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The Dual Role of HLA-C in Tolerance and Immunity at the Maternal-Fetal Interface

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To establish a healthy pregnancy, maternal immune cells must tolerate fetal allo-antigens and remain competent to respond to infections both systemically and in placental tissues. Extravillous trophoblasts (EVT) are the most invasive cells of extra-embryonic origin to invade uterine tissues and express polymorphic Human Leucocyte Antigen-C (HLA-C) of both maternal and paternal origin. Thus, HLA-C is a key molecule that can elicit allogeneic immune responses by maternal T and NK cells and for which maternal-fetal immune tolerance needs to be established. HLA-C is also the only classical MHC molecule expressed by EVT that can present a wide variety of peptides to maternal memory T cells and establish protective immunity. The expression of paternal HLA-C by EVT provides a target for maternal NK and T cells, whereas HLA-C expression levels may influence how this response is shaped. This dual function of HLA-C requires tight transcriptional regulation of its expression to balance induction of tolerance and immunity. Here, we critically review new insights into: (i) the mechanisms controlling expression of HLA-C by EVT, (ii) the mechanisms by which decidual NK cells, effector T cells and regulatory T cells recognize HLA-C allo-antigens, and (iii) immune recognition of pathogen derived antigens in context of HLA-C.

Keywords: human, pregnancy, decidua, regulatory T cells, effector T cells, decidual NK cells, trophoblast, HLA-G

HIGHLIGHTS

- Expression of HLA-C, HLA-E, and HLA-G by EVT in the absence of HLA-A and HLA-B expression, requires trophoblast specific MHC class I transcriptional regulators.
- The expression of a polymorphic paternally inherited HLA-C antigen by EVT provides a target for maternal NK cells and T cells, the HLA-C cell surface expression levels influence how this response is shaped.
- Maternal CTL responses to fetal HLA-C and minor Histocompatibility Antigens (e.g., HY) are generated by many individuals, but during healthy pregnancy HLA-C mismatches are associated with immune tolerance.
- Multiple types of decidual Treg play a role in mechanisms of fetus-specific and non-specific immune tolerance. Further investigation of the function and specificity of decidual Treg has exceptional therapeutic potential for treatment of a wide variety of inflammatory disorders, including pregnancy complications.
- The balance between the transient dysfunction of decidual CD8⁺ T cells and dNK that are permissive of placental and fetal development, and reversal of this dysfunctional state to provide immunity, is crucial in understanding the ethology of pregnancy complications and prevention of congenital infections.
- Interactions of activating KIR with HLA-C reduces the risk of pregnancy complications, possibly through providing specific immunity to viral and bacterial pathogens.

INTRODUCTION

Human Leukocyte Antigen-C (HLA-C) was first discovered by antigen-antibody analysis in the early 1970s (1), but its history started ~10 million years ago on the precursor of the polygenic and polymorphic segment of human chromosome 6, which encodes the Major Histocompatibility Complex (MHC) molecules. HLA-C was formed by a duplication of the HLA-B gene and HLA-C homologs are only present in chimpanzees, gorillas, bonobos, and humans (2). Like the other classical MHC class Ia molecules HLA-A and HLA-B, HLA-C is a highly polymorphic hetero-trimer consisting of an alpha heavy chain, β 2-microglobulin and a peptide antigen (3, 4). Over 5,600 alleles and 3,400 protein variants have thus far been identified for HLA-C (IMGT/HLA sequence database: <http://www.ebi.ac.uk/imgt/hla/stats.html>) (5). HLA-C is a major determinant for NK cell activity (6, 7) and in 1983 a first report demonstrated the importance of HLA-C in pregnancy and placentation (8). HLA-C has also been associated with many diseases, including viral infections, cancer, autoimmune diseases and transplant failure (9–11). In this review, we critically discuss new insights into: (i) the mechanisms controlling the expression of HLA-C in the absence of HLA-A and HLA-B expression by placental extravillous trophoblasts (EVT); (ii) the mechanisms by which decidual NK cells, effector T cells and regulatory T cells recognize paternal HLA-C allo-antigens during pregnancy and its relevance in the development of pregnancy complications; and (iii) discuss

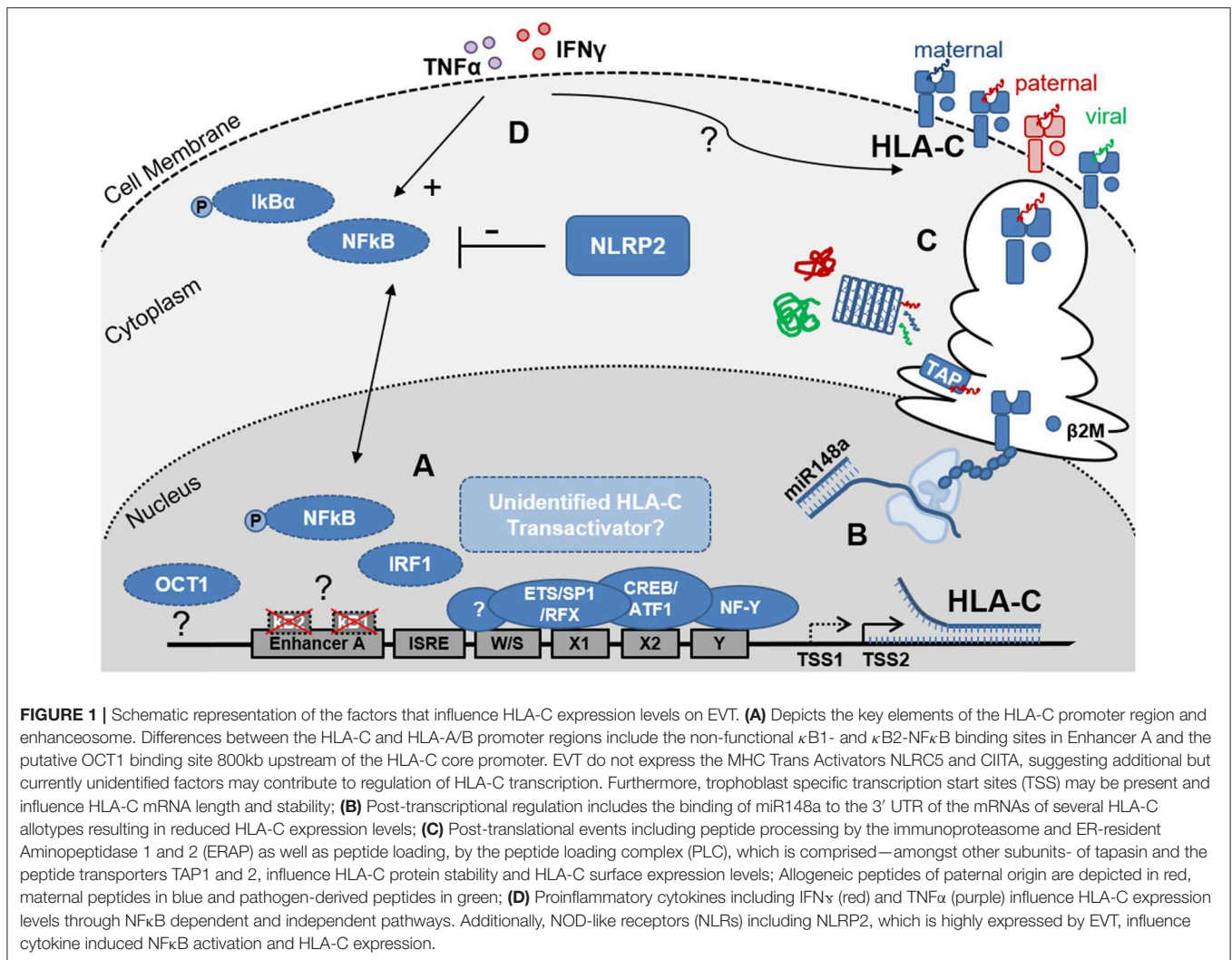
the relevance of immune recognition of pathogen derived antigens in context of HLA-C to establish placental immunity.

