Cutting Edge: Requirement of Class I Signal Sequence-Derived Peptides for HLA-E Recognition by a Mouse Cytotoxic T Cell Clone

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The human nonclassical MHC class I molecule HLA-E has recently been shown to act as a major ligand for NK cell inhibitory receptors. Using HLA-E-expressing transgenic mice, we produced a cytotoxic T cell clone that specifically recognizes the HLA-E molecule. We report here that this T cell clone lyses HLA-E-transfected RMA-S target cells sensitized with synthetic class I signal sequence nonamers. Moreover, this T cell clone lyses human EBV-infected B lymphocytes, PHA blasts, and PBL, formally demonstrating the surface expression of HLA-E/class I signal-derived peptide complex on human cells. Furthermore, these data show that HLA-E complexed with class I signal sequence-derived peptides is not only a ligand for NK cell inhibitory receptors, but can also trigger cytotoxic T cells (CTL). The Journal of Immunology, 1999, 162: S662–S665.

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Materials and Methods

**Mice**

All mouse strains used in this study were bred and maintained in our own colony. HLA-E heavy chain TGM (E-TGM) were obtained by microinjection of genomic DNA fragments containing the HLA-E (E*01033) gene (12) into the pronucleus of fertilized eggs [(C57BL/6 × SJL) × BALB/c]. Founder mice were mated to mice of an already established C57BL/10 transgenic line expressing human β₂-microglobulin (M-TGM), thereby yielding double TGM carrying both human genes (EM-TGM). To obtain HLA-E transgenic lines expressing H-2<sup>b</sup> and H-2<sup>d</sup> haplotypes, the mice were typed for H-2, and EM-TGM carrying H-2<sup>b</sup> or H-2<sup>d</sup> were repeatedly backcrossed to C57BL/10 background mice with H-2<sup>b</sup> (B10) or H-2<sup>d</sup> (B10.D2) haplotypes. The mice used in the present study originated from line 81. Cell surface expression of HLA-E transgenic molecules and their alloantigenic function have been described elsewhere (14).

**In vivo generation of CTL**

Recipient mice were injected with 10<sup>7</sup> irradiated (25 Gy) spleen cells in the hind footpads. After 3 days, cell suspensions were prepared from draining lymph nodes and cells were cultured in vitro for four more days in the absence of any stimulating cells in culture medium containing Con A-stimulated rat spleen cell supernatant as a lymphokine source (50 U IL-2/ml). The culture medium was MEM-a-medium (Life Technologies, Gaithersburg, MD) supplemented with 100 U/ml penicillin (Life Technologies), 2 mM glutamine (Life Technologies), 5 × 10<sup>−3</sup> M 2-ME (Sigma, St. Louis, MO) and 10% heat-inactivated FCS (Life Technologies).

**CTL clones**

A CTL clone (TER-1) was isolated by limiting dilution from in vivo-induced CTL and maintained in culture by periodic (every 11 days) stimulation. For this, 0.5 × 10<sup>6</sup> CTL were mixed with 10 × 10<sup>6</sup> irradiated spleen cells originating from TGM expressing HLA-E molecules in a final volume of 10 ml IL-2-containing culture medium (described above). Five days after stimulation the surviving cells were washed and cultured (10<sup>6</sup> spleen cells originating from TGM expressing HLA-E molecules in a final volume of 10 ml IL-2-containing culture medium). Cells were used for functional assays 5 days after their last stimulation.

**Cell-mediated lympholysis assay**

Five thousand ⁵¹Cr-labeled target cells were incubated with effector cells at various E:T ratios in round-bottom wells for 4 h. The percentage of specific ⁵¹Cr release was calculated as: (experimental − spontaneous release)/(maximum − spontaneous release) × 100.

**Peptide-induced stabilization of HLA-E molecules**

RMA-S cells transfected with human β₂-microglobulin and HLA-E heavy chain genes were cultured at 26°C for 18 h. Saturating amounts of peptides (Europgentec, Brussels, Belgium) were added to the culture to a final concentration of 25 μM (class I leader) or 100 μM (BZLF-1) peptides and incubated at 37°C for 1 h.

**Flow cytometry**

Cells were used as positive controls for target cell lysis.

