Trends in Immunology

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Review

HLA-G: At the Interface of Maternal–Fetal Tolerance

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During pregnancy, semiallogeneic fetal extravillous trophoblasts (EVT) invade the uterine mucosa without being rejected by the maternal immune system. Several mechanisms were initially proposed by Peter Medawar half a century ago to explain this apparent violation of the laws of transplantation. Then, three decades ago, an unusual human leukocyte antigen (HLA) molecule was identified: HLA-G. Uniquely expressed in EVT, HLA-G has since become the center of the present understanding of fetus-induced immune tolerance. Despite slow progress in the field, the last few years have seen an explosion in our knowledge of HLA-G biology. Here, we critically review new insights into the mechanisms controlling the expression and function of HLA-G at the maternal–fetal interface, and discuss their relevance for fetal tolerance.

The Pregnancy Paradox

Every one of us is here today thanks to successful pregnancy. However, pregnancy has puzzled immunologists ever since Peter Gorer and George Snell laid out the laws of transplantation. A developing fetus can be seen as a semiallogeneic graft expressing paternally-derived antigens, yet it is nurtured for many months without suffering rejection by the maternal immune system, a paradox first formulated by Peter Medawar [1]. Initially, this phenomenon was postulated to be due to systemic unresponsiveness to cells expressing foreign fetal antigens [1]. However, later work established that pregnant mothers do develop fetal antigen-specific cytotoxic T cell and antibody-mediated responses during gestation [2,3]. These maternal immune responses to fetal antigens are subdued by the induction of regulatory T cells (Tregs) specific for fetal antigens, as well as modulation of effector T cells and NK cells at the maternal–fetal interface [4–7]. Curiously, Medawar, who coined the term 'immune privilege' in the late 1940s and received the Nobel Prize in 1960 for the discovery of acquired neonatal tolerance, never considered acquired immune tolerance as an explanation for the pregnancy paradox. Accumulating evidence suggests that fetal immune tolerance is established locally at the placenta, a transient organ consisting of fetal trophoblasts and the maternal decidua, which develops from the uterine mucosa. During implantation, extravillous trophoblasts (EVT) arise from the tips of anchoring villi and invade the decidua, defining the boundary between mother and fetus: the maternal–fetal interface. Far from being devoid of immune cells, the decidua harbors multiple populations of maternal immune cells, all of which extensively interact with fetal-derived trophoblasts (Figure 1). In fact, up to 40% of decidual cells are leukocytes [8].

The Emergence of a Tolerance-Inducing MHC Molecule: HLA-G

Several mechanisms protecting invading EVT from rejection by maternal leukocytes have been described (Box 1). EVT evade maternal immune surveillance while inducing immune tolerance by expressing a unique set of MHC molecules. Classical MHC class I molecules, HLA-A, HLA-B, and HLA-C, are ubiquitous highly polymorphic proteins dedicated to peptide presentation to cytotoxic T cells. Unlike most cells, EVT are devoid of HLA-A and HLA-B expression,

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HLA-G-expressing extravillous trophoblasts (EVT) play an important role in embryo implantation and establishment of maternal–fetal immune tolerance. A cis-regulatory element 12 kb upstream of the HLA-G locus, Enhancer L, is required for HLA-G expression.

During interactions with EVT, decidual natural killer (dNK) cells can physically acquire HLA-G by trogocytosis. Signaling from dNK endosomes promotes a tolerogenic NK signature while maintaining the potential for antiviral immunity at the maternal–fetal interface.

Naturally occurring HLA-G polymorphisms that may result in reduced HLA-G levels have been found to correlate with pregnancy complications such as miscarriage, preterm birth, preeclampsia, and recurrent spontaneous abortions (RSA).

Conversely, aberrant HLA-G expression in cancers has been reported to correlate with tumor progression, metastasis and poor clinical outcome, possibly constituting a mechanism co-opted by tumors to evade immune surveillance.

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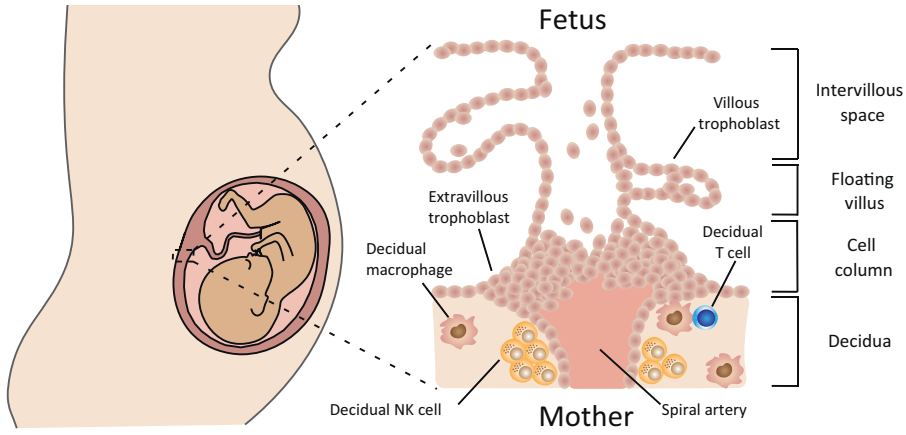


Figure 1. Anatomy and Cellular Composition of the Human Maternal–Fetal Interface. The human placenta has an outer layer composed of HLA-G$^+$ extravillous trophoblasts (EVT) and floating villi containing MHC-negative villous trophoblasts (VT). EVT are formed in cell columns and invade the decidua, mediating placental attachment of the fetus. Importantly, EVT progressively replace endothelial cells on the walls of uterine spiral arteries, increasing their caliber. This process, which ensures proper blood flow to the intervillous space to nourish the fetus, requires the presence of decidual NK cells, the most numerous leukocytes at the maternal–fetal interface [9,10]. Moreover, NK cells are thought to control viral infections in the placenta [11]. Decidual macrophages, the second most abundant immune cell type in the decidua, also play a crucial role in tissue remodeling during implantation, and present protein and lipid antigens to decidual T cells [12,13].

expressing only HLA-C and the nonclassical non (or low) polymorphic MHC class I molecules HLA-E and HLA-G [14].

