

# Characterization of Monoclonal Antibodies Recognizing HLA-G or HLA-E: New Tools to Analyze the Expression of Nonclassical HLA Class I Molecules

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**ABSTRACT:** Nonclassical major histocompatibility complex (MHC) class I human leukocyte antigen E (HLA-E) and HLA-G molecules differ from classical ones by specific patterns of transcription, protein expression, and immunotolerant functions. The HLA-G molecule can be expressed as four membrane-bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7) proteins upon alternative splicing of its primary transcript. In this study, we describe a new set of monoclonal antibodies (mAbs) called MEM-G/01, -G/04, -G/09, -G/13, MEM-E/02, and -E/06 recognizing HLA-G or HLA-E. The pattern of reactivity of these mAbs were analyzed on transfected cells by flow cytometry, Western blotting, and immunochemistry. MEM-G/09 and -G/13 mAbs react exclusively with native HLA-G1 molecules, as the 87G mAb. MEM-G/01 recognizes (similar to the 4H84 mAb) the denatured HLA-G heavy chain of all isoforms, whereas MEM-G/04 recog-

nizes selectively denatured HLA-G1, -G2, and -G5 isoforms. MEM-E/02 and -E/06 mAbs bind the denatured and cell surface HLA-E molecules, respectively. These mAbs were then used to analyze the expression of HLA-G and HLA-E on freshly isolated cytotrophoblast cells, on the JEG-3 placental tumor cell line, and on cryopreserved and paraffin-embedded serial sections of trophoblast tissue. These new mAbs represent valuable tools to study the expression of HLA-G and HLA-E molecules in cells and tissues under normal and pathologic conditions. *Human Immunology* 64, 315–326 (2003). © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Science Inc.

**KEYWORDS:** nonclassical HLA class I molecules; HLA-E; HLA-G; monoclonal antibodies; pregnancy; transplantation

## ABBREVIATIONS

FITC fluorescein isothiocyanate

ILT immunoglobulin-like transcript

KIR killer cell immunoglobulin-like receptor

mAb monoclonal antibody

MHC major histocompatibility complex

NK natural killer

PE phycoerythrin

PBL peripheral blood lymphocytes

## INTRODUCTION

Nonclassical major histocompatibility complex (MHC)

class Ib, human leukocyte antigen E (HLA-E), and

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HLA-G molecules are homologous to classical MHC class Ia, HLA-A, -B, and -C molecules, and display limited polymorphism [1, 2]. HLA-E and HLA-G molecules appear to exhibit a distinct pattern of expression. Although the HLA-E molecule is widely distributed in adult and fetal tissues [3], HLA-G expression is restricted to trophoblast cells [4] and some medullary thymic epithelial cells [5]. The HLA-G primary transcript is alternatively spliced leading to at least seven different HLA-G isoforms, namely the HLA-G1, -G2, -G3, and -G4 membrane-bound and the HLA-G5, -G6, and -G7 soluble proteins [6–9]. The protein structure of the 39-kDa full-length HLA-G1 isoform is similar to that of HLA-E and classical HLA class I molecules, consisting of three globular domains associated with  $\beta_2$ -microglobulin. The HLA-G2, -G3, and -G4 isoforms are lacking the  $\alpha 2$ , both  $\alpha 2$  and  $\alpha 3$ , or the  $\alpha 3$  domains, respectively. The soluble forms of HLA-G, a full-length HLA-G5, and two shorter HLA-G6, and -G7 forms lacking the  $\alpha 2$ , and both  $\alpha 2$  and  $\alpha 3$  domains, respectively, are encoded by alternatively spliced transcripts that retain intron 4 (for HLA-G5 and -G6) or intron 2 (for HLA-G7) that contain a stop codon, thus precluding the translation of transmembrane and cytoplasmic domains.

HLA-E expression matches the distribution of classical HLA class I molecules and HLA-G. Indeed, the expression of HLA-E is necessarily dependent upon that of HLA class I molecules that, within their leader sequences, possess a peptide ligand for HLA-E [10, 11]. In contrast, HLA-G displays restricted tissue distribution and is found *in vivo* during pregnancy at the maternal–fetal interface in the placenta [4, 12]. Within the placenta, HLA-G is detected by immunohistochemistry using specific HLA-G mAbs in invading extravillous cytotrophoblast, and amnion epithelial cells [13]. Soluble forms of HLA-G have been found in embryo cells [14], amniotic fluids, and plasma [15]. The expression of the HLA-A and -B class I molecules is lacking in extravillous cytotrophoblast that do express low levels of HLA-C molecules [16]. Thus, HLA-E expression in these cells is principally dependent on that of both HLA-G and HLA-C. In this case, these HLA class I molecules might protect the fetus from attack by maternal natural killer cells (NK) and cytotoxic T lymphocytes (CTL). In addition to the modulation of both innate and adaptive immunity, engagement of HLA-G by its multiple receptors (such as CD8, KIR2DL4, ILT-2, and ILT-4) might contribute to others functions, including blastocyst implantation, trophoblast invasion, angiogenesis, and inflammation [17, 18].

Although HLA-G mAbs have been already validated at the previous International Preworkshop on HLA-G and HLA-E (Paris, July 2000) [19], new mAbs are still

needed to further analyze the biochemical and functional properties of these nonclassical HLA class I molecules as well as their tissue distribution in normal and pathologic conditions. This is particularly important for HLA-E because no mAb is currently available. We here describe a new set of mAbs recognizing either cell-surface or denatured HLA-G and -E molecules. The use of these mAbs was evaluated by flow cytometry, Western blotting, and immunochemistry analysis on transfected cell lines and tissues following the procedures agreed upon for the Preworkshop.

## MATERIALS AND METHODS

### Cell Lines, Transfectants, and Tissues

M8 is an HLA-A, -B, -C, -E positive (HLA-A1, -A2, -B12, and -B40/male), but HLA-G negative melanoma cell line [20]. The HLA-G1, -G2, -G3, -G4, and -G5 transfectants of M8 were obtained as previously described [21, 22]. The HLA class I negative K562 cell line (American Type Culture Collection [ATCC], Manassas, VA, USA) was transfected with the pcDNA3.1 vector containing the HLA-G1 cDNA (K562-HLA-G1) as previously described [23]. Cells were maintained in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum, 2-mM L-glutamine, 1  $\mu$ g/ml gentamicin, and fungizone (Sigma, St. Louis, MO, USA). HLA-G transfectants were cultured in media containing hygromycin B for pcDNA 3.1 constructs (M8-HLA-G1, -G2, -G3, -G5, and K562-HLA-G1) or geneticin for pRc-RSV constructs (M8-HLA-G4; Sigma). The pcDNA3.1/hygro vector was used to generate negative control cells (M8-pcDNA and K562-pcDNA). The LCL 721.221-AEH transfectant was a gift from D. Geraghty (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) and was obtained by transfection of an hybrid AEH gene in the lymphoblastoid cell line LCL 721.221, as previously described [10, 19]. Briefly, construct AEH consists of the HLA-A2 promoter sequence through the end of exon 1 encoding the leader peptide, fused to the HLA-E intron 1 sequence, and extending beyond the HLA-E 3' untranslated sequence. This hybrid proved to be efficient in directing cell-surface expression of the HLA-E molecule [10]. Lymph node cells from transgenic mice expressing human  $\beta_2$ -microglobulin (M-TGM) alone or together with the HLA-E<sup>G</sup> heavy chain (EM-TGM) [24], and mouse L cells expressing various HLA class Ia molecules were obtained as previously described [24]. The human HLA-G-positive choriocarcinoma cell line JEG-3 (ATCC) was cultured in DMEM (Sigma) supplemented with 10% heat-inactivated fetal calf serum. The cells used were routinely tested for and found to be free of mycoplasma.

