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Authors

Crisa, Laura
McMaster, Michael T
Ishii, Jennifer K
et al.

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Identification of a Thymic Epithelial Cell Subset Sharing Expression of the Class Ib HLA-G Molecule with Fetal Trophoblasts

By Laura Crisa,* Michael T. McMaster,‡ Jennifer K. Ishii,* Susan J. Fisher,‡ and Daniel R. Salomon*

*From the *Department of Molecular and Experimental Medicine and Immunology, The Scripps Research Institute, La Jolla, California 92037; and ‡Department of Stomatology, University of California San Francisco, San Francisco, California 94143*

Summary

HLA-G is the only class I determinant of the major histocompatibility complex (MHC) expressed by the trophoblasts, the fetal cells invading the maternal decidua during pregnancy. A unique feature of this nonclassical HLA molecule is its low polymorphism, a property that has been postulated to play an important role in preventing local activation of maternal alloreactive T and natural killer cells against the fetus. Yet, the mechanisms by which fetal HLA-G can be recognized as a self-MHC molecule by the maternal immune system remain unclear. Here we report the novel observation that HLA-G is expressed in the human thymus. Expression is targeted to the cell surface of thymic medullary and subcapsular epithelium. Thymic epithelial cell lines were generated and shown to express three alternatively spliced HLA-G transcripts, previously identified in human trophoblasts. Sequencing of HLA-G1 transcripts revealed a few nucleotide changes resulting in amino acid substitutions, all clustered within exon 3 of HLA-G, encoding for the $\alpha 2$ domain of the molecule. Our findings raise the possibility that maternal unresponsiveness to HLA-G–expressing fetal tissues may be shaped in the thymus by a previously unrecognized central presentation of this MHC molecule on the medullary epithelium.

MHC class I molecules are important recognition elements regulating presentation of self and nonself antigens to cytotoxic $CD8^+$ T cells. MHC class I molecules have evolved to form two different, though structurally related, groups of molecules known as class Ia (e.g., classical HLA-A, -B, and -C) and class Ib (e.g., nonclassical HLA-E, -F, and -G). Although polymorphism of MHC class Ia antigens plays an important role in establishing the diversity of immune responses (1), selective pressure has maintained a limited polymorphism of the class Ib MHC molecules, particularly at the peptide binding groove (2). Furthermore, in contrast with the ubiquitous distribution of class Ia molecules, MHC class Ib determinants have a developmentally regulated or tissue-restricted expression, suggesting immune functions specialized for binding specific antigens and/or for presentation to particular subpopulations of T cells.

Among members of the class Ib MHC molecules, human HLA-G is unique in that its expression is primarily restricted to the cell surface of the extravillous trophoblasts, the placental tissue of fetal origin invading the maternal decidua during implantation of the embryo (3, 4). Neither the polymorphic class Ia nor the class II HLA antigens are expressed by human trophoblasts (5, 6). It has been proposed that the selective expression of nonpolymorphic HLA-G at this site may protect the semiallogeneic fetal tissues from maternal immune rejection by preventing the activation of maternal alloreactive T and NK cells resident in the decidua (7–11). In fact, a direct role of HLA-G in inhibiting NK-mediated lysis of target cells has been demonstrated (11–13). As for decidual T cells, it is unknown whether they are restricted by, and therefore tolerant to, HLA-G. Nevertheless, in the mouse there is evidence that decidual T cells of the γ/δ lineage are oligoclonal (14) and restricted by nonpolymorphic MHC class Ib determinants (15). Interestingly, the same population of γ/δ T cells can be positively selected in the mouse fetal thymus (16). These studies suggest that selection events occurring in the thymus may influence the repertoire of T cell immune responses at the uterine epithelial surface.

An important question is whether maternal effector cells are controlled exclusively by the peripheral presentation of nonpolymorphic HLA-G molecules at the uterine–placental interface or whether central presentation of HLA-G

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molecules occurs in the thymus. Thymic selection events restricted by HLA-G may provide powerful mechanisms by which the maternal immune system could efficiently adapt to accept the fetal graft presenting such HLA determinants. Previous studies have reported very low levels of HLA-G messenger RNA (mRNA)[1] in the fetal thymus (17, 18), but no evidence has been provided for the expression of the protein, nor for the identity of the cells exhibiting such expression. In the present study, we therefore investigated whether HLA-G is expressed in the human thymus and, specifically, whether its expression localizes to antigen-presenting elements such as thymic epithelial cells.

Materials and Methods

Immunohistochemistry and Immunofluorescence Studies. Thymi were obtained from pediatric donors (1–12 yr old) undergoing elective surgery for cardiac malformations. Fetal thymi were obtained from Advanced Bioscience Resources (Alameda, CA). 10 μm cryostat sections of thymic tissues were dried and fixed in either 2% paraformaldehyde (immunocytochemistry) or absolute ethanol at −20°C (immunofluorescence). Sections were probed with the anti–HLA-G–specific IB8 mAb (4) followed by a biotin-conjugated Fab_2 anti–mouse IgM secondary Ab and either streptavidin alkaline phosphatase or streptavidin Lissamine-Rhodamine. For immunocytochemistry, sections were counter stained with brilliant blue and mounted in Gelmount (Biomeda, Foster City, CA). For two-color immunofluorescence studies, HLA-G–probed sections were further blocked with an excess of polyclonal mouse IgG and incubated in the presence of an FITC-labeled pan-cytokeratin mAb (clone KL1; Immunotech, Westbrook, ME) or an FITC-labeled isotype-matching IgG control. Sections were analyzed using an Olympus or a Zeiss Axiovert microscope equipped with a scanning laser confocal attachment (MRC 1024; Bio Rad Labs., Hercules, CA).