Initially discovered using HLA locus-specific Southern blot probes [15], HLA-G was soon revealed to be an uncommon MHC molecule (Table 1). First, it was found to be specifically

Table 1. Milestones in the Biology of HLA-G at the Maternal–Fetal Interface

Year	Milestone	Refs
1982	Detection of a novel HLA class I gene using Southern blot	[15]
1984	EVT express HLA molecules other than HLA-A or HLA-B	[21]
1986	Novel HLA molecule with short cytoplasmic tail found in trophoblasts	[22]
1987	6.0 kb *Hin*dIII restriction fragment cloned from HLA locus: HLA 6.0	[23]
1990	HLA6.0 is renamed HLA-G, the newest HLA class I gene	[24]
1990	HLA-G is uniquely expressed in EVT	[16]
1994	HLA-G is sufficient to inhibit decidual NK cell killing	[25]
1995	HLA-G presents peptides	[26]
1996	HLA-G is sufficient to inhibit peripheral NK cell killing	[17]
1999	KIR2DL4 is identified as an HLA-G receptor found in all KIR haplotypes	[20]
2000	The proximal promoter of *HLA-G* is defective	[27]
2001	*HLA-G* polymorphisms are associated with pregnancy complications	[28]
2002	HLA-G forms homodimers on the cell surface	[29]
2005	Crystal structure of HLA-G	[30]
2006	HLA-G is endocytosed by NK cells	[31]
2007	HLA-G can be transferred to immune cells via trogocytosis	[32]
2012	HLA-G induces senescence in peripheral NK cells	[33]
2016	A distant enhancer controls trophoblast HLA-G expression	[34]

Box 1. Trophoblasts Are Equipped with Immunomodulatory Molecules

Two seminal experiments carried out in the early 1960s established that trophoblasts possess intrinsic immune suppressive properties. First, while syngeneic tumors transplanted into the uterine horns of mice grew indefinitely, allogeneic tumors were rejected. Presensitization of the recipient with tumor antigens accelerated the rejection process irrespective of the recipient's pregnancy status, indicating that normal immune reactions can take place unimpeded in the uterus [92]. Second, studies using transplantation into the kidney capsule revealed that, while embryonic tissues were promptly rejected, trophoblasts proliferated and recruited new vessels, regardless of genetic matching [93]. Hence, trophoblasts must actively induce immune tolerance in order for successful pregnancy to take place. How is this accomplished?

Trophoblasts express a battery of immune inhibitory molecules predominantly targeting T cells. Fas ligand (FasL), TRAIL, and indoleamine 2,3-dioxygenase (IDO) – potent inducers of apoptosis of activated T cells – are all highly expressed by human trophoblasts [94–96]. Consistent with a role for these molecules in enforcing maternal–fetal tolerance, FasL-deficient pregnant mice display extensive leukocyte infiltration and killing at the maternal–fetal interface [97], and pharmacological inhibition of IDO leads to T cell-mediated rejection of allogeneic fetuses [98]. Interestingly, IDO deficiency in pregnant women has been linked to pre-eclampsia [99].

In addition to T cell apoptosis inducers, trophoblasts also express the immune checkpoint molecule PD-L1, a suppressor of TCR-mediated T cell activation. During human pregnancy, trophoblast PD-L1 expression increases during gestation, most dramatically at the onset of the second trimester. This upregulation matches the inception of maternal blood flow to the placenta and concomitant increase in the numbers of maternal T cells in decidua, consistent with a role for fetal PD-L1 in silencing maternal alloresponses to fetal antigens [100]. Indeed, PD-L1 blockade results in an 86% spontaneous fetal resorption rate in pregnant mice [101].

Immune tolerance induction by trophoblasts is accomplished not only by directly inhibiting effector T cells, but also by promoting regulatory T cell (Treg) induction and recruitment. Trophoblasts foster Treg differentiation directly, via PD-L1 [102] and human chorionic gonadotropin (hCG) secretion [103], as well as indirectly by stimulating decidual dendritic cells (dDC) with TSLP, which in turn induce Treg polarization via TGF-β [104].

Genome-wide gene expression analysis has revealed that EVT express many immunomodulatory molecules beyond the ones described here, such as LAIR-2 and CRTAM [38,105]. Exploring their function at the maternal–fetal interface will help paint a more complete picture of immune tolerance induction at the maternal–fetal interface.

expressed in EVT [16]. Second, only 51 *HLA-G* alleles encoding 16 different HLA-G proteins have been reported, a surprisingly low level of polymorphism for an HLA gene. As a comparison, *HLA-A* has 3356 known alleles. These two features, tissue-specificity and low degree of polymorphism, immediately suggested that HLA-G might play a role in immune tolerance induction at the maternal–fetal interface, prompting a period of intense investigation aimed at unraveling its function. At the time, *HLA-C* alleles had just been divided into two groups, C1 and C2, based on their recognition by inhibitory killer cell immunoglobulin-like receptors (KIRs) on NK cells. Surprisingly, transfection with HLA-G was sufficient to inhibit killing by both HLA-C1- and HLA-C2-specific NK cell lines [17]. The following year, HLA-G was found to confer protection against peripheral blood NK cells (pNK) from 20 different donors [18], further suggesting that HLA-G was a 'universal' NK cell inhibitory ligand. This was an intriguing possibility at the time; even though each NK cell receptor was known to recognize more than one MHC class I molecule, there was no single known molecule capable of inhibiting all NK cells [19]! A 'universal' receptor for HLA-G expressed across all NK cells tested was finally described in the late 1990s: KIR2DL4 [20]. These early studies thus established HLA-G as a *bona fide* trophoblast-restricted inhibitory ligand of the predominant immune cell type at the maternal–fetal interface in early pregnancy: NK cells [10].

Regulation of HLA-G Expression

How Is HLA-G Expression Restricted to Trophoblasts?

To date, HLA-G is the only known MHC gene whose expression in healthy tissues is exquisitely cell type-specific. Yet, despite decades of work delineating the transcriptional regulation of MHC genes, the mechanisms behind trophoblast-specific HLA-G expression are still not fully understood.

Early experiments using *HLA-G* transgenic mice led to the identification of a locus control region (LCR) located 1 kb upstream of the *HLA-G* promoter. Transgenic mice carrying either a full-length 6 kb *HLA-G* transgene or a 5.7 kb 5′ truncated version were created. HLA-G was specifically detected in trophoblasts in the placenta of embryos carrying full-length *HLA-G*. Surprisingly, however, the truncated version of the *HLA-G* transgene was also expressed in mesenchymal cells [35]. These experiments established that a 250 bp region 1 kb upstream of *HLA-G*, LCR, is critical to control tissue-specific HLA-G expression. Subsequent *in vitro* studies found that the LCR sequence binds trophoblast-specific transcription factor complexes [36]; their identity, however, remains to be determined. More recent efforts identified a conserved binding site for Ras-responsive element binding protein (RREB-1), a transcriptional repressor, within the LCR [37]. Transfection with RREB-1 was shown to repress reporter gene activity driven by a construct containing the LCR and the *HLA-G* proximal promoter, leading the authors to propose that RREB-1 represses HLA-G expression in HLA-G negative cells. However, RREB-1 is highly expressed in EVT [38], casting doubt on this hypothesis.

