp deletion, a G at the +3010 SNP, a C at the +3142 SNP, an A at the +3187 SNP, and a C at the +3196 SNP was much more frequent in the control group of primipara with no preeclampsia compared to the primipara group with severe preeclampsia (26.4% vs. 6.3%).

An HLA-G allele containing the 14 bp insertion, G*01:06, has been linked to preeclampsia in different studies [(74, 103, 104)]. The polymorphic 1 bp deletion of a cytosine residue at codon 130 which results in null allele (G*01:05N) described earlier, is associated with increased risk of preeclampsia in one study (111), and a reduced HLA-G level in maternal serum from normotensive African-American controls was observed in women bearing the null allele (111). However, this was not confirmed in another study (79). The 1597ΔC null mutation is rare in Europeans but more common in other global populations (79, 102, 112, 113), which emphasizes that ethnic difference or demographic factors should be considered in future study set-up, or when interpreting meta-studies on the association of HLA-G polymorphisms with preeclampsia.

Taken together, whether HLA-G genotypes and expression patterns might have a significant influence on the development of preeclampsia remains controversial. Further studies investigating an array of polymorphisms associated with preeclampsia in a larger scale are warranted, especially ones that set to investigate the mRNA and cell surface protein expressions simultaneously.

HLA-E ALLELIC POLYMORPHISMS

Two non-synonymous HLA-E alleles, E*01:01:xx:xx and E*01:03:xx:xx, have been identified (36, 114). They are distinguished by having either an arginine or a glycine at position 107 of the protein, and are so far the only HLA-E allelic variants to affect intracellular trafficking and surface expression (115). The frequency of these alleles is nearly equal in different populations, which indicates a balancing selection implying that a functional difference exists between the two alleles (116). One study showed that, although no difference was found between proteins in steady-state, the E*01:03:xx:xx allele exhibited higher surface expression than the E*01:01:xx:xx allele (117). In addition, the E*01:01:xx:xx and E*01:03:xx:xx alleles differ in their peptide binding affinities, E*01:03:xx:xx exhibiting a 10- to 100-fold higher affinity than E*01:01:xx:xx. A differential expression could have consequences for the inhibitory effect of HLA-E on NK cells and T cells. Indeed, the surface levels of HLA-E have been shown to affect inhibitory activity *in vitro* (22), and HLA-E polymorphisms have been associated with nasopharyngeal carcinoma (118), and recurrent spontaneous abortions (119). If HLA-E expression is hypothesized to be important in the context of pregnancy, an association of preeclampsia with HLA-E polymorphisms seems relevant to investigate. While no such study exists, one study showed that sera from early-onset, severe preeclamptic women could induce HLA-E surface expression in an EA.hy296 endothelial cell line *in vitro* (120). This upregulation was countered by addition of recombinant interferon (IFN)-γ. Soluble HLA-E was detectable in sera, but no difference was found between preeclamptic women and controls (120), indicating HLA-E surface expression on endothelial cells as a symptom of endothelial activation in preeclampsia, possibly mediated by other factors.

PATERNAL CONTRIBUTION TO PREECLAMPSIA

Preeclampsia is mostly considered a disease with maternal and fetal involvement, but there are some indications of paternal contributions as well. For example, preeclampsia is associated with an increased partner-specific CTL response in a mixed lymphocyte reaction (MLR), a finding that was not observed, when the MLR was performed with an unrelated partner, who fathered two previous uncomplicated pregnancies (121). This study indicates a maternal response directed against specific paternal antigens. In addition, the fetus is a natural allograft and the mother could carry killer immunoglobulin-like (KIR) allelic gene variants that mismatch with paternal HLA-C expressed on trophoblast cells. KIR receptors constitutes a highly polymorphic family of HLA class I receptors expressed on NK cells that is able to engage a cytotoxic NK cell response upon binding to HLA-C in the placenta. One study found that the combination of maternal KIR-AA and fetal HLA-C2, but not fetal HLA-C1, lead to increased risk of preeclampsia (122), but more studies are needed to confirm this.

A paternal contribution of the G*01:06 allele increases the risk of preeclampsia in multigravidae, at least according to one study (104). In the case-control study using family triads by Hylenius et al., an importance of paternal transmission of the 14 bp ins HLA-G allele to the offspring in the preeclampsia triads was observed, which supports the findings by Tan et al. (103). Another triad-study found that father/offspring pairs homozygous for the 14 bp del were significantly less frequent in early- compared to late-onset preeclampsia (106).

IMMUNE CELLS IN PREGNANCY AND PREECLAMPSIA

Initially, data from epidemiologic studies suggested that inappropriate activation of the immune system or immune maladaptation plays a critical role in the development of preeclampsia (123). *Ex*

vivo studies have since confirmed that immune cells play a central role in the pathophysiology of preeclampsia (124). An emerging theory is that a shift in immune cell functionality in uterine subpopulations reflects a maladapted maternal immune system, or a loss of tolerance mechanisms, which precedes the progress of placental oxidative stress and ischemia observed in preeclampsia (**Figure 3**) (125). Uncomplicated pregnancies are dependent on a delicate interplay between regulatory T cells and dNK cells that recognize and accept paternal antigens presented by the semi-allogenic fetus while simultaneously allowing vascular remodeling and placental growth (3). Although regulatory T cells and dNK cells have been the focus of most studies, it is likely that other immune cells like monocytes, DCs, and macrophages participate in upholding fetal tolerance (**Figure 3**). An aberrant/activated maternal immune system is associated with pregnancy complications like recurrent spontaneous abortions and preeclampsia. The expression of HLA-G receptors on decidual immune populations like NK cells, T cells, DCs, monocytes, and macrophages implicate HLA-G in the regulation of the uterine microenvironment (126, 127). However, direct effects of HLA-G on immune cell activation, recruitment, and function in the context of preeclampsia remain to be elucidated.

NK CELLS IN PREGNANCY AND PREECLAMPSIA

The early decidua is characterized by a unique population of dNK cells that constitute 50–90% of all leukocytes present in the uterine compartment in first trimester (1, 128). Compared to conventional pNK cells circulating the periphery, dNK cells exhibit a different repertoire of cytokines and receptors reflecting a more tissue-specific function (128, 129). dNK cells secrete vascular endothelial growth factor (VEGF), placental growth factor (PLGF), interleukin-8 (IL-8), and IFN-inducible protein-10 (IP-10) (129). In an *in vitro* migratory assay, dNK cell migration was correlated to the amount of the chemokines IL-8 and IP-10, when co-cultured with trophoblast cells (129), indicating a specific recruitment possibly mediated by the cognate CXCR1 and CXR3 chemokine receptors expressed on trophoblast cells. An aberrant production of cytokines and chemokines could have a great impact on the depth of trophoblast infiltration/invasion as seen in cases of preeclampsia.

In preeclampsia, pNK cells have en altered NKG2A and -C receptor expression (130), while dNK cells isolated from decidua at term show a higher expression of NKG2-associated receptor CD94 (131). HLA-G interacts with three inhibitory receptors, ILT2, ILT4, and KIR2DL4, as discussed earlier (132, 133). KIR2DL4 is not expressed on the surface of NK cells in steady-state, but surface expression can be induced after *in vitro* culture, and the expression and function is determined by genotype (134). KIR2DL4 seems not to be associated with preeclampsia. However, the presence of a fetal G*01:06 allele in combination with the maternal KIR2DL4*006 allele has been reported to be significantly associated with preeclampsia risk in multigravida pregnancies, suggesting a gene–gene interaction (135).

A recent study showed that a decidual population of $CD56^{high}CD27^{+}$ dNK cells accumulates in the first trimester of pregnancy and dampens the effects of inflammatory Th17 cells via IFN-γ secretion (136). In an $Nfil3^{-/-}$ mouse model of pregnancy where the mice lack NK cells entirely, and in an NK cell-depleted pregnant mouse group, they both demonstrated a significantly higher percentage of Th17 cells (136). In humans, the $CD56^{high}CD27^{+}$ dNK cells and their supernatants inhibited the expansion of Th17 cells – an effect reversed by addition of neutralizing anti-INF-γ (136).

There is still some controversy about NK numbers in preeclampsia. In peripheral blood, the prevalence of NK cells differ between preeclamptic cases and controls in some studies (137) but not in all (138). However, it is more likely that a difference should be found in the uterine environment within the dNK population. HLA-G has been shown to inhibit NK lysis in HLA-G transfected cell lines in a concentration-dependent manner (91, 139, 140), and the physiological relevance of this effect was demonstrated by a study showing that *ex vivo* NK cell functional responses to HLA-G differ between peripheral blood and decidua, where dNK cells were refractory to stimulation compared to pNK cells (141), further supporting the important role of HLA-G in sustaining pregnancy and its influence on dNK cells.