Isolation and Phenotypic Characterization of HLA-G$^+$ Thymic Stromal Cells. Thymic fragments were digested with collagenase (10 mg/ml) in the presence of DNase (Boehringer Mannheim, Indianapolis, IN), and thereafter treated with a nonenzymatic dissociation medium (Sigma Chemical Co., St. Louis, MO). This treatment yields single cells (thymocytes and stromal elements) as well as some incompletely dissociated cell clusters. This cell preparation was incubated in ice-cold PBS–2% FCS in the presence of IB8 mAb followed sequentially by a Fab_2 anti–mouse IgM secondary Ab (Cappel, Durham, NC), streptavidin-Tricolor (CALTAG Labs., Burlingame, CA) and biotin magnetic beads (Miltenyi Biotec Inc., Sunnyvale, CA). The cell preparation was passed through 300- and 40-μg nylon meshes to deplete cell aggregates, and applied to the magnetic column. Column-adherent cells were then eluted by removing the magnetic field and washing with several column volumes of buffer. Two-color flow cytometric analysis for HLA-G and epithelial cell adhesion molecule (Ep-CAM) was performed by staining with IB8 mAb as described above. After blocking with mouse IgG, cells were incubated with FITC-labeled anti-EpCAM mAb (clone 323A3; gift of Dr. G. Chamness, University of Texas, San Antonio, TX) or an FITC-labeled isotype-matching control IgG. Three-color flow cytometric analysis for HLA-G, -A, -B, -C, and CD3 was performed by sequential labeling with the IB8 mAb followed by an Fab_2 anti–mouse IgM secondary Ab (Cappel) and streptavidin-FITC (CALTAG Labs.), in combination with an RPE-conjugated anti–HLA-A, -B, and -C mAb (clone G46-2.6; PharMingen, San Diego, CA) and Cy-Chrome–conjugated anti–human CD3 mAb (clone 145-2C11; PharMingen). Flow cytometry was performed on a FACScan® (Becton Dickinson, San Jose, CA). For cell sorting experiments, thymic stromal cells were dissociated from the tissue as described above and let to recover by overnight culture in RPMI–10% FCS. The next day, cells were harvested and HLA-G^{high} epithelial cells purified by sequential enrichment on the magnetic column followed by fluorescence-activated cell sorting on a FacStar® (Becton Dickinson).

Generation of Thymic Epithelial Cell Lines. Thymic epithelial cell lines were generated by collagenase and trypsine digestion of thymic fragments followed by culture in serum-free conditions as previously described (19). The method yields epithelial cells with a uniformly bright cobblestone-like appearance by light microscopy which are 80–90% pure as determined by cytokeratin staining. Monolayers of thymic epithelial cells at the third or fourth passage (~4–6 wk of culture) were grown on glass coverslips, fixed in cytoskeleton-stabilizing buffer (20) containing 2% paraformaldehyde, and permeabilized in PBS–0.1% Triton X-100. After blocking in PBS–50 mM glycine–5% goat serum, cells were stained for HLA-G and cytokeratin as described above.

Western Blotting. Cell monolayers were lysed in 20 mM Tris/1% Triton X-100 and protease inhibitors. After removal of insoluble material by spinning at 10,000 *g* for 30 min, protein concentration was determined by the BCA protein assay (Pierce Chem. Co., Rockford, IL). 10–20 μg aliquots of total protein were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. After blocking, the membrane was probed with 1:100 dilution of the anti–HLA-G mAb 4H84 (culture supernatant) or mouse IgG. Antibody binding was detected using a peroxidase-conjugated secondary antibody and chemoluminescence (Kirkegaard & Perry Labs., Inc., Gaithersburg, MD).

PCR Amplification of HLA-G Transcripts and Sequencing. Single strand cDNA was transcribed from 0.5 μg of polyA$^+$ RNA using avian myeloblastosis virus reverse transcriptase and oligo dT-primers. One third of this reaction was used as template for PCR amplification of HLA-G transcripts expressed in TEC lines. Oligonucleotides used as primers for reverse transcriptase PCR (RT-PCR) of HLA-G transcripts were: 5′-AGGATGGTGGTCATGGCG (sense) located at the 5′ end of the cDNA and 5′-GGGCTGGTCTCTG-CACAAAGAGA (antisense) located within the 3′ untranslated region (21). Cyclophilin-specific primers were: 5′-ATGGTC-AACCCCACCGTGTTCTTC (sense) and 5′-CATAGATGG-

[1]*Abbreviations used in this paper:* EpCAM, epithelial cell adhesion molecule; FSC, forward side scatter; mRNA, messenger RNA; RT-PCR, reverse transcriptase PCR.

Figure 2. HLA-G expression pattern in the thymic medulla and identification of HLA-G$^+$ epithelial cells. Cryostat sections of human thymus showing staining for HLA-G protein as revealed by the HLA-G–specific mAb IB8 using an immunofluorescence detection method. (*A* and *B*) Medullary HLA-G$^+$ cells, often organized in railway-like structures, are seen to sharply delimit areas of tissue densely packed with thymocytes. (*C*) Two-color immunofluorescence analysis of thymic sections stained with a Lissamine-Rhodamine–labeled anti–HLA-G mAb (*red fluorescence*) and a FITC-labeled pan-cytokeratin mAb (*green fluorescence*) demonstrating the colocalization of HLA-G with cytokeratin-positive medullary epithelial cells (*yellow*). Cells with a dendritic-like morphology positive for HLA-G but negative for cytokeratins are also evident (*arrows*).