Mononuclear cytotrophoblast cells were isolated from

trophoblast tissues obtained from first-trimester terminations of normal pregnancies at 6–12 weeks gestation (local ethics committee approval was obtained) by suction curettage, as previously described [25]. Briefly, mononuclear cytotrophoblast cells were isolated using a trypsin-EDTA dispersion method, followed by a Ficoll gradient centrifugation step and a CD45-positive depletion with immunomagnetics beads. The trophoblast origin of these isolated cells was confirmed by their CD45-negative staining by flow cytometry and their expression of cytokeratin 7 (CK7) analyzed by immunocytochemistry [26, 27].

To perform immunohistochemistry analysis, human first-trimester placenta tissue as well as normal skin biopsies obtained following plastic surgery were either snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ , or fixed in 4% formalin and embedded in paraffin. Cryostat tissue sections (4  $\mu\text{m}$ ) were mounted on slides, air dried for 30 minutes, and fixed in cold acetone for 10 minutes and air dried. The paraffin-embedded tissues were cut at 4- $\mu\text{m}$  thickness, mounted on precleaned glass microscope slides, deparaffinized using toluene, rehydrated through a graded series of ethanol, and rinsed in distilled water.

### Monoclonal Antibodies

The mAbs of the MEM-G/ and MEM-E/series were made in the Prague laboratory by standard procedures from splenocytes of Balb/c mice immunized with bacterially produced extracellular domains of HLA-E or HLA-G, either denatured by 8-M urea or renatured in the presence of  $\beta_2\text{-m}$  and appropriate peptides; these antigens were provided to the Prague laboratory within a collaborative project by M. Valter and G. Pazmany from the laboratory of J.L Strominger (Harvard University, Cambridge, MA, USA). The hybridoma supernatants were screened for specific HLA-G or HLA-E reactivity by enzymeimmunoabsorbent assay (ELISA) and Western blotting (those directed against denatured molecules), or by flow cytometry using transfectants expressing on the surface HLA-G or -E as the only HLA class I molecules.

The primary antibodies MEM-G/01, -G/04, -G/08, -G/09, -G/10, -G/11, -G/12, -G/13, -G/14, -E/02, and -E/06 (all of IgG isotype) were used as ascitic fluids, except for the double-staining of trophoblast cells in which the MEM-G/09 conjugated with fluorescein isothiocyanate (FITC) was used (Exbio, Prague, Czech Republic). The 87G mAb, a purified mouse IgG2a recognizing both HLA-G1 and HLA-G5 isoforms (1  $\mu\text{g}/\text{ml}$  for flow cytometry), was kindly provided by D. Geraghty, and the 4H84 mAb, a mouse IgG1 reactive with both native and denatured HLA-G heavy chain (1/500 dilution of ascitic fluid for immunohistochemistry), was kindly provided by M. McMaster (University of Califor-

nia, San Francisco, CA, USA). Both 4H84 and 87G mAbs had been previously validated during the HLA-G, -E, -F International Preworkshop [19]. The HLA class I molecules were stained using either the W6/32 or B9.12.1 mAbs. For immunohistochemistry analysis, trophoblast cells were identified by their staining with an anti-CK7 mAb (Novocastra Laboratories, Beton Lane, UK) [26, 27].

### Flow Cytometry Analysis

For flow cytometry assays, cells were washed in PBS and stained with the corresponding primary mAb in PBS 2% heat-inactivated fetal calf serum for 30 minutes at  $4^{\circ}\text{C}$ . After washing, cells were subsequently stained with an F(ab')<sub>2</sub> goat anti-mouse IgG antibody conjugated with FITC or phycoerythrin (PE) (Beckman Coulter, Villepinte, France) for 30 minutes at  $4^{\circ}\text{C}$ . Control aliquots were stained with an isotype-matched antibody to evaluate nonspecific binding to target cells. Each antibody was first tested at several dilutions in order to determine the optimal dilution to be used. The cell-surface expression of HLA-G2, -G3, and -G4 on M8 transfectants was analyzed by flow cytometry using the 4H84 mAb gated on propidium iodide-negative cells, as previously described [22]. The freshly isolated cytotrophoblast cells were double-stained by the 87G mAb followed by an F(ab')<sub>2</sub> goat anti-mouse IgG antibody conjugated with PE, and the MEM-G/09 directly conjugated to FITC. Fluorescence was detected by using either a FACS Scan (Becton Dickinson, Palo Alto, CA, USA) or an EPICS XL4 flow cytometer (Beckman Coulter, Brea, CA, USA).

### Western Blot Analysis

Aliquots of total proteins from either M8-pcDNA, LCL 721.221, LCL 721.221-AEH, K562-pcDNA, and K562-HLA-G1 cells were separated in 12% SDS-PAGE. Cells were washed with PBS and lysed in lysis buffer (50-mM Tris-HCl, pH 7.4, 0.5% Chaps [Sigma], containing protease inhibitors [Complete; Roche Diagnostics, Meylan, France]). After centrifugation at 15,000g at  $4^{\circ}\text{C}$  for 20 minutes, supernatants were supplemented with 6 $\times$  Laemli buffer. All samples were heated for 5 minutes at  $95^{\circ}\text{C}$  before loading on a 12% SDS-PAGE. Proteins were then electroblotted onto nitrocellulose membranes (Hybond; Amersham, Buckinghamshire, UK) and the membranes blocked by incubation with PBS containing 0.2% Tween 20 and 5% nonfat dry milk. The membranes were then probed with the corresponding mAb overnight at  $4^{\circ}\text{C}$  and washed in PBS containing 0.2% Tween 20. The membranes were subsequently incubated for 30 minutes at room temperature with goat anti-mouse horseradish peroxidase (Amersham), and washed thoroughly. Signals were detected

using enhanced chemiluminescence reagent (ECL; Amersham).