T CELLS IN PREGNANCY AND PREECLAMPSIA

$CD4^{+}$ T cells, or T helper (Th) cells, can be subgrouped on the basis of their cytokine profile into Th1 and Th2 T cells. According to an early theory, successful pregnancy is biased toward a Th2 humoral response characterized by release of immunoregulatory cytokines such as IL-10 and TGF-β (142). Cytokines and other soluble factors like progesterone and indoleamine 2,3-dioxygenase (IDO) have been proposed to act on the Th1/Th2 balance, and a shift toward a Th1 response has been hypothesized to occur in preeclampsia (143). Furthermore, when cell lines are transfected with membrane-bound HLA-G1 and co-cultured with decidual or uterine mononuclear cells, several studies have observed a decrease in TNF-α and an increase in IL-10 (144–146). So, it seems plausible that HLA-G can mediate a shift from a proinflammatory Th1 cell-mediated response toward a Th2 response inducing tolerance. However, pregnancies in Th2 knockout mice proceed without complications, indicating how a higher complexity of the cytokine network in the placenta or other mechanisms may add to fetal tolerance (147). In the slipstream of the Th1/Th2 paradigm, a new has emerged: the Th1/Th2/Th17/T regulatory cells (Tregs) paradigm (148). Th17 cells are immunoregulatory cells that play a critical role in induction of inflammation and have been linked to autoimmune diseases and tissue transplant rejection, and possibly to pregnancy complications (148, 149). The Th1/Th2 balance and the capacity of Th17 cells to produce cytokines are modulated by TGF-β and IL-10 or by cell–cell interaction with $CD4^{+}CD25^{high}$ Tregs, described later (148). Although little is known about Th17 cells, recruitment and expansion of this subset seem to be promoted by proinflammatory cytokines like IL-1β and IL-6, and the highest percentage exists in the first trimester (136, 150). Interestingly, a novel role for Th17 cells in trophoblast proliferation and invasion was recently indicated (151). In this study, Th17 cells were recruited from the periphery in early pregnancy by CCL2-secreting decidual stromal cells, and inhibited apoptosis of trophoblast cells via an IL-17-dependant mechanism (151), suggesting a vital role for Th17 cells in normal pregnancy. However, an exaggerated production of IL-17 could have unwarranted

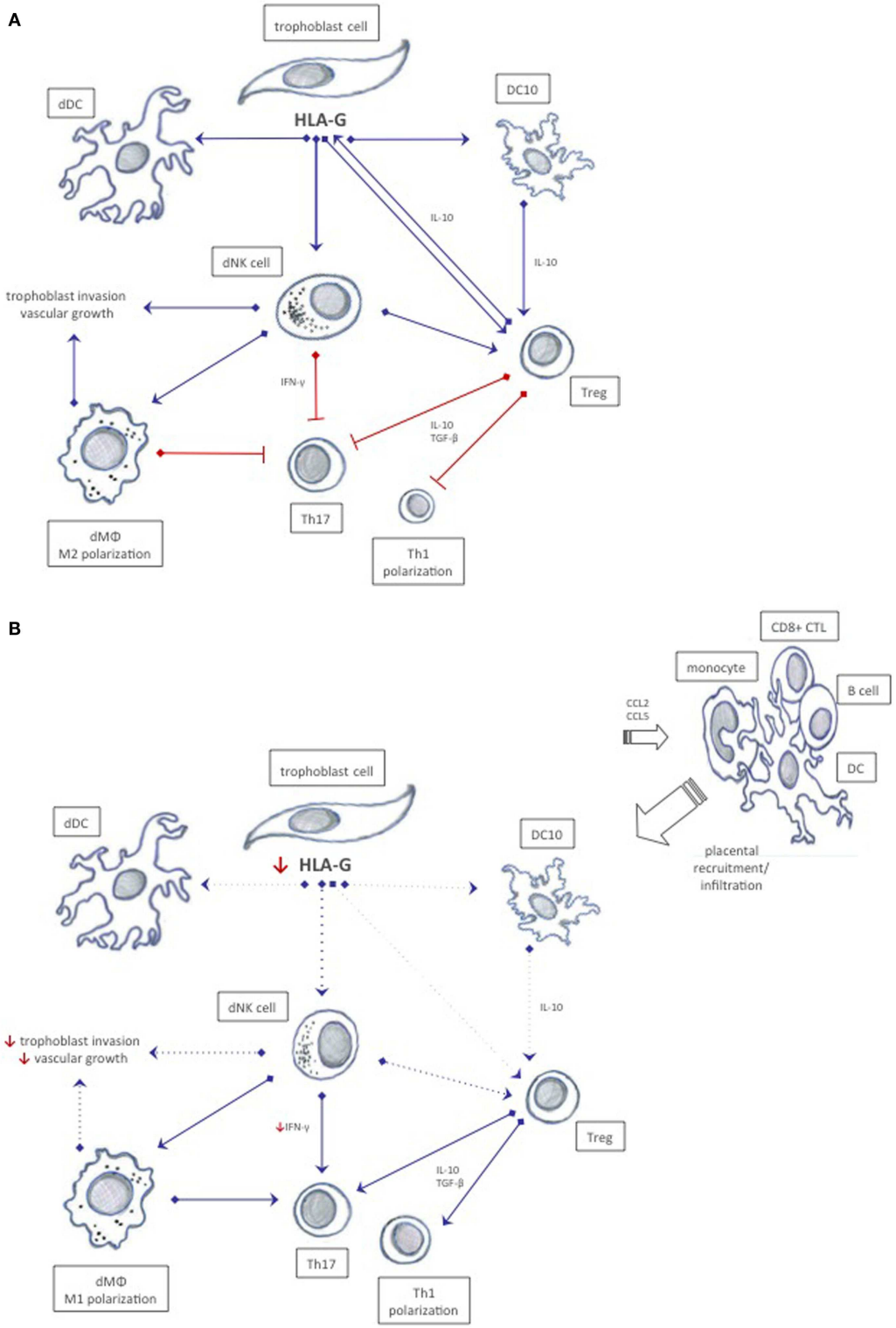

FIGURE 3 | Possible immune interactions between HLA-G and decidual immune cells in normal pregnancy and in preeclampsia. **(A)** In normal pregnancy, HLA-G expression is believed to ensure a tolerogenic uterine environment by inhibiting cytotoxicity, inducing release of anti-inflammatory cytokines, and by promoting proliferation of tolerogenic decidual immune cells that mutually stimulate each other to sustain tolerance. (**B**) In preeclampsia, a possible reduced soluble and membrane-bound HLA-G expression in trophoblast cells may affect immune cells expressing cognate receptors, and thus enhance immunity rather than tolerance. Increased CCL2 and CCL5 chemokines and inflammatory cytokines may recruit activated immune cells from the periphery further abrogating the tolerogenic milieu. Dotted lines represent reduced stimuli.

consequences. In preeclampsia, the prevalence of IL-17-producing CD4, CD8, and NK cells is elevated in peripheral blood compared with normotensive pregnant women (152), and the Th1/Th2 and Th17/Treg balance is shifted toward increased immunity determined by a Th1 response, elevated Th17 T cells and reduced Treg numbers, possibly affecting the uterine microenvironment conjointly with dNK cells (152). Furthermore, in preeclampsia, monocytes produce IL-1β and IL-6 that mediate terminal differentiation of Th17 cells possibly causing an exaggerated inflammatory response, which may consequently reduce Treg abundance and function (148).

Classical Tregs constitute a subset of T cells with suppressive properties. They are capable of inhibiting redundant immune responses in a very potent fashion, and aid in maintaining antigen-specific T cell tolerance important in pregnancy (153). In mice, the Treg population increases markedly during early gestation (154), and a similar effect is observed in pregnant women with a peak during the second trimester and a decline in numbers postpartum (155). Adoptive transfer studies in mice have demonstrated the physiological importance of $CD4^+CD25^+$ Tregs in pregnancy (156, 157). For example, when a total pool of $CD4^+$ T cells is depleted of the $CD4^+CD25^+$ Treg subpopulation and transferred into pregnant mice deficient of T cells, allogeneic mice fetuses are rejected, while syngeneic fetuses remain unaffected (156). In humans, isolated $CD4^+CD25^+$ cells are able to suppress autologous $CD4^+$ T cells stimulated by allogeneic DCs (155), and to inhibit IL-4 secretion against paternal but not unrelated allo-antigens *in vitro* (158).

In preeclampsia, the number of $CD4^+CD25^{high}$ Tregs is decreased in peripheral blood (150, 159) as well as in term placentas (160). However, not all studies confirm these findings (161). Assessing Treg numbers based on the co-expression of CD4 and CD25 solely has been questioned, and with the identification of the transcription factor forkhead box P3 (Foxp3), a more reliable marker for Tregs was found. In support of the findings associating $CD4^+CD25^{high}$ Treg numbers with preeclampsia, circulating levels of $CD4^+CD25^{high}FoxP3^+$ Tregs are decreased in preeclamptic women (138, 150, 162). Highly relevant in the context of identifying Tregs, a study by Santner-Nanan et al. compared $CD4^+CD25^{high}$, $CD4^+CD127^{low}CD25^+$, and $CD4^+Foxp3^+$ cells from preeclamptic women and controls, and found that the frequency of Tregs in all three "groups" was reduced in preeclamptic women (150). However, *ex vivo*-sorted Tregs had preserved their suppressive properties implying that a reduced number of Tregs rather than a lack of suppressive function occurs in preeclampsia (150). Furthermore, Santner-Nanan et al. also reported that the ratio of Tregs to Th17 was significantly increased in normal pregnancy but not in preeclampsia (150). The conversion of Tregs to Th cells has been documented in both mice and humans (163), and lately, this conversion has been suggested to occur as a part of the pathophysiology of preeclampsia (164).

Subsets of non-conventional Tregs more recently described include HLA-G-positive Tregs and tolerogenic $CD4^{low}$ and $CD8^{low}$ T cells. $CD4^+HLA\text{-}G^+$ Tregs lack classical Treg markers and are characterized by the constitutive expression of HLA-G (165). Functional characterization indicates that the suppressive properties of this subset rely on the immunoregulatory properties of HLA-G, which enables $CD4^+HLA\text{-}G^+$ Tregs to inhibit bystander immune activations by direct cell–cell interaction (166). In normal pregnancy, the prevalence of $CD4^+HLA\text{-}G^+$ T cells is high in decidua (167), while a recent study showed that the expansion of the HLA-G-positive T cell subset is impaired in preeclampsia (168). Furthermore, it was indicated that classical Foxp3 Tregs and $CD4^+$ T cells acquire HLA-G from monocyte-derived DCs via the process of trogocytosis where membrane fragments are dispatched from the DCs and transferred to the surface membrane of leukocytes (168).

Non-conventional regulatory T cell subsets, which are distinguished by lower surface expression of CD4 and CD8, have been identified in a transplantation study (169). Interestingly, regulatory activity by these $CD3^+CD4^{low}$ and $CD3^+CD8^{low}$ T cells was induced by soluble HLA-G and/or HLA-G1-expressing DCs (169). While these subsets have not been investigated in relation to pregnancy and preeclampsia, it is reasonable to believe that their suppressive activities mediating allograft acceptance could be relevant in a pregnancy setting, and match a hypothesis where HLA-G in the placental microenvironment influences the phenotype and function of local T cells.