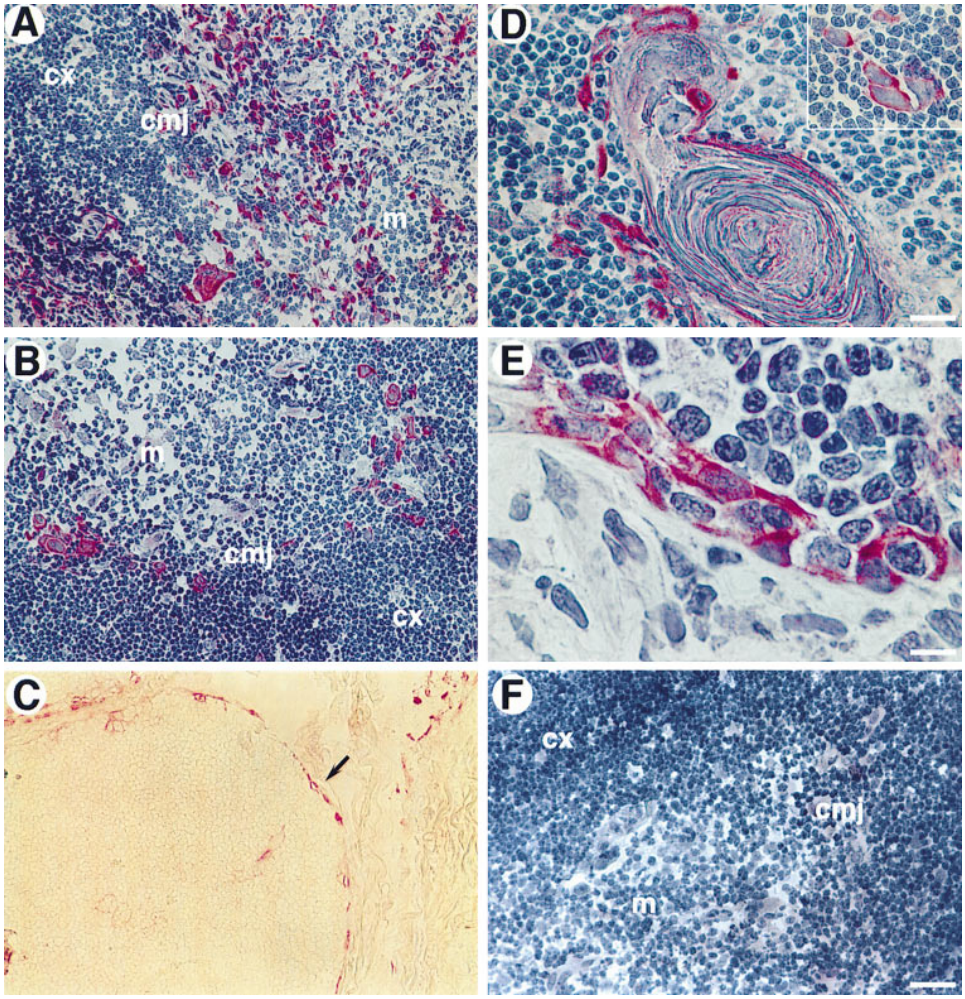
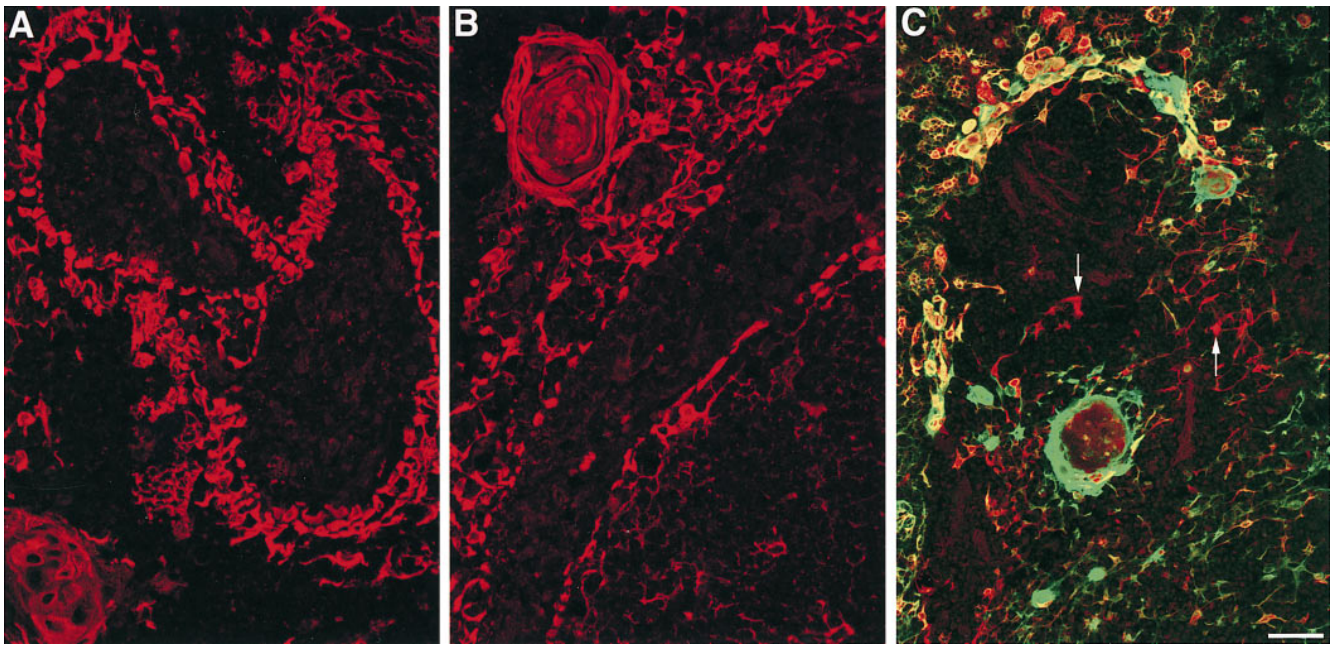


Figure 1. Expression of HLA-G by a subset of medullary and subcapsular thymic stromal cells. Cryostat sections of human thymus showing staining for HLA-G protein as revealed by the HLA-G–specific mAb IB8 (IgM) using an alkaline phosphatase detection method. Expression of HLA-G is primarily restricted to the (*A*) medullary (*m*), (*B*) cortico-medullary junction (*cmj*), and (*C*) subcapsular (*arrow*) regions of the thymus. Bar (*A*, *B*, *C*, and *F*) 60 μm. Within the medulla, HLA-G$^+$ cells appear as large stromal elements with big nucleoli (*D*, *inset*) often organized at the periphery of Hassal corpuscles (*D*, bar, 30 μm) or arranged in "railway"-like strings (*E*; bar, 12 μm). Background staining obtained using a control IgM antibody is shown in *F*.