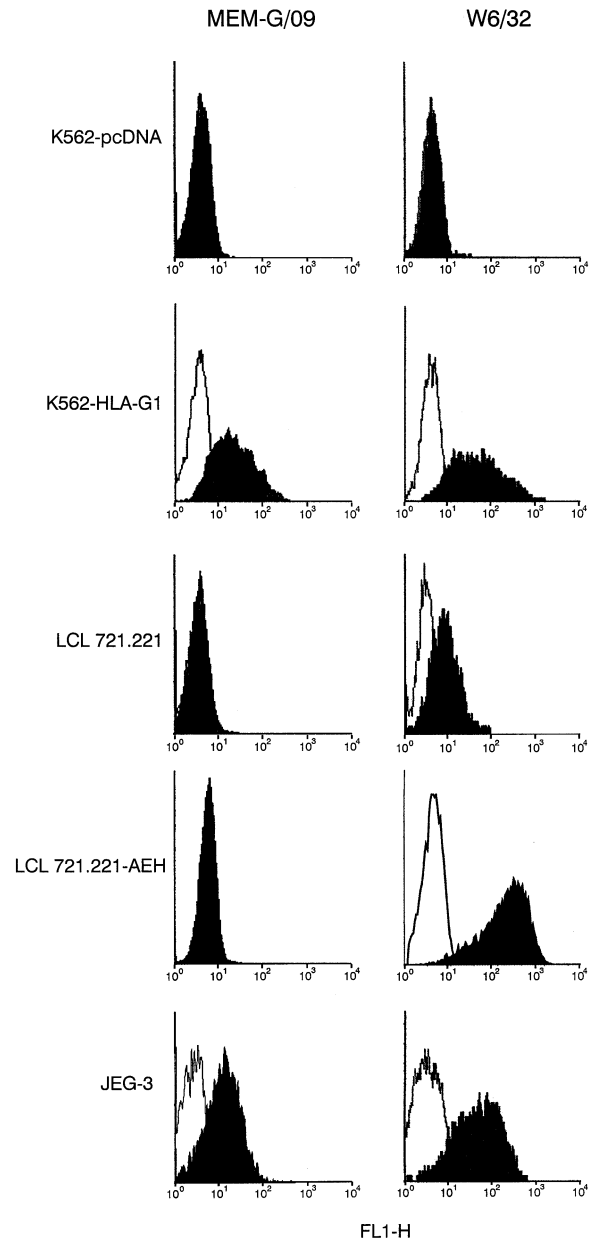
### Immunocytochemistry Analysis

Cryostat tissue sections were treated as previously described [19]. Deparaffinized tissue sections were subjected to epitope retrieval treatment by high temperature in 10-mM sodium citrate buffer (pH 6.0) using a commercial microwave to optimize immunoreactivity. Slides were then rehydrated for 5 minutes in PBS containing 0.05% saponin and 10-mM HEPES buffer. Endogenous peroxidase activity was quenched by treating sections for 5 minutes at room temperature with 3% hydrogen peroxide in water. Nonspecific binding was prevented by applying 30% human serum for 20 minutes before staining with the primary mAb for 30 minutes at room temperature. Each mAb was first tested at several dilutions in order to determine the optimal dilution to be used. An isotype-matched antibody was used under similar conditions to control nonspecific staining. Immunostaining was evaluated on tissues cells using the DAKO EnVision + System, peroxidase (AEC; Dako, Hamburg, Germany) as previously described [19]. Serial sections of trophoblast tissue were positively identified by their staining with an anti-CK7 mAb, and the anti-HLA-G mAbs, namely 87G (for frozen tissue sections) or 4H84 (for both frozen and paraffin-embedded tissue sections).

## RESULTS

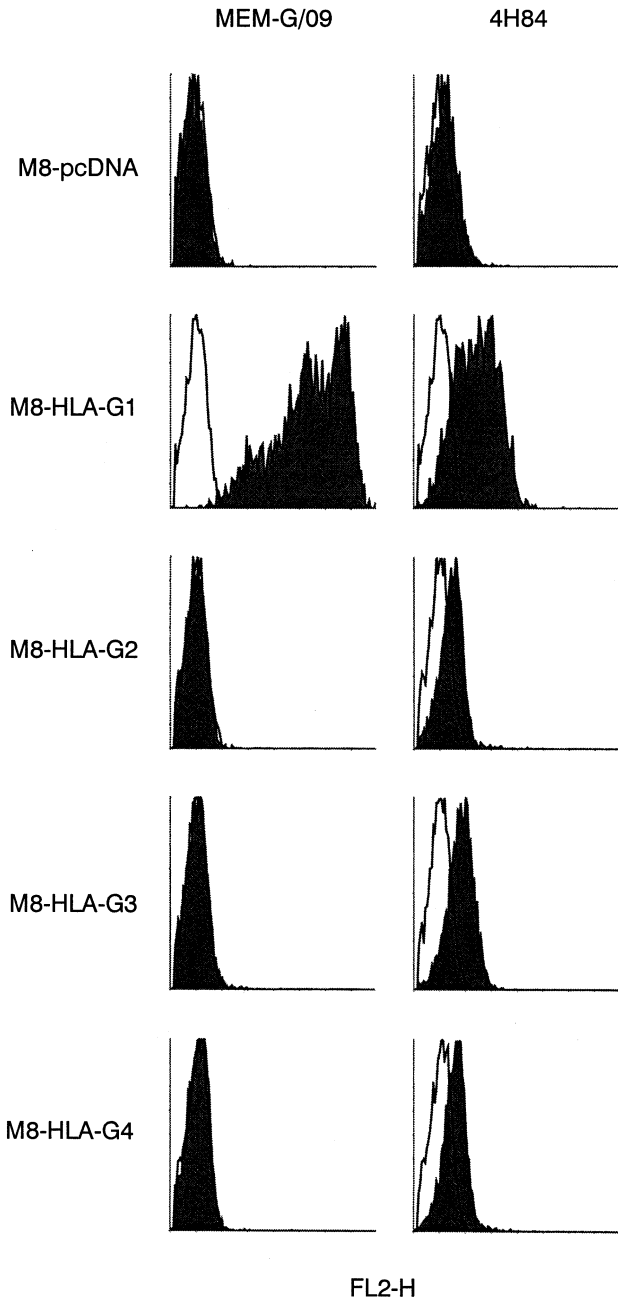
### Characterization of mAbs Specific for $\beta_2$ m-Associated HLA-G1 Cell Surface Molecule

The reaction pattern of the MEM-G/09 mAb, taken as a representative example, to cell-surface HLA-A, -B, -C, -G1, and -E molecules was defined by flow cytometry analysis. For this purpose HLA class I negative or positive cell lines were used in this study that were all previously characterized and validated in the International Preworkshop on HLA-G/-E (Paris, July 2000). As illustrated in Figure 1, the MEM-G/09 positively stained the HLA-G1 positive cell lines M8-HLA-G1 and K562-HLA-G1. At the cell surface of the K562-HLA-G1, HLA-G1 is the only HLA class I molecule expressed, as previously described [28] and here assessed by the absence of staining of K562-pcDNA with the pan HLA class I W6/32 mAb, thus exhibiting the reactivity of the MEM-G/09 mAb with HLA-G1. However, to be sure that no cross-reactivity with the other HLA-A, -B, -C, and -E molecules occurs, MEM-G/09 was tested against the following: the M8-pcDNA cell line that expresses spontaneously high levels of HLA-A, -B, -C, and -E molecules [21]; and the HLA class I negative LCL 721.221 cell line in which the cDNA of HLA-E was transfected leading to the LCL 721.221-AEH transfectant



