Tetrameric Complexes of Human Histocompatibility Leukocyte Antigen (HLA)-G Bind to Peripheral Blood Myelomonocytic Cells

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Summary

The nonclassical MHC class I molecule human histocompatibility leukocyte antigen (HLA)-G is selectively expressed on fetal trophoblast tissue at the maternal–fetal interface in pregnancy. It has long been suggested that HLA-G may inhibit maternal natural killer (NK) cells through interaction with particular NK cell receptors (KIRs). To investigate interactions of HLA-G, we constructed phycoerythrin-labeled tetrameric complexes of HLA-G refolded with a self-peptide. These HLA-G tetramers failed to bind to NK cells and cells transfected with CD94/NKG2 and killer immunoglobulin-like NK receptors. In contrast, HLA-G tetramers did bind to peripheral blood monocytes, staining a $CD16^{+}CD14^{mid}$ subset with greater intensity. On transfectants, HLA-G tetramers bound to inhibitory immunoglobulin-like transcript (ILT)2 and ILT4 receptors. However, staining in the presence of antibodies reactive with ILT receptors revealed that the interaction of HLA-G tetramers with blood monocytes was largely due to binding to ILT4. These results suggest that the primary role of HLA-G may be the modulation of myelomonocytic cell behavior in pregnancy.

Key words: immunoglobulin-like transcript • monocyte • natural killer cell • CD94 • killer immunoglobulin-like receptor

The MHC class Ib molecule HLA-G has been the subject of considerable interest for several years because of its unique tissue distribution. HLA-G is expressed primarily by a population of fetal extravillous cytotrophoblast cells that lie in direct contact with maternal uterine mucosa during pregnancy (1–3). Because of this location, many have speculated that HLA-G may act to modulate the maternal immune response towards the semiallogeneic fetus.

Fetal trophoblast tissue largely fails to express MHC class I HLA-A and -B or MHC class II molecules (4), perhaps escaping T cell–mediated recognition. However, many maternal NK cells cluster adjacent to trophoblast tissue (5), and it has often been proposed that HLA-G expression might be responsible for their inhibition. Indeed, several groups have provided functional evidence that transfection of HLA-G could inhibit NK cells expressing CD94/NKG2A/B NK cell receptors, implying that HLA-G was directly recognized (6–8). However, these results must now be reexamined in light of recent discoveries that nonclassical HLA-E is the ligand for CD94/NKG2A/B/C receptors and cell surface expression of HLA-E is upregulated upon acquisition of peptides derived from the leader sequences of other MHC class I molecules, including HLA-G (9–14). HLA-G has also been suggested to interact with NK cell receptors of the killer Ig-like receptor (KIR)[1] family, including KIR2DL1, KIR2DL3, and KIR3DL1 (15, 16), with known specificities for two groups of HLA-C and HLA-Bw4 molecules, respectively. KIR2DL4 (or p49, KIR103) has also been reported to recognize several MHC class I molecules including HLA-G (17).

Maternal T cells and macrophages are also present in appreciable numbers at the maternal–fetal tissue interface, suggesting other possible interactions for HLA-G. Indeed, another group of Ig superfamily receptors known as ILTs (Ig-like transcripts) (18–22), LIRs (leukocyte Ig-like receptors) (23–25), or MIRs (monocyte/macrophage Ig-like receptors) (26, 27) are expressed in many cell subsets, particularly myelomonocytic cells. Fusion proteins of certain members of this family

[1] *Abbreviations used in this paper:* DC, dendritic cell; Tet, tetramer; ILT, immunoglobulin-like transcript; KIR, killer immunoglobulin-like receptor.

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have been shown to bind to several MHC class I molecules including HLA-G on transfectants (19, 20, 25).

To investigate whether HLA-G was capable of interaction with such a diversity of receptors and to identify new ligands, we created HLA-G tetrameric complexes. Recombinant HLA-G was refolded in vitro with β2 microglobulin and a synthetic self-peptide, and conjugated with PE-labeled ExtrAvidin (Sigma Chemical Co.) molecules to create tetrameric complexes. Similar MHC class I tetramers have proven to be very powerful tools allowing visualization of interactions with TCRs and NK cell receptors in flow cytometric analyses (28).