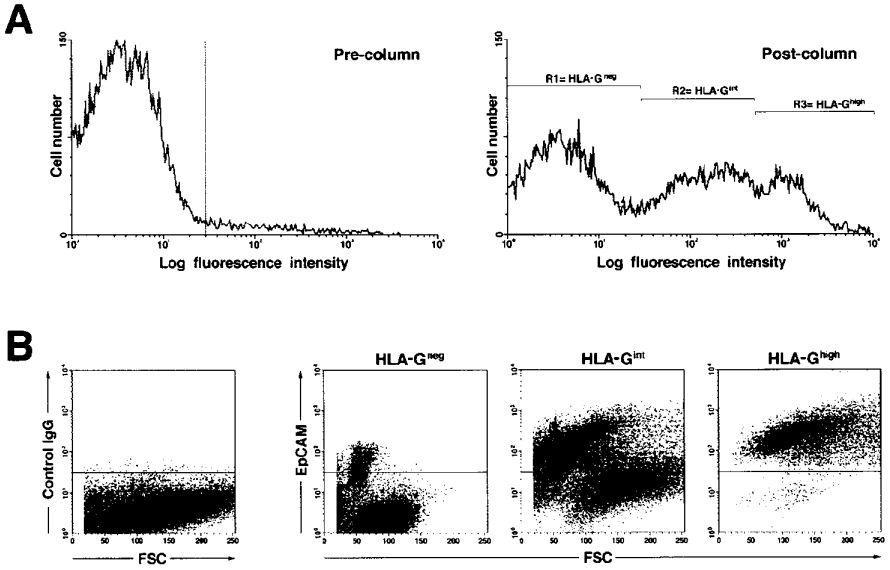


Figure 3. Surface expression of HLA-G on freshly isolated epithelial cells. A cell suspension enriched for HLA-G$^+$ cells was obtained by collagenase digestion and nonenzymatic dissociation of thymic fragments, followed by positive selection of IB8-labeled cells using an immunomagnetic bead separation technique. (*A*) Flow cytometric analysis of a single cell suspension stained for HLA-G before (*left*) and after (*right*) enrichment by positive selection on the magnetic column. The vertical line marks the upper limit of the background intensity of fluorescence obtained using a control IgM. (*B*) This HLA-G–enriched cell population was further probed for the expression of the surface epithelial marker EpCAM using an anti-EpCAM FITC-labeled mAb (clone 323A3) or a FITC-labeled isotype-matching control IgG, and analyzed by flow cytometry. The horizontal line marks the upper limit of the background fluorescence obtained with the control IgG (*left*). HLA-G^{high} cells (*A*, *R3*) comprise cells with a large FSC and coexpress high levels of EpCAM, supporting their epithelial nature. HLA-G^{int} cells (*A*, *R2*) comprise EpCAM$^{int\text{-}high}$ cells with an intermediate FSC as well as EpCAM^{low} cells with a large FSC. HLA-G^{neg} cells are EpCAM$^{low\text{-}neg}$.

ACTTGCCACCAGTGC (antisense). In the studies addressing the expression of HLA-E, -F, and -G in sorted HLA-G^{high} cells, separate PCR reactions were performed using the locus-specific oligonucleotides and the pan-HLA class I primers listed in Table 1. The locus specificity of primers used has been previously documented (22–25). PCR was carried out for 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. For semiquantitative analysis of PCR products, a linear range of amplification was identified corresponding to cycles 22, 24, 26, and 28 using 1/50 of the total cDNA reaction as template. PCR products were separated on a 1.8% agarose gel and visualized by ethidium bromide staining. Densitometric analysis of the bands was performed using National Institutes of Health Image software (version 1.59b9). For sequence analysis, bands corresponding to the PCR-amplified cDNAs were excised from low melting point agarose gels and directly cloned in a pCR™ II vector using a TA cloning system (Invitrogen, San Diego, CA). Fragments cloned from at least two separate colonies for each alternatively spliced form of HLA-G were sequenced from both ends using Sequenase (United States Biochemical Corp., Cleveland, OH).

Results and Discussion

Immunohistochemical analysis of human thymic sections using an HLA-G–specific mAb (clone IB8; reference 4) revealed a distinct pattern of expression restricted to medullary and subcapsular stromal cells (Fig. 1). HLA-G$^+$ cells were identified within the medulla (Fig. 1 *A*) and formed a distinct rim of cells at the corticomedullary junction (Fig. 1 *B*) and subcapsular region of the thymus (Fig. 1 *C*). At higher magnification, positive cells appeared as large oval shaped elements often surrounding Hassal corpuscles (Fig. 1 *D*, inset) or organized to form railway-like strings (Fig. 1 *E*). These latter structures sharply demarcated areas of the medulla (Fig. 2, *A* and *B*) that were densely packed with thymocytes. This distinct pattern of expression was observed in tissue specimens from pediatric donors (e.g., 1–12 yr). In contrast, fetal thymic tissues (14–22 wk of gestation) revealed only few HLA-G$^+$ cells scattered randomly throughout the medulla (not shown). Two-color confocal microscopy was used to colocalize the expression of HLA-G with cytokeratin. This analysis revealed that most HLA-G$^+$ cells are epithelial (Fig. 2 *C*). Nevertheless, a few HLA-G$^+$ cells with a dendritic morphology and negative for cytokeratin were observed in the medulla (Fig. 2 *C*, *arrows*).