Work using human cells initially focused on the classical promoter sequence conserved across all *HLA* genes and responsible for basal and induced gene expression. This region harbors well-defined regulatory motifs: enhancer A, interferon-stimulated response element (ISRE) and the SXY module (S, X1, X2 and Y elements). Enhancer A is bound by NF-κB, downstream of TNF-α signaling, while the ISRE binds IRF1, mediating IFN-γ-induced *HLA* gene upregulation [39]. SXY sequences are recognized by ATF1 and CREB1 transcription factors, as well as by the RFX complex. The current paradigm states that these transcriptional regulators are assembled in an enhanceosome complex via association with the transactivator NLRC5, resulting in MHC-I transcription [40]. However, the original wave of studies on *HLA-G* transcription quickly revealed that the classical promoter of *HLA-G* is not functional alone due to variations in its regulatory motifs [39] (Figure 2A). These results explain why most cell types do not express HLA-G and hint at the requirement for an additional enhancer active in trophoblasts to allow HLA-G expression in these cells.

HLA-G Transcription Is Controlled at a Distance

By the mid-2000s, long-range chromatin interactions had been shown to play a role in MHC gene regulation. Sequence analysis of the MHC class II locus revealed the existence of intergenic conserved regulatory factor X (RFX)-binding sites in addition to the X-Y elements present in the classical promoters of MHC class II genes. One of these intergenic elements, XL9, is located between the *HLA-DRB1* and *HLA-DQA1* genes, which are separated by 44 kb. Surprisingly, XL9 was found to bind not RFX factors, but the insulator CCCTC-binding factor (CTCF), triggering association with the nuclear matrix [43]. In subsequent studies, CTCF was found to mediate a long-range chromatin interaction between the promoters of *HLA-DRB1* and *HLA-DQA1*. Importantly, disrupting this interaction by depleting CTCF led to a marked reduction in the expression of these genes [44]. At the time, there was no report of enhancer looping in the MHC class I locus.

Traditionally, enhancer discovery has relied on examining features predictive of enhancer activity, such as chromatin accessibility, DNA and chromatin covalent modifications, and sequence conservation between species (Box 2). However, substantial differences in regulatory sequences between species limit the ability to derive conclusions from model organisms regarding human gene regulation. In particular, the MHC locus differs significantly between mouse and humans [45], and *HLA-G* lacks a clear ortholog in mice.

Recently, trophoblast-specific HLA-G expression was investigated using a high-throughput unbiased approach: a massively parallel reporter assay (MPRA) [46]. MPRA allows the direct functional interrogation of a locus without any prior knowledge about its regulatory landscape

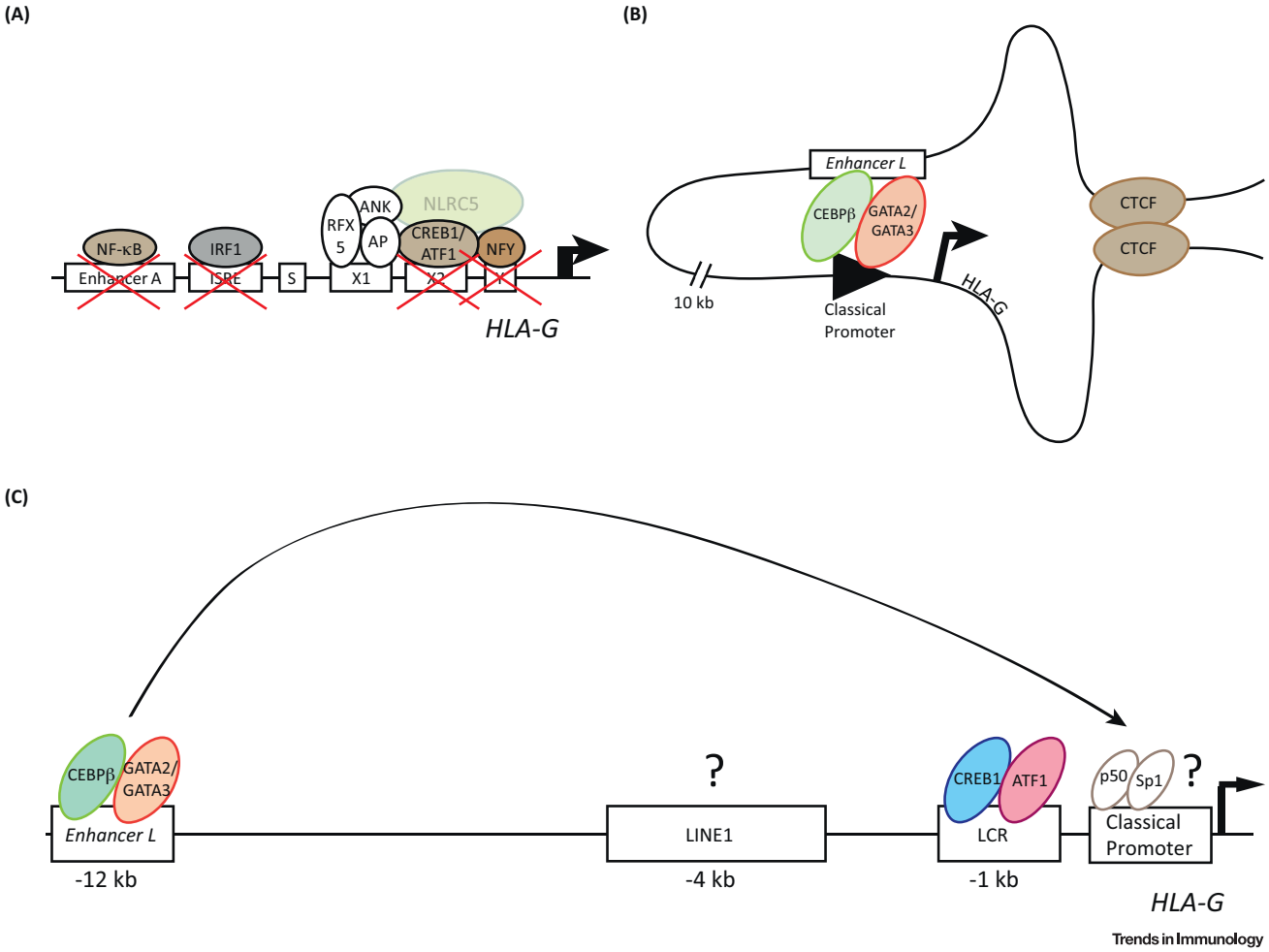


Figure 2. Transcriptional Regulation of *HLA-G*. (A) Despite its similarity with other MHC class I gene promoters, the classical promoter of *HLA-G* is defective, harboring mostly nonfunctional regulatory elements [39]. Specifically, the *HLA-G* promoter Enhancer A does not bind the p65 subunit of NF-kB, thus being unresponsive to tumor necrosis factor (TNF)-α; the interferon-stimulated response element (ISRE) is partially deleted and consequently unresponsive to IFN-γ, and the SXY module contains defunct X2 and Y elements [27]. In addition, neither NLRC5 nor CIITA are expressed in extravillous trophoblasts (EVT) [38]. (B) Long-range chromatin interactions between Enhancer L and the *HLA-G* classical promoter are mediated by CEBP and GATA transcription factors. The insulator CCCTC-binding factor (CTCF) may play a role in defining the boundaries of Enhancer L activity. (C) Schematic summarizing the main regulatory elements upstream of *HLA-G*. All distances, in thousands of base pairs (kb), are relative to the transcription start site. Functional CREB/ATF binding sites have been identified within the locus control region (LCR) [41], possibly compensating for the defective X2 element in the *HLA-G* classical promoter [39]. LINE1, a recently described long interspersed nuclear element upstream of *HLA-G*, is thought to repress HLA-G expression in nontrophoblast cells via hairpin loop formation [42]. How this looping is prevented in HLA-G$^+$ trophoblasts, however, remains unknown. The curved arrow represents chromatin looping, while the bent arrow denotes the transcription start site.

