Expression and Release of HLA-E by Melanoma Cells and Melanocytes: Potential Impact on the Response of Cytotoxic Effector Cells[1]

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HLA-E are nonclassical MHC molecules with poorly characterized tissue distribution and functions. Because of their capacity to bind the inhibitory receptor, CD94/NKG2A, expressed by NK cells and CTL, HLA-E molecules might play an important role in immunomodulation. In particular, expression of HLA-E might favor tumor cell escape from CTL and NK immunosurveillance. To address the potential role of HLA-E in melanoma immunobiology, we assessed the expression of these molecules ex vivo in human melanoma biopsies and in melanoma and melanocyte cell lines. Melanoma cell lines expressed no or low surface, but significant intracellular levels of HLA-E. We also report for the first time that some of them produced a soluble form of this molecule. IFN-γ significantly increased the surface expression of HLA-E and the shedding of soluble HLA-E by these cells, in a metalloproteinase-dependent fashion. In contrast, melanocyte cell lines constitutively expressed HLA-E molecules that were detectable both at the cell surface and in the soluble form, at levels that were poorly affected by IFN-γ treatment. On tumor sections, a majority of tumor cells of primary, but a low proportion of metastatic melanomas (30–70 and 10–20%, respectively), expressed HLA-E. Finally, HLA-E expression at the cell surface of melanoma cells decreased their susceptibility to CTL lysis. These data demonstrate that HLA-E expression and shedding are normal features of melanocytes, which are conserved in melanoma cells of primary tumors, but become dependent on IFN-γ induction after metastasis. The biological significance of these findings warrants further investigation. *The Journal of Immunology,* **2006, 177: 3100–3107.**

The HLA-E molecules belong, together with HLA-F and HLA-G members, to the family of human MHC class Ib molecules. This family essentially differs from classical MHC class Ia molecules (HLA-A, -B, and -C), by a limited polymorphism, and by a low and restricted cell surface expression. Although it is transcribed in all human tissues, cell surface expression of HLA-E was rarely found, impeding a precise delineation of its function.

The membrane-bound HLA molecules are trimolecular complexes comprised of a 43-kDa H chain, noncovalently associated with a peptide, and a 12-kDa β_2-microglobulin (β_2m)[4] (1). Defective cell surface expression of HLA-E was attributed in part to the limited availability of peptides capable of binding the α-chain peptide groove, preventing the stabilization, intracellular transport, and cell surface expression of the molecule (2, 3). HLA-E binds a restricted set of autologous- or pathogen-derived nonamer peptides. Earlier studies suggested that most of these derive from the leader sequence of classical MHC or of HLA-G molecules (4). However, HLA-E also binds peptides derived from stress-associated autologous (5, 6) and pathogen molecules (7–9). As for other MHC molecules, the nature of the bound peptides restricts the recognition of HLA-E by receptors. When bound to signal peptides from classical MHC class I molecules, HLA-G or CMV, HLA-E efficiently triggers the inhibitory NKR:CD94/NKG2A. Similar but partly different set of peptides permits the triggering of the activatory homologue CD94/NKG2C receptor (10–14). In contrast, HLA-E association with a heat shock protein (hsp) 60-derived signal sequence peptide in stressed cells prevents these interactions. Other HLA-E peptide complexes are ligands of HLA-E-restricted T cells (7, 15).

The biological significance of HLA-E remains poorly defined. Because most NK cells and $\gamma\delta$ T cells, and a significant fraction of $\alpha\beta$ CD8 T cells express the inhibitory NKR CD94/NKG2A, current concepts about the function of HLA-E essentially rely on its capacity, when present on target cells, to inhibit the activities of CD94/NKG2A-expressing effector cells. This was documented in vitro by the demonstration that inducing HLA-E expression by target cells leads to a significant inhibition of both lysis and cytokine secretion by CD94/NKG2A-expressing NK or CTL (16–18). Ag-presenting function of HLA-E has been reported (7, 8, 15, 19). Nonetheless, studies in the mouse suggest that it might have been underlooked, considering the role of Qa-1 (the HLA-E mouse homologue)-restricted T cells in regulating adaptive immunity to both self and foreign Ags (20–24). Data reported by Chess and colleagues (25, 26) support the existence of such regulatory T cells in humans.

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Received for publication January 12, 2006. Accepted for publication June 20, 2006.

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[1] This work was supported by grants awarded by the Ligue Nationale contre le Cancer (labellisation 2005). L.D. and E.G. were recipients of fellowships awarded by the Ligue Départementale de Loire Atlantique and the Association pour la Recherche contre le Cancer.

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[4] Abbreviations used in this paper: β_2m, β_2-microglobulin; hsp, heat shock protein; MMP, matrix metalloproteinase; RFI, ratio fluorescence intensity; sHLA-E, soluble HLA-E.

0022-1767/06/$02.00

HLA-E expression by tumors was indirectly shown by the observation that lysis, by autologous or allogeneic T cells, of IFN-γ-treated carcinoma cells might be increased or restored by blocking the CD94/NKG2A receptor. This leads us to postulate that HLA-E expression might represent a mechanism of tumor escape from immune surveillance (27, 28). Recently, the availability of anti-HLA-E Ab allowed a direct assessment of HLA-E expression by tumor cells. Surprisingly, most carcinoma cell lines tested lacked significant HLA-E expression, contrasting with a frequent expression of this molecule by lymphomas (29, 30).

The aim of the present study was to document HLA-E expression by human melanoma cells in vitro and in vivo, by immunohistochemistry, cytometry, and Western blotting, using recently available HLA-E-reactive mAbs, to ascertain the potential contribution of the CD94/NKG2A receptor in regulating the antitumor immune responses.