To determine whether HLA-G is expressed on the cell surface, a suspension of thymic stromal cells was prepared in two steps, a collagenase digestion followed by a nonenzymatic dissociation of the thymic tissue. Cells were then stained with the IB8 mAb and enriched by positive selection with a magnetic bead–column technique. Flow cytometric analysis of the cells before purification on the column demonstrated a low percentage (~1%) of HLA-G$^+$ cells (Fig. 3 *A*, *left*). However, two discrete populations of cells expressing intermediate and high levels of HLA-G (HLA-G^{int} and HLA-G^{high}, respectively) on the cell surface could be detected after enrichment on the magnetic column (Fig. 3 *A*, *right*). HLA-G^{int} and HLA-G^{high} cells represent 33 and 17% of the enriched population, respectively.

To characterize both quantitatively and qualitatively the thymic epithelial cells expressing HLA-G, the column-enriched preparation was further stained with an FITC-labeled antibody specific for EpCAM, a cell-surface epithelial marker that preferentially labels the medullary epithelium (26, 27). Fig. 3 *B* shows that HLA-G^{high} cells are strongly positive for EpCAM. As determined by their forward side scatter (FSC) values, these cells are relatively large, consistent with the characteristics of most medullary epithelial cells previously described by immunohistochemical and electron microscopy studies (28, 29). HLA-G^{int} cells also comprise an EpCAM$^+$ population of large to intermediate size cells,

Table 1. *Expression of HLA-E, -F, and -G Transcripts in Sorted HLA-G^{high} Thymic Epithelial Cells*

Primers	Location	PCR fragment	Expression (% of pan-HLA class I)*
		bp	
HLA-E			
5′-TCCGAGCAAGAATCAAATGATG	Exon 3	700	41.5 ± 12
5′-GTGTGAGGAAGGGGGTCATG	3′-untranslated		
HLA-F			
5′-TATTGGGAGTGGACCACAGGGTAC	Exon 2	842	31.4 ± 10
5′-GGCACAAGTGCAATTCTGCTAC	3′-untranslated		
HLA-G			
5′-TTGGGAAGAGGAGACACGGAACAC	Exon 2	866	3.3 ± 2.4
5′-GGGCTGGTCTCTGCACAAAGAGA	3′-untranslated		
pan-HLA class I			
5′-TCCCACTCCATGAGGTATTTC	Exon 2	777	100
5′-CACATGGCAGGTGTATCTCTG	Exon 4		

Messenger RNA from HLA-G^{high} thymic epithelial cells purified by fluorescence-activated cell sorting, was amplified by PCR using either locus-specific oligonucleotides or pan-HLA primers corresponding to conserved sequences in all class I genes. Each PCR reaction was performed using mRNA from 5×10^3 cells. PCR fragments obtained in the linear range of amplification were separated by agarose gel electrophoresis, stained by ethidium bromide, and analyzed by densitometry. The pixel intensity of each nonclassical HLA transcript is expressed as percentage of that measured for the total HLA transcript, amplified with pan-HLA primers.
*Data represent mean ± SEM of three independent cell sorting experiments.

as well as a population of large EpCAM^{low} cells. Thus, ~40% of the cells eluted from the column are EpCAM$^+$ and the majority of these EpCAM$^+$ cells (e.g., ~82%) express HLA-G. These results demonstrate that the HLA-G$^+$ subset comprises cells with the medullary epithelium phenotype, as also shown by the in situ immunofluorescence (Fig. 1). Finally, HLA-G$^-$ cells comprise two populations of relatively smaller cells, one EpCAM$^+$ and one EpCAM$^-$.

To assess whether the epithelial cell expression of HLA-G and classical class Ia HLA antigens are mutually exclusive, an enriched population of HLA-G$^+$ cells obtained as described above was stained by three-color immunofluorescence for CD3, HLA-G, and a monomorphic determinant on classical class Ia antigens. Flow cytometric analysis of gated CD3$^-$ cells demonstrate that ~95% of the cells expressing high to intermediate levels of HLA-G, coexpress high levels of classical class Ia HLA (mean fluorescence log intensity = 1,300 versus control = 10). The minority population of CD3$^-$ HLA-G$^-$ cells comprise cells with low levels of HLA-A, -B, and -C (mean intensity of fluorescence = 60), a phenotype consistent with the presence of MHC class Ia^{low} cortical stromal elements within this population (30, 31). Only a few cells (~5%) were detected that were HLA-G^{high} HLA-A, -B, and -C$^-$. Although these data demonstrate that HLA-G and classical class Ia HLA molecules are coexpressed on thymic epithelium, they raise an additional question regarding the expression of other nonclassical HLA molecules such as HLA-E and -F. Of these nonclassical HLA molecules, HLA-E has been reported to be expressed in the cytotrophoblasts (23). Because specific antibodies for HLA-E and -F are not available, we determined the expression of these HLA molecules at the transcriptional level. For this purpose, HLA-G^{high} thymic epithelial cells were purified by fluorescence-activated cell sorting from pediatric thymi. mRNA from 2–3 × 10^5 HLA-G^{high} cells was then used as template for PCR amplification of HLA-E, -F, and -G transcripts using locus-specific primers (Table 1). PCR reactions were also performed using a pair of pan-HLA primers for amplification of all HLA class I mRNAs. The linear range of amplification was determined for each of the four primers and PCR transcripts, visualized by ethidium bromide staining, were analyzed by densitometry. The results of this analysis are summarized in Table 1. HLA-E and -F–specific transcripts were easily detected by PCR, representing 41.5 ± 12% and 31.4 ± 10% of the total class I HLA mRNA. Unexpectedly, low levels of the full-length HLA-G transcripts (e.g., 3 ± 2.4% of total HLA) were detected in the sorted HLA-G^{high} cells. Alternatively spliced forms of HLA-G (see below) were undetectable. It is unlikely that these results reflected an inefficient amplification of HLA-G mRNA, since the same set of primers readily amplified HLA-G mRNAs from fresh thymic tissue and thymic epithelial cell lines (see below). Rather, these results suggest that HLA-G mRNAs in sorted cells may be either unstable or truly expressed at low levels relative to other class I HLA transcripts. This latter possibility implies that the high levels of expression of the HLA-G protein, demonstrated in situ and by flow cytometry, may be maintained in thymic epithelial cells by a highly efficient translation of HLA-G mRNA or by a low turnover of the cell surface protein.

