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Soluble HLA-I/Peptide Monomers Mediate Antigen-Specific CD8 T Cell Activation through Passive Peptide Exchange with Cell-Bound HLA-I Molecules

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Accumulating evidence that serum levels of soluble class I HLA molecules (sHLA-I) can, under various pathological conditions, correlate with disease stage and/or patient survival, has stimulated interest in defining whether sHLA-I can exert immunological functions. However, despite a mounting number of publications suggesting the ability of sHLA-I to affect immune effectors in vitro, the precise underlying mechanism still remains controversial. In this article, we address potential functions of both classical and nonclassical sHLA-I, using soluble recombinant HLA-I/peptide monomers, and clearly demonstrate their ability to trigger Ag-specific activation of CD8 T cells in vitro. Furthermore, we provide strong evidence that this behavior results from the passive transfer of peptides from monomers to T cell-bound HLA-I molecules, allowing for fratricide representation and activation. Hence, we proposed a unifying model of T cell activation by HLA-I/peptide monomers, reappraising the potential involvement of sHLA-I molecules in the immune response. *The Journal of Immunology, 2014, 192: 000–000.

The human MHC encodes two sets of class I molecules: class Ia or classical molecules, (HLA-A, -Bm and -C) characterized by a high degree of polymorphism and a broad tissue expression (1), and class Ib or nonclassical molecules (HLA-E, -F, and -G) presenting a more restricted polymorphism and expression profile (2). Both classical and nonclassical HLA-I molecules need to accommodate endogenously processed peptides and to associate with the invariant β2-microglobulin (β2m) chain to be stably expressed at the cytoplasmic membrane (3). Cell-surface HLA-I/peptide/β2m complexes can then be scanned by CD8 T lymphocytes and NK cell receptors, allowing immune effectors to distinguish healthy cells from altered ones (i.e., stressed, infected, or transformed ones) (4, 5).

Besides being expressed at the membrane of most nucleated cells, HLA-I molecules are also present in body fluids in soluble forms [soluble class I HLA molecules (sHLA-I)], mostly generated by proteolytic cleavage of membrane-bound molecules, that can be either free or associated with peptide and/or β2m (6, 7). Of interest, although serum sHLA-I levels are rather stable in physiological conditions, they are modified in various pathological conditions, including inflammation, autoimmunity, infections, transplant rejection, and cancer, and can even correlate with disease stage, patient survival, and/or prognosis (7–9). Moreover, several studies have reported, in vitro, the ability of sHLA-I molecules to modulate the activity of NK cells and T lymphocytes expressing cognate NK or TCRs, suggesting that sHLA-I molecules may play significant immunoregulatory roles in vivo (10–16).

Although accumulated evidence points out the immunological properties of sHLA-I molecules, the precise underlying mechanisms remain poorly understood and debated. Most studies argue that sHLA-I effects result from the triggering of their cognate NK or TCRs (or even with the CD8 coreceptors) (10–16). However, most sHLA-I molecules are found in monomeric forms, and should therefore not be able to mediate multivalent (i.e., productive) engagement of their receptors. An alternative mechanism relying on the transfer of class I MHC–derived peptides toward cell-bound MHC molecules has been suggested and could reconcile these observations with the well-accepted model of multivalent engagement (17, 18).

We previously reported the ability of both endothelial (19) and tumor cells (20, 21) to produce sHLA-E molecules, especially in proinflammatory conditions, and documented a significant increase of serum sHLA-E levels in melanoma patients, compared with healthy donors (20). Moreover, we and others (19, 22) observed that naturally produced and recombinant sHLA-E monomers decrease the allogeneic and antitumor activity of NK and CD8 T cells expressing the related inhibitory NK receptor CD94/NKG2-A. In this study, taking advantage of an HLA-E–restricted CD8 T cell clone (23), we investigated whether sHLA-E functions rely on engagement of their cognate receptors or, alternatively, on peptide transfer from sHLA-E onto cell-bound HLA-E molecules. To check whether these mechanisms are shared by classical HLA-I molecules, the same experiments were conducted in an HLA-A2 model.

In agreement with previous reports, results clearly showed that both classical and nonclassical sHLA-I monomers could efficiently activate Ag-specific CD8 T cells. In addition, we demonstrated that...
the underlying mechanism relied on a passive peptide transfer from sHLA-I complexes to membrane-bound HLA-I molecules, hence prompting reappraisal of the ability of sHLA-I molecules to modulate immune responses in vivo.