Materials and Methods

mAbs

The CD94-specific mAb Y9 used in the cytotoxic assays was a hybridoma supernatant provided by A. Moretta (Genova, Italy). The anti-HLA class I Ab (W6/32) and the irrelevant mouse IgG1 Ab, used as an isotype control, were purchased from Beckman Coulter. The MEM-E/02 mAb (Serotec), which binds the denatured HLA-E protein, was used for Western blotting and immunohistology, and the MEM-E/07 mAb (provided by V. Horejsi, Prague, Czech Republic), which binds native HLA-E proteins, was used for flow cytometry (31). Mouse anti-human Melan-A/MART-1 mAb A103 was purchased from DakoCytomation and used in immunochemistry assay.

Cell lines

Foreskin-derived normal melanocytes 00M10, 00M33, 01M03, 01M10, 01M11, 01M20, and 97M10 were a gift from M. Regnier (L'Oréal Laboratory, Clichy, France) (32). Melanoma cell lines (M28, M74, M83, M102, M117, M119, M132, M140, M200, M204) were established in our laboratory from metastatic tumor fragments, as previously described (33). Some melanoma cell lines were obtained from other laboratories: IPC277/5 (C. Aubert, Marseille, France); DAGI (J. Doré, Lyon, France); FM25 and FM29 (J. Zeuthen, Copenhagen, Denmark); G-mel (A. Houghton, New York, NY), MW75 (D. Schadendorf, Mannheim, Germany); and Mel17 (S. Perez, Athens, Greece). All cell lines were cultured in RPMI 1640 with 10% of FCS.

T cell clones

Melanoma-reactive CD8 αβ T cell clones specific for Melan-A/MART-1_{26-35} (M77.84 and MEL2.46) and NA17-A_{1-9} (M17.221, H2, CDM39.91A, and CDM39.91H) epitopes were derived in our laboratory either from tumor-infiltrating lymphocytes or from peptide-stimulated PBMC. The melanoma-reactive Vγ9Vδ2 T cell clone C4.112 was obtained from colon tumor-infiltrating lymphocytes. T cell clones were obtained by limiting dilution and expanded, as described previously (34–36).

HLA-E allele typing

Melanoma cell lines were analyzed for *HLA-E* alleles in relation to amino acid position 107 (G vs R). An HLA-E cDNA sequence spanning exons 2–4 was amplified by RT-PCR with the following primers: 5′-TGCGCG GCTACTACAATCAG-3′ and 5′-TGTGTCTTTGGGGGCTCCAG-3′. PCR were run at 94°C for 1 min, at 68°C for 1 min, and at 72°C for 45 s for 30 cycles. PCR products were purified and sequenced using Dye Terminators and the ABI-PRISM 377 DNA Sequencer (Applied Biosystems).

Stable transfection of melanoma cell line DAGI by the HLA-E cDNA

Melanoma cell line DAGI was transfected by electroporation using AMAXA system, according to manufacturer's instructions, with a full-length human HLA-E cDNA (a gift from V. Braud, Valdonne, France) inserted in pcDNA-3 eucaryote expression vector. The transfected cell line DAGI was cultured in RPMI 1640 supplemented with 10% FCS and 0.6 μg/ml neomycin (Sigma-Aldrich), and was cloned by limited dilution to establish stable HLA-E-transfected cell lines.

Immunohistochemistry

In vivo expression of HLA-E was studied in human normal skins (5), cutaneous melanomas (4), and metastatic melanomas (5 melanoma-invaded lymph nodes, 1 liver, and 1 stomach metastasis) fixed in 10% formalin and routinely processed for paraffin embedding. Four-micrometers-thick paraffin sections were subjected to epitope retrieval in microwave oven using citrate buffer (pH 6.0), and then stained using a two-step visualization system based on a peroxidase-conjugated dextran backbone, which avoids endogenous biotin detection (Dako EnVision System; DakoCytomation). Tissue sections were rinsed in buffer. Endogenous peroxidase activity was blocked with the peroxidase-blocking solution for 5 min, and sections were incubated at room temperature for 30 min with the anti-human HLA-E mAb MEM-E/02 at a dilution of 1/100 or with the anti-human Melan-A mAb A103 at a dilution of 1/50 to identify intraepidermal melanocytes. This was followed by incubation with secondary Ab coupled to the peroxidase-conjugated polymer for 30 min at room temperature. Immunostaining was visualized using the substrate system provided in the kit (diaminobenzidine/H_2O_2 substrate), and tissues were counterstained with hematoxylin.

Immunofluorescence analysis

For detection of surface HLA-E, 10^5 cells (melanoma cell lines or freshly isolated melanocytes) were stained with the specific or isotype control mAb for 30 min at 4°C. After washing, cells were incubated for 30 min with the secondary PE-labeled mAb and analyzed on a FACScan flow cytometer using CellQuest software (BD Biosciences). Ratio fluorescence intensity (RFI) has been calculated for HLA-E expression, as follows: mean fluorescence intensity with the specific mAb/mean fluorescence intensity with the negative control.

For detection of intracellular HLA-E, cells were fixed 10 min at room temperature in a solution of PBS 4% paraformaldehyde (Sigma-Aldrich) and washed before labeling. Reagent dilutions and washes were made with PBS containing 0.1% BSA and 0.1% saponin (Sigma-Aldrich).