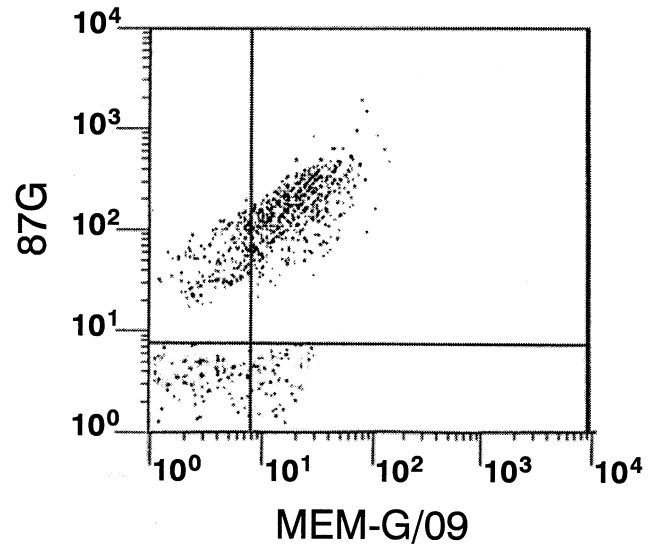
**FIGURE 1** Reaction patterns of the MEM-G/09 monoclonal antibody (mAb) to human leukocyte antigen (HLA) class I cell-surface molecules on different cell lines. K562, LCL 721.221 transfectants, and JEG-3 choriocarcinoma cell line were labeled by indirect immunofluorescence with the MEM-G/09 mAb at the optimal dilution of 1/500 (bold profiles), and with the W6/32 pan HLA class I mAb. Controls were the same cells stained with a mouse IgG1 isotypic control (light profiles). After washing, cells were stained with FITC-conjugated goat anti-mouse IgG.

expressing HLA-E at its cell surface [10, 29]. In both cases, no staining with MEM-G/09 was observed, revealing no cross-reactivity with the HLA class I molecules expressed by these cell lines (Figures 1 and 2). It



**FIGURE 2** Reaction patterns of the MEM-G/09 monoclonal antibody (mAb) to M8 transfectants expressing the different human leukocyte antigen G (HLA-G) isoforms. The presence of HLA-G isoform at the M8 transfectant-cell surface was confirmed by their staining with 4H84 diluted 1/100 on propidium iodide-negative cells in order to exclude intracellular HLA-G staining by 4H84 mAb on dying or dead cells. We thus gated only viable nonpermeabilized cells.

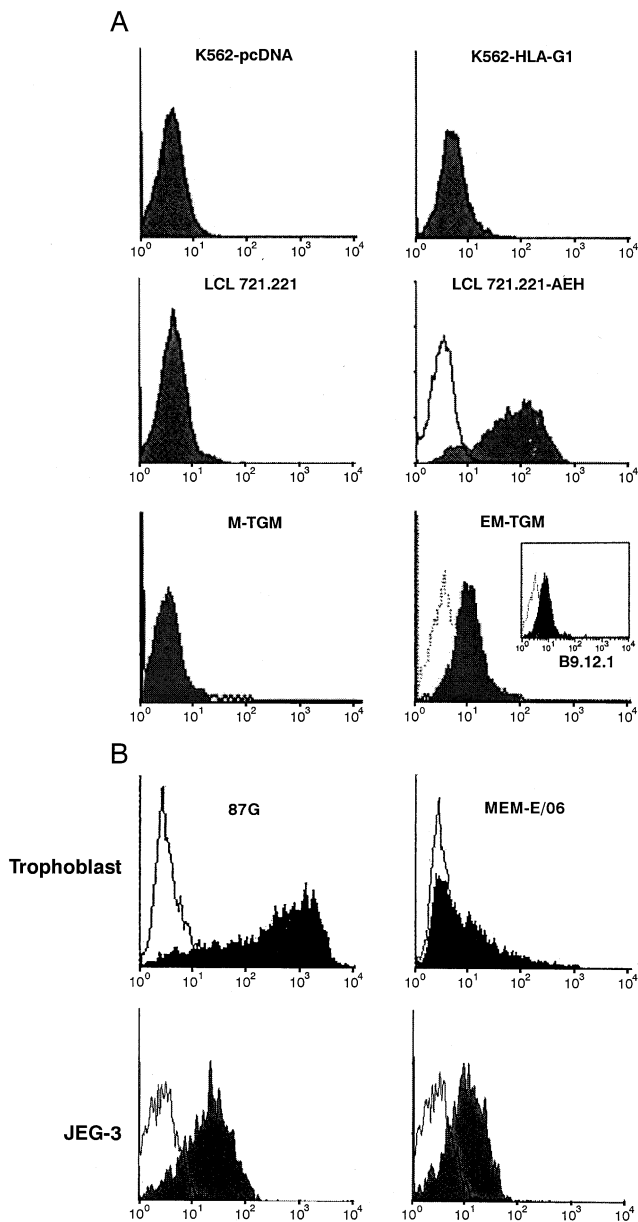
is of note that the LCL 721.221 cell line expresses endogenously HLA-E, which can reach the cell-surface at 26 °C [11] and may explain the W6/32-positive staining observed in Figure 1. MEM-G/09 was also tested on



**FIGURE 3** MEM-G/09 staining of the 87G-positive trophoblast population. Freshly isolated cytotrophoblast cells were labeled with the 87G monoclonal antibody (mAb) then with PE-conjugated goat anti-mouse IgG followed by the MEM-G/09 mAb conjugated to FITC.

peripheral blood lymphocytes (PBL) from 20 healthy adult volunteers donors without any staining (data not shown and [30]). Further, MEM-G/09 did not react with the M8-HLA-G2, -G3, and -G4 transfecting cell lines revealing no recognition of the corresponding HLA-G isoform, although these HLA-G isoforms were cell-surface expressed, as assessed by the 4H84-positive staining gated on nonpermeabilized cells (Figure 2). Among the other mAbs tested, the MEM-G/08, -G10, -G11, -G12, -G13, and -G14 specifically react as MEM-G/09, with  $\beta_2$ -m-associated HLA-G1 form but not with denatured HLA-G molecules under the conditions of Western blotting (data not shown). Optimal working dilution was determined for each mAb (*i.e.*, 1/500 of ascitic fluid).