Materials and Methods

T cell clones and cell lines

The human CMV-reactive CD8 αβ T cell clone specific for UL40.15–23 epitope (MART.22) was derived from the PBMCs of a kidney transplant patient (23). The human melanoma-reactive CD8 αβ T cell clone specific for NA17-A1–9 epitope (H2) was derived from peptide-stimulated PBMCs (21). T cell clones were expanded by stimulation with PHA-L (Sigma-Aldrich, Saint Quentin Fallavier, France) in the presence of irradiated feeder cells (24). The human mutant CEMx721 T2 (T2), the B-EBV 721.221 (0.221), and the adenocarcinoma HeLa cell lines, purchased from the American Type Culture Collection, were maintained in RPMI 1640 10% FCS.

Peptides and recombinant peptide/HLA-E monomers

The peptides (Millegen, Lahege, Belgium; purity > 80%) used were: HLA-A*0201–binding peptides, NA17-A1, (VLPDFVIRF) (25), and Melan-A26–35 (ELAGIGILTV) (26) derived from two human melanoma-associated Ags, HLA-E*0101/03–binding peptides, UL40.15–23 (VMAPRTLVL and VMAPRTPLL) derived from human CMV strains (Toiw and Toledo) (27, 28), and HLA-B*3501–binding self-peptide 37F (LPFDFTPGY) (29), HLA-A*0201/VLPDFVIRF, HLA-A*0201/ELAGIGILTV, HLA-E*0101/VMAPRTRLVL, and HLA-E*0101/VMAPRTLLL monomers were generated by the local recombinant protein facility (SFR Santé, Nantes, France), as previously described (30). Briefly, recombinant proteins were produced as inclusion bodies in Escherichia coli XA90’Lacq1, dissolved in 8 M urea, and refolded 5 d at 4˚C with the peptide of interest. The solution was then concentrated and the buffer changed on Amicon membranes 10 kDa (Millipore, Bedford, MA). Folded MHC/peptide complexes in 8 M urea, and refolded 5 d at 4˚C with the peptide of interest. The solution was then concentrated and the buffer changed on Amicon membranes 10 kDa (Millipore, Bedford, MA). Folded MHC/peptide complexes were biotinylated with the BirA enzyme (Avidity, Denver, CO) for 2 h at 4˚C.

CD69 and CD107a video imaging

Fura-2/AM loaded T cells (1 μM) (Invitrogen, Saint Aubin, France) for 1 h at room temperature in HBSS (Invitrogen) were resuspended in HBSS 1% FCS and seeded on Lab-Tek glass chamber slides (Nunc, Naperville, IL) coated with poly-L-lysine (Sigma-Aldrich). After addition of monomers (10 μg/ml equivalent to 0.2 μM) or anti-CD3 Ab (10 μg/ml) (UCHT1, Beckman Coulter, Marseille, France), measurements of intracellular Ca2⁺ were performed at 37°C with a DMI 6000 B microscope (Leica Microsystems, Nanterre, France). Cells were illuminated every 15 s with a 300-W xenon lamp, using 340/10 nm and 380/10 nm excitation filters. Emission at 510 nm was captured with a Cool Snap HQ2 camera (Roper, Tucson, AZ) and analyzed at single-cell level with Metafluor 7.1 imaging software (Universal Imaging, Downington, PA).

CD69 and CD107a upregulation

A total of 2 x 10⁵ T cell clones were stimulated for 6 h by antigenic or irrelevant monomers (10 μg/ml equivalent to 0.2 μM). Anti-CD3 stimulation (1 μg/ml coated) (OKT3; Beckman Coulter) was used as positive control. CD69 and CD107a markers were analyzed by flow cytometry using specific mAb (Becton Dickenson, Le Pont de Claix, France). Relative fluorescence intensity (RFI) was calculated as sample mean fluorescence divided by isotype control mean fluorescence.

