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A Signal Peptide Derived from hsp60 Binds HLA–E and Interferes with CD94/NKG2A Recognition

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Abstract

Human histocompatibility leukocyte antigen (HLA)–E is a nonclassical major histocompatibility complex (MHC) class I molecule which presents a restricted set of nonameric peptides, derived mainly from the signal sequence of other MHC class I molecules. It interacts with CD94/NKG2 receptors expressed on the surface of natural killer (NK) cells and T cell subsets. Here we demonstrate that HLA–E also presents a peptide derived from the leader sequence of human heat shock protein 60 (hsp60). This peptide gains access to HLA–E intracellularly, resulting in up-regulated HLA–E/hsp60 signal peptide cell-surface levels on stressed cells. Notably, HLA–E molecules in complex with the hsp60 signal peptide are no longer recognized by CD94/NKG2A inhibitory receptors. Thus, during cellular stress an increased proportion of HLA–E molecules may bind the nonprotective hsp60 signal peptide, leading to a reduced capacity to inhibit a major NK cell population. Such stress induced peptide interference would gradually uncouple CD94/NKG2A inhibitory recognition and provide a mechanism for NK cells to detect stressed cells in a peptide–dependent manner.

Key words: CD94/NKG2 • MHC class I • cellular stress • peptide interference • hsp60

Introduction

NK cells play a major role in innate immune responses and can provide immediate protection against certain types of infection. They are also involved in the shaping of subsequent adaptive immune responses via the secretion of cytokines. NK cells express both activating and inhibitory receptors, which interact with MHC class I molecules on target cells (1). The receptors belong to two major groups, comprising (a) molecules with C-type lectin like folds (CTLDs) and a type II membrane topology (e.g. CD94/NKG2; reference 2), and (b) receptors of the Ig-superfamily with a type I membrane orientation (3).

CD94/NKG2 receptors are expressed by a large proportion of NK cells, both in human and mouse, and interact with the nonclassical MHC class I molecule HLA-E and its murine homologue Qa-1^b, respectively (4, 5). NKG2A contains an intracellular immunoreceptor tyrosine-based

inhibitory motif (ITIM) mediating inhibitory signals (6), whereas NKG2C associates with the immunoreceptor tyrosine-based activating motif (ITAM) bearing adaptor molecule DAP-12, and mediates positive signaling (7). CD94/NKG2A/C receptors have been reported to discriminate between different HLA-E and Qa-1^b binding peptides (8–11), but the physiological significance of this selectivity remains unclear.

HLA-E is widely expressed in association with β_2-microglobulin and peptide on the surface of cells, albeit at low levels (12). The peptide loading of HLA-E is believed to be transporter associated with antigen processing (TAP)-dependent (13), although there are reports of TAP-independent presentation (14, 15). In contrast to classical MHC class I molecules, HLA-E displays a rather limited polymorphism, and its peptide binding cleft is primarily occupied by nonameric peptides derived from the signal sequence of certain HLA-A, –B, –C, and –G molecules (5). These peptides share a common motif: methionine at position 2, and leucine or isoleucine at position 9 (16). Analysis of the crystal structure of HLA-E has provided an elegant explanation for its peptide selectivity (17). Qa-1^b also primarily presents peptides derived from the signal sequence of some

mouse MHC class I molecules, with identical anchor residues at positions 2 and 9 (18, 19). It has, however, recently been demonstrated that both HLA-E and Qa-1^b can bind a diverse array of peptides derived from random peptide libraries (8, 20). Furthermore, Qa-1^b can present peptides derived from mouse and bacterial heat shock protein 60 (hsp60),* and these complexes can be detected by T cells via their antigen-specific TCR (21).

Hsp60 is present in all living cellular organisms (22, 23). In eukaryotic cells it serves a vital function as a mitochondrial chaperone, and in bacteria as an intracellular protein involved in the assembly and disassembly of multi-subunit protein complexes (24). Increased levels of hsp60 are induced in response to a variety of stress stimuli, e.g. temperature increase, nutrient deprivation, exposure to toxic chemicals, inflammatory responses, and allograft rejection (22, 25, 26). Hsp60 is believed to play an important role in the protection of cells from the consequences of these harmful stimuli. At the same time it may render these cells more susceptible to attack by hsp60-directed innate and adaptive immune responses.