B CELLS IN PREGNANCY AND PREECLAMPSIA

In normal pregnancy, the almost complete absence of B cells in decidua suggests that no B cells are localized, recruited nor activated by fetal allo-antigens (170). Like for other leukocytes, ILT2 is also expressed on the surface of B cells (133). A recent study in mice showed that ILT2–HLA-G engagement on B cells inhibits both naïve and memory B cell function *in vivo* and *in vitro* at the level of proliferation, differentiation, and Ig secretion (171). The inhibitory effects of HLA-G were independent of the form of B cell activation, suggesting that the presence of T cells could be less important. Moreover, HLA-G mediates phenotypic and functional downregulation of CXCR4 and CXCR5 chemokine receptors on germinal center B cells (171). *In vivo* support for HLA-G as a negative B cell regulator was provided in a xenograft mouse model, which showed a significantly altered antibody secretion pattern (171).

A specific subpopulation of $CD19^+CD5^+$ B cells that secrete autoantibodies is identified in preeclampsia (172), indicating a dysfunctional immune regulation or B cell activation mediated by fetal allo-antibodies. Furthermore, a recent study on the interactions between Tregs and B cells indicated that a negative correlation between Tregs and memory B cells exists in peripheral blood of preeclamptic women (173). Although the Treg population was reduced numerically, interestingly, the suppressive effects on autologous B cell proliferation were unaffected (173).

DCs AND MONOCYTES IN PREGNANCY AND PREECLAMPSIA

In the periphery, DCs play a crucial role in linking innate and adaptive immunity by virtue of their exceptional ability to capture, process and present antigens to naïve T cells, and by mediating cross-talk with a broad range of immune cells. In the decidua, however, DCs are scarce, making up app. 1% of the decidual immune population (174). A decidual subset of tolerogenic DCs that express high levels of HLA-G was recently identified. These cells spontaneously secrete high amounts of IL-10 and are named

DC-10. DC-10s can be differentiated *in vitro* from peripheral blood monocytes with proinflammatory cytokines including granulocyte macrophage-colony stimulating factor (GM-CSF), IL-4, and IL-10 (175, 176). DC-10s are able to induce immunosuppressive $CD4^+$ T cells, and their potency to do so was demonstrated when a single stimulation of $CD4^+$ T cells with DC-10 promoted a fraction of anergic T cells that contained up to 15% of already differentiated inducible Tregs (177, 178). In a transplantation study, engagement of ILT4 on DCs by HLA-G-tetramers resulted in maturation/activation, and prolongation of allogeneic graft survival (179). The local milieu in the placenta is likely to moderate the function and activity of local immune cells, but evidence points to a systemic effect as well. As an example, the TLR expression and cytokine profile in circulating DCs is dysregulated in preeclampsia, and they demonstrate a weaker response to TLR-stimulation compared with controls (180). In addition, a recruitment of mature and immature DCs to the decidua is observed in preeclampsia (181).

MACROPHAGES IN PREGNANCY AND PREECLAMPSIA

The majority of decidual leukocytes in the first trimester consist of NK cells and second to these are the tissue-specific macrophages, which make up 20–25% (1, 182, 183). These decidual macrophages are characterized by their immunosuppressive abilities, and two different subsets have so far been identified in the feto-maternal interface; single-positive $CD14^-CD68^+$ and double-positive $CD14^+CD68^+$ macrophages (183, 184), which, however, still need to be characterized. The abundance of decidual macrophages in the first trimester indicates vital tissue-specific functions and thus, an important role in maintenance of normal pregnancy (185). In support of this notion, they are co-localized with evading trophoblast cells and found in the vicinity of spiral arteries, where they are believed to modulate the immune response to pathogens, to mediate vascular remodeling and promote trophoblast invasion (186, 187).

Studies have shown that decidual macrophages may contribute to the development of preeclampsia, primarily by a shift in the cytokine profile leading to poor spiral artery remodeling (186, 188, 189). In addition, increased macrophage infiltration in the decidua is observed in preeclampsia (181). Upon proinflammatory stimuli, monocytes, macrophages, and DCs are recruited to the decidua by specific chemokines, especially CCL2 and CCL5 (190). In accordance with the observed increase in infiltration of macrophages in preeclampsia, CCL2 and CCL5 expression is increased in preeclamptic decidua (181). An excessive release of GM-CSF in preeclamptic placentas contributes to macrophage differentiation, further increasing the production of proinflammatory cytokines (191). TNF-α, PAI-1, and inducible nitric oxide synthase secreted by decidual macrophages inhibit trophoblast invasion and migration, and thus, spiral artery remodeling (192, 193). Macrophages regulate angiogenesis by secreting VEGF, which binds to fms-like tyrosine kinase-1 (Flt-1), both of which are dysregulated in preeclampsia (194). In addition, decidual macrophages express IL-2 and ILT4, and HLA-G may thus regulate their functional properties (82, 133). This was indicated in a study showing that upon co-culture with transfectants expressing HLA-G homodimers, cytokine production was greatly increased in CD14-positive decidual macrophages (195).

Similar to the concept of Th1/Th2 polarization in effector T cell function, macrophages are characterized according to their effector phenotype and cytokine repertoire, subgrouping them into classical activated macrophages, M1, or alternatively activated macrophages, M2 (185, 196). M1 secretes IL-12 and TNF-α upon stimuli from LPS or IFN-γ, while M2 upon stimuli with IL-4 secretes the tolerogenic cytokines IL-10 and IL-13 (196). However, the existence of a M1/M2 balance in the placenta and the possible implication of this in preeclampsia still need to be investigated. Recently, increased numbers of $CD14^+$ cells were identified in preterm preeclamptic placentaes, and – supporting the importance of a M1/M2 balance – a lower CD163+/CD14+ ratio (M2), and a higher CD209+/CD14+ ratio (M1) were observed in preeclamptic placentas compared with controls (197).

CONCLUSION AND PERSPECTIVES

A vast amount of evidence highpoint an involvement of immune cell populations in pregnancy, and preeclampsia is indeed characterized by an aberrant immune system. While studies show that a broad continuum of immune cells are affected, or more specifically activated, to induce unwanted immunity rather than tolerance against the semi-allogeneic fetus in preeclampsia (123, 125), an important question is whether this occurrence precedes the abrogated placentation and endothelial activation and inflammation observed. Associations between cytokine production and repertoire and vascularization support this theory. Given that immune maladaptation is an early event in the etiology of preeclampsia, we speculate whether one or few immune populations are responsible for altering the local, and possibly systemic, cytokine milieu resulting in a more general change in the function and abundance of other immune cells not typically present in the uterine environment – like B cells and DCs. This would require an immune population that acts as a "linker" between the innate and adaptive immune system, and in addition, an immune population with specific receptors for HLA class Ib expressed by trophoblast cells. A simple answer would be that the dNK cells constitute this "linker" population. However, the explanation may not be that straightforward. Transplantation studies have offered new insights into tolerance mechanisms provided by other immune cells. These include tolerogenic $CD4^{low}$ and $CD8^{low}$ T cells, HLA-G-expressing T cells and HLA-G-expressing DCs, and in this context, the key perspective may not be abundance, since these cells are present in low numbers, but instead tolerance potency.

Immunological memory is another important aspect that needs to be addressed. According to epidemiological findings, primiparity is the strongest risk factor for preeclampsia occurring in up to 75% of cases (123, 198, 199). Furthermore, in multiparas, a change of partner increases the risk to the level of the first pregnancy, although the idea of a partner-specific effect has been challenged as merely a consequence of a long interval since the last pregnancy, which is also a risk factor of preeclampsia (123, 200). Memory T cells, which induce tolerance to paternal antigens, may explain these epidemiological findings (123, 201). In mice, an accelerated expansion of maternal $CD4^+Foxp3^+$ Tregs specific for fetal antigens support that multiparas are protected by a regulatory memory

for fetal antigens (201, 202). Recent data have also revealed that exposure to seminal fluid may induce paternal-specific tolerance (203) and short cohabitation, use of condoms and insemination with donated spermatozoa are risk factors of preeclampsia (123) suggesting that absence of semen exposure could fail to induce adequate tolerance, resulting in preeclampsia. Paternal allo-antigens and soluble factors like TGF-β, prostaglandins and HLA-G are present in seminal fluid, and could well prove important for Treg expansion, differentiation, and immunological memory (42, 203, 204).

While some decidual cell populations, including Tregs and DC-10, may be licensed for tolerance induction or immune modulation even before conception, it is likely that their differentiation and proliferation is co-dependent on the HLA class Ib molecules both in the initial stages and throughout the course of pregnancy. Indeed, reviewing the idea that a "linker" is needed to affect vascularization and different immune populations simultaneously, and given that aberrant dNK function and numbers are not sufficient to account for the pathophysiology observed in preeclampsia alone, this "linker" may well be represented by HLA-G. The low expression of HLA-G in preeclampsia, and the sum of *in vivo* and *in vitro* studies showing a broad array of immune interactions/cross-talk with, and through, HLA-G and cognate receptors, supports this hypothesis. Why is it then that genetic variation in HLA-G, although nicely shown to influence the transcription and expression of HLA-G *in vitro* still lacks strong association with preeclampsia in some studies? One answer could be that we still lack knowledge of some fundamental aspects of HLA-G biology. What significance can be attributed the alternative splicing of HLA-G mRNA transcripts, and what are their isoform-specific functions? What is the significance of higher-order HLA-G- and HLA class Ib protein-assemblies and HLA-G-positive exosomes, and are they detected with conventional assays? These questions have not been actively addressed so far, and some investigators have indicated that due to the low abundance of G2 and G4-7 mRNA transcripts in the placenta, the physiological effects are provided essentially by HLA-G1 (89, 205). Conflicting with this notion is the immune regulatory capacity of the HLA-G5 isoform that, despite the fact that this transcript is scant in the placenta, has proven potent as an immunosupressor in several studies (59, 87). Another explanation for the lack of association between HLA-G genetics and preeclampsia could be due to different methodological approaches, small-scale studies on different ethnic populations, or explained by the fact that preeclampsia is a multifactorial disease that presents with different degrees of severity, and additionally, in an early- and late-onset form, possibly with distinct etiologies (206).