Materials and Methods

Antibodies. 42D1 mAb (rat IgG2a) has been described previously (20) and 27D6 mAb (rat IgM) was another clone obtained from the same fusion. TC16-28C8 and TC16-40H2 mAbs (both rat IgG1) were produced by immunizing female Lewis rats with a human Fcγ1 fusion protein of ILT6, a receptor that naturally lacks a transmembrane domain.

Reactivities of 40H2, 28C8, 42D1, and 27D6 are detailed in the legend to Fig. 5, based on flow cytometry experiments using the panel of cell surface ILT receptor transfectants available for this study (as shown in Fig. 4).

Cells. Baf3 cells transfected with CD94/NKG2A (29), CD94/NKG2C/DAP12 (29), or KIR receptors (30); Jurkat cells transfected with ILT3 (21); RBL cells transfected with ILT4 or ILT5 (20); and P815 cells transfected with ILT1 (22) have been described previously. Baf3 cells transfected with ILT2 were generated as previously described (29). RBL cells transfected with ILT8, an activating receptor similar to ILT1, were produced as previously described (20).

HLA-G Tetramers. Using HLA-G*01012 cDNA (1) as a template, extracellular domains (amino acids 1–276) of HLA-G1 were amplified by PCR using primers: 5′-ctcgagcatatgggTtcTcaTtcTatgCgTtatttTagcgcAgcAgtTtcTcgTccAggccgcggg-3′ and 5′-atgcagggatccctgcttccatctcagcatgagggg-3′ and cloned into a pGMT7 vector derivative containing a BirA recognition and biotinylation site in frame at the COOH-terminus (11). The NH_2-terminal primer contained several synonymous nucleotide substitutions (capitalized) designed to optimize protein expression from *Escherichia coli* strain BL21 pLysS. HLA-G tetramers were created essentially as previously described (11), using synthetic peptide RIIPRHLQL (or KIPAQFYIL where indicated) (Genosys) previously shown to interact with HLA-G (31, 32). Dilutions for flow cytometry staining contained ~14 μg/ml of refolded HLA-G/β2 microglobulin.

HLA-E*0101 and HLA-B*2705 tetramers were refolded with peptides VMAPRTLFL and KRWIILGLNK, respectively (11, 33).

Flow Cytometry. Staining of PBMCs and transfectants was performed using standard protocols. For PBMCs, PBS 0.05% NaN_3 buffer was supplemented with 10% human serum for blocking and primary incubation, and 1–2% human serum for washes and secondary incubations. PBMCs were stained on ice immediately after Ficoll-Hypaque separation or frozen and thawed immediately before use. Cells were analyzed on a FACScan™.

Results and Discussion

HLA-G Tetramers Bind to Myelomonocytic Cells from Peripheral Blood. We constructed HLA-G tetrameric complexes refolded with a synthetic self-peptide (RIIPRHLQL) derived from human histone H2A (31, 32). These PE-labeled HLA-G tetramers were used to stain PBMCs from healthy individuals. No significant HLA-G tetramer binding was observed on $CD56^+$ NK cells, $CD3^+$ T cells, or $CD19^+$ B cells within the gated lymphocyte population (Fig. 1). In contrast, when an electronic gate was set on myelomonocytic cells, significant HLA-G tetramer interaction was observed. $CD14^{high}$ cells, representing the majority of monocytes, stained weakly, with intensity of staining varying between individuals (Fig. 1 and data not shown). In addition, a subset of cells within the myelomonocytic population exhibited considerably brighter HLA-G tetramer staining (Fig. 1). These cells ranged from $CD14^{high}$ to $CD14^-$. In freshly isolated PBMCs from six individuals, this HLA-G Tet^{bright} subset represented 5–12% of cells within the myelomonocytic gate, or 1–2.8% of total PBMCs. Almost indistinguishable patterns of staining were obtained with an HLA-G tetramer refolded with a second peptide (KIPAQFYIL) (data not shown) also known to bind to HLA-G (31). However, interactions with myelomonocytic cells were not unique to HLA-G, as tetramers of other MHC class I molecules (including HLA-A*0201, HLA-A*6802, HLA-B*3501, and HLA-E*0101) exhibited similar staining, although often with considerably less intensity (data not shown).