51Cr release assay

Cytotoxicity was assessed in a standard chromium release assay. Target cells were labeled for 1 h with 100 μCi Na⁵¹ CrO₄ (NEN Life Science, France), washed, and incubated with monomers for 1 h. The 10⁵ target cells were then mixed with T cells at different effector/target ratio (10:1 to 2:1). After 4 h, supernatant radioactive content was measured on a β plate counter (EG&G Wallac, Eivy, France). The percentage of lysis was calculated as follows: (sample release – spontaneous release)/(maximum release – spontaneous release) x 100.

TNF-α intracellular staining

A total of 10⁵ T cells were stimulated for 6 h in the presence of brefeldin A (10 μg/ml) (Sigma-Aldrich) with peptides or monomers (0.2 μM) in autoradiopportunity assays or with 2 x 10⁶ peptide- or monomer-loaded target cells. For loading, target cells were incubated with peptides or monomers for 1 h and washed before incubation with T cells. In some experiments, target cells were fixed for 30 s with glutaraldehyde (0.05%; Sigma-Aldrich) prior to incubation with peptides or monomers. When T cells were used as target cells, they were stained for 1 h with calcein acetoxymethyl ester (1 μM) (Invitrogen). Cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich), labeled with anti–TNF-α mAb (Becton Dickinson) and analyzed by flow cytometry. Results are expressed as percentages of TNF-α-producing T cells.

Annexin V and 7-aminocynoinycin D double staining

A total of 10⁵ T cells were stimulated for 6 h with monomers (0.2 μM) or anti-CD3 mAb (OKT3, 1 μg/ml), then stained with Annexin V-FITC and 7-aminocynoinycin D (7-AAD) (Becton Dickinson) and analyzed by flow cytometry. Results are expressed as the percentages of death cells, including apoptotic (annexin V+ 7-AAD⁻) and secondary necrotic (annexin V⁻ 7-AAD⁺) cells.

Competition binding experiments

T cell clones were stimulated in the presence of brefeldin A (10 μg/ml) with a constant concentration (0.2 μM) of specific monomers and increasing concentrations of competitor or irrelevant peptides. After 6 h, cells were analyzed for TNF-α production.

Dose and kinetic responses

T cell clones were stimulated for 6 h with various concentrations (from 10⁻⁷ to 10⁻⁶ M) of monomers or peptides. For kinetic studies, T2 cells were loaded for 15–120 min with monomers or peptides and washed before incubation with T cells. T cell activation was then assessed by TNF-α staining.

Results

Activation of CD8 T cell clones by classical and nonclassical shLA class I monomers

We assessed the ability of soluble recombinant HLA-I/peptide monomers (HLA-E*0101/VMAPRTLLL and HLA-A*0201/ VLPDFVIRF) to activate CD8 T cells expressing cognate TCRs. To this end, experiments were carried out in parallel on two human CD8 T cell clones: a CMV-reactive clone, MART.22, specific for the UL40.15–23 HLA-E–restricted VMAPRTLLL epitope (23), and a melanoma-reactive clone, H2, specific for the NA17-A1–9 HLA-A*0201–restricted VLPDFVIRF epitope (21). To prevent protelyotic degradation of HLA–peptide complexes, all experiments were carried out in serum-free AIM V medium. Moreover, to exclude that HLA-I/peptide monomer activity could rely on their adventitious immobilization on plastic, we preincubated culture wells with a saturating dose of BSA (1 mg/ml).

As shown in Fig. 1, we observed that incubation with antigenic sHLA-I/peptide monomers (10 μg/ml) triggered an increase in intracellular free calcium (Ca²⁺) concentration in both MART.22 (Fig. 1A) and H2 (Fig. 1B) clones. In contrast, no significant Ca²⁺ increase was detected with monomers refolded with irrelevant peptides (HLA-E*0101/VMAPRTLLL for MART.22 clone and HLA-A*0201/ELAGIGILTV for H2 clone). We next analyzed several activation and function markers after 6 h of incubation with anti-CD3 mAb or sHLA-I/peptide monomers (Fig. 2, Supplemental Fig. 2 for MART.22 T and H2 clones, respectively). T cells stimulated by antigenic soluble monomers displayed an activated phenotype characterized by an increased expression of the early activation marker CD69 (Fig. 2A, Supplemental Fig. 2A). In addition, we observed the induction of TNF-α production (48% and 87% of TNF-α–producing T cells for MART.22 and H2 clones, respectively) (Fig. 2B, Supplemental Fig. 2B) and CD107a surface mobilization reflecting T cell degranulation (Fig. 2C, Supplemental Fig. 2C). Finally, we showed that incubation of CD8 T cells with
soluble monomers induced significant T cell apoptosis (36% and 46% Annexin V+7AAD+ for MART.22 and H2 clones, respectively) (Fig. 2D, Supplemental Fig. 2D). In agreement with Ca2+ flux experiments, T cell activation was not observed with irrelevant monomers. Thus, these data indicated that sHLA-I monomers were able to induce Ag-specific activation of CD8 T cell clones.