Western blot analysis

Melanoma cell lines or melanocytes were incubated or not with IFN-γ (500 U/ml) for various times. In some experiments, after 24 h of culture with IFN-γ, cells were incubated for an additional 4 h with chloroquin (100 μM), leupeptin (100 μM), PMSF (1 mM), EDTA (0.5 mM), or Galardin (10 μM) (Calbiochem). A total of 3×10^6 cells was then washed and incubated in 300 μl of lysis buffer (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 0.05% Triton X-100, and protease inhibitors (Sigma-Aldrich)) for 15 min on ice. Supernatants and cell lysates were collected after centrifugation, respectively, for 10 min at 1,500 × *g* or 12,000 × *g* at 4°C. Melanoma supernatants or lysates were separated in 12% SDS-PAGE. The gels were blotted onto nitrocellulose membranes (Hybond; Amersham), and the membranes were blocked by incubation with TBS containing 5% nonfat dry milk. The membranes were then probed with the anti-HLA-E mAb MEM-E/02 overnight at 4°C, washed in TBS containing 0.1% Tween 20, and incubated for 2 h at room temperature with peroxidase-conjugated sheep anti-mouse IgG Ab (Ozyme). After washing, membranes were stained with ECL reagent (Roche) and exposed to x-ray film. Blot quantification was performed by densitometry analysis with Kodak Digital Science Image Station 440 CF and Kodak Digital Science Image Analysis 1D software (Eastman Kodak). Results are expressed as arbitrary units.

^{51}Chromium microcytotoxicity assay

Melanoma cell lines, pretreated or not with IFN-γ (500 U/ml) for 48 h, were labeled with 100 mCi $Na_2{}^{51}CrO_4$ (Oris Industrie) for 1 h at 37°C. One thousand labeled target cells were incubated with T cell clones or NK cells at different E:T ratios.

In some experiments, the effector cells were preincubated either with the anti-CD94 Y9 mAb or with an isotype control (1 μg/ml) at 4°C for 20 min.

Results

Immunohistochemistry analysis of HLA-E expression in melanoma cells and melanocytes

HLA-E expression by melanoma cells and melanocytes was investigated by immunohistochemistry on sections of normal skins ($n = 5$) and of melanoma tumor samples ($n = 11$) using the mAb MEM-E/02 that reacts specifically with the denatured H chain of human HLA-E. In normal skin sections, a majority of melanocytes (identified by colabeling with an anti-Melan-A Ab) was strongly labeled (Fig. 1, *a* and *b*). Keratinocytes of all epidermal layers

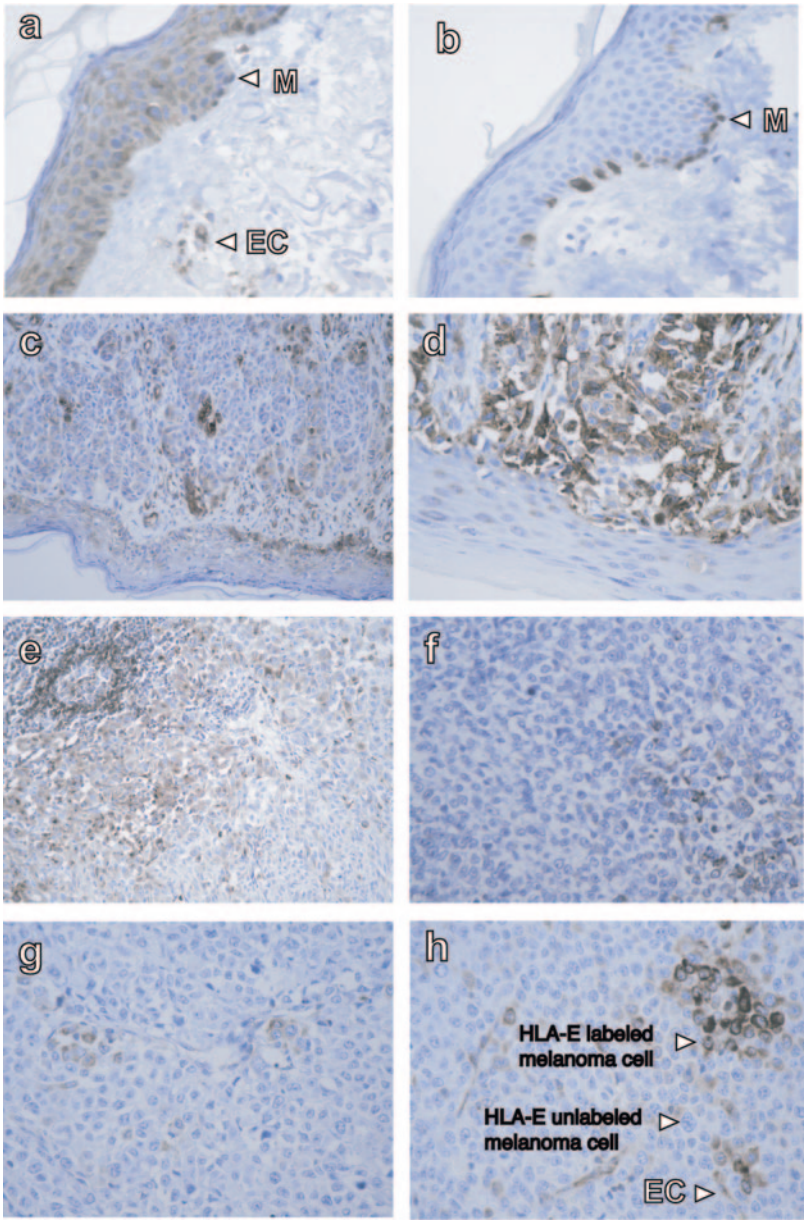

FIGURE 1. In vivo HLA-E expression in melanoma lesions. Immunoperoxidase staining, with the HLA-E-specific mAb (MEM-E/02), of surgically removed tissues was performed: *a*, normal skin; *c* and *d*, primary cutaneous melanomas; *e* and *f*, melanoma-invaded lymph nodes; *g*, gastric; and *h*, hepatic metastatic melanomas. Staining of normal skin with Melan-A-specific mAb (A103) (*b*) was used for melanocyte identification. M, melanocyte; EC, endothelial cell.