HLA-G Tetramers Brightly Stain a Distinct $CD16^+CD14^{mid}$ Monocyte Subset. To further characterize the cells staining intensely with HLA-G tetramers, the expression of a number of other cell surface markers was examined in three individuals. Levels of CD13, CD32 (FcγRII), and CD33 on HLA-G Tet^{bright} cells were comparable or slightly lower than most monocytes (Fig. 2). The expression of CD33 and CD13 on the HLA-G Tet^{bright} subset was consistent with these cells having a myeloid origin. The HLA-G Tet^{bright} cells appeared to form a distinct subgroup, expressing much higher CD16 (FcγRIII), lower CD64 (FcγRI), lower CD11b, higher CD11c, higher CD45RA, and slightly lower CD45RO levels than the majority of monocytes (Fig. 2). Similarly, HLA-G Tet^{bright} cells showed slightly higher levels of costimulatory CD86 (B7-2) and CD40 molecules and MHC class II (anti–HLA-DR or anti–pan-class II) compared with typical monocytes (Fig. 2 and data not shown). This phenotype is very similar to a previously described $CD16^+CD14^{mid}$ monocyte subset (34). Ziegler-Heitbrock has suggested that these $CD16^+$ $CD14^{mid}$ cells may be differentiating to become tissue macrophages (34). Intracellular staining for CD68, which is highly expressed by macrophages, did reveal a marginally brighter signal in HLA-G Tet^{bright} cells (data not shown). However, the HLA-G Tet^{bright} subset failed to stain with antibodies to scavenger receptor A or mannose receptor found on tissue macrophages (data not shown). Many of these patterns of marker expression are also suggestive of a peripheral blood dendritic cell (DC) phenotype (35–37). Expression of CD16, however, is inconsistent with prior descriptions of blood DCs (35–37). HLA-G Tet^{bright} cells also fail to express DC-associated markers CD1a and CD83 (data not shown). Nonetheless, the HLA-G Tet^{bright} subset

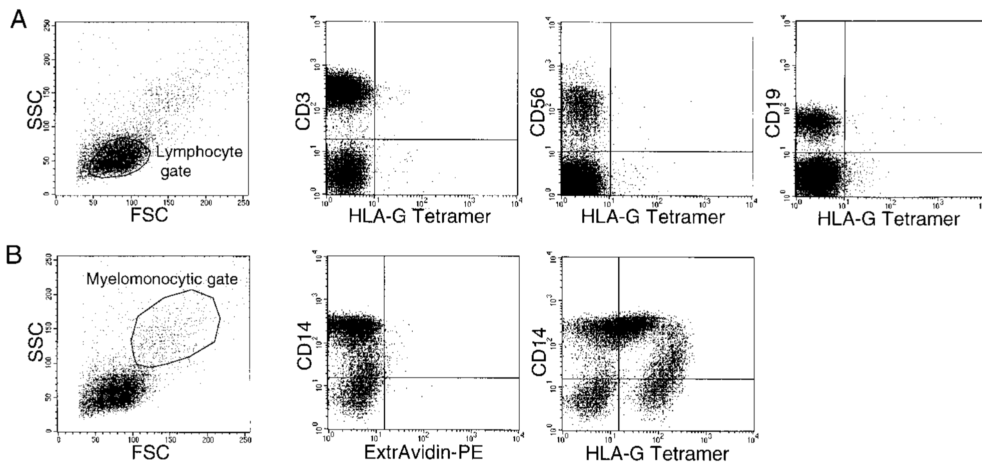

Figure 1. HLA-G tetramers bind to peripheral blood myelomonocytic cells. PBMCs from a healthy individual were stained with PE-labeled HLA-G tetramers or ExtrAvidin-PE control and anti-CD3, -CD56, -CD19, or -CD14 directly labeled mAb. An electronic gate based on forward and side light scatter properties was set on lymphoid cells (A) or myelomonocytic cells (B). Patterns in A were not significantly different from ExtrAvidin-PE control.

could represent a stage in either the macrophage or DC differentiation pathways.