(Box 2). MPRA tiling of the *HLA-G* locus led to the discovery of a trophoblast-specific enhancer of HLA-G expression located 12 kb upstream of *HLA-G*: Enhancer L [34]. Unlike previously described regions, Enhancer L is active specifically in HLA-G$^+$ cells. Furthermore, CRISPR/Cas9-mediated deletion of Enhancer L in the trophoblast-like HLA-G$^+$ JEG3 cell line and in primary EVT revealed that it is absolutely required for HLA-G expression. Interestingly, CTCF binding sites immediately upstream of Enhancer L and downstream of the *HLA-G* coding sequence likely establish an insulated chromatin domain for *HLA-G* transcriptional regulation (Figure 2B). Mechanistically, Enhancer L loops into the core promoter of *HLA*-G upon association with transcription factors previously implicated in trophoblast development and function, CCAAT/enhancer binding proteins (CEBP)β and GATA2/3, activating *HLA-G* expression (Figure 2B, C).

Box 2. Toolbox for Enhancer Discovery: From Correlation to Function

DNase hypersensitivity sites sequencing (DNase-seq): treatment of nuclei with DNase I preferentially fragments nucleosome-depleted genomic DNA, revealing DNase hypersensitive sites (DHS), which are highly correlated with active enhancers. DNase-seq can also predict transcription factor occupancy, as binding of each class of transcription factors leaves a characteristic cleavage pattern ('footprint') within a DHS [106].

Assay for transposase-accessible chromatin sequencing (ATAC-seq): exploits the preferred integration of a transposable element, Tn5 transposase, into nucleosome-free regions of chromatin. ATAC-seq provides similar information to DNase-seq, yet requires three to five orders of magnitude less cells [107].

Chromatin immunoprecipitation sequencing (ChIP-seq): can be used for genome-wide mapping of histone modifications correlated with active enhancer regions, namely H3K4me1 and H3K27Ac, as well as factors ubiquitously associated with enhancers, such as p300 [108]. ChIP-seq is limited to prior knowledge on which factors may bind a region of interest.

Chromosome Conformation Capture (3C): three-dimensional chromatin interactions are detected using cross-linking, enzymatic digestion, and PCR followed by sequencing. Circularized chromatin conformation capture (4C), a 'one-to-all approach', can guide the discovery of new regulatory DNA segments that loop into the promoter of a target gene [109].

Reporter gene assay: a reporter gene, typically firefly luciferase, is inserted downstream of a promoter next to the DNA element being tested. This method quantitatively measures transcriptional transactivation by a DNA fragment in cis. Yet, it cannot provide information on long-range chromatin interactions in cis or trans that are typical of an enhancer element.

Massive parallel reporter gene assay (MPRA): simultaneously probes large numbers of DNA fragments that are synthesized and cloned into a barcoded library of uniquely sequence tagged vectors. Similar to a conventional reporter gene assay, MPRA only provides information on the transactivation activity of a DNA fragment in cis. However, it allows the interrogation of a multikilobase genomic region in one experiment by tiling the respective locus. Moreover, by mutating a specific interrogated sequence at any given position, it can reach base pair resolution [46].

Multiplexed editing regulatory assay (MERA): simultaneously interrogates large numbers of DNA elements in their endogenous chromatin context by systematically introducing mutations using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9. Introduction of a GFP reporter downstream of the gene of interest coupled with FACS sorting allows for the identification of regions required for optimal gene expression – enhancers [110]. Ultimately, precise genomic excision of a desired DNA element followed by RNA-seq unequivocally identifies its target gene [34].

How did *HLA-G* expression become trophoblast-specific? Remarkably, the presence of Enhancer L is also unique to two of the great apes (chimpanzees and gorillas) and may have been introduced by retrotransposon insertion coinciding with the acquisition of placental HLA-G expression. Consistent with this hypothesis, the only great ape lacking Enhancer L in its genome is the orangutan, whose *HLA-G* classical promoter has functional X2 and Y motifs and is thus likely to be ubiquitously expressed. This mechanism of *de novo* acquisition of tissue-specific expression was likely reinforced by the fact that Enhancer L controls HLA-G expression via the binding of CEBP and GATA factors, as deficiency in CEPBα, CEBPβ, GATA2, or GATA3 leads to profound placentation defects [47,48].

Several questions remain. Are there additional trophoblast-restricted transcription factors controlling *HLA-G* expression? Do they act via Enhancer L? Intriguingly, EVT express neither NLRC5 nor CIITA, yet constitutively express three MHC class I genes: HLA-C, HLA-E, and HLA-G [38]. How are these three genes expressed simultaneously in the absence of HLA-A and HLA-B transcription? According to our current model, HLA-G expression is accomplished by a mechanism fundamentally different from the one previously described for classical MHC class I gene expression: looping of a distant enhancer mediated by CEBP and GATA transcription factors into the proximal promoter. Yet, this unique looping mechanism does not exclude the possibility that a transactivator other than NLRC5 and CIITA assembles a transcriptional complex at the *HLA-G* promoter similar to the well-studied MHC enhanceosome.

Posttranscriptional Regulation of HLA-G Expression

In addition to transcriptional regulation, posttranscriptional mechanisms also influence HLA-G expression. Shortly after the discovery of *HLA-G*, a 14 bp insertion/deletion (indel) polymorphism was identified in its 3′ untranslated region (3′ UTR) [49]. One decade later, the 14 bp genomic insertion was found to lead to increased *HLA-G* mRNA stability [50].