Activation by soluble monomers relied on autopresentation of antigenic peptide by T cells

The data presented above could be interpreted as the result of productive engagement of TCR by sHLA-I/peptide monomers, but this hypothesis would disagree with the well-accepted multivalent engagement of receptor model. Thorough analysis of Ca2+ flux

FIGURE 1. Induction of calcium mobilization upon activation of CD8 T cells with sHLA-I monomers. HLA-E– (A) and HLA-A2–restricted (B) T cells were stimulated by antigenic (HLA-E*0101/VMAPRTLLL and HLA-A*0201/VLPDFIRC, respectively) or irrelevant (HLA-E*0101/VMAPRTLVL and HLA-A*0201/ELAGIGILTV, respectively) monomers (0.2 μM). Anti-CD3 stimulation (1 μg/ml) was used as a positive control. Graphs represent the kinetics of intracellular Ca2+ levels (340/380 nm ratio). Values correspond to the mean of emission measured among all T cells present in the field. Results are representative of two independent experiments.

FIGURE 2. CD8 T cell activation after stimulation by sHLA-E monomers. HLA-E–restricted T cells were stimulated by antigenic HLA-E*0101/VMAPRTLLL or irrelevant HLA-E*0101/VMAPRTLVL monomers (0.2 μM). Anti-CD3 stimulation (1 μg/ml) was used as positive control. (A) Surface expression of CD69 activation marker (RFI). (B) TNF-α production. Data are expressed as mean percentage of T cells producing cytokine. (C) Surface expression of CD107a degranulation marker (RFI). In histograms (A–C) specific mAbs are presented by solid lines and isotype controls by dotted lines. (D) Detection of apoptosis by Annexin VFITC and 7-AAD staining. Data are expressed as percentages of dead cells (Annexin V+). Results are representative of three independent experiments.
experiments suggested that T cell activation by soluble monomers required cellular contact because T cells in direct physical contact displayed higher calcium influx than did isolated T cells (Fig. 3). Thus, these results suggested that, rather than arising directly from monomeric engagement of the TCR, the underlying activation mechanism is related to induction of Ag autopresentation by T cells, as previously suggested in rodent models (17, 31).

To document this proposed representation mechanism, we tested whether T cell clones preincubated with sHLA-I/peptide monomers (10 μg/ml equivalent to 0.2 μM, for 1 h) can present the antigenic peptides on their surface and activate T cell clones that have not been directly exposed to the Ags. To differentiate “target” (directly exposed to Ag) and “effector” cells (indirectly exposed to Ag), we previously stained the effector population with calcein acetoxymethyl ester (Fig. 4A). As illustrated in Fig. 4B and 4C, T cells preincubated with their antigenic peptide, both in a free or in HLA-complexed form, were able to elicit the production of TNF-α by effector T lymphocytes, thus suggesting that soluble monomer–induced activation depends on peptide representation and fratricide responses.

To further substantiate these data, we addressed the requirement of cognate HLA-I expression to mediate T cell activation. To this end, we assessed whether soluble monomers could sensitize target cells, expressing, or not, the restricting HLA-I molecule, for recognition by CD8 T cells. We show that preincubation of T2 and 0.221 cells (HLA-E+), but not HeLa cells (HLA-E−), with HLA-

FIGURE 3. CD8 T cell activation by sHLA-I monomers relies on cellular contact. HLA-E– (A) and HLA-A2–restricted (B) T cells were stimulated by antigenic monomers (10 μg/ml). Kinetic profiles of intracellular Ca2+ were performed on T cells with or without contact. Values correspond to the mean of emission measured among all T cells present in the field. Results are representative of two independent experiments.