Then, the most strongly reactive anti-HLA-G mAb, MEM-G/09, was used to analyze HLA-G expression on the JEG-3 choriocarcinoma cell line (Figure 1) and on freshly isolated cytotrophoblast cells (Figure 3) by flow cytometry. Results indicate that MEM-G/09 mAb bind to JEG-3 cells, as well as the pan HLA class I W6/32 mAb (Figure 1) and the 87G mAb considered as the reference mAb for the detection of HLA-G1 cell-surface molecules by flow cytometry (Figure 4B). Purified cytotrophoblast cells were double-stained by the 87G and MEM-G/09 mAbs, demonstrating that both mAbs recognize the same trophoblast cells (Figure 3). The trophoblast origin of these purified cells was confirmed by their positive staining with anti-CK7 mAb and the efficacy of depletion of maternal leukocytes contaminants was as-



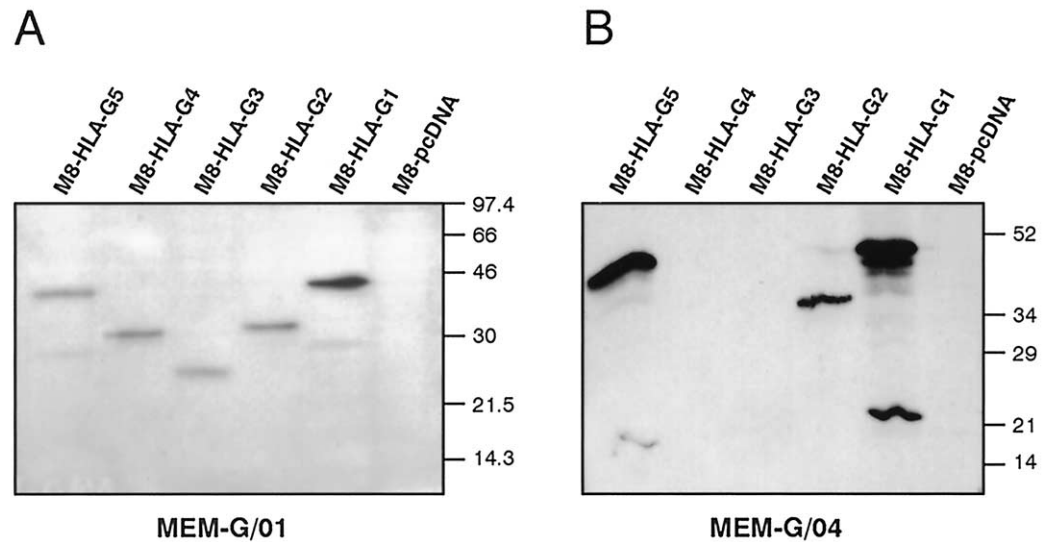
**FIGURE 4** Reaction patterns of the MEM-E/06 monoclonal antibody (mAb) to cell-surface human leukocyte antigen (HLA) class I molecules on transfected cell lines. (A) K562, LCL 721.221 transfectants, and lymph node cells from transgenic mice expressing human  $\beta_2$ -microglobulin (M-TGM) alone or together with the HLA-E<sup>G</sup> heavy chain (EM-TGM) were labeled by indirect immunofluorescence with the MEM-E/06 mAb at the optimal dilution of 1/100 (bold profiles). The EM-TGM cells were also labeled with the B9.12.1 pan HLA class I mAb (insert). Controls were the same cells stained with a mouse IgG1 isotypic control (light profiles). After washing, cells were stained with FITC-conjugated goat anti-mouse IgG. (B) Freshly isolated cytotrophoblast cells and JEG-3 cells were also stained with the MEM-E/06 mAb as described above and in the same experiment, with the 87G mAb. The MEM-E/06 mAb was concomitantly validated on the LCL 721.221-AEH transfectant and the M8-HLA-G1 transfectant.

essed by the absence of staining by anti-CD45 mAb (data not shown).

#### Characterization of mAbs Specific for Denatured HLA-G Heavy Chain

Three of the mAbs tested, namely MEM-G/01, -02, and -04, did not bind HLA-G cell surface molecules when analyzed by flow cytometry but reacted with denatured HLA-G heavy chain by Western blot analysis. Their fine specificity towards the HLA-G isoforms was characterized by SDS-PAGE (in 12% gel, reducing conditions) and Western blotting of detergent lysates from M8-HLA-G1, -G2, -G3, -G4, and -G5 transfectants. Lysate from the M8-pcDNA cell line was used to evaluate the eventual cross-reactivity of these mAbs with the denatured HLA-A, -B, -C, and -E molecules. Results presented in Figure 5 illustrate the pattern of reactivity of the MEM-G/01 and MEM-G/04, respectively. Both mAbs react specifically with the denatured HLA-G heavy chain. However, the MEM-G/01 recognizes an antigenic determinant present on all HLA-G isoforms because bands at 39, 31, 23, 30, and 37 kDa were detected for M8-HLA-G1, -G2, -G3, -G4, and -G5, respectively. The MEM-G/04 reacts with an epitope only present on the HLA-G1, -G2, and -G5 denatured heavy chains because bands were revealed only in the corresponding transfectant lysate. Additional smaller bands were observed for HLA-G1 and HLA-G5 in the lysate of the corresponding M8 transfectant (Figure 5). We have previously described such bands that correspond to glycosylated proteins [22]. Whether these smaller migrating bands are due to *in vivo* protein degradation remains to be determined. Finally, no band was observed for M8-pcDNA revealing that both MEM-G/01 and -G/04 mAbs do not recognize denatured HLA-A, -B, -C, and -E molecules. In a previous study [30] MEM-G/01 was tested by Western blotting on PBMC lysates obtained from ten healthy adult volunteers donors and no band was revealed, confirming the absence of cross-reactivity with classical HLA class I and nonclassical HLA-E molecules. Another study [31] has also tested MEM-G/01 by Western blot analysis on several denatured recombinant proteins including HLA-A2, -B8, -Cw3, -Cw4, -Cw6, Cw7, -E, -G, and on cell lysates of LCL721.221 cells transfected with each of these HLA class I molecules, indicating that HLA-G was the only protein detected by this mAb. MEM-G/02 exhibited similar reactivity pattern as MEM-G/01. The optimal working dilution was of 1/1000, 1/500, and 1/500 of ascitic fluid for MEM-G/01, -G/02, and -G/04, respectively.

**FIGURE 5** Reaction patterns of the MEM-G/01 (A) and MEM-G/04 (B) monoclonal antibodies (mAbs) to denatured human leukocyte antigen G (HLA-G) molecules on transfected cell lines. Aliquots of total proteins from M8 transfectants were separated in 12% SDS-PAGE. The gel was blotted onto nitrocellulose membrane that was then probed with the MEM-G/01 or MEM-G/04 mAb at the optimal dilution of 1/1000 or 1/500, respectively. Numbers at the right of the figure refer to  $M_r$  in kilodaltons.