were also stained with variable intensity. Furthermore, in agreement with the results from others (S. Coupel, A. Moreau, M. Hamidou, V. Horejsi, J. P. Soulillou, and B. Charreau, unpublished observations), endothelial cells of all types of vessels were also strongly stained (Fig. 1*a*). On sections of melanoma tumor samples, a variable fraction of tumor cells was labeled. This fraction ranged between 30 and 70% in primary tumors ($n = 4$) (Fig. 1, *c* and *d*) and from 10 to 20% in tumor-invaded lymph nodes ($n = 5$) and in visceral metastasis ($n = 2$) (Fig. 1, *e–h*). HLA-E-labeled tumor cells localized more frequently at the periphery of metastatic tumor lobules. These findings indicate that a significant fraction of melanocytes and melanoma cells expresses HLA-E and suggest that expression by tumor cells is down-regulated during melanoma progression.

IFN-γ enhances or induces cell surface expression of HLA-E by melanoma lines

To analyze the impact of HLA-E expression by melanoma cells on the lysis susceptibility of these cells to cytotoxic effectors, we then looked for HLA-E expression by human melanoma cell lines. HLA-E expression was investigated by flow cytometry in 17 metastatic melanoma cell lines, and as controls in 7 short-term cultured melanocytes, using the MEM-E/07 mAb that recognizes native HLA-E and strongly cross-reacts with HLA-B7 (31) (data not shown). Therefore, we used only cell lines from non-HLA-B7 donors, as shown by lymphocyte HLA class I typing (data not shown). All melanoma cell lines were either not labeled or labeled at low levels on their surface (as indicated by the RFI ranging from 1 to 4). In contrast, most of them expressed significant amounts of intracellular HLA-E (RFI ranging from 1.5 to 10 for all but 2 cell lines) (Fig. 2*c*). Cell surface, but not intracellular HLA-E expression was significantly increased for most cell lines upon IFN-γ treatment (Fig. 2). These HLA-E expressions significantly differed from those of classical HLA class I molecules, investigated by W6/32 staining. These molecules, in contrast with HLA-E, were expressed at significantly higher levels on the membrane than inside melanoma cells (Fig. 2, *b* and *c*). Furthermore, their up-regulation by IFN-γ on the cell surface was dissociated from that of HLA-E in some cell lines (FM29, M28) and vice versa on other cell lines (G-mel, M83). Therefore, spontaneous and IFN-γ-induced