The involvement of HLA class Ib in preeclampsia remains controversial. The function of HLA-F is unknown, and despite findings showing that HLA-E is involved in immune suppression, soluble HLA-E levels seem not associated with preeclampsia. More studies, not only focusing on the two non-synonymous alleles classically investigated, are needed. The functional significance of HLA-G in pregnancy is more complex than HLA-E and -F. However, the high expression of HLA-G compared to HLA-E and -F in the placenta, and the presence of HLA-G in semen, the endometrium, in the matured cumulus–oocyte complex, as well as the rise in soluble level after conception imply an important role for HLA-G in early pregnancy (42, 45). Furthermore, the dual role of HLA-G in immune regulation and spiral artery remodeling underscores its importance and multifaceted activities. So far, aberrant HLA-G expression is a likely contribution to preeclampsia. As isoform-specific functions are possible to exist, more studies on this are highly warranted.

The etiology of preeclampsia is multifactorial and involves interactions between immune cells and HLA class Ib molecules, possibly as early as during conception or embryogenesis (46). And since an interaction in essence is a mutual or reciprocal action or influence, any one unfavorable genetic or immunological contribution either from the mother, the father, or the fetus, may tip the steady-state immune balance in a direction unfavorable for pregnancy – consequently leading to preeclampsia. Further in-depth investigation will help to elucidate the precise mechanism of HLA class Ib receptor recognition and signaling, and the role of these interactions in successful reproduction.

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HLA-G orchestrates the early interaction of human trophoblasts with the maternal niche

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Extravillous trophoblasts (EVTs) play a central role in educating maternal leukocytes, endometrial stromal and endothelial cells to generate a receptive decidual microenvironment tailored to accept the semi-allogeneic fetus. HLA-G, a non-classical HLA class I molecule endowed with immune-regulatory functions, is primarily expressed on EVTs lining the placenta and on the naturally occurring tolerogenic dendritic cells, named DC-10, which are enriched in the human first trimester decidua. Decidual DC-10 are involved in HLA-G-mediated tolerance at the maternal–fetal interface. EVTs not only establish a tolerogenic microenvironment through the interaction with maternal innate and adaptive cells but also orchestrate placenta vascular and tissue remodeling, leading to a successful pregnancy. Here, we discuss the potential implications of the HLA-G-mediated cross-talk among the cells present at the maternal–fetal interface, and its role in maintaining a positive relationship between the mother and the fetus.

Keywords: HLA-G, trophoblasts, dendritic cells, IL-10, T regulatory cells, vascular remodeling

INTRODUCTION

The maternal–fetal interface is composed of fetal trophoblasts intermingled with maternal leukocytes, stromal, and endothelial cells that comprise the decidua. During implantation, trophoblasts, derived from the trophoectoderm surrounding the blastocyst, differentiate into the syncytiotrophoblasts that infiltrates the endometrium, and the cytotrophoblasts at the embryo side. The layer of syncytiotrophoblasts in contact with the decidua represents the extravillous trophoblasts (EVTs) (**Figure 1**). EVTs orchestrate bi-directional cross-talk between the mother and the fetus by providing structural and biochemical barriers, serving as an endocrine organ that support and regulate placental and fetal development and growth, and modulating maternal innate and adaptive immune responses (1).

The evidence that, after embryo implantation, defective development and function of EVTs can lead to fetal loss and pregnancy-associated pathological conditions, including pre-eclampsia and intrauterine growth restriction (2–4), sustains the important role of EVTs in orchestrating the decidual modification for successful pregnancy. The expression of HLA-G, a non-classical HLA class I molecule, on EVTs contributes to trophoblast invasiveness, decidual cell differentiation, vascular remodeling, and maintenance of a local immunosuppressive state. A proper understanding of regulatory mechanisms that control EVTs interaction with the maternal niche is a critical issue in reproduction.

STATE OF THE ART

HORMONAL REGULATION AT THE MATERNAL–FETAL INTERFACE

The endometrial microenvironment, constituted by luminal and glandular epithelial cells, stromal cells, fibroblasts, vascular smooth muscle cells, endothelial cells, leukocytes, endometrial stem cells, and dynamic leukocyte populations, undergoes cyclical changes regulated by sex hormones. In the absence of pregnancy, the endometrium is sloughed off at menstruation. In the post-menstrual proliferative phase, under estradiol stimulation, it undergoes rapid regeneration into a fertile soil capable to accept the embryo (5). During the secretory phase, the blood flow increases, the arteries branches, and the glands enlarge and start to secrete fluids rich in glycogen used by the embryo as an energy source in its early stages of growth. These processes are driven by the post-ovulatory rise of progesterone that inhibits the pro-proliferative effect of estradiol and, in mammals, induces a radical transformation of the endometrium (pre-decidualization) that heralds the limited period of endometrial receptivity, ("implantation window") during which embryo attachment can take place (6). Pre-decidualization is primarily defined by the transformation of endometrial stromal cells into secretory epithelioid-like decidua cells and is characterized by massive influx of maternal innate immune cells and vascular remodeling (7).

In the presence of the embryo, the human chorionic gonadotropin (hCG) sustains the full decidualization of the

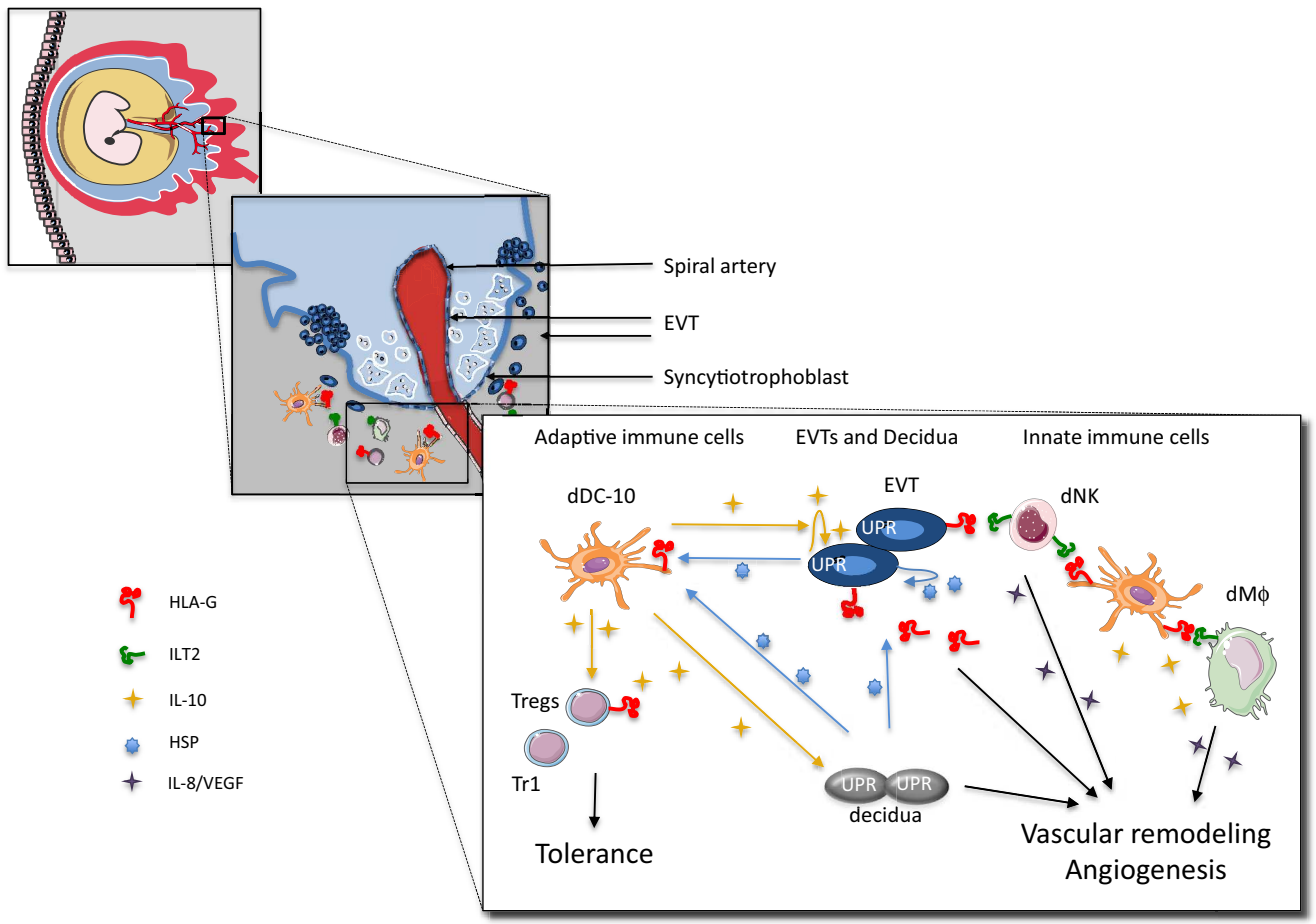

FIGURE 1 | Proposed model for cross-talk among embryo trophoblasts, decidual leukocytes, and stromal cells at the maternal–fetal interface in human first trimester pregnancy. EVTs express and secrete HLA-G, and release IL-10 (and TSLP), which instruct dAPCs to become tolerogenic DC (i.e., dDC-10 or TSLP-modulated dDC) secreting IL-10 and promoting the induction of a variety of Tregs (i.e., Tr1 cells, $CD4^+CD25^+FOXP3^+$ Tregs, and $CD4^+HLA\text{-}G^+$ Tregs). Induced Tregs inhibit effector T cells, and, via IL-10 secretion, promote HLA-G expression on EVTs. EVTs via HLA-G directly promote dNK cell activation and the release of angiogenic factors. dDC-10 is $HLA\text{-}G^+$ and can interact with either dNKs or dMΦ via ILT2, and promote their activation and pro-angiogenic effects. dDC-10 themselves secrete also pro-angiogenic factors supporting neo-vascularization. HSPs secreted by the maternal cells and trophoblasts contribute to the regulation of HLA-G expression on dAPCs and EVTs. Finally, IL-10 modulates the UPR pathway and regulates vascular uterine remodeling by $HLA\text{-}G^+$ EVTs.

endometrium via stimulation of progesterone production. hCG is the most specific embryo-derived signal observed in humans and the *hCG* gene is transcribed as early as the two-cell stage (8, 9). Being released before embryo implantation, hCG also acts on endometrial cells in a paracrine way by inducing their differentiation characterized by secretion of prolactin, leukemia inhibitory factor (LIF), and IL-6 (10, 11). Furthermore, hCG promotes angiogenesis by increasing vessel sprouting of endothelial cells and secretion of vascular endothelial growth factor (VEGF) (12, 13). The immunomodulatory properties of hCG are multiple (13): it regulates decidual natural killer (dNK) cell proliferation, contributing to the remodeling of decidual spiral arterioles (14, 15); it induces CXCL8 production by monocytes (16); it influences tolerogenic dendritic cells (DCs) proliferation and differentiation (17); and it contributes to recruitment of T regulatory cells (Tregs) (18).