HLA-G Tetramers Bind to ILT2 and ILT4 Receptors. To search for the receptors responsible for the observed staining of PBMCs, we stained a number of transfectants with HLA-G tetramers. We began with cells transfected with several NK cell receptors previously suggested to interact with HLA-G (6–8, 15, 16). As shown in Fig. 3 A, HLA-G tetramers did not bind to transfectants expressing high levels of CD94/NKG2A or CD94/NKG2C/DAP12. Similarly, we did not observe any binding of HLA-G tetramers to cells transfected with KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2, or KIR2DL4 members of the KIR family (Fig. 3 B). These findings correlate with the failure of HLA-G tetramers to stain NK cells from peripheral blood. It remains possible that interactions might be missed if they were of low affinity or peptide dependent, or required glycosylation unattained on HLA-G expressed in *E. coli.* However, the efficient interactions of similarly constructed HLA-E tetramers with CD94/NKG2A and C receptors and HLA-B*2705 tetramers with KIR3DL1 (Fig. 3) considerably weaken such arguments. In addition, the previously reported recognition of HLA-G by NK clones expressing CD94/NKG2 receptors (6–8) can be explained by interactions with HLA-E, which is upregulated upon acquisition of leader sequence peptides from HLA-G (9–14).

We next investigated HLA-G tetramer interactions with receptors of the ILT (or LIR or MIR) family (18–27). HLA-G tetramers efficiently stained transfectants expressing ILT4 and ILT2 receptors (Fig. 4). HLA-G tetramer binding, however, was quite dependent upon the density of receptor. Significantly higher expression of ILT2 was necessary to observe efficient HLA-G tetramer binding, suggesting that this interaction may have relatively lower affinity compared with ILT4 (Fig. 4 and data not shown). However, such interactions were not unique to HLA-G, as