HLA-G is known as a nonclassical MHC class I gene with a low degree of polymorphism. The genetic sequences encoding exons 2 and 3, which represent the α1 and α2 domains (peptide binding domains) of the HLA-G protein are indeed relatively nonpolymorphic, when compared to the corresponding sequences in the *HLA-A*, *HLA-B*, and *HLA-C* genes, which are highly polymorphic. Nevertheless, the 3′ UTR of *HLA-G* displays a high degree of sequence variation (Figure 3). More than 40 haplotypes of the 3′ UTR of *HLA-G* have been described, but 7 of them (UTR-1 through UTR-7) account for nearly 90% of all *HLA-G* genes sequenced to date [51]. Those with a frequency greater than 1% are thought to have been maintained by balancing selection as a result of heterozygote advantage. Interest in this variability stems from the fact that it may affect surface HLA-G expression on EVT; for example, UTR-1 is a high expressor, while UTR-2 is an intermediate level expressor [51]. The variations include the 14 bp indel and single nucleotide polymorphisms (SNPs). The 14 bp indel has been associated with stability of the *HLA-G* mRNA and/or its translation, as modified by the binding of microRNAs (miRs) and/or by alternative splicing of the gene. Importantly, some polymorphisms have been associated with the binding of miRs and the decreased production of HLA-G protein. The mechanism behind this observation is the disruption of miR binding sites. miR148a and miR152, whose expression is suppressed specifically in trophoblasts, downregulate *HLA-G* levels [52]. Consistent with this model, overexpression of miR148 or miR152 enhanced NK cell cytotoxicity and diminished binding to the NK cell receptor ILT2 [52]. Interestingly, the largest conglomeration of miRNA genes in the human genome, the chromosome 19 miRNA cluster (C19MC), is almost exclusively expressed in trophoblasts [53]. Within these miRNAs, there exist miRNAs predicted

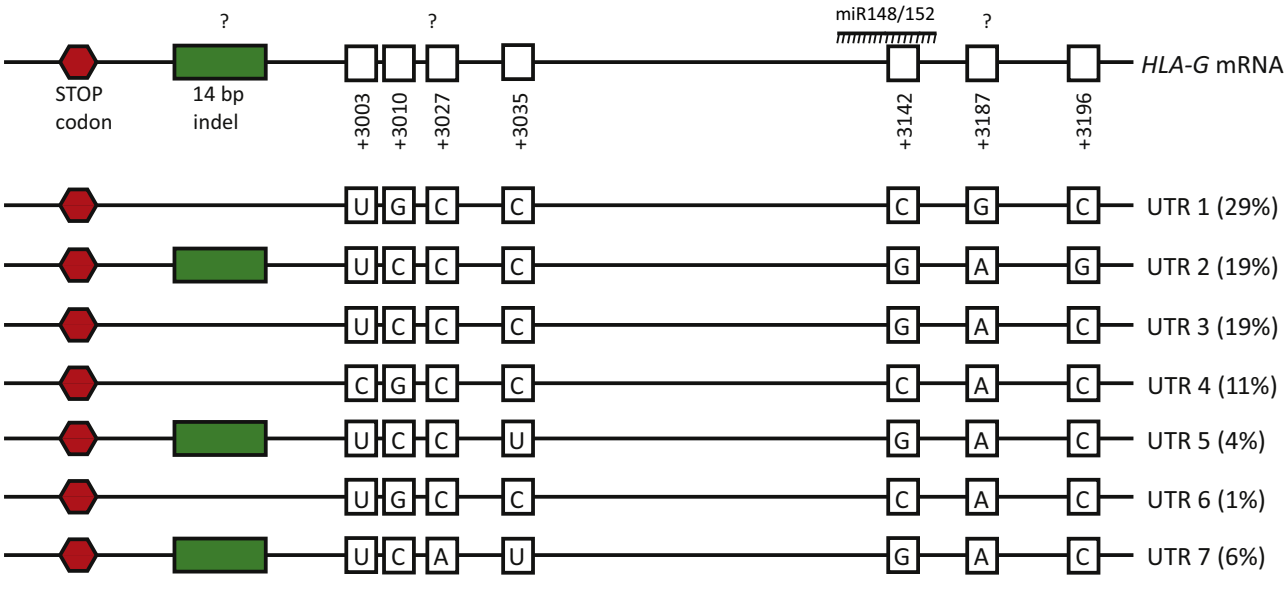


Figure 3. Polymorphisms of the 3′ Untranslated Region (UTR) of *HLA-G*. Seven *HLA-G* 3′ UTR haplotypes account for approximately 90% of the entire population [51]. Partly overlapping binding sites for miRNAs miR148 and miR152 have been identified in a region containing the *HLA-G* 3′ UTR single nucleotide polymorphism (SNP) at position +3142. miR148 and miR152 have been shown to inhibit *HLA-G* expression by binding to these sites [52]. Additional miRNA binding may occur elsewhere in the *HLA-G* 3′ UTR, most likely where other SNPs have been described, as indicated by question marks. Adapted from [51].

Box 3. HLA-G As an Immunomodulatory Molecule Co-opted by Cancer

While the possibility that tumors co-opt HLA-G expression to evade immunosurveillance is intriguing, supporting evidence is controversial. The first report of HLA-G expression in cancers, in 1996, was obtained from studies in hematopoietic cells; while no HLA-G transcript was detected in hematopoietic stem cells, thymocytes or natural killer cells, a minute fraction of analyzed leukemias expressed HLA-G [111]. Two years later, detection of HLA-G expression was extended to a solid tumor type, melanoma [90]. Given its role in inducing immune tolerance at the maternal–fetal interface, it was immediately suggested that HLA-G was co-opted by some malignant tumors to evade immune surveillance. Consistent with this hypothesis, an HLA-G transcript positive melanoma cell line was protected against NK cell lysis, while two HLA-G transcript negative melanoma cell lines were lysed. Nevertheless, the authors failed to detect full-length HLA-G protein expression in melanoma samples with HLA-G transcript. A second experiment using the antibodies HCA2 and 4H84 pulled down lower molecular weight bands identified by the authors as shorter HLA-G splice isoforms [90]. However, subsequent studies revealed cross-reactivity of both antibodies with HLA-A, HLA-B, and HLA-E [112,113], casting doubt on HLA-G protein expression, and thus function, in metastatic melanoma.

Comprehensive analysis of tumor samples from six different origins and 31 tumor cell lines did not find HLA-G surface expression in any of the samples, despite detecting HLA-G transcription in most samples [114]. While some studies found no evidence of HLA-G protein expression in melanoma, either cell lines or primary tumor samples [115], others have found HLA-G expression in a subset of melanomas characterized by high levels of HLA-G transcription [116]. It should be emphasized that these independent studies were performed on the same type of tumor, melanoma, using the same antibody to detect HLA-G expression, 87G. Functionally meaningful HLA-G expression may thus be restricted to a small subset of melanomas.