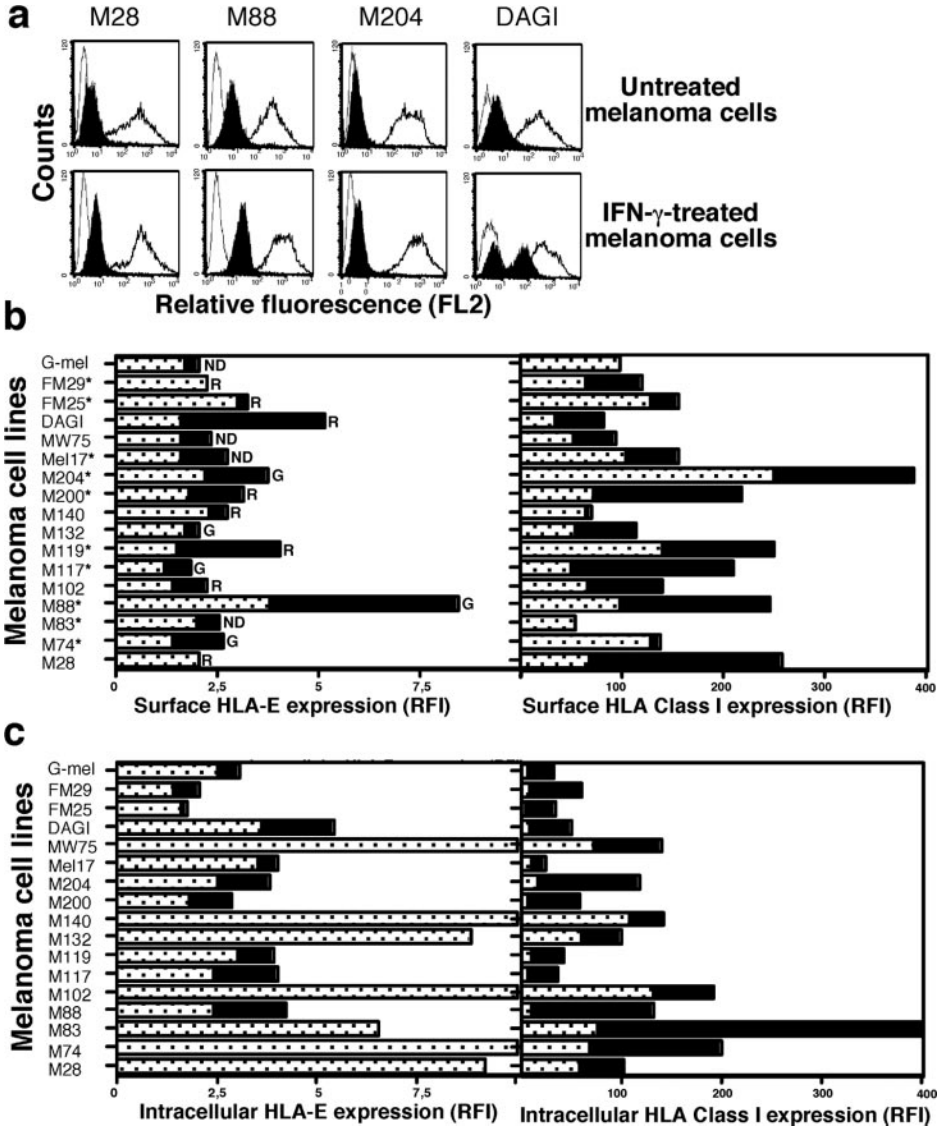

FIGURE 2. In vitro HLA-E cell expression by melanoma cell lines. *a*, Representative flow cytometry profiles of four melanoma cell lines (as indicated on the *top* of the histograms) treated or not with IFN-γ. Cells were stained with isotype control (light profiles), with the anti-HLA-E/HLA-B7 MEM-E/07 mAb (black profiles), or with the anti-HLA class I mAb W6/32 (bold profiles). *b*, Relative HLA-E (*left*) and total HLA class I (*right*) surface expression of 17 melanoma cell lines, untreated (▨) and IFN-γ treated (■). Results are expressed as RFI. Asterisks represent the cell lines that produced sHLA-E upon IFN-γ treatment (see Fig. 4). *HLA-E*^{G} and *HLA-E*^{R} alleles are indicated, respectively, by G and R letters. *c*, Relative HLA-E (*left*) and total HLA class I (*right*) intracellular expression in 17 melanoma cell lines, untreated (▨) and IFN-γ treated (■). Results are expressed as RFI.

expressions of HLA-E and of classical HLA class I molecules by melanoma cell lines do not correlate, as reported before by Palmisano et al. (30), Also in accordance with this study, we failed to observe any correlation between HLA-E expression and β_2m transcription in 10 melanoma cell lines (data not shown). Finally, we asked whether HLA-E expression by melanoma cell lines might be

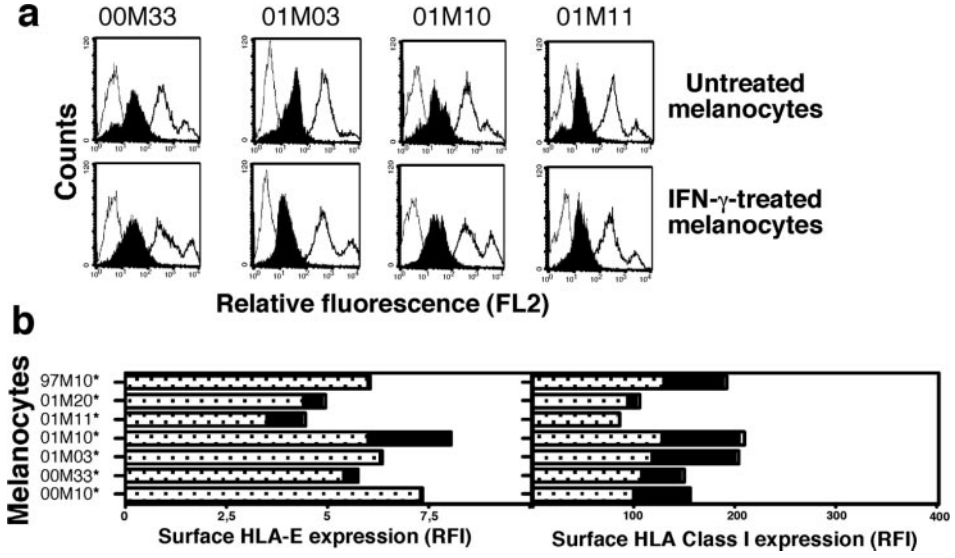

FIGURE 3. In vitro HLA-E cell surface expression by melanocyte cell lines. *a*, Representative flow cytometry profiles of four short cultured melanocytes (01M10, 00M33, and 01M11) labeled by the MEM-E/07 mAb (black profiles), with the W6/32 mAb (bold profiles) or with the isotype control (light profiles). *b*, Relative HLA-E (*left*) and total HLA class I (*right*) expression of seven freshly isolated melanocyte cell lines, untreated (▨) and IFN-γ treated (■). Results are expressed as RFI. Asterisks indicate the cell lines that produced sHLA-E upon IFN-γ treatment (see Fig. 4).

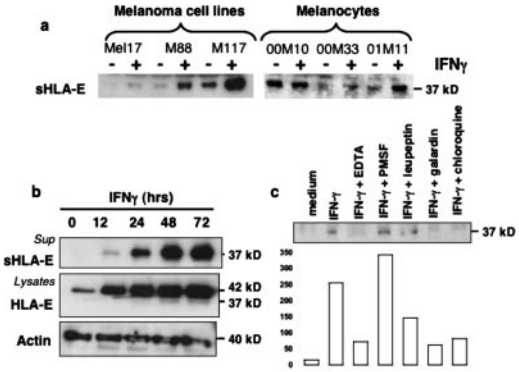

FIGURE 4. Detection of sHLA-E by Western blot analysis in culture supernatants and lysates of melanoma cell lines and melanocytes. *a*, The 48-h culture supernatants of melanoma cell lines and of short-term melanocyte cultured pretreated or not with IFN-γ were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with mAb MEM-E/02. Immunoblots were reprobed with anti-actin mAb to compare protein loading in samples. *b*, Kinetic analysis of the release of sHLA-E from melanoma cells. Culture supernatants (*upper panel*) and lysates (*lower panels*) of the IFN-γ-treated M200 cell line were collected after 12, 24, 48, and 72 h and analyzed, as previously described. *c*, The mechanism for the IFN-γ-induced generation of the sHLA-E was evaluated, as follows: M200 cells were incubated with IFN-γ for 24 h and cultured for an additional 4 h with chloroquine, leupeptin, PMSF, EDTA, or Galardin. Culture supernatants and cells were analyzed, as previously described. Blot quantification was performed by densitometry analysis and expressed as arbitrary units.

correlated with the allele-expressed *HLA-E *0101* (*HLA-E^G*) and **0103* (*HLA-E^R*), as suggested by some groups (2, 37, 38). Determination of the *HLA-E* allele expressed was performed by RT-PCR and sequencing in 13 melanoma cell lines (data not shown). The results did not support such a correlation (Fig. 2*b*).