Results and Discussion

To characterize primary in vivo-induced anti-HLA-E CTL, and especially those involved in graft rejection, we have used an in vivo approach based on our previous observations (15) that injection of allogeneic cells into the hind footpads leads to the development of CTL within the draining lymph nodes. Spleen cells from double EM-TGM carrying H-2<sup>b</sup> haplotypes were injected into the hind footpads of nontransgenic H-2-compatible mice. After 3 days, cell suspensions were prepared from draining lymph nodes and cells were cultured for four more days in the absence of any stimulating cells. After this period, required to allow full differentiation of sensitized CTL precursors (16), lymph node cells were tested for the presence of CTL in a ⁵¹Cr-release assay, using Con A blasts as target cells (Fig. 1A). CTL generated in this way lysed Con A blasts from both H-2<sup>b</sup> and H-2<sup>d</sup> EM-TGM, indicating that the elicited CTL recognized HLA-E as an intact molecule and not as an HLA-E-derived peptide presented by a mouse MHC molecule. No lysis of target cells that originated from M-TGM was observed. A CTL clone (TER-1) was derived by limiting dilution and maintained by periodic stimulation with irradiated H-2<sup>b</sup> EM-TGM spleen cells. The TER-1 clone was found to be cytotoxic (Fig. 1B) for target cells from both H-2-matched (H-2<sup>b</sup>) and H-2-mismatched (H-2<sup>d</sup>) EM-TGM, but not from M-TGM. The results (Fig. 1B) revealed very efficient killing (65% at an E:T ratio of 0.6:1) of HLA-E-positive targets. To further characterize its specificity, the TER-1 clone was tested on mouse fibroblasts (H-2<sup>b</sup>) expressing various HLA class I molecules (-A3, -A11, -A26, -A29, -B7, -B27, -Cw3, and -Cw7) or on P815 cells (H-2<sup>d</sup>) expressing HLA-A2 molecules. A FACS analysis was performed using the HLA class I-reactive mAb (B9.12.1), which confirmed the cell-surface expression of HLA class I molecules on these mouse transfecants (data not shown). K<sup>+</sup> and L<sup>d</sup>-reactive CTL (Fig. 2) were used as positive controls for target cell lysis.

![FIGURE 1. Anti-HLA-E cytotoxic T cell response elicited in mice. Anti-HLA-E cytotoxic activity was evaluated by a 4-h ⁵¹Cr release assay using as targets Con A blasts from TGM expressing HLA-E molecules (EM; full symbols) or only human β₂-microglobulin (M; empty symbols) carrying H-2<sup>b</sup> (bb), H-2<sup>d</sup> (dd), or H-2<sup>b</sup>/H-2<sup>d</sup> (bd) haplotypes. A Primary in vivo induction of HLA-E-reactive cytotoxic T cells in mice of the H-2<sup>b</sup> haplotype immunized with H-2-matched spleen cells expressing HLA-E molecules (see Materials and Methods). B Lysis of HLA-E-expressing mouse target cells by the TER-1 CTL clone.](http://www.jimmunol.org/)

![FIGURE 2. Classical HLA class I molecules are not recognized by the TER-1 clone. Cytotoxic reactivity of the TER-1 clone (solid lines) was tested in a 4-h ⁵¹Cr-release assay using as targets either mouse fibroblasts (H-2<sup>b</sup>) expressing various HLA class I molecules (-A3, -A11, -A26, -A29, -B7, -B27, -Cw3, and -Cw7) or P815 cells (H-2<sup>d</sup>) expressing HLA-A2 molecules. HLA-E-expressing target cells (x) were used as a positive control for TER-1 cytotoxic activity. K<sup>+</sup> and L<sup>d</sup>-reactive CTL (dotted lines) were used as positive controls for target cell lysis.](http://www.jimmunol.org/)
Synthetic peptides were then used to sensitize Tap-defective RMA-S target cells transfected with human β2-microglobulin and HLA-E heavy chain genes, thereby defining the epitope recognized by the TCR of the TER-1 clone. As expected, synthetic nonamers derived from residues 3–11 of the signal sequences of HLA-A2 (VMAPRTLVL), -A3 (VMAPRTLLL), -B7 (VMAPRTVLL), and H-2Db (AMAPRTLLL) bound to HLA-E molecules, resulting in increased expression of HLA-E on the surface of the processing-defective RMA-S mutant (Fig. 3A), and sensitized RMA-S cells to lysis by the TER-1 clone (Fig. 3B). HLA-E cell-surface expression on RMA-S transfectants could also be enhanced by adding the BZLF-19–47 nonamer derived from the EBV protein BZLF-1 (Fig. 3A). However, HLA-E complexed with the BZLF-19–47 peptide was not recognized by the TER-1 clone. These results indicate that TER-1 cells bearing an alpha receptor (data not shown) specifically recognize HLA-E gene products complexed with MHC class I signal sequence-derived peptides.