#### Characterization of mAb Specific for HLA-E Cell Surface Molecule

To determine the specificity of the MEM-E/06 mAb, we have tested different HLA-E positive and negative cell lines by flow cytometry. In this regard, the LCL 721.221, M-TGM (lymph node cells originated from transgenic mice expressing only  $\beta_2$ -microglobulin) K562-pcDNA, and K562-HLA-G1 cells were used as HLA-E negative controls, whereas LCL 721.221-AEH (HLA-E<sup>R</sup> transfectant) and EM-TGM (expressing  $\beta_2$ -microglobulin together with HLA-E<sup>G</sup> molecule) were used as HLA-E positive cell lines, as previously described [10, 23, 24], and here assessed by their staining with the pan class I W6/32 (Figure 1) or B9.12.1 mAb (Figure 4A). No staining was observed on LCL 721.221, M-TGM, K562-pcDNA, and K562-HLA-G1 cells, indicating that the MEM-E/06 mAb does not recognize  $\beta_2$ -microglobulin and HLA-G1 cell-surface molecules. In contrast, this mAb stained positive with the HLA-E transfectant LCL 721.221-AEH and EM-TGM cells, demonstrating that MEM-E/06 mAb reacts with native HLA-E associated with the  $\beta_2$ -microglobulin at the cell surface and recognizes both HLA-E alleles, namely HLA-E<sup>R</sup> (in LCL-721.221-AEH) and -E<sup>G</sup> (in EM-TGM). The MEM-E/06 optimal working dilution to be used in flow cytometry was defined as 1/100 of ascitic fluid. The MEM-E/06 did not work under Western blotting conditions (data not shown).

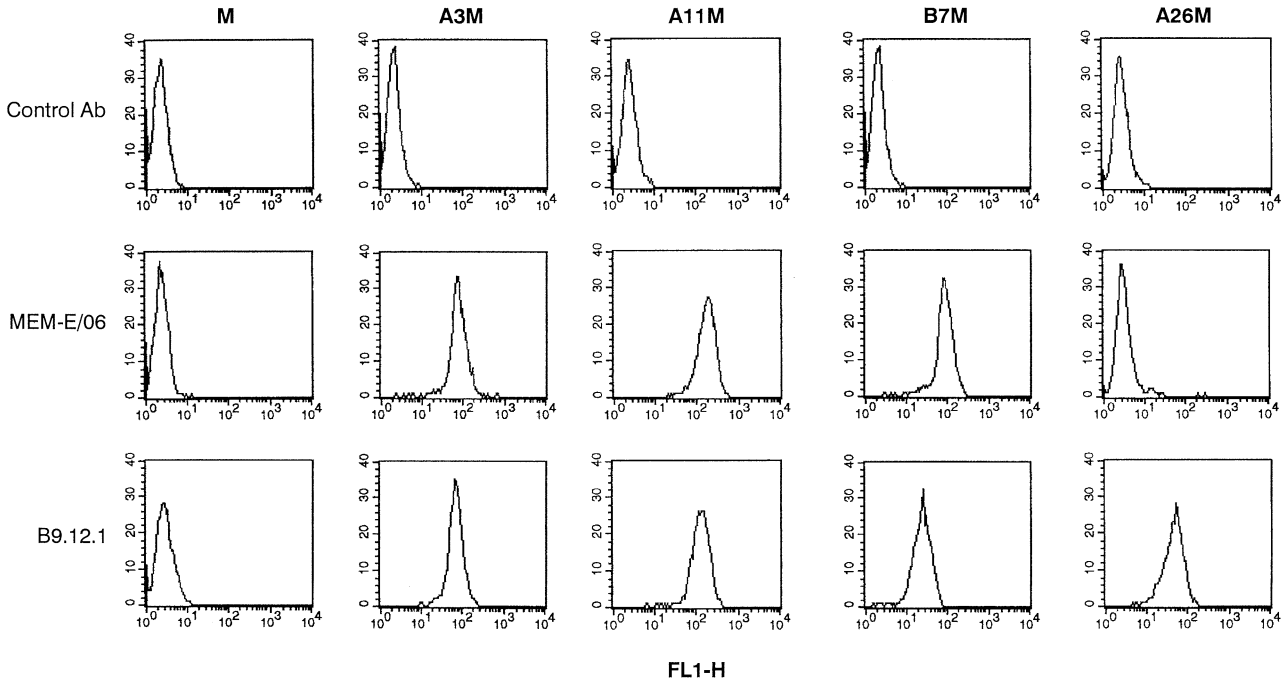
We also analyzed the HLA-E expression on the JEG-3 choriocarcinoma cell line and on freshly isolated cytotrophoblast cells by flow cytometry (Figure 4B). The MEM-E/06 stains positive with the JEG-3 cell

line, confirming the concomitant expression of HLA-E and HLA-G at its cell surface. As expected, the freshly isolated cytotrophoblast cells present a high expression level of cell-surface HLA-G1 molecules, as attested by the 87G staining. Such results contrast with the low level of HLA-E cell-surface expression observed with the MEM-E/06 mAb that otherwise positively stained the LCL 721.221-AEH cell line tested in the same experiment (Figure 4A).

Then, the HLA-E specificity of MEM-E/06 mAb (diluted 1/50) was tested by flow cytometry on mouse L cells expressing various HLA class I molecules used and described as previously [24]. The HLA class I expression was assessed on these L cells by their positive staining with the B9.12.1 mAb (Figure 6). As illustrated in Figure 6 with the A26M cells, taken as a representative example, no reactivity of the MEM-E/06 was observed for the mouse cells transfected with human  $\beta_2$ -microglobulin alone or together with either the HLA-A26, -A29, -B27, -Cw3, or -Cw7 heavy chain. However, MEM-E/06 exhibits a cross-reactivity with HLA-A3, -A11, and -B7 molecules (Figure 6). By comparing the HLA-E and the others HLA class I protein sequences, any differential sequence could be observed that may explain the MEM-E/06 cross-reactivity.