The pre-ovulatory peak of estrogen is important for proliferation of the uterine epithelium in preparation for implantation, while rising progesterone after ovulation is required for implantation of the embryo and decidual differentiation. Together with hCG, progesterone and estradiol are also essential for the programing of a local tolerogenic environment (19). Progesterone polarizes T-cell responses toward an anti-inflammatory phenotype, favoring T(helper)h2 while dampening Th1 and Th17 cells, and inducing Tregs via thymic stromal lymphopoietin (TSLP) (20–22). The increased concentration of progesterone at the maternal–fetal interface may play a role in regulating HLA-G gene expression (23). Progesterone induces up-regulation of HLA-G in primary cultures of first trimester cytotrophoblasts through the binding to an alternative progesterone response element in the *HLA-G* promoter (24).

Estradiol regulates the immune system by affecting T and B cells, and down regulating NK cell cytotoxicity (25). Interestingly, estradiol helps to regulate fetal tolerance during pregnancy by expanding Tregs and their suppressive function (26, 27).

Dendritic cells, by expressing specific receptors, are susceptible to stimulation with hCG, progesterone, and estradiol. Pregnancy hormones can either activate or reduce the stimulatory activity of monocyte-derived DCs. Consistent up-regulation of IL-10 production by human DCs has been observed upon stimulation with pregnancy hormones [as reviewed in Ref. (28)].

HLA-G-EXPRESSING TROPHOBLAST AT THE MATERNAL-FETAL INTERFACE

HLA-G has well-recognized immunomodulatory activities, is low polymorphic [reviewed in Ref. (29)], and has limited tissue distribution [reviewed in Ref. (30)]. HLA-G was the first HLA class I molecule identified on EVTs (31). EVTs, forming the placental interface with the maternal systemic circulation, do not express HLA class I, but as they differentiate to invade the decidua and contact maternal decidual leukocytes, they begin to express HLA-G (32). All EVTs, syncytiotrophoblasts (33), interstitial and endovascular trophoblasts, and placental bed giant cells are HLA-G positive [reviewed in Ref. (34)].

By alternative splicing of the primary transcript, four membrane-bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7) isoforms can be generated [reviewed in Ref. (35)]. In addition, a soluble isoform, named shed HLA-G1, is released after proteolytic cleavage of the membrane-bound HLA-G1 by metalloproteinases (36, 37). Through the interaction with the inhibitory receptors immunoglobulin-like transcript (ILT)2 and ILT4, and the killer immunoglobulin-like receptor (KIR)2DL4, HLA-G regulates innate and adaptive immune responses and participates in promoting tolerance [reviewed in Ref. (38)].

During the last decade, it has become evident that the expression of HLA-G on EVTs is not primarily involved in protecting the fetus from the attack by maternal cells, but it plays an important role in tissue remodeling. HLA-G expressed or secreted by EVTs controls their decidual and endovascular invasion. EVTs can express membrane-bound or shed HLA-G1, and soluble HLA-G2, -G5, and -G6 (39–43) (**Table 1**). Studies in placental sections demonstrated that β2m-bound HLA-G is expressed by all EVTs, whereas more distal EVTs at the invasion front express the free heavy chain (FHC) HLA-G (40). It has been proposed that the selective expression of FHC–HLA-G, which is not recognized by ILT2 (44), may limit the inhibition of dNKs while allowing these cells to secrete factors required for successful pregnancy. *In vitro* studies showed that treatment of primary trophoblasts with HLA-G5 stimulates cell invasion and increases the production of metalloproteinases and urokinase, known to remodel the endometrial extracellular matrix (45, 46). Moreover, the interaction between HLA-G on EVTs and dNKs leads to CXCL8 and CXCL10 secretion that in turn, via stimulation of CXCR1 and CXCR3, promote EVTs invasiveness (14). Thereby, HLA-G-expressing EVTs regulate decidual invasion in both autocrine and paracrine manner.

The presence of soluble HLA-G in embryo culture supernatants positively associates with embryo implantation (58–60). The interaction of HLA-G with ILT2 on endometrial stromal cells

Table 1 | Expression pattern of HLA-G-related molecules on cells at the maternal–fetal interface.

Cell types		HLA-G isoforms (reference)	HLA-G receptors (reference)		
			ILT2	ILT4	KIR2DL4
EVTs		HLA-G1 (39, 40) shed HLA-G1 (40, 42) HLA-G2 (42) HLA-G5 (41) HLA-G6 (43)	Neg (47)	Neg (47)	n.t.
Syncytiotrophoblasts		HLA-G5 (33)	Neg (47)	Neg (47)	n.t.
Endothelial cells	Maternal endothelium	n.t.	Neg (47)	Neg (47)	n.t.
	Fetal vessels	n.t.	Neg (47)	n.t.	n.t.
Endometrial stromal cells		n.t.	Pos (47)	Neg (47)	n.t.
dNK	Total $CD56^+$	Neg (48)	Pos^{low} (49)	Neg (49)	Pos (49–51)
$CD4^+$	Total $CD4^+$	n.t.	Pos (52)	n.t.	Pos (52)
	$CD4^+HLA\text{-}G^+$	HLA-G1 (53, 54)	n.t.	n.t.	n.t.
$CD8^+$	Total $CD8^+$	n.t.	n.t.	n.t.	n.t.
	$CD8^+HLA\text{-}G^+$	HLA-G1 (53)	n.t.	n.t.	n.t.
Macrophages	$CD14^+CD163^+$	Neg (55)	Pos (50, 56)	Pos (50, 56)	n.t.
DCs	DC-$SIGN^+$	HLA-G1 (57)	n.t.	Pos (57)	n.t.
	DC-10	HLA-G1 (53)	Pos (53)	Pos (53)	n.t.

The indicated markers have been tested on cells at the maternal–fetal interface and demonstrated to be expressed (Pos) or not (Neg).
The indicated markers have not been tested yet (n.t.).

(47) might contribute to the remodeling of uterine vascularization, and EVT migration and invasion (61, 62). Moreover, the interaction between EVTs and resident dNKs that express both ILT2, although at low levels, and KIR2DL4 (49, 50) guarantees the correct arterial remodeling (**Table 1**). In contrast to peripheral NK, dNKs are poorly cytotoxic and secrete, in addition to IFN-γ, the pro-angiogenic factors VEGF, placental growth factor (PLGF), angiopoietin 1 and 2, and transforming growth factor (TGF)-β1 (14, 63–66). These molecules promote the uterine vascular changes necessary for maximizing maternal blood flow through the placenta. Moreover, the perivascular localization of dNKs in a microenvironment enriched in EVT-derived soluble HLA-G enables the formation of uterine spiral arteries (67). *In vitro* studies show that the interaction between HLA-G5 and shed HLA-G1, with KIR2DL4 in the early endosome of activated NKs promotes phenotypical and physiological changes leading to cellular senescence, which sustains the secretion of pro-angiogenic mediators (49, 51). Exposure of macrophages (MΦ) isolated from the first trimester decidua to HLA-G-expressing cell lines induces secretion of IL-6, CXCL8, and TNF-α that activate dNK-mediated vascular remodeling (50). Hence, the cross-talk between HLA-G-expressing/secreting EVTs and decidual innate cells coordinate the tissue remodeling necessary for a successful pregnancy.

It cannot be overlooked that EVTs-derived HLA-G also induces tolerogenic immune responses leading to semi-allogeneic fetus acceptance. In addition to dNKs, MΦ, DCs, effector and regulatory T cells, and B cells infiltrate the decidua (52, 68, 69), which are likely to be important determinants in tolerance induction. dMΦ are characterized by low levels of CD86 coupled with the expression of the immunomodulatory molecule indoleamine 2,3-dioxigenase (IDO) (70), and by IL-10 production (50, 71, 72). Gene expression profiling demonstrated that dMΦ from the first trimester of pregnancy express genes functionally related to immunomodulation and tissue remodeling (73). *In vitro* studies showed that exposure of U937 cells to HLA-G5 or HLA-G6 modulates IL-10 and TGF-β secretion (74). Based on these data, and on the fact that dMΦ express ILT2 and ILT4 (50, 56) (**Table 1**), it was postulated that, in the presence of dNK-derived IFN-γ, dMΦ in contact with HLA-G$^+$EVTs and exposed to soluble HLA-G are induced to secrete IL-10 and TGF-β, which limit T-cell responses and promotes tolerance (74).

Plasmacytoid (BDCA-2$^+$) and myeloid (BDCA-1$^+$ and BDCA-3$^+$) DCs have been also identified at the maternal–fetal interface (53, 75, 76). In early human pregnancy, DC-SIGN$^+$ dDCs, characterized by low expression of CD86 and DEC-205, were described (77). DC-SIGN$^+$ dDCs might be involved in re-programing the local immune response since they are associated with GM-CSF- and IL-10-secreting large granular lymphocytes that inhibit their maturation, and possibly favor tolerogenic responses (78). It has been shown that a population resembling DC-SIGN$^+$ dDCs that express ILT4 can be differentiated *in vitro* (57, 76), suggesting that these cells can be also modulated by HLA-G$^+$ decidual resident cells (**Table 1**). Our group identified a peculiar subset of tolerogenic DCs at the maternal–fetal interface in the first trimester of pregnancy. These DCs, termed DC-10, express HLA-G and ILT4 and secrete IL-10, thus are potentially involved in promoting tolerance (53) (**Table 1**). Future investigation is warranted to define whether dDC-10 and DC-SIGN$^+$ dDCs are distinct populations of tolerogenic APCs, or cells at different stages of differentiation.