Here we demonstrate that HLA-E can present a peptide derived from the signal sequence of human hsp60 (hsp60sp). This leads to loss of recognition by CD94/ NKG2A inhibitory receptors, suggesting a mechanism by which CD94/NKG2A$^+$ NK cells can recognize stressed cells in a peptide-dependent manner.

Materials and Methods

Cell Culture. K562 (human HLA class I–negative erythroleukemia), and 721.221 (human HLA class I low B-lymphoblastoid cell) were maintained in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Two human CD94/NKG2A$^+$ (but killer Ig-like receptor [KIR]$^-$) cytotoxic NK cell lines (NKL; provided by Dr. M. Robertson, Indiana University School of Medicine, Indianapolis, IN), and Nishi (a gift from Dr. H. Wakiguchi, Department of Pediatrics, Kochi Medical School, Kochi, Japan) were grown in IMDM supplemented with 7% pooled heat-inactivated human AB$^+$ serum, 200 U IL-2/ml (PeproTech), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Ba/F3 cells cotransfected with CD94 and NKG2A, CD94, DAP-12, and NKG2C-green fluorescent protein (GFP) or CD94 and DAP-12 have been described previously (7). HB-120 (pan-HLA class I–specific hybridoma) was obtained from American Type Culture Collection, and was cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, sodium pyruvate, HAT, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies).

Peptides, HLA-E Stabilization, and Cell Culture Stress Assays. Synthetic peptides, purchased from Research Genetics, were dissolved in PBS. The peptides used were B7sp (VMAPRTVLL), hsp60sp (QMRPVSRVL), B7 R5V (VMAPVTVLL), hsp60 V5R (QMRPRSRVL), and P18I10 (RGPGRAFVTI) (all from

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*Abbreviations used in this paper: GFP, green fluorescent protein; hsp60, heat shock protein 60; hsp60L, hsp60 leader sequence aa 1-26; hsp60sp, heat shock protein 60 signal peptide QMRPVSRVL; KIR, killer Ig-like receptor.

Research Genetics). Cells and their HLA-E–transfected derivatives were incubated with synthetic peptides (3–300 μM) at 26°C for 15–20 h in serum-free AIM-V medium (GIBCO BRL) at a concentration of 1–3 × 10^6 cells/ml. Cells were then harvested, washed in PBS, stained with mAbs, and analyzed by flow cytometry. Cells were subjected to stress by allowing them to grow at increasing cell density. Briefly, cell cultures were set up at the cell concentration of 0.2 × 10^6 cells/ml at different time points for a period of up to 6 d. At the end point, cell concentration and viability were determined by trypan blue exclusion. The expression of cell-surface HLA class I molecules was assessed by flow cytometry. Cell cultures with viability >90% and at three different densities were selected as targets for cytotoxic assays.

HLA-E Tetramer Production. HLA-E tetrameric complexes were generated as described previously (5, 27). Briefly, HLA-E and β$_2$–microglobulin (β$_2$m) were overexpressed in *Escherichia coli* BL21 pLysS, purified from inclusion bodies, solubilized into a 8 M urea solution, and then refolded by dilution in vitro with synthetic peptides (B7sp, hsp60sp, B7 R5V, or hsp60 V5R; Research Genetics). Complexes of the HLA-E heavy chain, β$_2$m and peptide were purified by size exclusion chromatography on a Superose12 column (Amersham Biosciences), biotinylated with BirA enzyme (Avidity) according to the instructions of the manufacturer, then quickly frozen and stored at −80°C. Tetrameric HLA-E complexes were generated by mixing biotinylated monomers with streptavidin–phycoerythrin (Molecular Probes) at a 4:1 molar ratio. Similar quality of the different tetramers was verified by gel-shift assays, as well as by staining a pan-HLA specific hybridoma (HB-120).