FIGURE 4. CD8 T cell activation by sHLA-I monomers relies on autopresentation of monomer-derived peptide. (A) Experimental design and protocol. Calcein staining was used to differentiate target and effector CD8 T cells. Calcein+ T cells were pulsed 1 h with free or HLA-complexed peptide (0.2 μM), washed, and cocultured with calcein− T cells. Activation of calcein+ T cells was assessed by TNF-α staining. Results are representative of three independent experiments.
that fixation itself did not alter Ag presentation by T cells. As shown in Fig. 6, fixation of target T cells did not significantly alter their capacity to activate effector T cells, demonstrating that internalization was not involved in monomer-mediated activation. In support of these results, the use of endocytosis inhibitors, such as cytochalasin D and dimethyl amiloride, abolishing phagocytic and pinocytic activities, respectively, had no effect on the efficiency of monomer-mediated activation (Supplemental Fig. 3 and data not shown). These results invalidate the hypothesis of an active mechanism, supporting the assumption of an exogenous passive transfer of monomer-derived peptide toward cell-bound HLA-I molecules of T cells.

On the basis of these results, we generated single-chain trimers \[\text{(SCT)}\] a single polypeptide chain with a linear composition of antigenic peptide, \(\beta_2\)-m, and H chain connected by flexible linkers \[32, 33\] to address whether covalent binding of peptide within HLA-I monomers could affect their ability to activate T cells. First, one must demonstrate that immobilized SCTs are able to stimulate T cell clones. We observed that immobilized SCTs are able to activate T cells. We observed that immobilized HLA-A*0201/VLPDFIRC SCTs were able to activate, even modestly, H2 T cell clones (8% of TNF-\(\alpha\)-producing cells), but no activation at all could be observed with soluble forms of SCT (Supplemental Fig. 4). Thus, these data suggest that T cell–induced activation is strictly dependent on the ability of monomer-derived peptide to

**FIGURE 5.** CD8 T cell activation by sHLA-I monomers relies on cognate membrane HLA expression. Target cells (T2 [HLA-A2+, HLA-E+], 0.221 [HLA-A2+, HLA-E+], and HeLa [HLA-A2+, HLA-E+]) were pulsed with either HLA-E*0101/VMAPRTL or HLA-A*0201/VLPDFIRC monomers (0.2 \(\mu\)M), washed, and cocultured with HLA-E– (A and B) and HLA-A2–restricted (C and D) T cells. Cytotoxicity was assessed by a chromium release assay (A and C) and TNF-\(\alpha\) (B and D). Results are representative of three independent experiments.
dissociate from HLA-Ia complexes. Unfortunately, we could not confirm this result for nonclassical HLA-E because the preassembled nature of these SCTs makes them ineffective to activate CD8 T cells, even immobilized.

To formally demonstrate that monomer-induced activation relied on a passive peptide transfer toward surface HLA-I molecules, we tested whether this mechanism could be inhibited by competitor peptides (i.e., nonantigenic peptides binding the same HLA-I molecules). As shown in Fig. 7, addition of HLA-E– and HLA-A2–binding competitor peptides induced a dose-dependent inhibition of monomer-induced T cell activation, whereas irrelevant peptides (i.e., HLA-B35–binding peptides) had no impact. These data demonstrated that stimulatory properties of soluble monomers result from passive peptide transfer onto T cell endogenous HLA-I molecules and can be prevented in the presence of high concentrations of competing peptides.

Finally, we evaluated the efficiency and kinetic of this passive peptide transfer. To this end, we performed titration assays comparing T cell–induced activation upon direct incubation with soluble monomers, or with the corresponding peptides at equivalent molar concentration. As shown in Fig. 8A and 8C, TNF-α production by T cells could be detected from 0.05 μM of both soluble monomers, and free peptides indicated that they were equally efficient in inducing CD8 T cell activation. Kinetic experiments were conducted by stimulating T cell clones with T2 cells that have been loaded for various times (from 0 to 2 h) with soluble monomers or peptides. Again, the two kinetic profiles were very similar, with 15 min of monomer/peptide loading being sufficient to induce 50% of maximal T cell response (Fig. 8B, 8D). These data clearly showed that sHLA-I/peptide monomers and peptides exhibited a similar ability to activate CD8 T cells.