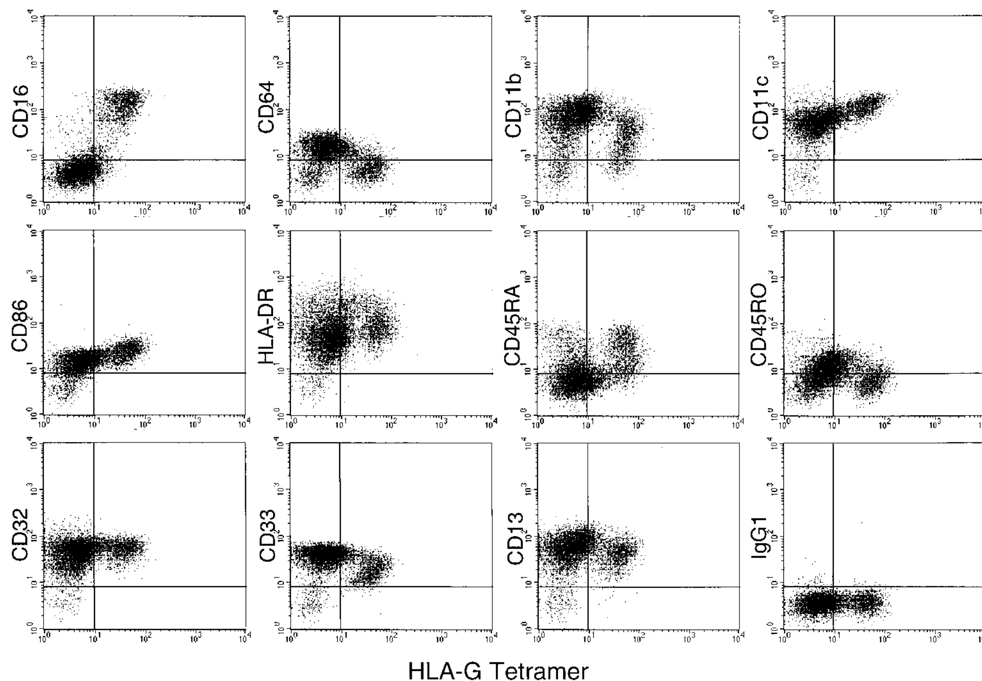

Figure 2. HLA-G tetramers intensely stain a distinct CD16^{+} CD14^{mid} monocyte subset. PBMCs from a healthy individual were stained with PE-labeled HLA-G tetramers and several FITC-labeled or unconjugated mAbs as indicated. Only cells within a myelomonocytic light scatter gate are shown. Results with FITC-labeled or unconjugated IgG1 and IgG2a control mAbs were very similar to unlabeled IgG1 shown. Unlabeled mAbs were detected with FITC-conjugated F(ab′)$_2$ goat anti–mouse Ig. Additionally, an HLA-G tetramer created with a different peptide (KIPAQFYIL) displayed a very similar pattern of staining (data not shown).

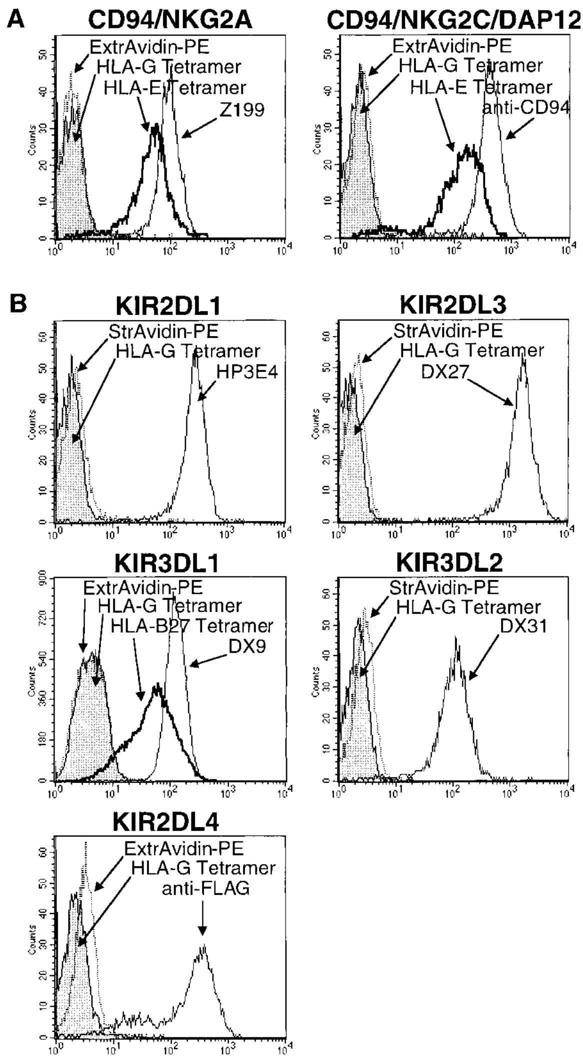

Figure 3. HLA-G tetramers do not bind to NK receptors. Baf3 cells transfected (A) with CD94 and NKG2A or CD94, NKG2C, and DAP12, or (B) with several KIR receptors were stained with HLA-G, HLA-E*0101, or HLA-B*2705 tetramers, or ExtrAvidin-PE or Streptavidin-PE controls. Expression of transgenes was verified with mAbs recognizing CD94, KIRs, or the FLAG peptide epitope, as indicated.

tetrameric complexes of several other MHC class I molecules (including HLA-A*0201, A*6802, B*3501, B*2705, and HLA-E*0101) also stained the ILT2 and ILT4 transfectants (data not shown and reference 20). Binding of certain molecules, such as HLA-E, appeared less efficient. These results are consistent with the findings that fusion proteins of ILT2 and ILT4 could interact with cells transfected with certain MHC class I molecules including HLA-G (19, 20, 25).