Recent studies by independent groups collectively reveal various *HLA-G* polymorphisms as predictors of susceptibility to and clinical outcome of different cancers [117]. Moreover, HLA-G protein expression has now been detected in several types of primary solid tumors in addition to melanoma, including cervical cancer and breast cancer, [118,119]. In all cases, HLA-G expression was associated with disease progression and poor prognosis, warranting further research to determine the relevance of HLA-G expression in malignancies.

to selectively bind each MHC class I gene, including HLA-G, as well as combinations, suggesting that miRNAs may play a pre-eminent role in establishing EVT's unique MHC expression pattern. Of note, many clinical conditions thought to involve reduced immunosuppression are associated with variants with reduced *HLA-G* expression. These include pregancy-related disorders such as preterm birth, pre-eclampsia, miscarriage, and recurrent spontaneous abortion, as well as autoimmune disorders such as asthma and type 1 diabetes [54–58]. Although many of these studies may not be entirely convincing individually, the mass of published work suggests that it is an important area for further study.

Finally, extracellular factors, their receptors, and downstream signaling molecules are likely to provide an additional layer of regulation of HLA-G expression, as several pregnancy-related hormones have been shown to modulate HLA-G expression, notably progesterone [54]. Future studies further dissecting the regulation of *HLA-G* expression may enable us to specifically control HLA-G expression in any desired cell type, potentially leading to novel treatments for pregnancy disorders and transplant rejection. Moreover, they may shed light on the mechanisms of the *de novo* HLA-G expression observed in some cancers (Box 3).

New Insights into the Immunomodulatory Function of HLA-G

HLA-G Modulates dNK Functions from the Inside

Determining the mechanisms by which HLA-G induces immune tolerance at the maternal–fetal interface has been technically challenging. Unlike most immune genes, murine models are not an option to investigate HLA-G function, as there is no consensus on the identity (or even existence) of its murine orthologue. Currently, the only organisms where there is solid evidence for the presence of HLA-G are primates, much less versatile *in vivo* models than rodents [59]. Progress creating *in vitro* models of the human maternal–fetal interface has been hampered by scarcity of available human placental material from early pregnancy terminations, as well as by

the fact that primary EVT are difficult to obtain in large numbers and only survive a few days in culture.

Consequently, in the initial experiments that established the inhibitory properties of HLA-G, the NK-sensitive MHC-I deficient B cell line LCL721.221 was utilized. However, overexpressing HLA-G in these cells upregulated surface expression of another nonclassical MHC molecule found in trophoblasts, HLA-E. In fact, loading HLA-E with an HLA-G-derived peptide results in its highest affinity for the inhibitory NK receptor CD94/NKG2A [60]. Therefore, NK cell inhibition by EVT could be achieved by HLA-G directly or by HLA-E presenting an HLA-G peptide. Could a major purpose of trophoblast HLA-G expression be to boost HLA-E surface expression?

Currently, the most used *in vitro* model to study HLA-G is the choriocarcinoma cell line JEG3, which expresses HLA-G, HLA-E, and HLA-C, thus recapitulating the MHC expression pattern of EVT. Unexpectedly, masking MHC class I surface expression in JEG3 using either blocking antibodies or acid treatment does not render them sensitive to NK cells, arguing that MHC molecules are not essential to protect trophoblasts from NK cell lysis [61]. Cancer cells are notable for developing multiple strategies to evade immune surveillance, making primary EVT indispensable to dissect the role of HLA-G in maternal–fetal tolerance.

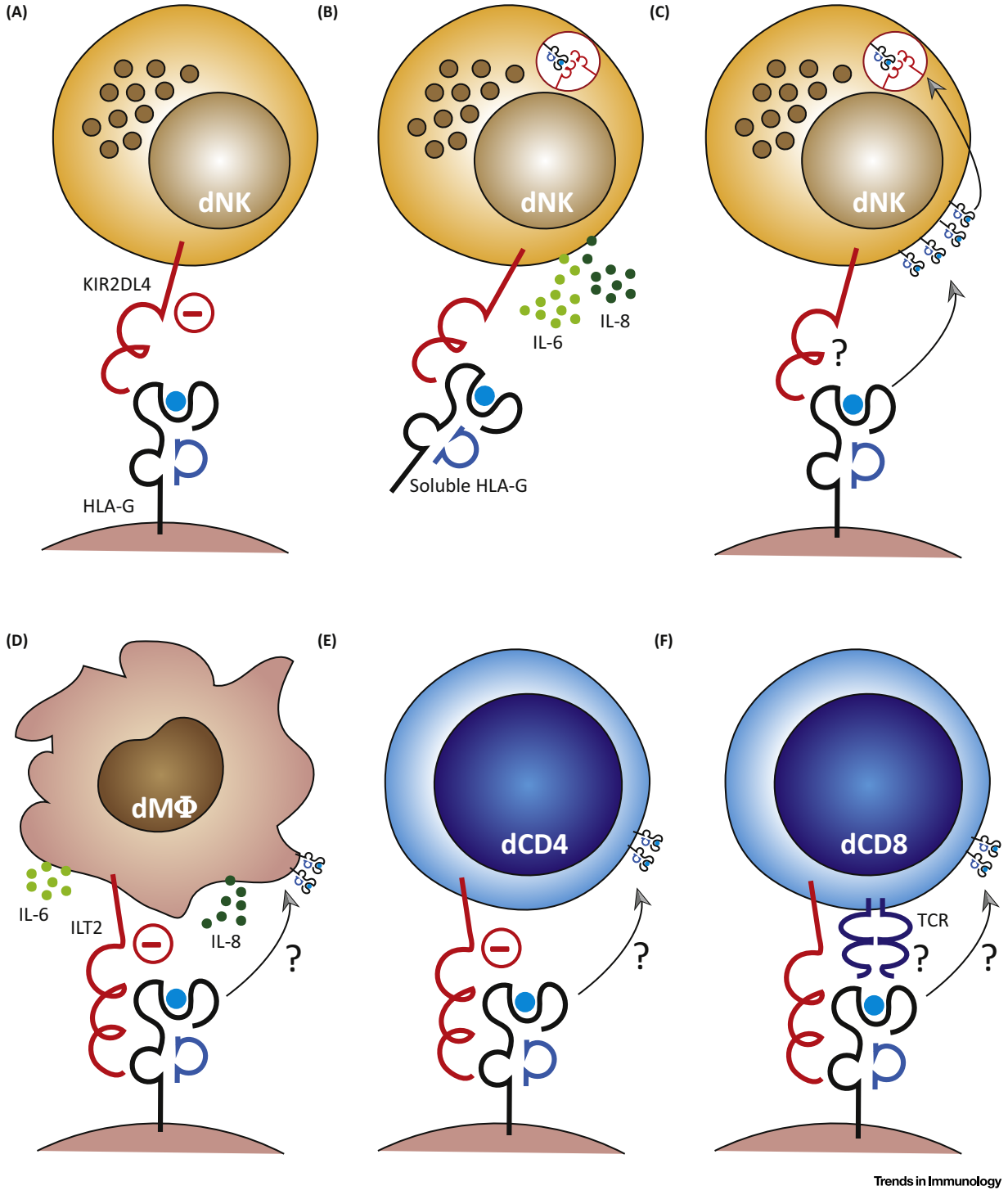
To date, experiments using primary human trophoblasts have yielded conflicting results. One study found that first trimester EVT remained insensitive to polyclonal dNK cell-mediated lysis following surface MHC blockade [62]. Yet, a different group showed that primary EVT were lysed by dNK when their total surface MHC-I expression was masked with a pan-MHC-I antibody, yet they remain protected when only classical MHC class I – HLA-C in this case – was blocked [63]. Technical problems such as suboptimal cell separation or incomplete antibody blockade may be responsible for these discrepancies.