Alternative splicing of the HLA-G mRNA has been de-

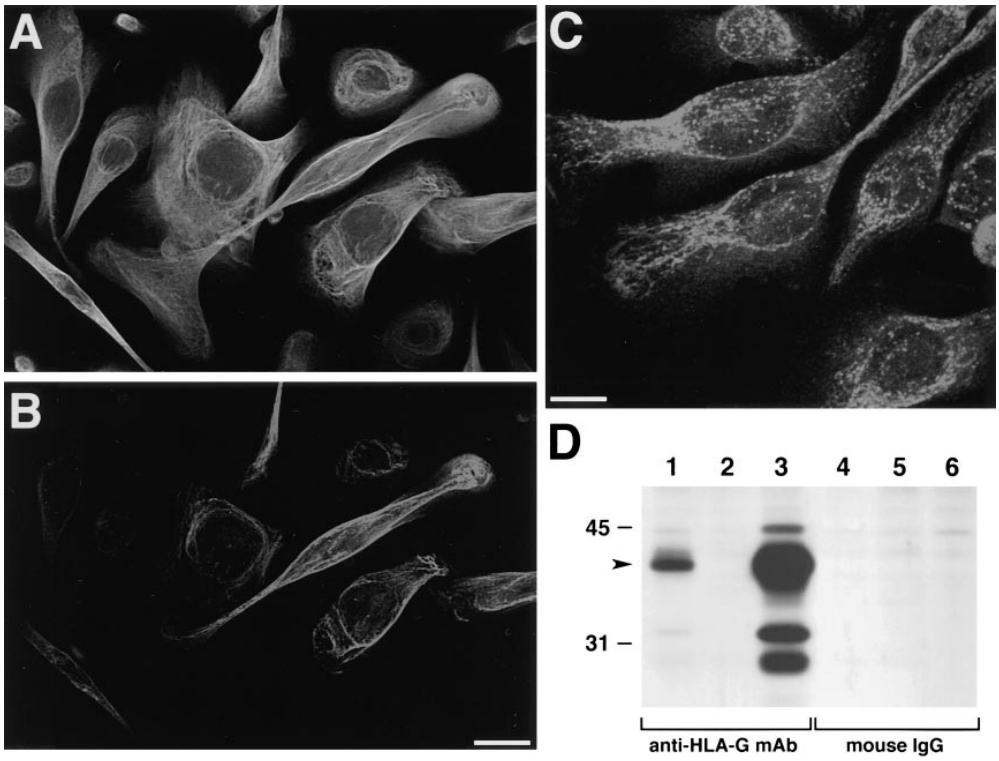


Figure 4. Expression of HLA-G in cultured human thymic epithelial cell lines. Confocal microscopy of primary thymic epithelial cells stained with an FITC-conjugated pan-cytokeratin mAb (*A*) and a Lissamine-Rhodamine–conjugated IB8 mAb (*B*), or 4H84 mAb (*C*). Analysis of single confocal planes from a z series revealed that HLA-G–specific staining is predominantly intracytoplasmic. Bars: (*B*) 45 μm; and (*C*) 22 μm. (*D*) Western blotting analysis of detergent lysates from a human TEC line (lanes *1* and *6*), Jurkat lymphoma cells (HLA-A, -B, -C^+, and -G^-; lanes *2* and *4*) and JEG-3 choriocarcinoma cells (HLA-A, -B^-, and HLA-G^+; lanes *3* and *5*). The membrane was probed with an anti–HLA-G IgG mAb specific for a peptidic sequence within the α1 domain of HLA-G (clone 4H84; McMaster, M.T., and S.J. Fisher, manuscript in preparation; lanes *1*–*3*) or mouse IgG (lanes *4*–*6*). Antibody binding was detected by chemiluminescence. 20 μg total proteins were loaded in lanes *1* and *6*; 10 μg of total proteins were loaded in the remaining lanes. A prominent band of ~38–40 kD is detected in TEC and JEG-3 extracts, but not in Jurkat's extracts (*arrow*).

scribed in human trophoblast and HLA-G–transfected cell lines (25, 32). The full-length transcript, named HLA-G1, gives rise to a molecule containing the α1, α2, and α3 domains, forming the basic structure of MHC class I heavy chain. A shorter HLA-G2 transcript excludes exon 3 encoding the α2 domain, thereby creating a protein containing an α1 domain joined directly to the α3 domain. A third transcript, HLA-G3, encodes a molecule in which the α1 domain is directly connected to the transmembrane region. Most recently, variants of HLA-G1 and -G2 mRNAs have been reported containing the intron 4 and encoding for soluble HLA-G proteins (33, 34). To investigate which of these transcripts are expressed by thymic epithelial cells, we developed human primary epithelial cell (TEC) lines from both fetal and pediatric thymi. Expression of HLA-G proteins in these cell lines can be detected in ~10–20% of cells, though confocal microscopy demonstrates that expression is primarily at an intracellular location (Fig. 4, *B* and *C*). Western blotting analysis of detergent extracts from TECs using an HLA-G–specific mAb (clone 4H84; McMaster, M.T., and S.J. Fisher, manuscript in preparation) demonstrates that the predominant HLA-G protein expressed has an apparent molecular weight of 38–40 kD (Fig. 4 *D*).