REGULATION OF HLA-C EXPRESSION BY EVT

Whereas nearly all nucleated cell types express the polymorphic MHC class Ia molecules HLA-A, HLA-B and HLA-C, as well as the invariant MHC class Ib molecule HLA-E, EVTs lack expression of HLA-A and HLA-B, while expressing HLA-C and HLA-E. In addition, EVT uniquely express the invariant MHC class Ib molecule HLA-G (12–14). Despite the distinct HLA expression phenotype of EVT, the regulatory mechanisms that prevent HLA-A and HLA-B expression while establishing HLA-C, HLA-E, and HLA-G expression on EVT have not been fully elucidated (15–17). NLRC5 (nucleotide-binding domain, leucine-rich repeat family, CARD domain-containing 5), an important transcriptional regulator of MHC class I genes (18–20), and CIITA, the MHC Class II Trans Activator (21), are not expressed by EVT, suggesting that other MHC regulators must be present to control HLA-C, HLA-E, and HLA-G expression in these cells (16). Regulation of HLA-C cell surface expression depends on transcriptional as well as post-transcriptional regulatory mechanisms and the resulting HLA-C cell surface expression levels were shown to impact CD8⁺ effector T cell (Teff) responses to viral infection as well as allogeneic Teff responses (9, 10, 22).

Transcriptional Regulation of MHC Class I

Both MHC class I and II contain highly conserved cis-regulatory elements in their promoter regions, about 250 base pairs (bp) upstream of the transcriptional start site (23). These include the W/S, X, and Y box motifs that assemble the MHC enhanceosome (24, 25) (**Figure 1A**). It has recently been shown that the W/S box is crucial for MHC class I transactivation by NLRC5 (24, 26), but no DNA-binding protein of the W/S box has been identified thus far. In the absence of a known DNA binding domain, NLRC5 acts as an MHC class I specific trans activator by binding to and cooperating with the trimeric RFX transcription factor protein complex consisting of RFX5, RFXAP, and RFXANK/B (24). While the RFX complex is also essential for enhanced transactivation of MHC class II genes by CIITA, NLRC5 engages a unique S box sequence within the W/S motif thereby gaining specificity for a selected set of target genes (26). The MHC class I promoter also includes Enhancer A and an Interferon Stimulated Response Element (ISRE) to which transcription factors of the NF κ B/Rel family and IRF1 bind, respectively (23, 27). Enhancer A and the ISRE element are important for both constitutive and cytokine-induced MHC class I expression (27, 28). In addition, both NLRC5 and CIITA are highly inducible by IFN γ stimulation. Thus, IFN γ and other proinflammatory cytokines not only influence HLA expression directly through Enhancer A and ISRE elements but also act by upregulating NLRC5 and CIITA expression levels. Additional transcriptional control mechanisms beyond the immediate promoter region include long range enhancer-promoter interactions and the ability of



CIITA and NLRC5 to influence chromatin opening through their interaction with chromatin modifiers, such as the histone acetyl transferases p300/CBP-binding protein (p300/CBP), general control of amino acid synthesis 5 (GCN5) and p300/CBP-associated factor (PCAF), thus enabling RNA polymerase II to initiate MHC gene transcription (29–31). These and other transcriptional regulators forming the MHC class I and II enhanceosome have been reviewed (25, 28, 32).

Differences Between HLA-A, HLA-B, and HLA-C Promoter Structures

HLA-C has lower cell surface expression levels compared to HLA-A and HLA-B (33, 34). Furthermore, HLA-A and HLA-B have higher nucleotide diversity in the promoter region (1.8 and 1.9%, respectively) compared to the HLA-C promoter (0.9%), but no relationship has been found between promoter similarity and expression levels (35). Additional differences between the HLA-A, HLA-B and HLA-C promoter regions are found in Enhancer A. HLA-A contains two functional NF κ B binding sites, κ B1 and

κ B2. In the HLA-B and HLA-C promoters, κ B2 contains major nucleotide variations that largely disable the NF κ B binding. The transactivation of HLA-B by NF κ B is facilitated by binding of transcription factor Specificity Protein 1 (SP1) to κ B2. Interestingly, NF κ B binding occurs neither at the κ B1, nor at the κ B2 site of the HLA-C Enhancer A (27, 36). Thus, direct NF κ B transactivation seems to be restricted to the HLA-A and HLA-B genes. Using primary EVT and the EVT model cell line JEG3 we recently demonstrated that while IFN γ stimulation did indeed upregulate cell surface expression of HLA-C by an almost 5-fold change, IFN γ stimulation, expectedly, did not induce NF κ B phosphorylation. In contrast, TNF α stimulation did induce NF κ B phosphorylation in these cells and also increased HLA-C levels by 3-fold (16). These observations suggest that both, NF κ B-dependent and NF κ B-independent mechanisms may play a role in fine tuning the upregulation of HLA-C in trophoblasts upon cytokine stimulation.