dNK constitute up to 90% of decidual leukocytes in early pregnancy and, while there is a sharp decline in their numbers during gestation, they remain present until term pregnancy [10]. It has become increasingly clear from both human and mouse studies that dNK significantly differ from pNK in phenotype and function. In a healthy state, freshly isolated dNK are poorly cytotoxic, being rather geared towards producing high levels of cytokines and angiogenic factors [7,64]. However, placental viral infections as well as cytokines can trigger dNK to become cytotoxic and proinflammatory similarly to pNK [11,65]. How is the balance between tolerance and antiviral immunity by dNK achieved at the maternal–fetal interface?

Recently, HLA-G-induced immune tolerance has been found to occur via a peculiar cell biological process: trogocytosis. Named after the Greek word *trogo* (to nibble), trogocytosis consists of membrane protein transfer between cells. Despite having been first observed in immune cells in the early 1970s [66,67], the prevalence and exact mechanism of trogocytosis are unclear, although some understanding has been obtained [67–69]. Transendocytosis, a cell biological process related to trogocytosis, allows immune cells to acquire proteins produced by other cells via endocytosis. However, unlike transendocytosis of CD80 and CD86, where CTLA4 expression on the recipient cell has been shown to be necessary and sufficient to capture its costimulatory ligands from an interacting cell [70], the existence and identity of a receptor for HLA-G trogocytosis remain elusive. It is thus possible that HLA-G trogocytosis is receptor-independent. Still, the relevance of this phenomenon is quickly becoming widely accepted. Mounting evidence indicates that HLA-G trogocytosis is correlated with a poor prognosis in cancer. In myeloma patients, T cells are the preferential recipients of HLA-G from malignant myeloma cells [71]. Strikingly, in every system analyzed thus far, HLA-G trogocytosis confers upon the recipient cells a transient immune suppressive phenotype.

Key Figure

Multiple Mechanisms of HLA-G-Mediated Immune Modulation



(See figure legend on the bottom of the next page.)

Following its demonstration using surrogate cells [79] HLA-G trogocytosis between primary human EVT and dNK has recently been demonstrated, and is thought to play a role in balancing immune tolerance and antiviral immunity at the maternal–fetal interface [11]. Intracellular HLA-G protein was detected in a large fraction of dNK in the absence of *HLA-G* mRNA, suggesting that HLA-G trogocytosis could involve most dNK. Of note, cytokine activation of dNK resulted in the disappearance of internalized HLA-G in parallel with restoration of cytotoxicity; surface HLA-G was reacquired by dNK upon coincubation with EVT [11]. Internalized HLA-G correlates with very low cytotoxic activity of freshly isolated dNK. This phenomenon results from the failure of these dNK to mobilize the microtubule organizing center (MTOC) and its associated cytotoxic granules to the synapse between dNK and target cells [7]. However, the mechanism of polarization and of its failure as well as the mechanism of its activation remain unknown.

But why evolve a system where HLA-G is transferred between cells, when HLA-G is expressed on the cell surface and directly binds to inhibitory receptors on multiple immune cells? One possible answer may lie in the fact that KIR2DL4, the main HLA-G receptor expressed by dNK, resides mostly intracellularly in endosomes and signals from endolysosomes upon binding to HLA-G [72]. Furthermore, treating pNK with a soluble form of HLA-G triggers the expression of a gene signature characteristic of dNK, resulting in proinflammatory and proangiogenic cytokine production [33]. However, these studies have, for the most part, been carried out with recombinant soluble HLA-G protein and pNK, which are distinct from membrane-bound HLA-G and dNK [73]. Therefore, although they do not necessarily reflect events that occur at the maternal–fetal interface, they clearly point in an important direction for future work. One can envision that HLA-G trogocytosis allows for prolonged KIR2DL4-mediated signaling in dNK, leading to the secretion of cytokines and other small proteins with important roles in placental and fetal development, as well as establishment of immune tolerance. Strikingly, it has been recently shown that the HLA-G receptor Ig-like transcript 2 (ILT2, also known as LILRB1) can be transferred from monocytes to activated $CD4^+$ T cells and is fully functional in the recipient cells, mediating inhibition of these ILT2 positive T cells by HLA-G [74]. Trogocytosis may thus contribute to widespread HLA-G-mediated immune inhibition at the maternal–fetal interface involving decidual immune cells other than dNK.

HLA-G Interacts with Multiple Placental Immune Cell Subsets

The realization that most leukocytes present at the maternal–fetal interface during the first trimester of gestation are dNK has led the field of reproductive immunology to dedicate substantial effort to understanding their role in pregnancy. Yet, other immune cell types are present in decidua and play important roles during pregnancy (Figure 1). A growing body of evidence suggests that HLA-G modulates the activity not only of NK cells (Figure 4A–C), but also of macrophages [75] (Figure 4D), T cells [76] (Figure 4E, 4F), and B cells [77]. In addition to KIR2DL4, HLA-G is now known to bind to ILT2 and ILT4, inhibitory receptors found on some subsets of dNK, T cells and myeloid cells [78].

Figure 4. (A) Direct NK cell inhibition. HLA-G is a well-established inhibitory ligand of NK cells, acting via KIR2DL4, a receptor broadly expressed across NK cell subsets. (B) NK cell reprogramming via endosomal signaling. Upon interaction with KIR2DL4 resident in early endosomes, HLA-G (here shown in its soluble form) induces the secretion of cytokines and growth factors characteristic of dNK and required for successful maternal–fetal interface remodeling, such as IL-6 and IL-8. (C) Trogocytosis. Acquisition of HLA-G via intercellular membrane transfer (lower curved arrow) occurs in the majority of dNK. The involvement of KIR2DL4 or other HLA-G receptor(s) in this process, however, remains unclear. The acquired HLA-G molecules are then recruited by KIR2DL4-containing endosomes (upper curved arrow), triggering downstream signaling events. (D) Modulation of macrophages. Upon binding to ILT2 and ILT4 (not pictured), HLA-G induces the secretion of cytokines required for successful maternal–fetal interface remodeling by decidual macrophages. (E,F) Direct T cell inhibition. A fraction of $CD4^+$ and $CD8^+$ T cells also express surface ILT2, which suppresses T cell proliferation upon interacting with HLA-G. Moreover, trogocytosis of HLA-G by T cells confers on them a suppressive phenotype. The identification of HLA-G-restricted T cells remains elusive. Note: Even though HLA-G trogocytosis has been shown *in vitro* for NK cells [79], monocytes [80], and activated T cells [32,71], the only decidual immune cell subset where this phenomenon has been demonstrated to date is dNK cells that appear to acquire HLA-G from filopodia that express it at a high level [11]. dNK, decidual natural killer cell; dMΦ, decidual macrophage; dCD4, decidual $CD4^+$ T cell; dCD8, decidual $CD8^+$ T cell.