It is not surprising that Tregs are present in the decidua during pregnancy. An increased frequency of CD4$^+$FOXP3$^+$ Tregs in the peripheral blood of pregnant women has been shown (79) and the accrual of these cells has been described in human decidua with controversial results (53, 76, 80, 81). Recent evidence indicated that CD4$^+$FOXP3$^+$ Tregs might be generated *in situ* (57). A population of CD4$^+$ T cells expressing HLA-G, termed CD4$^+$HLA-G$^+$ T cells, representing up to 20% of the decidua-infiltrating CD4$^+$ cells, have been recently reported (53, 54) (**Table 1**).

OPEN ISSUES

TROPHOBLAST-MATERNAL APCs CROSS-TALK: ROLE OF HLA-G-MEDIATED SIGNALS

For the acceptance of the semi-allogeneic fetus, a crucial role is played by the trophoblasts themselves. In addition to express/secrete HLA-G, EVTs release immune-modulatory mediators (i.e., IL-10 and TSLP), which are involved in promoting a pro-tolerogenic microenvironment. Our group characterized the tolerogenic DC-10 that are present *in vivo* and are inducible *in vitro* in the presence of IL-10. DC-10 are mature myeloid cells that spontaneously secrete IL-10 in the absence of IL-12, and express HLA-G, ILT2, ILT3, and ILT4. Importantly, DC-10 promote the induction of adaptive T regulatory type 1 (Tr1) cells via the IL-10-induced HLA-G/ILT4 pathway (82). Later, we demonstrated that DC-10 accumulate in human decidua during the first trimester of pregnancy (53). Based on this observation, we postulate that dDC-10 may represent the naturally occurring HLA-G-expressing DCs involved in re-programing the immune response toward tolerance. The recent observation that the frequency of dDC-10 in women with spontaneous abortion is lower compared to that observed in pregnant women sustains this hypothesis (our unpublished data). One of the important questions regarding dDC-10 is whether they are recruited in decidua during pregnancy or are induced *in situ*. Recently, it was demonstrated that the secretion of TSLP by EVTs induces CD11c$^+$ dDCs to express co-stimulatory molecules and HLA-DR and to secrete IL-10 and TGF-β (83). TSLP-instructed DCs via TFG-β secretion induce CD4$^+$CD25$^+$FOXP3$^+$ Tregs that inhibit effector T cells, and promote HLA-G expression on EVTs (83). Thus, the decidual microenvironment, enriched in TSLP and IL-10, produced by both EVTs and immune cells, sustains the expression of HLA-G on EVTs. In this scenario, the cross-talk between HLA-G-expressing EVTs and decidual myeloid cells might favor the generation of a set of tolerogenic DCs, including dDC-10 and TSLP-modulated CD11c$^+$ dDCs, which co-operate in promoting tolerance via the generation of different subsets of Tregs: Tr1, CD4$^+$CD25$^+$FOXP3$^+$, or CD4$^+$HLA-G$^+$ cells. As discussed above, EVT-derived HLA-G directs dMΦ toward a tolerogenic path, which contributes to the inhibition of effector T cells and to the induction of Tregs. The hypothesis that decidual tolerogenic APCs drive the differentiation of Tregs is supported by the higher frequency of peripherally induced Tregs (defined as Helios$^-$ iTreg) compared to the thymic-derived Tregs in decidua (57). Our group recently demonstrated that co-expression of CD49b and LAG-3 identified Tr1 cells *in vivo* (84); thus, the use of these biomarkers in conjunction with the expression of FOXP3,

Helios, and HLA-G will better define Treg cell composition at the maternal–fetal interface and define their relationship and relative contribution in tolerance induction.

Tolerogenic DCs can also contribute to sustain the pro-angiogenic milieu in the decidua. dDC-10 through the HLA-G can interact with dNKs or dMΦ via ILT2 and promote their activation and the release of the angiogenic factors. Moreover, dDC-10 themselves secrete IL-8 and VEGF (our unpublished data), supporting their pro-angiogenic functions. Since dMΦ, dDC-10, and TSLP-modulated CD11c$^+$ dDCs are characterized by the ability to secrete IL-10, they can also support the up-regulation of HLA-G on EVTs and on other decidual infiltrating cells (85), hence facilitating the establishment of an appropriate vascular bed at the maternal–fetal interface.

TROPHOBLAST-DECIDUA CROSS-TALK: ROLE OF HLA-G-MEDIATED SIGNALS

The pre-decidualization program entails the production of a plethora of transcription factors, cell cycle regulators, cytokines, and the activation of diverse signaling pathways (86). Full decidualization is then achieved upon embryo arrival. In view of the increased requirements for protein secretion during embryo implantation, cytoplasmic and endoplasmic reticulum (ER) stress responses are activated at the maternal–fetal interface. Cytoplasmic stress responses are characterized by the rapid stress-induced synthesis of heat shock proteins (HSPs) that allow cells to restore protein homeostasis and to be protected against molecular damage (87). Stress-induced HSPs are not only essential for regulating the state of intracellular folding, assembly, and translocation of proteins but are also potent modulators of the immune responses. Moreover, HSPs are necessary for placental development. Targeted deletion of HSP90 results in embryonic lethality (88). In primary decidualizing, endometrial stromal cells treated with embryo supernatants, genome wide expression profiling revealed that HSP70 was strongly increased (89).

The range of functions attributed to HSPs has expanded to encompass functions outside the cell (90). Extracellular HSPs may be able to play a role as danger signals (91). In this context, HSPs may interact with pattern recognition receptors, and activate pro-inflammatory signaling and transcription. Specifically, extracellular HSP60 was shown to allow communication between immune cells and other cells in the body (92), and HSP70 can be released from cells after acute stress in different cells, including cultured rat embryo cells (93), and peripheral blood mononuclear cells (94). Notably, HSPs can activate NKs and Tregs (95, 96). Evidence for regulation of HLA-G by HSPs is still scanty. *HLA-G* transcription was found to be induced upon heat shock in tumor cell lines, by heat shock transcription factor 1 (HSF1) binding to a heat shock element (HSE) present in *HLA-G* but not in other *HLA class I* genes (97). Moreover, mice mutant for *Hsf1* have a thin spongiotrophoblast layer and die *in utero* (98). Further investigation is warranted to define if maternal/fetal-derived HSPs might contribute to the regulation of HLA-G expression on dDC-10 and EVTs.

Protein folding in the ER is essential to ensure normal cell function. Disruption of ER homeostasis causes accumulation of misfolded proteins in the ER, a condition referred to as ER stress. ER stress activates the unfolded protein response (UPR) to restore protein homeostasis within the ER. However, if ER stress is persistent and excessive, the ER homeostasis cannot be re-established and the UPR will induce apoptosis. Intriguingly, IL-10 is emerging as a novel modulator of the ER stress (99). Intestinal epithelial cells isolated from IL-10$^{-/-}$ mice exhibit increased expression levels of BiP, a prototypic marker for ER stress, suggestive of an increased ER stress in the absence of IL-10. Further observations revealed that IL-10 attenuates tunicamycin-induced ER stress through suppression of BiP (100). These studies consistently suggest a novel role for IL-10 in modulating ER stress (101). Under ER stress, which occurs during normal development of labyrinthine trophoblasts in the mouse placenta, transcriptional regulation of VEGF is mediated by the three master regulators of the UPR: IRE1a, PERK, and ATF6 (102). The modulation of the UPR pathway by IL-10, produced by dMΦ, dDC-10, and TSLP-modulated CD11c$^+$ dDCs, might represent an additional mechanism to regulate vascular uterine remodeling and placentation.

PERSPECTIVES

The existence of mechanisms by which fetal and maternal cells simultaneously attract and modulate each other is intriguing. Upon blastocyst implantation into the uterine wall, trophoblasts differentiate into EVTs that possess the ability to coordinate the cross-talk at the interface via the expression of HLA-G. Accumulating evidence indicate that EVTs play a key role in orchestrating a number of molecular and cellular decidual modifications by (i) regulating cell-migration in the decidua, (ii) supporting the induction of the pro-angiogenic decidual microenvironment necessary for effective vascular remodeling, (iii) inhibiting effector innate and adaptive immune responses, and (iv) promoting a tolerogenic loop in which resident cells are instructed to become tolerogenic. These functions are regulated through the finely tuned specific interactions of HLA-G-expressing EVTs with maternal innate immune cells, adaptive immune cells, and non-immune cells (**Figure 1**). The interplay among these cells supports the development of an appropriate maternal–fetal niche. Pregnancy hormones are essential to fully support the niche, although their role in regulating HLA-G expression has not been investigated yet (29).

We suggest that the integration and exchange between fetal and maternal blood vessels at the interface is likely to be contributed by multiple mechanisms, including trophoblast interaction with dNKs and resident/recruited APCs, as well as by the IL-10-driven tolerance and regulation of the UPR pathway in decidual and trophoblast cells.

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The Potential of HLA-G-Bearing Extracellular Vesicles as a Future Element in HLA-G Immune Biology

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The HLA-G molecule is a member of the non-classical HLA class I family. Its surface expression is physiologically restricted to the maternal–fetal interface and to immune privileged adult tissues. Despite the restricted tissue expression, HLA-G is detectable in body fluids as secreted soluble molecules. A unique feature of HLA-G is the structural diversity as surface expressed and as secreted molecules. Secreted HLA-G can be found in various body fluids either as free soluble HLA-G or as part of extracellular vesicles (EVs), which are composed of various antigens/ligands/receptors, bioactive lipids, cytokines, growth factors, and genetic information, such as mRNA and microRNA. Functionally, HLA-G and its secreted forms are considered to play a crucial role in the network of immune-regulatory tolerance mechanisms, preferentially interacting with the cognate inhibitory receptors LILRB1 and LILRB2. The HLA-G mediated tolerance is described in processes of pregnancy, inflammation, and cancer. However, almost all functional and clinical implications of HLA-G *in vivo* and *in vitro* have been established based on simple single ligand/receptor interactions at the cell surface, whereas HLA-G-bearing EVs were in minor research focus. Indeed, cytotrophoblast cells, mesenchymal stem cells, and cancer cells were recently described to secrete HLA-G-bearing EVs, displaying immunosuppressive effects and modulating the tumor microenvironment. However, numerous functional and clinical open questions persist. Here, we (i) introduce basic aspects of EVs biology, (ii) summarize the functional knowledge, clinical implications and open questions of HLA-G-bearing EVs, and (iii) discuss HLA-G-bearing EVs as a future element in HLA-G biology.