HLA-G tetramers did not bind to cells transfected with ILT1, ILT3, ILT5, or ILT8 (Fig. 4), confirming previous reports that failed to observe interactions of fusion proteins with MHC class I molecules (20, 21, 24).

HLA-G Tetramer Staining of Monocytes Is Predominately Due to Interaction with ILT4. To determine if the HLA-G tetramer staining of blood monocytes was the result of interaction with these ILT receptors, we stained PBMCs with HLA-G tetramers in the presence of ILT-reactive mAbs. The addition of 40H2 mAb, recognizing several members of the ILT family, caused an enhancement of HLA-G tetramer binding to both CD14^{high} and CD14^{mid} monocyte subsets, providing direct evidence for the involvement of ILT receptors (Fig. 5). Bivalent 40H2 rat IgG1 probably cross-linked receptors thus facilitating formation of multivalent tetramer interactions. 28C8 mAb, which reacted only with ILT2 and ILT4 of the panel of cell surface ILT receptors available for the study (shown in Fig. 4), almost completely blocked HLA-G tetramer binding to monocytes (Fig. 5). Thus, the interactions responsible for monocyte staining were narrowed to ILT2 and ILT4, consistent with the staining on transfectants, although binding to other receptors sharing very similar antigenic determinants could not be completely excluded. Further experiments with 42D1 and 27D6 mAbs, which recognize ILT4 but not ILT2, revealed that the majority of HLA-G tetramer staining of monocytes was the result of binding to ILT4. 42D1 mAb enhanced HLA-G tetramer staining of monocytes (Fig. 5) and 27D6 mAb almost completely abrogated HLA-G tetramer binding (Fig. 5). These results are consistent with the absence of HLA-G tetramer staining on

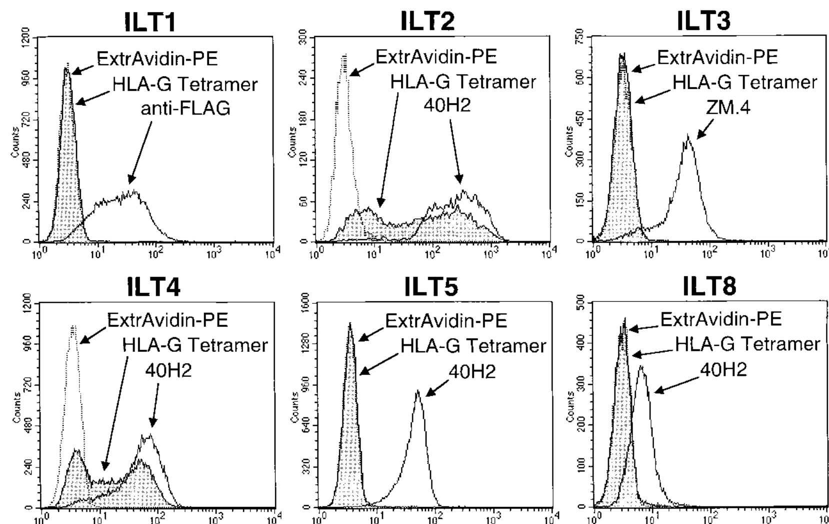

Figure 4. HLA-G tetramers bind to ILT2 and ILT4. HLA-G tetramers or ExtrAvidin-PE control were used to stain P815 cells transfected with ILT1; Baf3 cells transfected with ILT2; Jurkat cells transfected with ILT3; or RBL cells transfected with ILT4, ILT5, or ILT8. Expression was verified with ILT-reactive or anti-FLAG mAbs as shown.