Interestingly, HLA-G forms a homodimer that has been described in transfected cells, the HLA-G$^+$ choriocarcinoma cell line JEG3, and primary EVT [29,75,81,82], and is present in the crystal structure of the HLA-G/ILT2 complex [83,84]. This homodimer is assembled by disulfide bond formation between two HLA-G monomers as a result of Cysteine 42 in the HLA-G α1 domain, a position occupied by Serine 42 in all other HLA class I molecules. Interestingly, in at least one system, cytokine secretion occurred only in response to the HLA-G homodimer, but not to its monomeric form [75]. These observations raise the question of the biological function of different HLA-G structures. Do different HLA-G receptors respond to distinct forms of HLA-G, for example, the monomer or the homodimer? Are there other stable and functional HLA-G structures? Do they expand the repertoire of receptors that can engage with HLA-G?

Decidual macrophages (dMΦ) represent approximately 15–20% of decidual leukocytes. Mainly associated with immune suppression and tissue repair, dMΦ secrete high levels of factors that facilitate placental growth and trophoblast invasion. Nevertheless, dMΦ have also been found to produce proinflammatory cytokines in response to HLA-G binding to ILT2 [75] (Figure 4D). Distinct subsets of dMΦ have been identified that are dedicated either to antigen presentation or to tissue remodeling and repair at the maternal–fetal interface, having been shown to secrete proinflammatory cytokines and present antigens to decidual T cells [9,12,13,85].

Decidual T cells, in turn, comprise 5–15% of placental leukocytes during the first trimester of gestation, a number that can reach up to 70% at term pregnancy. In fact, high percentages of Tregs can be found in decidual tissue, where they suppress fetus-specific lymphocyte responses. Moreover, moderate numbers of activated effector memory CD8$^+$ T cells are also found at the maternal–fetal interface; their role in fetus- and virus-specific immune responses remains a topic of debate [5,86,87]. HLA-G was first shown to inhibit CD8$^+$ T cell cytotoxicity in 1999 [88]. The following year, these observations were extended by an independent group to inhibition of CD4$^+$ T cell proliferation [76]. How does HLA-G simultaneously work as a suppressor of NK and T cell responses? A fraction of both CD4$^+$ and CD8$^+$ T cells express ILT2, an MHC receptor on their surface with high affinity for HLA-G, which inhibits TCR-mediated activation by competing with CD8 for MHC binding [78,89] (Figure 4E, F). Strikingly, acquisition of membrane-bound HLA-G via trogocytosis confers a suppressive phenotype on CD4$^+$ T cells. These surface HLA-G expression positive T cells have been shown to inhibit allogeneic T cell responses as potently as conventional Tregs in the absence of FOXP3 expression [32]. The importance of this mechanism to quickly generate suppressive T cells at the maternal–fetal interface remains to be investigated.

Concluding Remarks

HLA-G remains a most enigmatic MHC class I molecule, and numerous questions remain to be answered (see Outstanding Questions). At the time of its discovery, many basic questions were only starting to be addressed: How many HLA genes are there? How do NK cells work? Does the immune system have access to the fetus? Following the initial spurt of studies characterizing HLA-G as a central molecule in maternal–fetal tolerance, inherent technical difficulties eventually halted progress in the field.

Yet, the last decade has witnessed a renaissance in HLA-G biology. We now know that the trophoblast-specific expression of HLA-G requires long-range chromatin interactions mediated by developmental transcription factors [34]. In addition, HLA-G functionality goes beyond that of a typical surface immune molecule: HLA-G, as well as its receptors, can be quickly transferred between cells via trogocytosis, conferring recipient cells an immunosuppressive phenotype [11,74,79]. Finally, there is the intriguing possibility that HLA-G may be at work in a context other than pregnancy – cancer [90]. HLA-G has been detected in tumor lesions, where it may facilitate immune surveillance escape (Box 3). Advances in human pluripotent stem cell

Outstanding Questions

What are the mechanisms of immune tolerance induction by HLA-G during pregnancy? As it becomes evident that decidual immune cells are markedly different from their peripheral blood counterparts, and HLA-G trogocytosis emerges as a widespread phenomenon at the maternal–fetal interface, dissection of the signaling pathways impinged on by HLA-G will likely reveal new modes of immune tolerance induction.

Have all HLA-G receptors been discovered? Many NK cell receptors remain orphan receptors, leaving open the possibility that some of them recognize HLA-G. Other decidual immune cells, such as $\gamma\delta$ T cells and innate lymphoid cells (ILCs), may also interact with HLA-G via unidentified receptors.

Are there pregnancy-specific extracellular factors modulating HLA-G expression at the maternal–fetal interface? Such signals might activate transcription factors or alter the chromatin conformation at the *HLA-G* locus, leading to *HLA-G* transcription. Indeed, several pregnancy-related hormones have been shown to upregulate HLA-G expression *in vitro,* notably progesterone.

Is there a functional homolog of HLA-G in the mouse? Currently, there are three murine nonclassical MHC candidates: Qa-2, H2-Bl, and H2-M3. However, some have argued that there is no orthologue of HLA-G in the mouse, as the significantly shorter gestation time and less profound trophoblast invasion might preclude its need.

What are the endosomal signaling events initiated by internalization of HLA-G? How do they lead to loss of cytotoxicity (tolerance)? Is failure to polarize the microtubule organizing center (MTOC) the only relevant event? What is the function of novel factors that may be produced by dNK as the result of HLA-G signaling?

Does HLA-G have a role in the protection of tumors from the host immune system and/or in the resistance of a fraction of tumors to immunotherapy with anti-CTLA4 and anti-PD-L1?

Is HLA-G indispensable for human pregnancy? There are reports of

differentiation into trophoblasts [91], decidual immune cell and EVT isolation, culture, and characterization [38], as well as genetic manipulation of primary EVT [34], will continue unraveling new mechanisms and functions for the only MHC molecule specifically purposed for tolerance – HLA-G.

individuals homozygous for mutations in *HLA-G* that prevent translation of full-length HLA-G protein. Yet, there is no known example of a living fully HLA-G-deficient human; the observed homozygous *HLA-G* mutations allow for the expression of truncated forms of HLA-G protein with inhibitory activity against NK cells (possibly by providing HLA-G leader peptide to HLA-E).

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