Antibodies and Flow Cytometry. mAbs used were: DX22 (anti-CD94; DNAX), anti-NKG2A (Z199, a gift from Dr. Lorenzo Moretta, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy), CD56 (B159, BD Biosciences), anti-MHC class I mabs (DX17, DNAX), and W6/32 (American Type Culture Collection). The 3H5 (anti-MICA) and 3D12 (anti-HLA-E) mAbs were provided by Drs. T. Spies and D. Geraghty, respectively (Fred Hutchinson Cancer Center, Seattle, WA). Anti-hsp60 (ML30) was provided by from Dr. J. Ivanyi (University of London, London, England). Anti-MICB (7C5) was generated in our laboratory by immunizing mice with P815 cells stable transfected with a pCDNA3 expression vector containing an NH$_2$-terminal CD8 leader peptide followed by a FLAG epitope and the extracellular, transmembrane and cytoplasmic MICB cDNA. Hybridoma 7C5 (anti-MICB) was selected and shown to bind 721.221 and P815 cells transfected with MICB*002 cDNA expression vectors, whereas untransfected or control transfected cells as well as MICA*005 transfected cells were negative (unpublished data). Second-step reagents were FITC- and PE-conjugated goat anti–mouse IgG (both from Dakopatts). DAK-GO1 was used as negative control mAbs for triple-color (Dakopatts). Cells were analyzed on a FACScan™, Becton Dickinson. Immunofluorescence staining was done using standard protocols. Briefly, K562 cells transfected with wild-type or mutant full-length hsp60 signal peptide-GFP were stained with the nuclear stain Hoechst33342 for 30 min at 37°C and the mitochondrial dye tetramethyl-rhodamine ethyl ester (TMRE) for 15 min at 37°C, followed by three washing steps. Cells were analyzed using a Nikon Eclipse E400 universal microscope connected to a Hamamatsu C4742–98 digital camera. Appropriate filters for immunofluorescence analysis of labeled cells were used and images were acquired using Jasc Paint Shop Pro 6.0 and imported into Adobe Photoshop™.

Expression Vectors and Generation of Transfected Cells. Synthesized sense and antisense DNA coding for the full-length hsp60

signal peptide flanked by a 5′Eco RI/3′ BamHI sites (5′-CGGA ATTCATGCTTCGGTTACCCACAGTCTTTCGCCAGATG AGACCGGTGTCCAGGGTACTGGCTCCTCATCTCACGG GCTTATGGATCCGC-3′) were purchased from Interactiva. The annealed and digested product was ligated into pEGFP-N3 expression vector (CLONTECH Laboratories, Inc.). The triplet coding for a Met-residue at position 11 in the hsp60 signal-peptide was mutated to a triplet coding for a gly-residue using the following oligonucleotide primer: 5′-CAGTCTTTCGCCAGGGGAGAC CGGTGTCCAG-3′ using a site-directed mutagenesis kit according to the manufacturer's recommendations (QuikChange™; Stratagene) and verified by sequencing. HLA-E*0101 and HLA-E*01033 cDNA encoding plasmids (pCDNA3) were provided by Drs. M. Ullbrecht and E. Weiss (Institut fuer Anthropologie und Humangenetik, Munich, Germany). 721.221 and K562 cells were transfected by electroporation (Gene Pulser; BioRad Laboratories) according to standard protocols. For transient cotransfection experiments with HLA-E and the chimeric GFP encoding plasmids we used a ratio of 10:1 (HLA-E:GFP). Transfected cells were selected in complete medium supplemented with 1 mg/ml G418 (BioRad Laboratories). Stable transfected cells were isolated by flow cytometry (FACScan™) on the basis of their green fluorescent properties.