We used the TER-1 clone as a very potent and highly specific cell probe to check for HLA-E cell-surface expression on human cells. We tested the TER-1 clone on a panel of EBV-transformed human B lymphocytes, all of which express the HLA-A3, -B7, and -Cw7 class I molecules that we have shown not to be recognized by this clone (Fig. 2). The HLA-E-reactive TER-1 clone efficiently lysed all human EBV line cells (Fig. 4A). No significant lysis was observed with HLA class I-negative cells (K562). The TER-1 clone was further tested on human PBL and on PHA blasts originating from two healthy donors, #298 (HLA-A2, 3; B7; Cw7) and #573 (HLA-A2; B7; Cw7). We show (Fig. 2) that classical class I molecules expressed on these targets were not recognized by the TER-1 clone. Efficient lysis of human cells was observed, with the exception of HLA class I-negative ones (Daudi; Fig. 4B). The addition of W6/32 mAbs, which recognize both classical and non-classical class I heavy chains associated with β2-microglobulin, including the HLA-E product (3), or the addition of mAbs, which recognize the αβ TCR, efficiently inhibited lysis of human and mouse targets (data not shown).

The results of HLA-E peptide-binding and cytotoxic assays conducted in mice, combined with the results of cytotoxic assays of human cells (Fig. 4), using the class I signal sequence-derived peptide-specific HLA-E-restricted T cell clone, provide proof of in vivo HLA-E cell-surface expression by lymphoid cells in man. Moreover, these results show that at least a significant portion of HLA-E molecules bear peptides derived from appropriate class I leader sequences. The allelic variation of the HLA-E polypeptide that has been described among caucasians (17) affects only amino acid position 107, which is occupied either by G (HLA-EG) or R.
with the receptor (4). In mice, the HLA-E homologue Qa-1 can interact conserved, implying that HLA-E may be capable of binding this natural features required for interaction with the CD8 receptor are all determined in complex with a prototypic ligand derived from highly available regarding identification of the HLA-E tetramer-binding action between this receptor and HLA-E. However no data are recognized CD94 (6), which is an NK cell receptor, indicating inter-

Our studies using CTL generated in mice show that HLA-E refolded around peptides derived from residues 3–11 of MHC class I molecule signal sequence mediates cognate recognition events by specific interactions not only with NK cell receptors, but also with the TCR of CTL. In the human, a soluble tetrameric form of HLA-E was shown (6) to bind to NK cells as well as to a small subset (mean 1.7%) of peripheral blood CD3+ T cells. Binding of the HLA-E tetramer to NK cells is abolished by mAbs that recognize CD94 (6), which is an NK cell receptor, indicating interaction between this receptor and HLA-E. However no data are available regarding identification of the HLA-E tetramer-binding receptor on T cells. The crystal structure of HLA-E was determined in complex with a prototypic ligand derived from highly conserved residues of the human MHC class I leader sequence (4), indicating that the basic architectural structure of HLA-E is similar to that of classical MHC class I molecules. In particular, the structural features required for interaction with the CD8 receptor are all conserved, implying that HLA-E may be capable of binding this receptor (4). In mice, the HLA-E homologue Qa-1 can interact with the αβ TCR (18–20). Our results (Fig. 3A), as well as those of Ullbrecht et al. (21), demonstrate that peptides derived from viral proteins bind to HLA-E in vitro. Using HLA-E-expressing TGM, we are currently investigating whether HLA-E might play a role as a restriction element for viral proteins in vivo. The exact role of HLA-E in immunological recognition and regulation is still pending. EM-TGM are not only a reliable source of cells expressing HLA-E molecules, but also provide a means to investigate the biological function of HLA-E molecules in vivo.

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