Interestingly, a NK cell-specific HLA-C promoter was shown to produce a large array of differentially-spliced transcripts that vary in their ability to be translated into HLA-C protein and

directly influenced the lytic activity NK acquire during their development (37). Here a polymorphism in the ETS/SP1-binding site in the HLA-C promoter was shown to influence HLA-C expression in NK cells, with individuals lacking an intact ETS-binding site having reduced HLA-C levels. Thus, the NK-intrinsic regulation of HLA-C, includes a distinct mechanism controlling its expression that influences NK development (37). Furthermore, identification of trophoblast-specific elements in the HLA-C core promoter further distinguished it from the HLA-A and HLA-B promoter (**Figure 1**) (38, 39). Here a specific transcriptional start site was observed in trophoblast cell lines ~30 bp upstream of the HLA-C transcriptional start site observed in other cell types. Enhanced HLA-C activity in trophoblast cell lines was mapped to the central enhanceosome region of the promoter, and mutational analysis identified changes in the ETS/RFX-binding region that generated a trophoblast-specific enhanceosome required for trophoblast-specific transcriptional activity (39). The mechanisms responsible for the specific expression of HLA-C in trophoblasts are likely to be distinct from HLA-G, which expression is controlled by a recently identified enhancer (enhancer L), 12 kb upstream of the HLA-G transcriptional start site and which is required for HLA-G expression in trophoblasts cell lines and primary EVT (39, 40). Here, the binding of CCAAT Enhancer Binding Protein Beta (C/EBP β) and GATA Binding Protein 2/3 (GATA2/3) transcription factors and long-range chromatin looping were implicated in HLA-G enhancer L function. In conclusion these studies suggest that distinct mechanisms and unique cell type specific HLA-C transcription factors control HLA-C expression in somatic cell types, NK cells and trophoblasts.

EVT Specific Factors Regulating HLA-C Expression in the Absence of HLA-A and HLA-B

While the transcriptional regulators that allow HLA-C, HLA-E, and HLA-G expression on EVT in the absence of HLA-A and HLA-B expression have not been identified thus far, a few studies have implicated other members of the NLR-family of proteins, to which both NLRC5 and CTIIA also belong, that can influence HLA-C expression by EVT. First, NLRP12 (formerly known as Monarch 1) was shown to regulate both the classical (HLA-A, HLA-B, and HLA-C) and non-classical (e.g., HLA-E and HLA-G) MHC class I protein and RNA expression at the promoter level (41). However, NLRP12 doesn't seem to localize to the nucleus and thus it is unclear how NLRP12 influences the promoter complex to regulate MHC transcription. Interestingly, NLRP12 is downregulated by TNF α and IFN γ and may only play a role in constitutive MHC class I expression. Of note, NLRP12 is predominantly expressed by dendritic cells, monocytes and granulocytes and its expression was also increased in EVT and JEG3 compared to decidual stromal cells (DSC) (16).

Secondly, NLRP2 which is highly expressed by EVT, was shown to suppress cell surface expression of HLA-C without affecting HLA-E and HLA-G expression on EVT (**Figure 1D**) (16). Furthermore, knock out of NLRP2 increased phosphorylation of NF κ B in JEG3 and EVT upon TNF α

stimulation, demonstrating the immune suppressive properties of NLRP2 in these cells. Interestingly, however was the observation that in the NLRP2 knock out JEG3 clones, HLA-C induction by TNF α and IFN γ was reduced suggesting that the presence of NLRP2 facilitates cytokine-induced HLA-C expression. By fine tuning HLA-C expression levels on EVT, NLRP2 may contribute to shape immune cell responses to EVT and balance both immune tolerance and immunity (16). These observations also support the notion that NLRP12 and NLRP2 and possibly other proteins of the NLR family have additional functions beyond pattern recognition and induction of proinflammatory responses that includes the control of MHC expression (42).

HLA-C Cell Surface Expression Varies Widely Across Individuals in an Allele-Specific Manner

Variations in HLA-C cell surface expression levels have been shown to influence the efficacy of cellular immune responses to viral-, allo- and self-antigens (10, 11, 22). High HLA-C protein expression on the cell surface was associated with protection against the HIV-1 virus, increased cytotoxic T lymphocyte (CTL) responses and increased frequency of HIV escape mutations, suggesting that high HLA-C expression exerts a selection pressure on the virus (10). High HLA-C expression levels also correlate with increased risk of Crohn's disease (11), and in cases of unrelated haematopoietic transplantation, with poor outcome and graft-vs.-host disease (22). Particularly the observed differences in HLA-C restricted allo-responses and HLA-C restricted anti-viral responses may have clinical implications for pregnancy outcome, but the correlation between HLA-C expression levels and pregnancy outcome has not been investigated thus far.

Variation in HLA-C expression between different HLA-C alleles have been attributed to an atypical dimorphic binding site for OCT1 (also known as POU2F1), located ~800 bp upstream of the HLA-C transcriptional start site. OCT1 is a member of the POU transcription factor family with unusual importance in embryogenesis (43). The dimorphism results from a single SNP (rs2395471) which seems to affect the affinity of OCT1 for the site and hence HLA-C promoter activity and HLA-C protein levels (33, 44). OCT1 was shown to bind with lower affinity to the G than to the A allele, resulting in lower HLA-C promoter activity and lower HLA-C cell surface levels. This polymorphism is responsible for up to ~36% of the difference in HLA-C cell surface levels. These observations did not address, however, whether OCT1 is essential for HLA-C expression or may only serve as factor that fine-tunes the level of HLA-C transcription.

Another sequence polymorphism in the 3' untranslated region (UTR) of HLA-C, involves the binding site for miR-148a. HLA-C alleles that have an intact miR-148a binding site (e.g., C*07 and C*03), have low protein expression due to miRNA mediated inhibition (**Figure 1B**). In contrast, about seven HLA-C alleles (including HLA-C*05 and HLA-C*08) escape miR-148a binding due to a deletion in the miR binding site, and these proteins are expressed at higher levels. This miR-148a binding

variation is unique for HLA-C, as all HLA-A molecules have an intact binding site for miR148a and all HLA-B molecules escape the miR148a binding (11, 45, 46). Other variations in HLA-C cell surface expression did not correlate with mRNA expression levels and were attributed to post-transcriptional and structural diversity of the HLA-C proteins (34). Most importantly, differences in the peptide binding groove and the diversity of peptides bound by different HLA-C alleles (**Figure 1C**) affected protein stability and thus impacted HLA-C cell surface expression levels (34).

RECOGNITION OF FETAL HLA-C ALLO-ANTIGENS BY MATERNAL NK AND T CELLS

While the expression of a polymorphic paternally inherited HLA-C antigen by EVT provides a target for maternal NK cells and T cells to recognize and respond to, the HLA-C cell surface expression levels influence how this response is shaped. As HLA-C⁺ HLA-G⁺ EVT invade maternal uterine tissues they encounter maternal leukocytes of which ~70–80% are decidual NK cells (dNK), 5–15% are decidual T cells, and ~10–15% are decidual macrophages (dMΦ) in first trimester pregnancy. These proportions change dramatically during the course of pregnancy so that at term pregnancy, T cells are the predominant decidual lymphocyte population comprising 40–70% of CD45⁺ leucocytes, but dNK and dMΦ remain present in relatively high proportions of ~20–50 and ~10–15%, respectively (47–49).