Keywords: extracellular vesicles, HLA-G, sHLA-G, LILRB1, LILRB2, KIR2DL4, exosome, HLA-G-bearing EV

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INTRODUCTION

HLA-G is a non-classical HLA class I molecule. It is a potent suppressive molecule that impairs effector functions of immune cells belonging to the innate and adaptive immune system. Under physiological conditions, its surface expression is restricted to the maternal–fetal interface and to immune privileged adult tissues (1). However, secreted soluble forms of HLA-G are detectable in a variety of body fluids such as peripheral blood and amniotic fluids (2), malignant ascites (3, 4),

pleural effusions (5), cerebrospinal fluid (6, 7), and sperm (8). Neo-ectopic or aberrant expression of HLA-G has frequently been related to malignancies (9–13), viral infections (14–19) including liver-related hepatitis B (16) and C (18) virus infections, autoimmune disorders (20–22), inflammatory diseases (23), complications (24, 25), and transplantation outcomes (26, 27).

A unique feature of HLA-G is that it exists in multiple structures, either expressed on the cell surface or in a secreted form. These different forms can mainly be attributed to alternative splicing of the primary transcript and differential association with β2-microglobulin (β2m). Four isoforms (HLA-G1, G2, G3, and G4) are membrane-expressed and three isoforms express either intron 4 (HLA-G5 and -G6) or intron 2 (HLA-G7) but lack the transmembrane and cytoplasmic domains, resulting in their secretion. With the exception of HLA-G3 (28), all HLA-G structures can create disulfide bounds between two unique cysteine residues at positions 42 (Cys42–Cys42 bonds) and 147 (Cys42–Cys147 bonds) (29, 30). The structures displaying the full-length extracellular domain (HLA-G1 and HLA-G5) are probably the most frequently detected. The structural diversity is further enhanced in that all membrane-expressed structures can also be shed from cell surface by metalloproteases (31) or can be secreted *via* extracellular vesicles (EVs) (32).

Regarding function, HLA-G and the soluble counterparts preferentially exert their immune modulating or suppressing functions by interaction with the two inhibitory receptors, leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) and LILRB2. LILRB1 is expressed on subpopulations of T-cells, B-cells, and Natural Killer (NK) cells. Monocytes/macrophages/dendritic cells (DC) express both receptors. These two receptors distinguish between β2m-associated and β2m-free HLA-G: LILRB1 interacts with HLA-G molecules associated to β2m, whereas LILRB2 specifically recognizes β2m-free HLA-G (33, 34). HLA-G dimers bind to LILRB with a higher affinity and avidity than monomers, resulting in more efficient LILRB-mediated signaling (35, 36). Additionally, HLA-G has been described to be the sole ligand for the killer immunoglobulin-like receptor 2DL4 (KIR2DL4), exhibiting both an activating and an inhibitory signaling domain. Moreover, soluble forms of HLA-G are able to trigger apoptosis in $CD8^+$ T and NK cells (37) as well as in CD160-bearing endothelial cells (38).

Based on the functionality of receptors and their expression profile, membrane-expressed and soluble forms of HLA-G molecules are involved in immune regulation in pregnancy, inflammation, and cancer. Thus, HLA-G can be considered as an immune checkpoint molecule (39). However, most functional implications of HLA-G *in vivo* and *in vitro* have been deduced from the HLA-G1 and HLA-G5 structures and from a rather simple point of view on single ligand/receptor interaction. Interaction of target cells with HLA-G-bearing EVs has typically not been considered. Here, we (i) introduce basic aspects of EVs biology, (ii) summarize the current knowledge and open questions of HLA-G-bearing EVs, and (iii) discuss HLA-G-bearing EVs as a future element in the HLA-G biology.

BASIC ASPECTS OF EV BIOLOGY

Common Features of Extracellular Vesicles

Extracellular vesicles are phospholipid bilayer-enclosed vesicles, which are released by most cell types, including immune cells, tumor cells, stroma cells, trophoblast cells, and adult and embryonic stem cells (40). Depending on the cell of origin, state, and micro-environment, EVs are highly heterogeneous in size, membrane composition, and molecular content. According to biogenesis, EVs are specified as exosomes (70–150 nm), microvesicles (100–1000 nm), and apoptotic bodies (AB) (>500 nm). Exosomes correspond to intraluminal vesicles (ILVs), formed from inward budding of small-sized plasma membrane and enclosed in multivesicular bodies (MVB). Exosomes are released into extracellular space after fusion of MVB with the plasma membrane (41). In contrast, microvesicles (MV) are formed by outward budding and sission of the plasma membrane. AB are generated from plasma membrane blebs of cells undergoing apoptosis. Oncosomes, which are generated by the shedding of plasma membrane blebs of non-apoptotic cancer cells (42), and form an atypically large EV population (1,000–10,000 nm). Several proteins are currently used as markers for EVs, including tetraspanins, different heat shock proteins, adhesion molecules, cytoskeletal proteins, and members of endosomal sorting complexes required for transport of exosomes like TSG101 (43, 44). However, so far, no specific markers have been identified allowing for the identification of particular EV subpopulations (44).

Different cell types release differently assembled EV. Furthermore, it is tempting to speculate that even individual cells release different EV types. Importantly, the cell of origin controls the molecular composition and cargo (45–48). EVs harbor various types of antigens, cell surface-expressed receptors or ligands including classical and non-classical HLA-G (32, 49–53), bioactive lipids such as prostaglandins (54) and leukotrienes (55). Additionally, EVs can serve as transport cassettes or a disseminated storage pool of bioactive effector molecules, e.g., cytokines transcription factors, growth factors, oncogenic proteins, and genetic information such as mRNA, microRNA (56–59). Here, the lipid membrane of EVs protects their contents against enzyme degradation present in body fluids and thereby facilitate the transfer of their cargo over a short or long distance.

Modes of Interaction between Extracellular Vesicles and Target Cells

The composition of EVs is responsible for the biodistribution, for the interaction of EVs to target cells or to extracellular matrix. Membrane fusion of EVs to target cells allows the transfer of bioactive molecules, including, e.g., CCR5 (60) and EGFRvIII (61), modifying the recipient cell phenotype. However, the direct fusion of EVs with the plasma membrane of effector cells requires a similar fluidity of the two fusing membranes. This can be achieved in an acidic micro-environment, which naturally occurs inside tumors (62–65) or at neutral pH in the presence of syncythin (66).

Besides membrane fusion, EVs can be internalized by different pathways including phagocytosis, clathrin- and caveolin-mediated endocytosis, or micropinocytosis (67). With the exception of the latter, the uptake and internalization of EVs are mostly receptor-mediated, e.g., *via* Hsp90 receptor or scavenger receptor CD36 (66). The expression of adhesion molecules on EVs probably facilitates the specific uptake of EVs, and their internalization by their cognate receptors being expressed on certain tissue or cell populations (68). The internalization of EVs results in the delivery and enrichment of bioactive molecules into the target cell's endosomes. Hence, these molecules may be forwarded to other cell compartments, where they may contribute to an intracellular signaling mechanism.

The Immunological Potential of Extracellular Vesicles

The communication and immune modulation by EVs take place among cells within same entity or between different types of cells. Various effector cells of the innate and adaptive immune system, including T cells and NK cells, antigen presenting cells (APCs), and mast cells have been reported to donate or to acquire ligand/receptor/genetic information *via* EVs. Due to the complex and often antagonistic composition, EVs can mediate gene expression modification, immune activating or immune suppression, introducing homeostasis or immune tolerance by the induction of T cell apoptosis, impairment of DC maturation, or the prevention of NK and T cell cytotoxicity (68–76). Furthermore, the molecular transfer of miRNA by EVs can alter the expression profile of the recipient cell (71). Tumor-derived EVs can stimulate immune suppression and tumor progression in different ways including the inhibition of tumor-specific T cell function and proliferation (77), the promotion of regulatory T cells subsets (78), and transfer of oncogenic receptors (61).

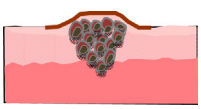
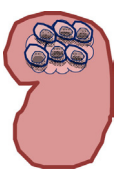
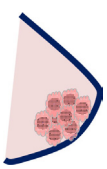
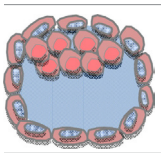
THE CURRENT STATUS AND OPEN QUESTIONS OF HLA-G-BEARING EXTRACELLULAR VESICLES

HLA-G-Bearing EVs and Cancer

Without any doubt, the neo-ectopic expression of HLA-G molecules either on the surface of tumor cells or released as soluble forms can be considered a critical factor for cancer progression. Albeit high blood levels of soluble forms of HLA-G have concordantly been related to cancer, the prognostic relevance of soluble HLA-G in the blood has not always been established as an independent marker in terms of disease progression and survival (39). To date, the source of soluble HLA-G is known. In addition, it is not clarified whether HLA-G-bearing EVs or free soluble HLA-G ($sHLA\text{-}G_{free}$) are produced by tumor cells and whether both subcomponents contribute to immune evasion of tumor cells.

Secreted HLA-G-expressing EVs (**Table 1**) have been detected for the first time in supernatants of a melanoma cell line, originated from a HLA-G-positive melanoma lesion (32). Both, the

TABLE 1 | Source of HLA-G-bearing EVs with potential immunological and clinical relevance.