All melanocyte cell lines were surface labeled by the anti-HLA-E mAb with RFI about twice higher than those of melanoma cell lines (3 to 7). This labeling was not or poorly increased by IFN-γ. When observed, IFN-γ-induced expression of HLA-E appeared limited to the small fraction of melanocytes that lacked HLA-E before treatment (Fig. 3).

Melanocytes and melanoma cell lines produce a soluble form of HLA-E

We then asked whether, as classical and some nonclassical MHC class I molecules (39, 40), HLA-E might be produced in a soluble form. To this end, we performed Western blot analysis using the MEM-E/02 mAb, specific for HLA-E on lysates or culture supernatant of melanoma and melanocyte cell lines, pretreated or not with IFN-γ. In the absence of IFN-γ treatment, a 37-kDa band, of faint to high intensity, was detected in the supernatants of about one-half of the melanoma cell lines (Figs. 4*a* and 2*b*). A same band, but always of high intensity, was systematically present in melanocyte cell line supernatants (Figs. 4*a* and 3*b*). IFN-γ treatment poorly affected the production of soluble HLA (sHLA)-E by melanocytes, but induced, or increased, its production by 14 of 22

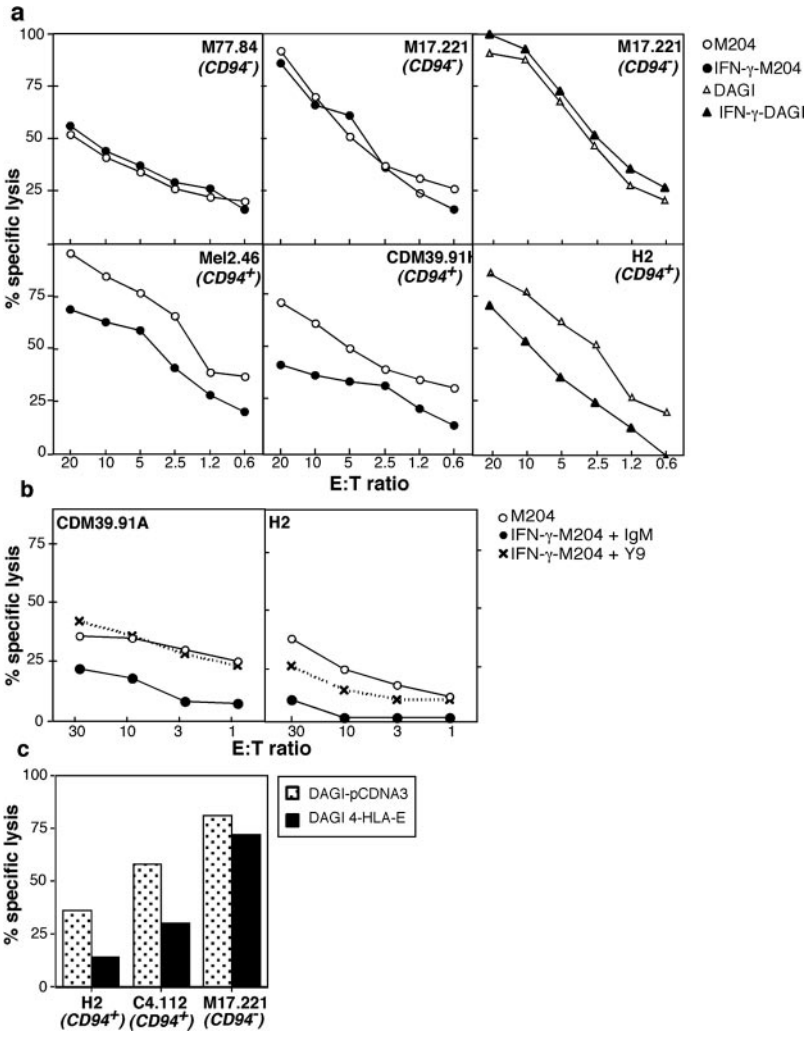

FIGURE 5. Consequence of membrane HLA-E expression by melanoma cells on their susceptibility to Ag-specific αβ T cell clone-mediated lysis. *a*, CTL clones recognizing the Melan-A/MART-1 (Mel2.46 and M77.84) or NA17-A (CDM39.91H and M17.221) Ags were used as effectors. CTL clones represented on the *lower panel* express the CD94/NKG2-A receptor, while those represented on the *upper panel* did not. Melanoma cell lines M204 and DAGI, untreated (open) or treated (filled) with IFN-γ for 48 h, were used as targets. *b*, The same experiment was performed for two CD94/NKG2-A$^+$ NA17-A-specific T cell clones (CDM39.91A and H2) in the presence of the blocking anti-CD94 mAb Y9 (black cross). *c*, Influence of HLA-E transfection of melanoma cell lines on their susceptibility to lysis by Ag-specific T cell clones. Lysis of control (▨) or HLA-E-transfected (■) melanoma cell line DAGI by CD8 αβ T cell clones specific for NA17-A$_{1-9}$ (M17.221 CD94$^-$ and H2 CD94$^+$) and Vγ9Vδ2 T cell clone C4.112 CD94$^+$ was evaluated in a 4-h ^{51}Cr release assay.

melanoma cell lines (Fig. 4*a*). Kinetic analysis showed that IFN-γ-induced production of sHLA-E by melanoma cells was detectable as soon as after 24 h of treatment and increased thereafter until 72 h (Fig. 4*b*). In melanoma cell lysates, a major band of 42 kDa was also present, corresponding to the full-length HLA-E protein (Fig. 4*b*).