HLA-C Recognition by Decidual NK Cells (dNK)

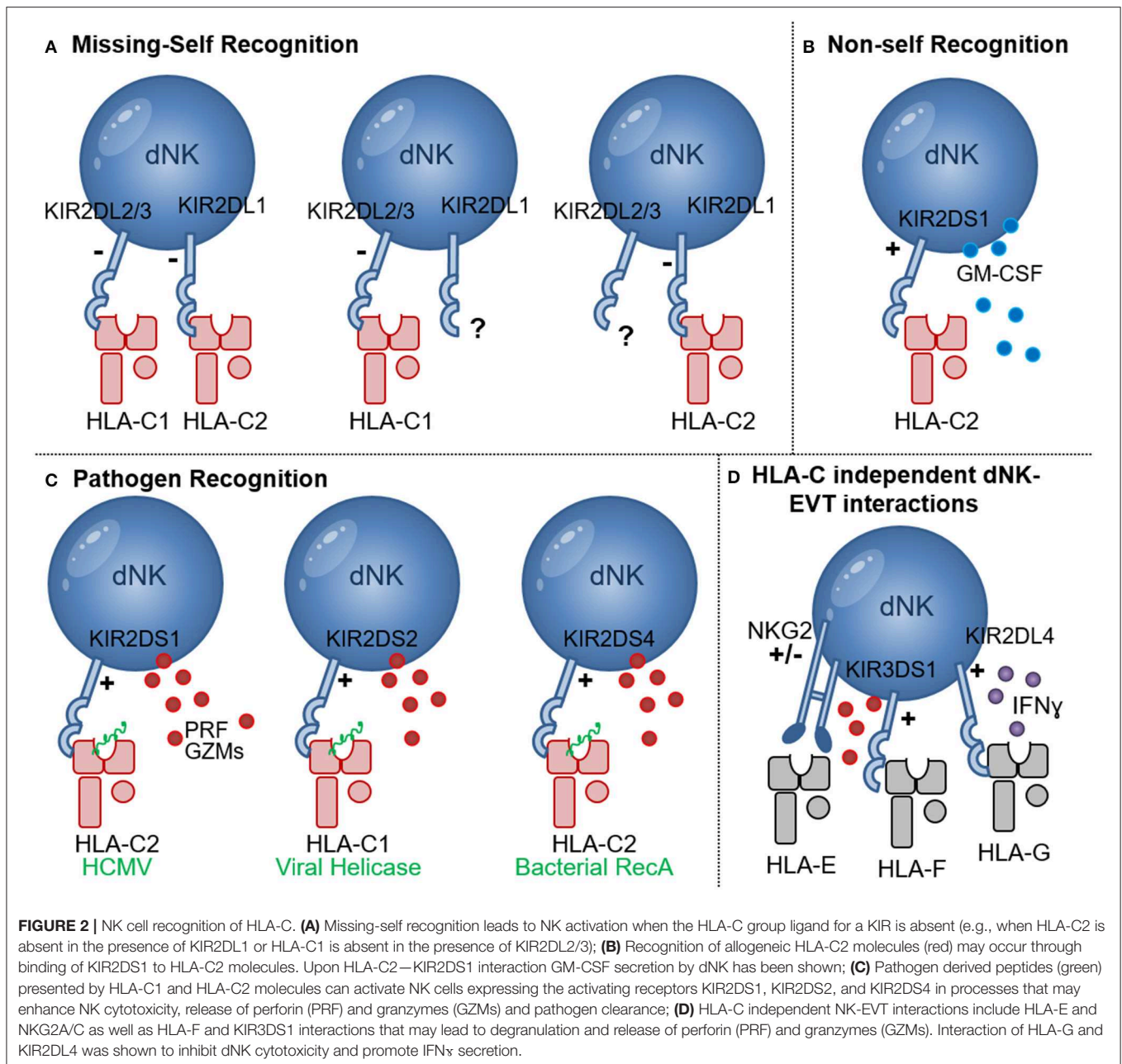
NK cells specifically recognize two groups of HLA-C allotypes, HLA-C1, and HLA-C2 group alleles, based on natural amino acid substitutions at position 80 of the HLA-C heavy chain, here HLA-C1 has asparagine and HLA-C2 group molecules have a lysine (50). NK cells were shown to carry killer cell Ig-like receptors (KIRs) with discrete specificity for HLA including HLA-C1, using KIR2DL2 and KIR2DL3 and HLA-C2 group allotypes using KIR2DL1 and KIR2DS1. Other KIRs were shown to recognize some HLA-A and HLA-B allotypes (7, 51). Due to rearrangements in the KIR gene cluster, which included duplications and deletions, everyone possesses a different combination and different number of inhibitory and activating KIR genes (52, 53). Additionally, within each individual, the process of how NK cells mature strongly depends on whether within this individual HLA allotypes for the KIR are present or not. Furthermore, the HLA-C protein expression levels directly influenced the lytic activity NK acquire during their development (37). Thus, during pregnancy maternal KIR haplotypes, NK cell education as well as the maternal and fetal HLA-C allotype combinations, differ in each pregnancy and shape the interactions of dNK with invading EVT.

dNK are specifically shown to have a KIR expression profile that is skewed in their high ability to recognize the HLA-C allotypes HLA-C1, utilizing the inhibitory KIR2DL2/3 receptors, and HLA-C2 using inhibitory KIR2DL1 and the

activating KIR2DS1 receptors (**Figure 2**) (54). In 2004 Hiby et al., demonstrated that combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success (55). In this study it was shown that mothers lacking most or all activating KIR, named the KIR-AA genotype, in combination with a fetus expressing HLA-C belonging to the HLA-C2 group, were at an increased risk of preeclampsia. Later it was shown that responses generated by the activating KIR2DS1 receptor binding to HLA-C2 resulted in secretion of cytokines, such as Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), which enhances migration of primary trophoblasts *in vitro* (56). However, the association between KIR-AA genotype and HLA-C2 and the increased risk of pregnancy complications has not been consistently reported (57, 58). Furthermore, another study didn't confirm the secretion of GM-CSF by KIR2DS1⁺ dNK during *in vitro* co-culture with HLA-C2⁺ EVT (59). KIR2DS1⁺ dNK acquired more HLA-G, compared to KIR2DS1⁻ dNK, during co-culture with primary EVT in a process called trogocytosis (60). dNK acquired HLA-G from EVT through direct cell-cell contact in which actin-ring formations, typical of an immune synapse, were formed between dNK and EVT. This however didn't result in EVT lysis by dNK. Additional genetic studies have further demonstrated that the presence of KIR2DS5 was associated with lower risk of developing pregnancy complications in African women, and KIR2DS5 genotypes that recognize HLA-C2 allotypes are common among Africans and absent from Europeans (61). In contrast, the protective effect of KIR2DS1 seems to be characteristic of European populations (61, 62). The presence of activating KIR was also associated with an increased birth weight (63). Although all studies described here point toward an increased interaction of KIR2DS1⁺ dNK with HLA-C2⁺ EVT, more detail on the mechanism underlying the protective effects of KIR2DS1 in pregnancy is required. Other lines of investigation should also include the possibility that HLA-C allo-recognition by dNK contributes to limiting EVT invasion and preventing deep invasion and placentation that is associated with placenta accreta, increta, and percreta, conditions that involve abnormal adherence of the placental trophoblasts to the uterine myometrium which can lead to fatal bleeding if not clinically managed (64).