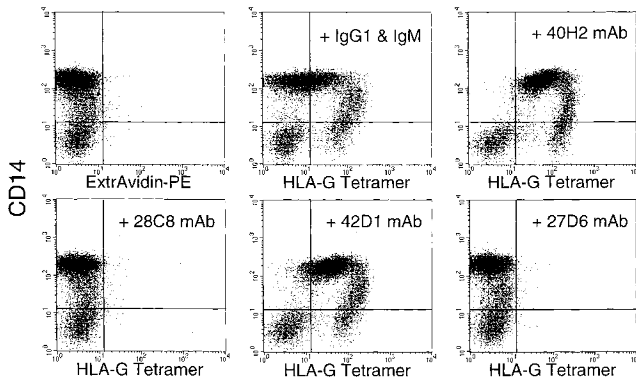

Figure 5. HLA-G tetramer staining of monocytes is largely due to interaction with ILT4. PBMCs from a healthy individual were stained with anti-CD14-FITC, HLA-G tetramer-PE, and various unlabeled mAbs reactive with ILT receptors. Only cells within a myelomonocytic light scatter gate are shown. Based on flow cytometry stains of the ILT transfectants shown in Fig. 4, 40H2 recognizes ILT2, ILT4, ILT5 and ILT8; 28C8 recognizes ILT2 and ILT4; and 42D1 and 27D6 recognize ILT4.

peripheral blood B cells, T cells, and NK cells that express some ILT2 (19, 25). Indeed, the pattern of tetramer staining matches the restricted expression of ILT4 on myelomonocytic cells (20, 25). In two-color flow cytometry analyses, HLA-G tetramer binding correlated with ILT4 staining (42D1) (data not shown) and $CD16^{+}$ and $CD14^{mid}$ monocytes exhibited higher expression of ILT4 compared with typical $CD14^{high}CD16^{-}$ monocytes (data not shown and reference 25). If HLA-G Tet^{bright} cells are indeed differentiating to become macrophages (34) or DCs, ILT4 expression may be modulated in preparation for the tissue phenotype. Alternatively, ligation or lack of ligation of ILT4 may be involved in the control of these differentiation pathways.

In conclusion, in this study we demonstrate an interaction of HLA-G tetrameric complexes with peripheral blood monocytes that results from binding to ILT4 receptors. We failed to observe any evidence of interaction of HLA-G tetramers with CD94/NKG2 or KIR NK cell receptors. This suggests that a dominant role of HLA-G may be the modulation of monocyte, macrophage, or DC behavior in pregnancy. ILT4 possesses inhibitory ITIM motifs in its cytoplasmic domain, and its ligation can inhibit Ca^{2+} fluxes and tyrosine phosphorylation events in myelomonocytic cells in response to several stimulatory signals (20, 25). Thus, HLA-G may provide important inhibitory signals capable of modulating antigen presentation, phagocytosis, antibody-dependent cell-mediated cytotoxicity, or cytokine production by the numerous maternal macrophages present at the maternal–fetal tissue interface.

Interactions with ILT4 are not unique to HLA-G, however, as tetramers of several classical MHC class I molecules and HLA-E also bound to monocytes and transfectants, although in some cases with considerably lower efficiency. Fetal trophoblasts are deficient in HLA-A and -B classical MHC class I expression (4). Thus, it is possible that expression of HLA-G on this tissue only replaces the inhibitory signals to macrophages normally provided by classical class I molecules in other tissues in the body.

Although the pattern of HLA-G tetramer staining of PBMCs closely mirrored the restricted expression of ILT4 on myelomonocytic cells (20, 25), HLA-G tetramers were also able to stain transfected cells expressing very high levels of ILT2 inhibitory receptors. However, transfectants with lower ILT2 expression did not efficiently bind HLA-G tetramers (data not shown). Thus, the failure of HLA-G tetramers to stain B, T, and NK cells, which express some ILT2 (19, 25), likely reflects a relatively lower binding affinity compared with ILT4. However, interactions of HLA-G with ILT2 receptors may allow functional inhibition of many cell subsets (19).

Finally, we can not rule out that HLA-G also serves as a restriction element for maternal T cells, because HLA-G tetramers refolded with self-peptides would not be expected to interact with antigen-specific T cell receptors.

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