We next investigated whether sHLA-E release by melanoma cells involved a proteolytic shedding, as described for classical (41) and for transfected nonclassical HLA class I molecules (42, 43). The M200 cell line pretreated for 20 h with IFN-γ was cultured for 4 h with chloroquine (lysosomal inhibitor), leupeptin (Ser/Thr proteinase inhibitor), PMSF (thiol proteinase inhibitor), EDTA (metalloproteinase inhibitor), or Galardin (broad spectrum matrix metalloproteinase (MMP) inhibitor) before HLA-E quantification in culture supernatant by Western blot. All inhibitors but PMSF markedly reduced the amount of sHLA-E detected (Fig. 4*c*). Therefore, the strong inhibition of IFN-γ-induced sHLA-E production by metalloproteinase inhibitors indicates that this form arises in great part from a proteolytic cleavage of membrane HLA-E by MMP.

IFN-γ-induced expression of HLA-E by melanoma cell lines decreases their susceptibility to CTL lysis

Because IFN-γ-treated melanoma cell lines expressed significant levels of membrane-bound HLA-E, we addressed the consequence of this expression on melanoma cell susceptibility to Ag-specific CTL lysis. HLA-A2-restricted αβ T cell clones, specific for Melan-A/MART-1 or NA17-A Ags, expressing or not CD94/NKG2-A, efficiently killed untreated melanoma cell lines (M204 and DAGI) expressing the relevant Ag. However, upon IFN-γ treatment, lysis of these cell lines by the CD94/NKG2-A-expressing clones was significantly decreased, while lysis by clones lacking this receptor was unaffected (Fig. 5*a*). Showing that lysis inhibition, induced by IFN-γ, was mediated in great part by an increased HLA-E/CD94/NKG2A interaction, it was suppressed in the presence of an anti-CD94 mAb (Y9) (Fig. 5*b*). Also supporting a role of membrane HLA-E on lysis inhibition by CD94/NKG2A-expressing clones, the transfection of melanoma cell lines by HLA-E cDNA significantly decreased the cytotoxic response of CD94/NKG2-A-expressing T cell clones (H2 and C4.112), but not that of CD94/NKG2-A-lacking clone (M17.221) (Fig. 5*c*). These results concur with previous studies (27, 28) to show that IFN-γ induces an inhibition of CTL-mediated antitumor responses through HLA-E up-regulation.

Discussion

In this study, we showed that significant fractions of melanoma tumor cells and melanocytes spontaneously expressed HLA-E in vivo. This expression was confirmed in vitro on metastatic melanoma and melanocyte cell lines. Using these cell lines, we further showed that some of them spontaneously produced sHLA-E and that IFN-γ significantly up-regulated both surface expression and shedding of HLA-E by most melanoma cell lines.

To our knowledge, this is the first report showing HLA-E membrane expression and secretion or shedding by melanocytes. These expressions appeared constitutive both on tissue sections and in culture, suggesting their importance for melanocyte biology. Membrane HLA-E can play two distinct roles. One is the presentation of self or viral peptides leading to HLA-E-restricted T cell responses (7, 8, 15, 19) or, as shown in mouse models, to immune regulation (20, 25, 44). Although studies suggested that melanocytes exhibit Ag-processing and -presenting capacities (45), we have no information concerning the potential impact of HLA-E expression on this poorly documented function. The second is the capacity to modulate cytotoxicity and/or cytokine production by immune effector cells, through the triggering of inhibitory CD94/NKG2A or activating CD94/NKG2C NKR, depending on the nature of the HLA-E-bound peptides (13, 46). Therefore, the impact on immune effector cell responses of membrane HLA-E expression by melanocytes will await identification of the peptides bound to these molecules. Tentatively, HLA-E expression by melanocytes might be important for the homeostasis of these cells regarding their frequent exposition to UV-dependent stress. Stress signals may induce the expression of activating NKR ligands such as MHC class I-related chain A (47). This would sensitize stressed melanocytes to NK cell lysis. A permanent expression of HLA-E might be important to limit this risk. However, nonprotective HLA-E-binding peptides, such as those derived from the hsp60 signal sequence, may also be induced by stress (5). This would uncouple CD94/NKG2A inhibitory recognition and so license NK cells to kill.

We confirmed previous reports showing that metastatic melanoma cell lines spontaneously expressed no or very low levels of surface HLA-E (29, 30) and showed that this expression profile did not seem to be influenced by the allele variants expressed, as suggested in some studies (30). However, other studies reported that the *HLA-E*^{G} allele was expressed on the cell surface at a higher level than the *HLA-E*^{R} allele in transfected cells (2, 37, 38). In contrast, we showed that these cell lines contained significant intracellular levels of this molecule, and that IFN-γ poorly affected this expression, whereas it significantly increased membrane expression. These data suggest that most metastatic melanoma cell lines produce significant amounts of HLA-E molecules, confirming transcription studies (48), but that these molecules are retained inside these cells. This might be due to a limiting availability of binding peptides. IFN-γ might induce HLA-E expression by supplying HLA-E-binding peptides, derived from the signal sequence of newly produced classical MHC class I and HLA-G molecules, allowing the transfer of intracellular HLA-E to the membrane. Such an expression of surface HLA-E from an intracellular store was observed in stressed cells, due to an influx of hsp60-derived peptides into the HLA-E presentation pathway (5). IFN-γ might also induce HLA-E membrane expression by up-regulating HLA-E transcription through STAT1 and GATA-1 response elements (49, 50).