HLA-C Specific CD8⁺ T Cell Responses

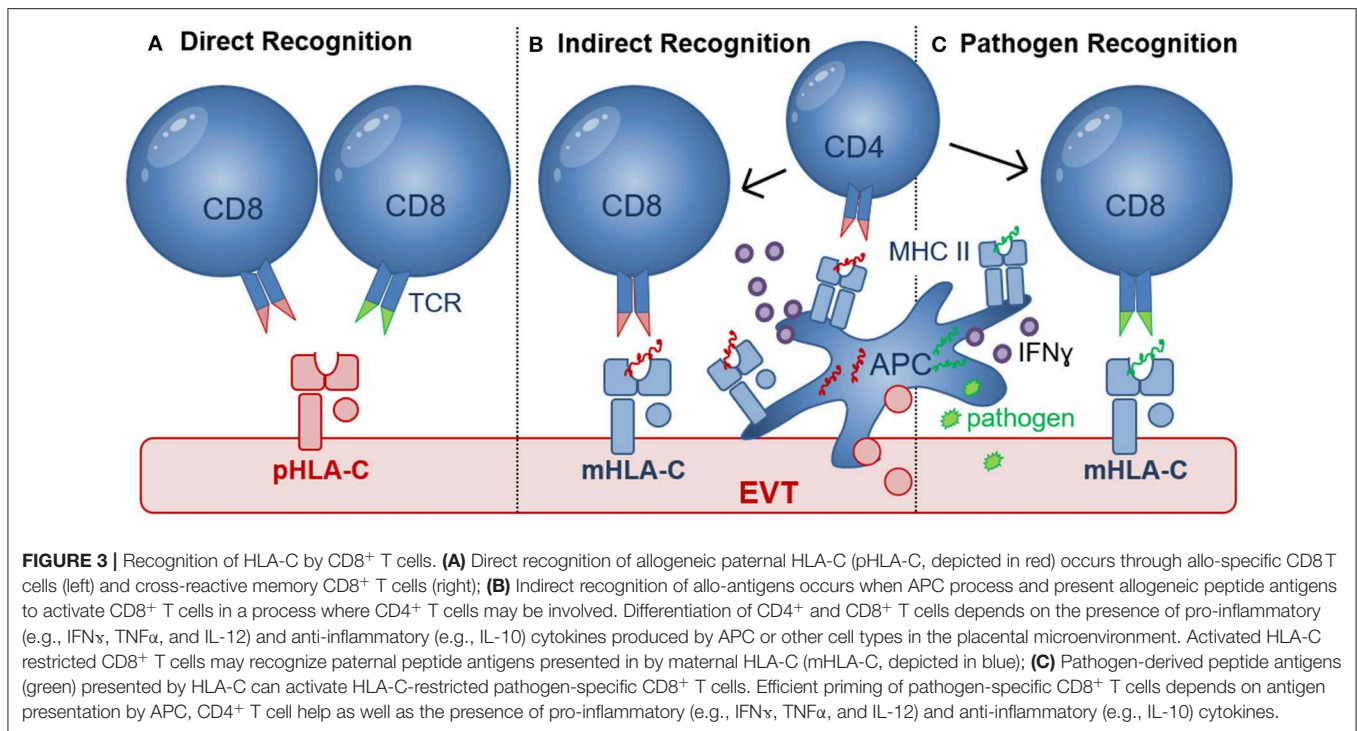
Maternal decidual CD8⁺ T cells are key cells that can directly recognize allogeneic HLA-C molecules of paternal origin during pregnancy (**Figure 3**) (65). Recognition of allogeneic HLA molecules largely depends on, (i) the differences in amino acid motifs (between donor/recipient) in the $\alpha 1$ and $\alpha 2$ domains of the HLA molecule which are relevant for HLA-TCR binding, (66, 67); (ii) the selection of peptides presented by the foreign MHC molecules (68); (iii) the TCR repertoire of the responder T cell pool; and (iv) the HLA cell surface expression levels on the target cells (9, 22). Previously, HLA-C has been shown to elicit a direct cytotoxic response by CD8⁺ T cells during allogeneic organ and hematopoietic stem cell (HSC) transplantation (66, 69). However, the percentage of donor/patient pairs with a detectable CTL response was lower in the HLA-C mismatched group (~38%) compared to donor/patient pairs with a single HLA-A or HLA-B



mismatch (~65%) (66, 69). Furthermore, the HLA-C cell surface expression level significantly influenced CTL reactivity, with the donor cells expressing the highest HLA-C levels eliciting stronger CTL responses (9, 22). The expression level of the patient's mismatched HLA-C allotype was also linked to transplant outcome (9, 22). The importance of recognition of fetal HLA-C in pregnancy was further demonstrated in a recent study suggesting that HLA-C antibodies may contribute to the etiology of miscarriage (70). Furthermore, fetus-specific CD8⁺ CTL responses to minor Histocompatibility antigens (mHags) were initiated in maternal peripheral blood during uncomplicated pregnancies (71–73). Here mHAg-specific responses had HLA-A and HLA-B restriction, but HLA-C-restricted mHAg-specific

responses have not been investigated thus far. Combined, these studies demonstrate that CTL responses to allogeneic HLA-C and mHAg are generated and can be detected in peripheral blood in high proportions of pregnant woman. However, during pregnancy a maternal-fetal HLA-C mismatch is associated with immune tolerance possibly due to the tolerogenic microenvironment at the maternal-fetal interface (74, 75).