Amplification of HLA-G–specific sequences from mRNA of TEC lines by RT-PCR demonstrated 1,200-, 900-, and 500-bp DNA fragments (Fig. 5 *A*) consistent with mRNA transcripts for the membrane-bound HLA-G1, -G2, and -G3 alternatively spliced isoforms, respectively (25, 32). Amplification of these transcripts was consistently obtained in six independent TEC lines. Semiquantitative analysis of these PCR products obtained in the linear range of amplification from mRNA of three TEC lines revealed that the HLA-G2 and -G3 transcripts represent 20.5 ± 3.9% and 4.4 ± 3.5% (mean ± SEM) of the HLA-G1 mRNA, respectively. PCR amplification of HLA-G transcripts from mRNA of fresh thymic tissue obtained from three separate donors revealed a similar pattern of frequency of the HLA-G2 and -G3 transcripts relative to the HLA-G1 transcript (e.g., 29.8 ± 4.1% and 0.57 ± 0.2%, respectively). Sequence analysis of HLA-G1 and -G2 cDNAs isolated from two pediatric thymic epithelial cell lines confirmed an intact primary structure and correct splicing of the corresponding transcripts. In particular, HLA-G1 cDNAs revealed a nucleotide sequence consistent with the splicing of exons 1, 2, 3, 4, 5, 6, and 8 of the HLA-G gene (21; Fig. 5 *B*). In contrast, HLA-G2 cDNAs revealed a sequence consistent with the splicing of exons 1, 2, 4, 5, 6, and 8. Similar analysis of HLA-G1 and -G2 cDNAs obtained from a fetal thymic epithelial cell line revealed an intact primary structure of the HLA-G1 transcript, but an HLA-G2 mRNA containing a 33-nucleotide deletion within exon 4. Fig. 5 *B* shows the location of the sequence variations identified by our analysis, as compared to the cDNA sequence of the full-length HLA-G reported by Shukla et al. (35). Notably, within the