Based on decreased susceptibility of tumor cell lines to CTL lysis, following their IFN-γ treatment and on lysis restoration by CD94/NKG2A-blocking mAb, it was previously suggested that IFN-γ up-regulates HLA-E expression on tumor cells (27, 28). In support of this, we show that HLA-E expression in melanoma cell lines induced by both IFN-γ treatment and stable HLA-E transfection significantly decreased the tumor cell recognition by Ag-specific clones expressing the CD94/NKG2A receptor. Nonetheless, this decrease was moderate. This might be due to the high avidity of the CTL clones used in our study and/or to the relatively low level of membrane HLA-E induced on metastatic melanoma cell lines by IFN-γ treatment. Therefore, such an inducible HLA-E expression might probably interfere essentially with the response of low avidity T cells. Furthermore, the extremely low level of spontaneous HLA-E expression by most of these cell lines precludes that it might affect significantly their susceptibility to immune effector cells.

Confirming the in vivo relevance of the poor spontaneous expression of HLA-E by metastatic melanoma cell lines, small fractions of tumor cells were labeled on metastatic tumor sections. In contrast, in primary melanoma tumors a high fraction of tumor cells was highly labeled. This shows that the constitutive expression of HLA-E by melanocytes is retained in early tumor cells, but

disappears later, becoming dependent on IFN-γ stimulation, at the metastatic stage. The potential consequence of spontaneous HLA-E expression by primary melanomas is unknown. Surface expression might provide a significant immune escape mechanism to primary melanoma tumors. This potential role should be addressed by determining the prognostic value of HLA-E expression in primary tumor tissue. The mechanism of the down-regulation of HLA-E expression in metastatic tumors is unclear. Classical HLA class I molecule down-regulation is known to occur in many cancers (51, 52). The loss of expression of specific alleles, signal peptides of which are critical for HLA-E stabilization, might be involved in HLA-E down-regulation during melanoma progression. In addition, MMP involved in cutaneous melanoma progression by degradation of basement membranes and extracellular matrix might also contribute to HLA-E down-regulation in metastatic tumors by inducing the shedding of cell surface HLA-E.

Using a mAb that reacts with the denatured form of HLA-E, we showed for the first time that melanocyte and melanoma cell lines could produce a sHLA-E chain of 37 kDa. This size could correspond to that of the extracellular portion of the membrane-bound HLA-E chain, suggesting that it is produced by the cleavage of membrane HLA-E by a membrane-bound protease. Small MHC molecules (35–37 kDa) are usually viewed as breakdown products of larger membrane-anchored molecules produced by proteolysis, and MMPs have been shown to be proteases involved in such shedding for classical MHC class I and MHC class I-related chain proteins (39, 41, 53, 54). MMP contribution in the production of sHLA-E molecules was also demonstrated, in HLA-E-transfected cells (42), and was established in this study, for the IFN-γ-dependent production of sHLA-E by metastatic melanoma lines. Although we did not study the mechanism of sHLA-E production by melanocytes, the secretion by these cells of several MMP has been reported (55), suggesting that these proteases might also be responsible for the HLA-E shedding by these cells.

Our data suggest that sHLA-E may be produced spontaneously by primary melanoma tumors, and also by some metastatic ones, especially in the context of an immune response involving IFN-γ production. The potential impact of this is unknown and should be addressed in cancer patients. For MHC class I-related chain A, another nonclassical MHC class I molecule, tumor shedding led to detectable amounts of this molecule in the serum of cancer patients, which impaired the expression of the receptor NKG2D by CTL and NK cells of these patients (39), leading to general immunosuppression. It will be important to look for the presence of sHLA-E in the serum of cancer patients, compared with healthy donors. As stated above, it will be necessary also to analyze the structure of sHLA-E and bound peptides and to look for the potential interference of this molecule with the function of membrane-bound HLA-E. Our data also showed that melanocytes permanently produce sHLA-E, at least in culture, and that this production was not or poorly affected by IFN-γ. As for tumor-derived sHLA-E, the biological significance of this production remains to be addressed and will rely on the existence of correctly folded HLA-E chains appropriately bound to β_2m and on the nature of the bound peptides, which may differ from those present on melanoma-derived sHLA-E molecules.

In conclusion, we have shown in this study that melanocytes and primary melanoma tumors spontaneously express significant levels of HLA-E molecules, intracellular, membrane bound, or secreted. The cell surface expression by melanocytes might play a role in the homeostasis of these cells and/or in some contribution of these cells to tolerance mechanisms to self Ags. Membrane HLA-E expression by primary melanoma might contribute to the escape of these tumors from immune surveillance, through inhibiting the lysis by NK cells and by tumor Ag-specific CTL, as suggested before (18, 56), and shown in this study. Furthermore, we showed the existence of a soluble 37-kDa form of HLA-E permanently produced by melanocytes and, under the effect of IFN-γ, by metastatic melanoma cells. More studies are warranted to establish the role of these sHLA-E. If appropriately conformed, and according to the peptides bound, these molecules might interfere with the biological functions of membrane HLA-E, such as modulation of the CD94/NKG2 receptor functions and/or with the activity of HLA-E-restricted T cells. In this respect, if HLA-E-restricted regulatory T cells, evidenced in the mouse (20–24, 57), also exist in humans, it will be important to address the impact of sHLA-E molecules on the function of these cells.

Disclosures

The authors have no financial conflict of interest.

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