Discussion
To elucidate the minimal requirements for T cell activation, previous studies reported that peptide class I MHC monomers, in contrast to peptide class II monomers, were sufficient to induce activation of T cells (12, 34, 35). Stimulatory capacity of HLA-I monomers was initially attributed to a productive TCR–CD8 heterodimerization, implying that CD8 T cell activation could arise from monomeric engagement. However, subsequent investigations have suggested an alternative mechanism, enabling reconciliation with previous observations of the consensual model of multivalent engagement of receptors. Using the 2C and OT-1 TCR mouse models, these studies provide evidence that stimulatory capacities of monomers were strictly dependent on the CD8 T cell ability to express the cognate MHC-I molecule. Therefore, an unexpected mechanism involving a peptide transfer from monomers to T cell endogenous MHC-I molecules has been proposed, thus leading to Ag autopresentation (17, 31). Similarly, using both a classical (HLA-A*0201) model and a nonclassical (HLA-E) model, we observed that monomer-induced CD8 T cell activation required cellular contacts and that CD8 T cells that had been previously exposed to monomers acquired the ability to activate CD8 T cells of the same antigenic specificity. Hence, rather than arising from monomeric engagement of the TCR, CD8 T cell activation apparently stems from the T cell ability to present monomer-derived peptide to each other, allowing fratricide responses. In support of this mechanism, we documented that HLA-I monomers could also sensitize target cells for recognition by CD8 T cells, but only if the target cells expressed the appropriate HLA-I molecules.

The aforementioned process raised the question of how such a peptide transfer could occur. Previous studies suggested that monomers could be internalized into T cell endocytic compartments (possibly after binding to TCR and/or CD8 molecules), where monomer-derived peptides could be reloaded onto endogenous HLA-I molecules (17, 31). However, we showed that cell fixation or use of endocytosis inhibitors did not alter monomer-derived peptide transfer, excluding this possibility and thereby suggesting that peptide is transferred exogenously. In support of these data, we uncovered evidence that monomer-derived peptide transfer could be efficiently inhibited in the presence of exogenous competitor peptides. Moreover, we observed, in the HLA-A*0201
model, that the covalent linking of peptides onto HLA-I monomers (referred to as SCTs) preclude their ability to activate CD8 T cells. Altogether, these observations strongly support a mechanism of passive external loading of monomer-derived peptide onto surface HLA-I molecules.

Consistent with previous observations (17, 31), we also documented that the transfer of monomer-derived peptides was as efficient and fast as loading of free peptides. This finding could be explained by quantitative dissociation of monomers and subsequent peptide release into the medium. However, this is incompatible with estimated HLA-I/peptide complex lifetime, as we could detect only minor dissociation of soluble monomers when incubated in assay conditions (Supplemental Fig. 1). Thus, rather, we hypothesize that the release of peptide from monomers probably occurs close to the cell membrane, allowing an immediate reloading on surface HLA molecules.

Several lines of evidence suggest that sHLA-I molecules are immunologically functional and may play a significant role in vivo (9). In this regard, others and we (19, 22) previously documented in vitro that sHLA-E molecules can modulate the effector functions of NK and CD8 T cells expressing the cognate inhibitory NK receptor CD94/NKG2-A (19, 22). We performed this study to find out whether this behavior relies on the ability of sHLA-E to directly interact with their receptors, as mostly suggested (15, 16), or on an indirect mechanism of peptide transfer, as described for classical MHC-I molecules in murine models (17, 31). In this article, we clearly demonstrated that sHLA-E molecules have the potential to exert immunoregulatory functions through their ability to provide peptides that can be transferred to cell-bound HLA-E molecules, hence raising the question of their potential involvement in the global immune response. Thus, we propose that prior observations made with CD94/NKG2-A+ cells are more likely to stem from a similar mechanism of peptide transfer toward surface HLA-E molecules of targets or immune cells than from the direct triggering of NK receptors.

In conclusion, we reported that both classical and nonclassical HLA-I/peptide monomers can efficiently activate Ag-specific CD8 T cells in vitro, via a mechanism that involves the passive transfer of monomer-derived peptide to cell-bound HLA-I molecules. These findings support the peptide-representation theory previously investigated in rodent models and provide a molecular basis for the capacity of sHLA-I molecules to modulate immune effectors expressing cognate receptors and thus open up the possibility that this mechanism could occur in vivo.
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