Maternal decidual CD8⁺ T cells form a minority of leukocytes present in first trimester decidual tissue (~2–7% of CD45⁺ cells) but their proportion increases up to ~30% in term pregnancy decidua. Decidual CD8⁺ T cells mainly consist of highly differentiated CD8⁺ effector-memory T cells, suggesting that antigens are present at the maternal-fetal interface that can

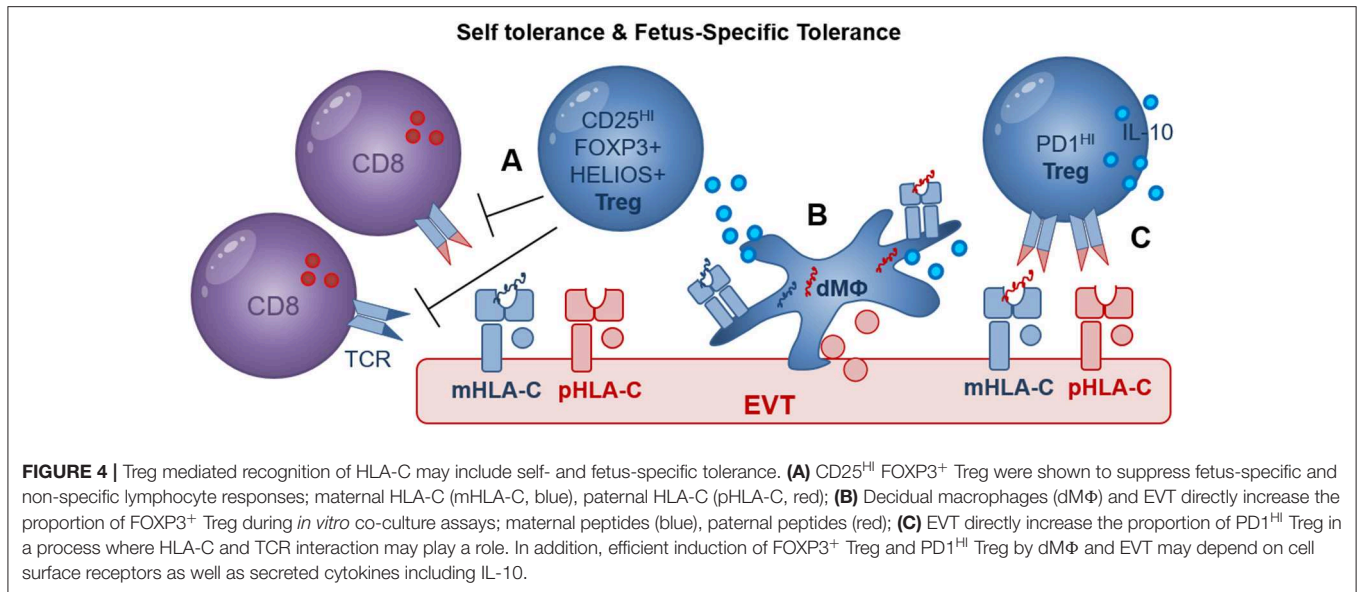


attract antigen-specific CD8⁺ T cells responses (76). Decidual CD8⁺ T cells were shown to have high expression of the co-inhibitory molecules Programmed Cell Death 1 (PD1), Cytotoxic T-Lymphocyte Associated Protein-4 (CTLA4), and Lymphocyte Activation Gene-3 (LAG3) and low expression of cytolytic molecules suggesting that the decidual microenvironment reduces CD8⁺ effector T cell function to maintain placental tolerance (76, 77). However, upon stimulation *in vitro* decidual CD8⁺ T cells degranulated, proliferated, produced IFN γ , TNF α , perforin, and granzyme B, demonstrating that decidual CD8⁺ T cells are not permanently suppressed and retain the capacity to respond to proinflammatory events, such as infections (77). The balance between transient dysfunction of decidual CD8⁺ T cells that are permissive of placental and fetal development, and reversal of this dysfunctional state, is crucial for placental tolerance and immunity to placental infections. In mice activation of T cells with direct specificity for paternal allogeneic MHC expressed by mouse trophoblast cells was observed in one study (78), but activation of allo-reactive T cells was not detected in another (79). Thus, both human and murine studies demonstrate that some but not all maternal-fetal MHC mismatches induce T cell responses. More importantly, these studies reveal that the presence of maternal CD8⁺ T cells with a direct specificity for fetal MHC was not associated with pregnancy complications or pregnancy failure.

HLA-C Recognition and Establishment of T Cell Tolerance

Maternal regulatory T cells (Treg) are of unique importance in establishing immune tolerance to invading EVT and preventing

detrimental inflammatory responses to fetal and placental antigens. Adoptive transfer of CD4⁺CD25⁺ Treg depleted lymphocytes to pregnant mice resulted in an increased resorption rate in allogeneic but not in syngeneic matings and CD3⁺ T cells were observed in placental tissues (80). Similarly, in another murine study mitigation of FOXP3⁺ Treg induction during pregnancy was used and resulted in antigen-specific fetal loss (81) and adoptive transfer of Treg from normal pregnant mice to abortion prone mice prevented fetal resorption in the abortion prone mouse model (82). Thus, these studies demonstrate that Treg play a key role in preventing rejection of allogeneic fetuses and maternal allo-specific lymphocytes can mediate fetal rejection in the absence of Treg. CD4⁺CD25^{HI}FOXP3⁺ Tregs are found at high levels in human decidual tissues in first trimester pregnancy and term pregnancy decidua basalis and decidua parietalis tissues (83–85). Decidual Treg and were shown to suppress fetus-specific and non-specific responses (Figure 4) (47). Most interestingly, HLA-C mismatched pregnancies had increased levels of CD4⁺CD25^{dim} activated T cells and increased levels of functional decidual CD4⁺CD25^{HI} Tregs, compared to HLA-C matched pregnancies (74). This suggests that maternal T cells may specifically recognize fetal HLA-C, and that this activation promotes Treg differentiation. In this study it was suggested that indirect presentation of HLA-C antigen by APC may be responsible for the increase in CD4⁺(83) CD25^{HI} Tregs. But recent studies demonstrate that *in vitro* co-culture of naive CD4⁺ T cells with EVT, directly increased the proportion of CD4⁺FOXP3⁺ Tregs, compared to CD4⁺ T cells cultured alone (59, 86, 87). Moreover, HLA-G⁺ EVT, but not dMΦ, also increased the proportion of PD1^{HI} Treg, in a process that may depend on HLA-C and TCR interactions (88). Here the



PD1^{HI}IL-10⁺ Treg suppressed CD4⁺ T cell proliferation and also increased IL-10 expression by CD4⁺ and CD8⁺ T cells possibly resulting in a positive feedback loop sustaining T cell suppression while inducing additional IL-10 secreting Tregs (88).

The question of whether decidual Treg are natural Treg (nTreg) generated in the thymus with specificity for self-antigens or induced Treg (iTreg) generated in the periphery with specificity for paternal antigens remains to be answered (89, 90). Co-culture of CD4⁺ T cells with EVT or dMΦ both significantly increased the expression of FOXP3 and HELIOS, advocating for either a local expansion of FOXP3⁺ and HELIOS⁺ nTreg or a possible *de novo* induction of FOXP3⁺ and HELIOS⁺ iTreg (88). Clonally expanded CD4⁺CD25^{HI}CD127⁻CD45RA⁻ Treg populations were also observed in term pregnancy decidua (91) but this study did not investigate the specificity of Treg suppression for self- or fetal-antigens. Evidence supporting the clinical relevance of Treg in pregnancy includes studies demonstrating decreased proportions of decidual FOXP3⁺ and HELIOS⁺ Tregs in cases of unexplained miscarriage compared to miscarriage with karyotype abnormalities (92, 93) as well as in preeclampsia compared to healthy control pregnancies (94–96). Further investigation of the multiple decidual Treg populations by studying their presence, functionality and specificity for HLA-C should reveal their role in fetus specific immune tolerance and in development of pregnancy complications.