#### Characterization of mAb Specific for Denatured HLA-E Heavy Chain

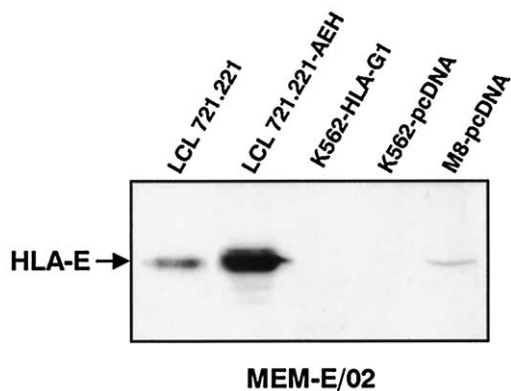
The MEM-E/02 mAb was found to react with the HLA-E denatured heavy chain by Western blot analysis but did not recognize native HLA-E molecule by flow cytometry (Figure 7). A band at 43 kDa corresponding to the molecular weight of the HLA-E heavy chain was



**FIGURE 6** Reactivity of the B9.12.1 (pan HLA class I) and MEM-E/06 monoclonal antibodies (mAbs; diluted 1/50) on mouse L cells transfected with human  $\beta_2$ -microglobulin alone (M) or together with either the HLA-A3 (A3M), -A11 (A11M), -B7 (B7M), or -A26 (A26M) heavy chain. Specific indirect fluorescence profiles with these mAbs were compared with those of background staining fluorescence of cells incubated only with FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse Ig (control Ab).

revealed in the lysate from LCL 721.221-AEH and at a lower level in the lysates from wild-type LCL 721.221 and M8-pcDNA cells. This latter result reveals that MEM-E/02 did not react with the denatured form of the particular HLA-A, -B, -C alleles present on the M8-pcDNA cell lysate. Moreover, no band was observed with

the HLA class I negative K562-pcDNA cells as well as with the K562-HLA-G1 cell lysate, leading us to conclude that the MEM-E/02 did not cross-react with denatured HLA-G1 form. The HLA-E specificity of MEM-E/02 was further investigated on a panel of cell lines expressing distinct sets of HLA-A, -B, and -C alleles and no additional band at 45 kDa could be detected by Western blot analysis (data not shown). The MEM-E/02 optimal working dilution to be used in Western blotting was defined as 1/10,000 of ascitic fluid.

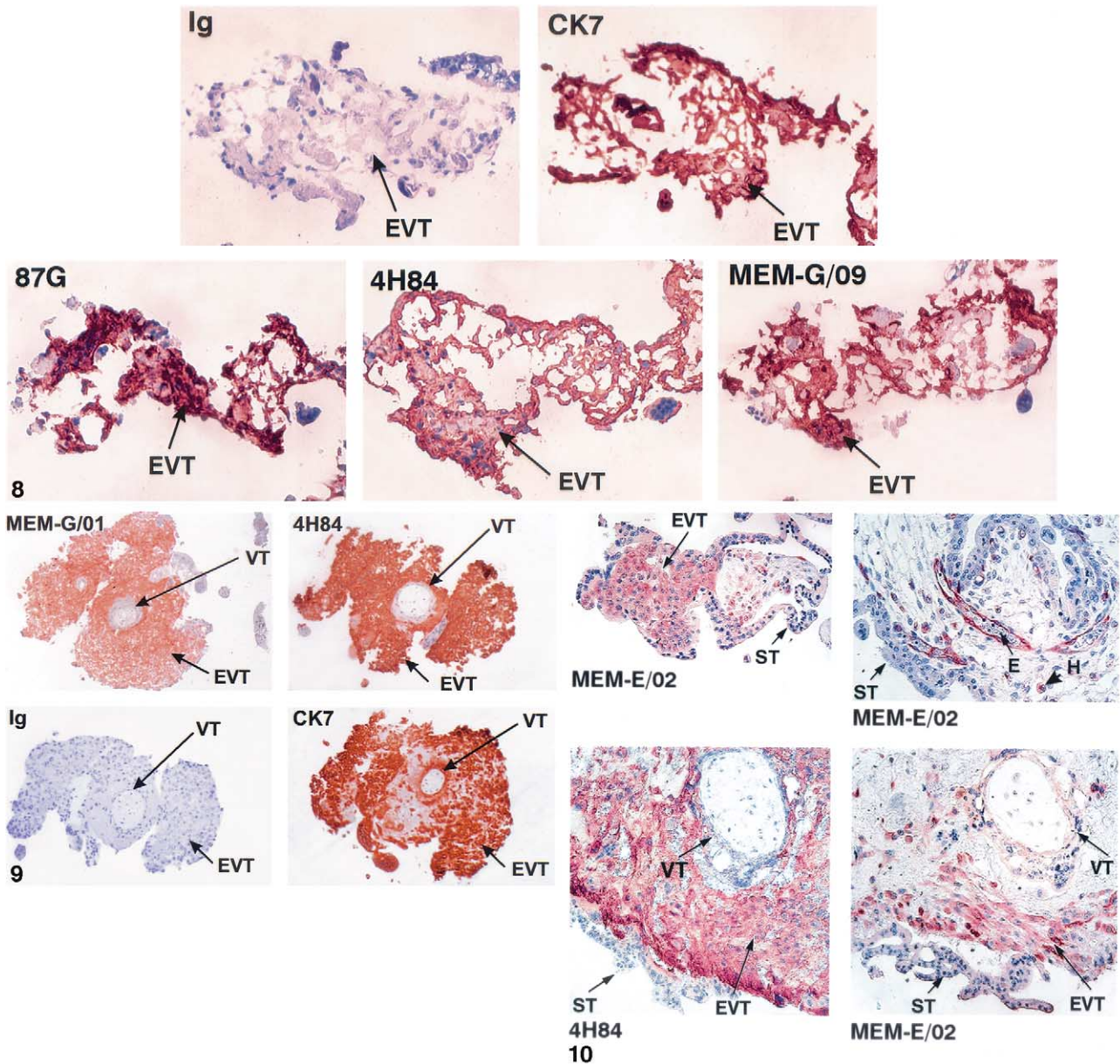


**FIGURE 7** Reaction patterns of the MEM-E/02 monoclonal antibody (mAb) to denatured human leukocyte antigen E (HLA-E) molecules on transfected cell lines. Aliquots of total proteins from the LCL 721.221, LCL 721.221-AEH, K562-pcDNA, K562-HLA-G1, and M8-pcDNA transfectants were separated in 12% SDS-PAGE. The gel was blotted onto nitrocellulose membrane that was then probed with the MEM-E/02 mAb at the optimal dilution of 1/10,000.

#### Analysis of HLA-G and HLA-E Expression in Human First-Trimester Placenta Tissue by Immunocytochemistry

First, the MEM-G mAbs that were defined above as reacting with  $\beta_2$ -m associated HLA-G1 molecules, namely the MEM-G/08 to G/14 antibodies, were able to detect HLA-G1 in M8-HLA-G1 cytopspined cells by immunocytochemistry (data not shown). Then we used the MEM-G/09 mAb at its optimal working dilution, defined as 1/500 of ascitic fluid, on cryopreserved serial sections of trophoblast tissue by immunohistochemistry under conditions defined previously. We positively identified trophoblast cells using anti-CK7 mAb and both 87G and 4H84 were used as HLA-G positive reference mAbs. As depicted in Figure 8, MEM-G/09 was able to detect HLA-G1 expression on extravillous trophoblast





**FIGURE 8** Immunohistochemistry analysis of cryopreserved serial sections of trophoblast tissue using the MEM-G/09 monoclonal antibody (mAb). Trophoblast tissue was stained with anti-cytokeratin 7 mAb (CK7) to assess trophoblast origin of the tissue, with the 87G and 4H84 reference mAbs to detect HLA-G, and with an isotype-matched Ab as negative control. EVT = extravillous trophoblast.