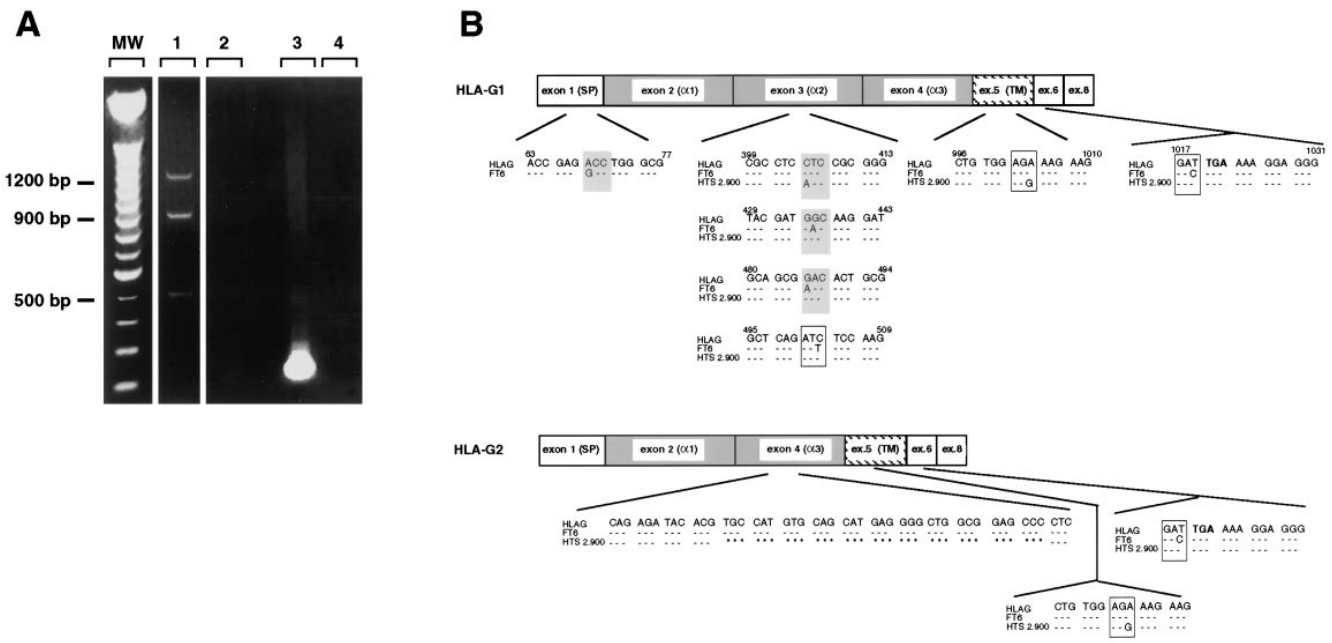


Figure 5. PCR amplification and sequence of HLA-G transcripts expressed by thymic epithelial cell lines. (*A*) Agarose gel electrophoresis of HLA-G alternatively spliced transcripts amplified by RT-PCR from mRNA obtained from a primary human thymic epithelial cell line. 1,200-, 900- and 500-bp bands are detected (lane *1*), consistent with the presence of the three main alternatively spliced forms of HLA-G, namely HLA-G1, -G2, and -G3 described by Ishitani et al. (32). In contrast, mRNA from freshly isolated thymocytes (lane *2*) demonstrates no HLA-G–specific transcripts. A positive control PCR reaction for thymocyte mRNA showing a 237-bp DNA fragment of cyclophillin is displayed in lane *3*. A negative control PCR reaction run using HLA-G–specific primers and no cDNA template is shown in lane *4*. Molecular weight markers corresponding to a 100-bp DNA ladder (GIBCO BRL, Gaithersburg, MD) are shown in the left lane (*MW*). (*B*) Primary structure and sequence variations identified within the HLA-G1 and -G2 cDNAs amplified by PCR from two of the three TEC lines that were sequenced, e.g., FT6 and HTS 2.900. Sequence variations are displayed with respect to their location within each exon and aligned with the published sequence of a full-length HLA-G cDNA expressed by placental trophoblasts reported by Shukla et al. (35). *SP*, signal peptide; *TM*, transmembrane domain. Stretches of nucleotide sequences are identified by base pair numbers for orientation. Dashes represent sequence identity; stars represent gaps. Codons containing point mutations are boxed. Mutations resulting in amino acid variations are also shadowed. Note the prevalence of point mutations resulting in amino acid changes within exon 4. The CTC→ATC (leu→ile) substitution corresponding to amino acid position 110 of the HTS 2.900 line has been previously reported (35). Though the GGC→GAC (gly→asp) substitution at amino acid position 120 of the FT6 line has not been reported, a variety of other amino acid substitutions have been identified at the very same position (36).

HLA-G1 transcripts, most nucleotide variations resulting in amino acid changes are clustered within exon 3, encoding for the α2 domain, located between the β-pleated sheet and the start of the α2 helix. Interestingly, such amino acid changes fall into the same stretch of sequence (e.g., amino acids 101–138) previously identified as a mutational hot spot by genomic sequence analysis of HLA-G alleles (36). The data indicate that, unlike classical HLA class I molecules in which polymorphic residues restricting peptide binding are present in both the α1 and α2 domains, polymorphism of HLA-G appears to be largely restricted to the α2 domain. The deletion which we report within HLA-G2 cDNA expressed by the fetal cell line includes the loss of cysteine residue 259 located in the α3 domain. It is conceivable that this sequence change results in a gross alteration of protein structure, suggesting that this transcript may either yield a protein with an altered function or behave as a pseudogene.

The present data demonstrate the compartment-specific expression of HLA-G molecules in the human thymus by medullary epithelial cells. These results raise the possibility that the T cell immune response of the mother to the fetus may be regulated by interactions of developing T cell progenitors with thymic HLA-G. The development of T cells restricted by this relatively nonpolymorphic MHC molecule during thymic ontogeny and its selective expression in the placenta during pregnancy, may both be required to ensure successful conception despite fetal–maternal disparities at polymorphic HLA loci. Thus, it is possible that cases of recurrent fetal miscarriage of immune etiology may be related to altered expression of HLA-G in the thymus.

The evidence that expression of MHC transgenes on medullary thymic epithelial cells result in antigen-specific T cell tolerance by anergy or unresponsiveness (37–39) rather than clonal deletion, suggests at least two mechanisms by which HLA-G expressed at this particular location in the thymus may influence maternal immune responses to fetal tissues presenting HLA-G. Mechanisms of tolerance by immune unresponsiveness may indeed be operative at the maternal–fetal interface since decidual cells are poor responders to mitogens and allogenic stimuli (9). An alternative mechanism by which thymic HLA-G may influence peripheral immune responses can be inferred by its expression in the thymic medulla, a location open to the recirculation of peripheral lymphoid cells (40). Thus, HLA-G may be involved in the uptake or presentation of circulating antigens (e.g., shed by the fetal cells of the placenta) to mature

peripheral T cells recirculating to this compartment. In support of this possibility, there is evidence that murine medullary, but not cortical, TECs can present soluble antigens (41), and that MHC class I–dependent pathways of presentation exist for exogenous antigens (42, 43).

Proposing a role for HLA-G in thymic-dependent T cell development depends upon the demonstration that HLA-G is expressed at the cell surface of thymic antigen-presenting cells. In this regard, our data represent the first evidence that this is the case. The antigen-presenting function of HLA-G is supported by the conservation of an MHC class I–like structure, at least for the HLA-G1 isoform (7), its ability to bind CD8 (44), and the presence of endogenous peptides bound in the groove (45). Interestingly, our data, as well as reports by others (36, 46), indicate that most sequence variability is clustered within the $\alpha 2$ domain of the molecule. This suggests that the $\alpha 1$, rather than $\alpha 2$, domain may impose greater structural constraints for binding of relatively invariant peptidic motifs to HLA-G. If indeed few peptide variants are presented by this MHC class I molecule as predicted by its low polymorphism, the predominant expression of HLA-G on a subset of medullary epithelial cells implies that a qualitative difference may exist in the repertoire of peptides presented by medullary and cortical TECs in the human thymus. Interestingly, the expression of a nonpolymorphic MHC class II molecule has been described on a subset of medullary TECs in the mouse thymus (47–49). It is possible that the medulla is specialized for the presentation of specific antigenic peptides in the context of nonpolymorphic MHC molecules.

We propose that in contrast with the universal expression of classical polymorphic MHC molecules, the targeted expression of the olygomorphic HLA-G to the maternal thymus and the fetal trophoblasts may have been selected and conserved for the immunological protection of pregnancy. Compartimentalization of the oligomorphic HLA-G to the thymic medulla would not interfere with selection events on polymorphic MHC molecules expressed in the cortex, the latter being required to impart a broad spectrum of immune responses to environmental antigens.

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Address correspondence to Dr. Laura Crisa, Department of Molecular and Experimental Medicine - SBR5, The Scripps Research Institute, 10550 North Torrey Pines, La Jolla, CA 92037. Phone: 619-784-2023; FAX: 619-784-2243; E-mail: crisa@scripps.edu

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