PATHOGEN RECOGNITION IN THE CONTEXT OF HLA-C

The maternal immune system must establish tolerance for fetal and placental antigens and provide protective immunity to fight infections (65). While NK cells utilize KIR and HLA-C interactions to clear pathogen infected target cells, when EVT are infected, maternal HLA-C is also the only molecule that can present pathogen-derived peptides to antigen-specific memory

CD8⁺ T cells and provide adaptive immunity (65, 97). Although pregnant women can generate protective immune responses to a variety of pathogens, when infections occur during pregnancy, they can cause severe maternal and fetal morbidity (98–101). Human placenta has no microbiome, but many pathogens infect the placenta before transmission to the fetus occurs (102, 103). Pathogens that can directly infect trophoblasts and other placental cells, include but are not limited to: Human Cytomegalovirus (HCMV), Hepatitis C virus (HCV), Herpes Simplex virus (HSV), Human Papillomavirus (HPV), Zika Virus (ZIKV), Rubella Virus, Varicella Zoster Virus (VZV), parvovirus B19, *Listeria Monocytogenes* (*L. monocytogenes*), *Toxoplasma gondii*, *Ureaplasma urealyticum*, *Mycoplasma hominis* (100, 104–111). Besides the severe congenital disease that occurs when pathogens transmit from mother to fetus, infections during pregnancy are also associated with recurrent spontaneous abortions (RSA), preterm birth, intrauterine growth restriction and preeclampsia (112–116). Very limited experimental data is present on how maternal immune cells respond to pathogens and provide immunity at the maternal-fetal interface.

HLA-C and KIR Interactions Enhances dNK Responses to Infection

In addition to the protective effects of HLA-C and activating KIR interactions during pregnancy complications [discussed in section HLA-C Recognition by Decidual NK Cells (dNK)], individuals who carry activating KIR also have a significantly improved outcome during viral infections including HCMV, HIV, HCV, and HPV infections (117–121). dNK (but not pNK), expressing KIR2DS1 had an increased ability to degranulate in response to HCMV-infected HLA-C2⁺ DSC during *in vitro* co-cultures (122). Engagement of KIR2DS1 with HLA-C2 molecules was shown to be dependent on peptides presented by HLA-C2 (123). Importantly, modulation of HLA-C2 by HCMV peptides stimulated KIR2DS1 recognition by NK cells, providing

a molecular basis for the increased degranulation response of KIR2DS1⁺ dNK to HCMV infection (**Figure 2C**) (124). Newer studies investigating the ligands binding to other activating KIR demonstrated roles for HLA-C in presenting bacterial and viral peptides to increase KIR binding and NK cell cytotoxicity. The activating receptor KIR2DS4 detects a highly conserved peptide sequence motif derived from bacterial recombinase A (RecA) when it's presented by HLA-C*05:01, an HLA-C2 group molecule (**Figure 2C**) (125). This study predicts that over 1,000 bacterial species, including *Helicobacter*, *Chlamydia*, *Brucella*, and *Campylobacter* species, could activate NK cells through KIR2DS4. Thus, human NK cells also contribute to immune defense against bacteria through recognition of a conserved RecA epitope presented by HLA-C*05:01 (125). In addition, KIR2DS2 recognized conserved peptide epitopes of viral helicases, in the context of HLA-C*01:02, an HLA-C1 group molecule (**Figure 2C**) (126). Viral helicases from hepatitis C virus and a number of flaviviruses including Dengue, Zika, and Yellow fever viruses presented in the context of HLA-C*01:02 to KIR2DS2⁺ NK cells, was sufficient to inhibit HCV and Dengue virus replication. The study illustrates that a KIR receptors have evolved to activate NK cells in response to multiple pathogenic viruses (126). Targeting short, but highly conserved, viral and bacterial peptides provide non-rearranging immune receptors with an efficient mechanism to specifically recognize multiple, highly variable, pathogens, a feature that is generally associated with rearranging T cell and B cell receptors. Interestingly however a combination of the activating receptor KIR2DS3 in combination with a single nucleotide polymorphism of the IL28B gene, significantly increased the risk of chronic disease in hepatitis C virus infection, possibly through a IL28 mediated inhibition of IFN γ by NK cells (127). Other studies also demonstrated that diminished inhibitory responses through KIR2D and HLA-C interactions, confers protection against HCV (128–130) and influences the development of severe influenza (131). Many HLA-C independent interactions between NK cells and EVT may contribute to inhibition and activation of dNK cytotoxicity, e.g., HLA-G and KIR2DL4 interaction favors IFN production but inhibits NK cytotoxicity (**Figure 2D**) (132), NKG2 receptors mediate responses through HLA-E as well as cellular stress ligands (133). Open conformers of HLA-F, which are HLA-F heavy chains devoid of peptide and/or β 2-microglobulin (β 2m), are high-affinity ligands of the activating NK-cell receptor KIR3DS1 and may play a role in limiting HIV-1 infection (119, 134). Furthermore, a recent report identified a unique subset of dNK expressing high levels of the activating HLA-E receptor NKG2C and the HLA-G receptor LILRB1 in multigravid woman compared to woman with a first pregnancies. These dNK secreted high levels of IFN- γ and VEGF α and may play a role in enhanced placentation in repeated pregnancies (135).

The mechanisms by which activating KIR and HLA-C interactions reduce the risk of pregnancy complications and enhance immunity to infections during pregnancy should be investigated in more molecular detail. Enhanced systemic NK mediated immunity to infection likely reduces systemic pathogen burden and can limit pathogens from infecting placental tissues.

The increased capacity of KIR2DS1 to clear HCMV-infected DSC in the placenta suggests that activating KIR also enhance dNK mediated placental immunity (122). However, the failure of dNK to kill EVT, even when infected with HCMV, may reduce the risk of immune rejection of EVT and placental tissues but promote the spread of infection and contribute to virus-induced placental pathology and development of complications later in pregnancy (97). These mechanisms may be related to how KIR2DS1, expressed by dNK, reduces development of severe pregnancy complications, such as miscarriages and preterm delivery. Increasing pNK and dNK responses by expansion of KIR expressing NK cells or utilizing chimeric antigen receptor NK cells are currently being investigated for treatment of cancer and chronic infections (136, 137). Utilizing these approaches to enhance HLA-C and KIR interactions in pNK and dNK has therapeutic potential to limit placental infection as well as mother to fetal transmission of a variety of pathogens. A focus on controlling infections during pregnancy may also help limit infection induced placental pathology and complications of pregnancy, such as miscarriage and spontaneous preterm birth that are related to infection (115, 138).