**FIGURE 9** Immunohistochemistry analysis of paraffin-embedded serial sections of trophoblast tissue using the MEM-G/01 monoclonal antibody (mAb). The MEM-G/01 mAb was used at the optimal dilution of 1/200. The 4H84 anti-HLA-G mAb was used as the reference mAb to detect HLA-G on these paraffin-embedded tissues, the anti-cytokeratin 7 mAb (CK7) as positive control, and a mouse IgG1 as negative control. EVT = extravillous trophoblast; VT = perivillous trophoblast.

**FIGURE 10** Immunohistochemistry analysis of paraffin-embedded trophoblast tissues using the MEM-E/02 monoclonal antibody (mAb). The working dilution of MEM-E/02 mAb was 1/200. The 4H84 anti-HLA-G mAb was used on serial sections for comparison to the pattern and the level of expression of HLA-E. EVT = extravillous trophoblast; VT = perivillous trophoblast; ST = syncytiotrophoblast; E = endothelial cells; H = Hofbauer cells.

similar to 87G and 4H84 mAbs, although no staining was observed in cryopreserved sections of normal skin biopsies obtained following plastic surgery used as neg-

ative control (data not shown). It should be noted that MEM-G/09 mAb did not work on paraffin sections.

In contrast, the MEM-G/01 mAb stains positively

paraffin-embedded serial sections of trophoblast tissue, as illustrated in Figure 9. The pattern of reactivity of the MEM-G/01 on this tissue was similar to that of the 4H84 mAb, established as the reference anti-HLA-G mAb upon this method. Indeed, the cells positively stained correspond to extravillous trophoblast (EVT), while the perivillous trophoblast (VT) was not stained. The staining by the anti-CK7 mAb confirms the trophoblast origin of the tissue [26, 27]. No staining was observed in paraffin sections of normal skin used as negative control (data not shown).

The MEM-E/02 mAb was also tested on paraffin-embedded sections of trophoblast (Figure 10). The structures stained by this mAb correspond at a lower level to those revealed by the use of the anti-HLA-G 4H84 and MEM-G/01 mAbs, namely EVT, whereas VT and syncytiotrophoblast (ST) are negatively stained (Figure 10). In addition, MEM-E/02 mAb clearly stained endothelial cells (E) and Hofbauer cells (H).

## DISCUSSION

The purpose of this study was to characterize mAbs capable of recognizing the nonclassical HLA class I molecules HLA-E and HLA-G. Although anti-HLA-G mAbs have been already characterized, production of new antibodies specific for HLA-G, and especially for HLA-E, remains an important challenge that will allow a better analysis of these nonclassical HLA class I molecules. We analyzed a set of new mouse mAbs obtained following immunization with either denatured or renatured (native, properly folded) HLA-G or HLA-E molecules. To evaluate the specificity of these mAbs, we performed three methods: flow cytometry, Western blotting, and immunochemistry. Flow cytometry demonstrated that the new mAbs MEM-G/08 through MEM-G/14 recognize surface-expressed native HLA-G1 molecules, but not HLA-G2, -G3, and -G4 isoforms, similar to the standard anti-HLA-G mAb 87G [32] and another mAb, G233 [33]. Note that 87G and MEM-G/09 have been described [34] as being also able to detect the soluble HLA-G5 form present in cell culture supernatant from transfected cells as well as in amniotic fluid and culture supernatants of first-trimester and term placental explants.

For Western blotting, the 4H84 mAb is used as a reference antibody that allows specific detection of all HLA-G isoforms [35]. Indeed, 4H84 specifically recognizes an epitope present on the  $\alpha 1$  domain of all HLA-G isoforms. Another antibody, namely HCA2, also detects the denatured HLA-G molecule in addition to HLA-A [36]. MEM-G/01 and MEM-G/02 mAbs gave a similar pattern of reactivity as 4H84 by recognizing all HLA-G isoforms. Interestingly, MEM-G/04 mAb recognizes

only HLA-G1, -G2, and -G5 isoforms, and is thus probably specific for an epitope located in the  $\alpha 3$ -domain.

The MEM-E/06 mAb was also selected as a good reagent to detect HLA-E expression at the cell surface. This mAb does not bind HLA-G1 cell-surface molecule. However, it must be considered that MEM-E/06 cross-reacts with some allelic forms of classical HLA class I molecules, namely HLA-A3, -A11, and -B7. Further investigation on the possible cross-reactivity of MEM-E/06 with other HLA class Ia alleles remains to be done. It has to be noted that the mAbs specific for HLA-E described to date are the 3D12 [10] and the V16 [24] mAbs. The DT9 mAb was also described as recognizing both HLA-E and HLA-C molecules [11]. We described here two new reagents recognizing HLA-E, namely the MEM-E/06 mAb, which reacts with HLA-E cell surface molecules, and the MEM-E/02, which appears to specifically recognize the denatured HLA-E heavy chain. Such tools will be useful for the detection of HLA-E in normal and pathologic cells and tissues.

The positive staining of EVT by MEM-E/02 confirms the previous study of King and coworkers [37], who demonstrated that HLA-E is expressed at the trophoblast cell surface as a consequence of binding signal sequence-derived peptides from HLA-G or HLA-C in a minor extent, both molecules expressed in trophoblast cells. Thus, expression of HLA-E on fetal endothelial cells may be due to the concomitant expression of classical HLA class I molecules.

In conclusion, we describe new mAbs representing great tools to study the expression of HLA-E and HLA-G molecules in cells and tissues. In this regard we could detect both HLA-G and HLA-E at the cell-surface of both freshly isolated cytotrophoblast cells and the JEG-3 placental cell line. Interestingly, although a high HLA-G expression level was found at the cell surface of cytotrophoblast cells, the HLA-E was very low in comparison to the levels of expression observed on the JEG-3 cell line that also spontaneously coexpressed HLA-E and HLA-G molecules. The flow cytometry analysis provides further information on the relative level of expression of HLA-E in cytotrophoblast cells, which is extremely heterogeneous and concerns few cells. This may have important implications on the respective role played by HLA-G and HLA-E at the maternal–fetal interface where these nonclassical HLA class I molecules have been proposed to mediate maternal–fetal tolerance. In this regard, Fuzzi and coworkers [14] recently reported the importance of soluble HLA-G antigens produced by early embryo for a successful pregnancy. Indeed, both HLA-E and HLA-G molecules may interact with inhibitory receptors (such as, respectively, CD94/NKG2A [37] and p49/KIR2DL4 [38, 39]) present on decidua NK cells that infiltrate the uterus in large numbers during pregnancy. As a remark,

a recent report demonstrates that KIR2DL4 activation leads to stimulation of IFN- $\gamma$  production in resting NK cells [40]. Finally, these mAbs are also useful tools to analyze HLA-G expression on tissues by immunohistochemistry, as shown here, on both cryopreserved and paraffin-embedded trophoblast tissue sections.

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