Cell type	EVs source	Potential target cell response	Function/mechanism	Clinical relevance	Reference
	Melanoma	Tolerance-inducing effect of melanoma derived HLA-G-bearing EVs on immune cells	Potential induction of inhibitory signaling by HLA-G1-bearing EVs *via* LILRB1/2 receptors	Unknown clinical relevance	(32)
	Kidney cancer	Inhibitory effect of HLA-G-bearing EVs on monocyte differentiation into mature DCs and reduced T cell proliferation	Inhibitory effect of HLA-G1-bearing EVs on monocyte differentiation and their maturation to DCs	Suppression of immune effector cells by HLA-G1-bearing EVs, leading to disease progression	(83)
	Breast cancer	Modulation of immune effector functions by circulating HLA-G-bearing EVs	Unknown function	Association of high circulating amounts of HLA-G-bearing EVs to disease progression	(84)
	Trophoblast	Modulation of immune effector functions by cytotrophoblast-derived HLA-G5-bearing EVs	Unknown function	Unknown clinical relevance, but potential biomarker for pregnancy-related disorders	(87)
	Mesenchymal stem/stromal cells (MSCs)	Induction of tolerance between graft and host immune cells by MSCs-derived EVs	Immunomodulation by synergistic additive effect of HLA-G, IL-10, and TGFβ	Potential therapeutic option for patients with therapy–refractory GvHD using MSC-derived HLA-G-bearing EVs	(91)

cell line cells and the secreted EVs express the full-length isoform HLA-G1. Up to now, it is not known whether HLA-G1-bearing EVs are functionally active to transduce inhibitory signals toward effector cells *via* the LILRB1/2 receptors, which may spread the tolerogenicity of HLA-G.

The first *in vivo* existence of HLA-G-bearing EVs was reported for ascites and pleural exudates derived from cancer patients (53). The EV fractions, however, contain ubiquitinated HLA-G molecules with atypically high HLA-G molecular sizes ranging from 50 to 75 kD. Generally, ubiquitination is a frequent post-translational protein modification, by which proteins are targeted to protein degradation or directed to other cellular locations (79, 80). Interestingly, EVs contain many polyubiquitinated proteins, which are not integrated into their membrane (81). Thus, the presence of secreted HLA-G5 or HLA-G6 cannot be excluded.

Very recently, we established the prognostic relevance of HLA-G-bearing EVs for neoadjuvant chemotherapy-treated (NACT) breast cancer patients for the first time (82). Both, the total amount of HLA-G_{tot} and the amount of sHLA-G_{free} were significantly increased in breast cancer patients. Before NACT, sHLA-G_{free} levels are exclusively related to estrogen receptor expression, whereas high amounts of HLA-G in EVs (sHLA-G_{EV}) enriched from peripheral blood samples are associated with the existence of circulating stem cell-like tumor cells. Strikingly, despite high amounts of sHLA-G_{tot}, its prognostic relevance could not be substantiated. However, different impacts on prognosis have been shown for the two subcomponents sHLA-G_{EV} and sHLA-G_{free}: high sHLA-G_{EV} levels are associated with disease progression, whereas high sHLA-G_{free} levels are related to an improved clinical outcome. This suggests that some of the sHLA-G_{free} molecules are impaired regarding LILRB1 recognition, and thereby are not qualified to exert inhibitory functions, as already demonstrated in rheumatoid arthritis patients (83). In conclusion, this study exemplifies the importance of stratifying soluble forms of HLA-G into free and EVs-bound molecules, as these two subcomponents can display diametrically opposed prognostic impact on disease progression likely due to the differential power of these compounds to contribute to an immune escape of tumor cells.

Further underlining the functional relevance of HLA-G-bearing EVs in cancer, a recent study demonstrated that (i) EVs released by renal cancer stem cells carry HLA-G with a HLA-G1 typical molecular weight, (ii) these HLA-G-bearing EVs impair the differentiation of monocytes to mature DCs, and (iii) the presence of these DCs reduces the T cell proliferation. Thus, HLA-G-bearing EVs mediate inhibitory effects on monocyte differentiation and their maturation to DCs (84).

HLA-G-Bearing EVs and Pregnancy

At the maternal–fetal interface, HLA-G and its soluble forms are expressed on both sides, on extravillous trophoblast cells lining the placenta and on tolerance-inducing DCs (DC-10) being enriched in the first trimester decidua (25, 85). Thus, HLA-G is thought to orchestrate the cross talk among embryo trophoblasts, decidual leukocytes, and stromal cells allowing the trophoblast invasiveness, decidual cell differentiation, vascular remodeling, and the reprograming of local maternal immune responses (86). Whether HLA-G-bearing EVs represent an additional instrument to mediate communication of these cells is currently unclear. Interestingly, both first trimester and term placentas have been reported to secrete HLA-G5 isoforms *via* EVs (87). In agreement with the reported immunolocalization of HLA-G (88) cytotrophoblast cells, but not differentiated syncytiotroblasts, are producing HLA-G5-positive exosomes. The observation of the presence of HLA-G5 in EVs raises the issue whether HLA-G5 is associated with the luminal or with the extravesicular EV side. As secreted molecules, the association of HLA-G5 with extravesicular EV sides would require a binding partner it can associate with. Alternatively, the association with the luminal side would require the transit of HLA-G into the cytoplasma after biosynthesis. Independently of the immunogenicity of EVs and of the secretion pathway directing HLA-G5 toward EVs, it is clear that HLA-G5 isoforms being inside of EVs are hidden, which provokes questions about the function of HLA-G5 in cytotrophoblast-derived EVs.

HLA-G-Bearing EVs and Mesenchymal Stem/Stromal Cells

Similar to trophoblast cells, mesenchymal stem/stromal cells (MSCs) express surface-expressed and soluble forms of HLA-G, which are involved in the suppression of T and NK cell functions (89). Besides HLA-G, MSCs exert the immune regulatory and modulatory activities through a variety of soluble mediators such as IL10, TGFβ, either as free soluble molecules or *via* immunological active EVs (90). The latter have been suggested to mediate synergistical effects of these molecules. In view of this, MSC-derived EVs, containing huge amounts of HLA-G, IL-10 and TGFβ, were used to treat a patient suffering from severe and therapy–refractory cutaneous and intestinal GvHD grade IV (91). After serial application rounds of MSC-EVs, a substantial improvement of the clinical GvHD symptoms has been achieved without any side-effects. Simultaneously, the allogeneic cytokine responsiveness of the patient's peripheral mononuclear blood cells was substantially reduced. Although a direct impact of HLA-G on the immune suppression has not been demonstrated, this study represents the first treatment in humans, in which HLA-G with the immune modulatory function of MSC-derived EVs has been applied. Thus, it triggered significant interest in applying EVs-based therapeutics in clinical trials (92).

New Perspectives of HLA-G-Bearing Extracellular Vesicles

Currently, the known functions of HLA-G are restricted to receptors expressed on the surface of effector cells of the innate and adaptive immune system. In this way, HLA-G inhibits the cytolytic function of NK cells (93, 94), the antigen-specific cytolytic function of cytotoxic T lymphocytes (CTL) (95) and γ/δ T cells (96), the allogeneic proliferative response (95), and proliferation of CD4+ T cells (97). HLA-G also impairs the maturation and function of DC (98, 99). Furthermore, HLA-G is related to regulatory cells including regulatory T cells (89, 100–102), regulatory DC (103), and myeloid-derived suppressor cells (104). Due to the differential composition of EVs, other compounds of the EVs may potentiate or abrogate the functional power of HLA-G.

Additionally, EVs harboring HLA-G may allow the interaction with target cells lacking the surface expression of HLA-G specific receptors.

Membrane fusion of EVs to target cells can represent a possible mode of how HLA-G can be transferred to target cells. In this context, it is noteworthy that a cellular translocation of HLA-G from APCs to activated T cells (102) and from tumor cells to T/NK cells has been reported (105, 106). The acquisition of HLA-G reverses the function of T and NK cells to regulatory cells impairing allo-immune responsiveness. Such a spatiotemporal mechanism is suggested to be an instrument for "emergency" immune suppression used by HLA-G-expressing tissues to protect themselves against aggressive immune intervention (102). It is tempting to speculate that EVs mediate a transfer of HLA-G to effector cells, which would abrogate at least the regional mode of action.

Independent of the pathway, internalization of HLA-G-bearing EVs provides the opportunity for HLA-G to participate in yet unknown intracellular pathways. Interestingly, both soluble HLA-G5 and shed HLA-G1 have been reported to be bound by the transiently expressed KIR2DL4 receptor and to be endocytosed into early endosomes of NK cells (107–109). This leads to the activation of a nuclear factor-κB-pathway and finally to the transcription of pro-inflammatory and proangiogenic factors. Thus, the sustained endosomal signaling by KIR2DL4/HLA-G may allow NK cell activation despite a potential dominant inhibitory receptor–ligand interaction at cell surface. In context with the secretion of HLA-G by fetal trophoblast cells, this NK cell-mediated mechanism has been discussed to be operative in the promotion of vascularization in maternal decidua during early pregnancy (107–109). Here the question arises, whether this KIR2DL4–HLA-G pathway becomes operative when fetal trophoblast cells secrete HLA-G-bearing EVs or whether other yet unknown receptors can mediate intercellular signaling. The investigation of molecular signature molecules on HLA-G-bearing EVs may help to provide an insight into the functional consequence and the intracellular signaling pathway after internalization (69).

Regarding the role of HLA-G in diagnosis, prognosis, and treatment, the cell-specific signature of HLA-G-bearing EVs may not only provide information about the potential target cells and about its potential interplay of the cognate receptor/ligand on target cells but also about the cells producing these EVs (110). In that way, the identification of the cellular source on HLA-G-bearing EVs, such as the detection of the tumor marker HER-2/neu, may offer unforeseen diagnostic opportunities to monitor the systemic health status/disease status and disease activity/progression.

CONCLUSION

It is well established that tumor cells, cytotrophoblast cells, and MSCs secret HLA-G-bearing EVs in addition to non-vesicular soluble HLA-G. All of these cell types are highly capable of promoting immune tolerance and tissue remodeling. Mechanisms and functional consequences of HLA-G-bearing EVs and their specific contribution to the biology of these cells have yet to be determined. So far, the classical concept of HLA-G function is based on the interaction of HLA-G with receptors being expressed on the cell surface membrane. EVs, however, may serve as a ticket for HLA-G to interact directly with cells or to enter into the inside of cells. The internalization of HLA-G may introduce new pathways or yet unknown cognate receptors, by which HLA-G contributes to intracellular communication. In that way, HLA-G-bearing EVs are likely to represent an important element in the biology of HLA-G.

AUTHOR CONTRIBUTIONS

VR: concept and design, drafting of manuscript. VR and PH: critical revision of the manuscript for important intellectual points, supervision. LK, FN, BW, LM, VR, and PH: drafting of manuscript.

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