HLA-C-Restricted Pathogen-Specific CD8⁺ T Cell Responses

The failure of dNK to respond to HCMV-infected EVT during *in vitro* co-culture (122), may leave decidual CD8⁺ T cells as the predominant effector cell to clear pathogen-infected EVT. HCMV sero-positivity was shown to dramatically alter the maternal CD8⁺ T cell repertoire during pregnancy (139) and similar to HIV, T cell responses to HCMV rely heavily on HLA-C-restricted responses because both viruses downregulate HLA-A and HLA-B upon infection (10, 140). During HIV and HCMV infection, HLA-C-restricted CD8⁺ CTL responses were shown to comprise as much as 54% of the total response in peripheral blood and were functionally and phenotypically identical to HLA-A- and HLA-B-restricted CTL (140, 141). Seropositive women during late pregnancy demonstrated an accumulation of highly differentiated HCMV-specific T-cells (139). Moreover, increased percentages of HCMV and EBV-specific CD8⁺ T cells were also found in decidual tissue compared with peripheral blood after uncomplicated pregnancy (142). These decidual virus-specific CD8⁺ memory T cells were able to produce IFN γ and were restricted to recognize viral peptides presented by HLA-A or HLA-B molecules. Thus, these decidual CD8⁺ T cells may provide cellular immunity for infected maternal cells that express HLA-A and HLA-B and limit the spread of infection to trophoblasts and/or the fetus. However, no data are currently available as to whether HLA-C-restricted virus-specific CTL are present and can provide immunity when EVT are infected (**Figure 3C**).

Virus Specific CTL Cross-React With HLA-C Allo-Antigens

A substantial proportion of virus-specific CD4⁺ and CD8⁺ memory T cells [for e.g., HCMV, Epstein–Barr virus (EBV), (VZV), and Influenza Virus] were shown to cross-react with

non-self allogeneic HLA molecules, including HLA-A, HLA-B, and HLA-DR molecules. In this case, the allogeneic HLA reactivity and virus specificity were mediated via the same TCR (143, 144). More recently, cross reactivity of a HLA-B*08:01-restricted EBV-specific peripheral blood T cell clone, showed significant alloreactivity against HLA-C*01:02 and in the same study cross reactivity of HLA-C*06:02-restricted HCMV-specific peripheral blood CD8⁺ T cell line against HLA-C*03:02 allele was detected (145). Although decidual CD8⁺ T cells contain higher proportions of virus specific CTL, these have not been investigated for cross reactivity (70) and no cross reactivity against HLA-E and -G molecules was detected (145). This demonstrates that cross reactivity of virus-specific CD8⁺ T cells against HLA-C can occur (Figure 3A), but cross reactivity to HLA-C allotypes was found to be about 10-fold lower than to HLA-A, HLA-B, and HLA-DR allotypes. Most importantly, cross reactivity has been shown to depend on both the peptides presented by allogeneic HLA molecules and the tissue and cell types used. EVT have been shown to express many ligands including Programmed Cell Death Ligand-1 (PDL1), Cytotoxic and Regulatory T Cell Molecule (CRTAM), B7H3, Polio Virus Receptor (PVR), and secrete inhibitory cytokines that may inhibit direct CD8 cytotoxicity (59). Furthermore, co-culture of blood CD8⁺ T cells or decidual CD8⁺ T cells with allogeneic EVT did not result in degranulation (59, 77). Further testing of cross reactive CD8⁺ T cells against HLA-C allotypes expressed on EVT in healthy pregnancy and pregnancy pathology will determine their role in development of pregnancy complications.

Viral Infections Influence Treg Stability and Enhance CTL Reactivity

Uncontrolled placental viral (and bacterial) infections also provide a pro-inflammatory milieu that can alter the stability and function of Treg and enhance alloreactivity (65, 146). In transplant recipients, infections have been associated with failure to induce transplant tolerance and allograft rejection even after long periods of transplant tolerance (146, 147). HCMV infection of EVT did not diminish the ability of EVT to increase FOXP3⁺ and PD1^{HI} Tregs (88), suggesting that HCMV infection does not alter the capacity of EVT to promote immune tolerance. This finding is in line with the observation that dNK fail to degranulate in response to HCMV-infected EVT and thus also maintain immune tolerance in the presence of infection (122). Future studies need to investigate the impact of other placental cell types (e.g., dMΦ and DSC) and changes in inflammatory factors of the placental microenvironment during infection on decidual Treg stability and Treg induction in the presence of infection.

OUTSTANDING QUESTIONS

- What are the regulatory mechanisms by which EVT express HLA-C, HLA-E, and HLA-G in the absence of HLA-A and HLA-B?

- Do high HLA-C cell surface expression levels enhance immunity to placental infection and/or generation of HLA-C reactive decidual T cell responses?
- Are decidual CD8⁺ T cells able to recognize and respond to viral, fetal and/or placental antigens in the context of HLA-C expressed by EVT?
- Do maternal regulatory T cells provide specific immune tolerance to fetal HLA-C mismatches in the placenta?
- What are the molecular and cellular mechanisms by which activating KIR and HLA-C interactions reduce the risk of pregnancy complications and enhance immunity to infections?

CONCLUDING REMARKS

The co-expression of maternally and paternally inherited HLA-C by EVT provides both a self and a non-self ligand for maternal decidual CD4⁺ Treg, CD8⁺ T_H1, and dNK to establish self- and fetus-specific immune tolerance. The maternally inherited HLA-C is the only molecule that can present pathogen-derived peptides to antigen-specific memory CD8⁺ T cells to provide adaptive immunity when EVT become infected. However, the expression of paternally inherited polymorphic HLA-C by EVT also provides a potential ligand for cytolytic CD8⁺ T_H1 and dNK, possibly resulting in detrimental inflammatory responses that are associated with pregnancy complications. Thus, HLA-C expression by EVT has a unique and dual role in maternal-fetal immune tolerance and immunity to placental infections (65). High HLA-C expression levels on the cell surface may enhance detrimental inflammatory and CTL responses to maternal-fetal HLA-C mismatches in pregnancy as was shown in transplant patients (9, 22). However, high HLA-C levels in pregnancy can also be beneficial and contribute to immune protection to a wide variety of infections and diminish infection related pregnancy complications (10). Further investigations, as to how EVT express HLA-C in the absence of HLA-A and HLA-B, as well as the role of individual HLA-C expression levels in shaping the maternal dNK, T_H1 and Treg responses to HLA-C will be key in understanding the development of pregnancy complications and preventing maternal to fetus transmission of infections.

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HP and TT: writing—original draft. TM, QL, JS, and TT: writing—review and editing. TT: visualization.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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