NK Cell–mediated Cytotoxicity Assays. NK cell–mediated cytotoxicity was measured using a 2 h standard ^{51}Cr radioisotope release assay. Briefly, target cells were incubated for 15–20 h at 26°C with the various peptides at concentrations ranging from 1–300 μM, and then labeled with ^{51}Cr. Peptides were washed away before setting up the assays, except in some experiments where the nonprotective hsp60sp, B7 R5V, and hsp60 V5R was kept throughout the assay to assure higher levels of HLA-E expression, as compared with targets incubated with the protective B7sp. In mAb blocking experiments, cells were preincubated with mouse serum, or an irrelevant isotype-matched antibody to block Fc-receptors. Blocking of either target or effector cells with mAbs was performed at 4°C, and the antibodies were also included during the assays.

Results

Hsp60sp Stabilizes HLA-E Cell Surface Expression. To identify peptides derived from human hsp60 with a potential to bind HLA-E, we searched the full length amino acid sequence of hsp60 for peptides displaying the HLA-E permissive motif (methionine at position 2 followed by either a leucine or isoleucine at position 9 at the COOH terminus). Among totally four such peptides (Fig. 1 and Table I), one was found within the hsp60 leader sequence (i.e., hsp60sp, QMRPVSRVL; Fig. 1 and Table I). In addition, hsp60sp not only bears a methionine at position 2 and a

leucine at position 9, but also shares amino acids at position 4 and 8 in common with some peptides known to efficiently bind to HLA-E. Thus, four out of the nine amino acids in hsp60sp are shared with some peptides found in HLA class I leader sequences (e.g. HLA-A*0201, and -A*3401, Table I). This homology suggested that hsp60sp may bind to HLA-E.

Studies on peptide and HLA-E interactions have demonstrated that the presence of an HLA-E binding peptide, either provided in a transfected cDNA expression plasmid, or by exogenous addition of synthetic peptides, is sufficient to stabilize and up-regulate HLA-E cell surface expression to levels detectable by flow cytometry (5, 28, 29). To test whether the hsp60-derived peptides were able to bind HLA-E, we stabilized HLA-E cell surface expression with the different synthetic peptides, by overnight incubation at 26°C. For this purpose we used MHC class I–deficient cell lines such as 721.221 (which lack HLA-A, –B, –C, and -G, but express HLA-E and -F intracellularly), as well as K562 cells transfected with HLA-E*01033 (K562-E*01033) or HLA-E*0101. The 721.221 cells and HLA-E transfected, but not untransfected, K562 cells express low levels of HLA-E at the cell surface during normal cell growth. These base levels of HLA-E expression suggest the presence of minute amounts of intracellular peptides, enough to stabilize nascent HLA-E molecules. As shown in Fig. 2, using hsp60sp, a substantial increase in HLA-E expression was observed in both HLA-E*01033 and HLA-E*0101 transfected K562 cells. The levels of HLA-E expression after loading with hsp60sp were comparable to the levels of cells loaded with a peptide derived from the leader sequence of HLA-B*0701 (B7sp, VMAPRTVLL; Fig. 2). However, at 37°C the HLA-E/hsp60sp complexes dissociated faster than the HLA-E/B7sp, reaching base levels after ∼5 h (data not depicted). In addition to hsp60sp, the hsp60.4 peptide (GMKFDRGYI) was also capable of stabilizing HLA-E molecules on transfected K562 cells as well as on 721.221 cells (data not depicted). This peptide has previously been shown to also bind to mouse Qa-1^b molecules (21). HLA-E stabilization was not observed with the other two hsp60-derived peptides (hsp60.2 and hsp60.3; Table I), possibly due to poor solubility in the assay medium.

Hsp60 Signal Peptide Gains Access to HLA-E Intracellularly and HLA-E/hsp60sp Levels Are Up-regulated during Cellular Stress. Hsp60 is a mitochondrial matrix protein, which is encoded within the genomic DNA (23, 30). It is synthesized as a precursor protein with an NH₂-terminal mito-


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MLRLPTVFRQ MRPVSRVLAP HLTRAYAKDV KFGADARALM LQGVDLLADA VAVTMGPKGR
TVIIEQSWGS PKVTKDGVTV AKSIDLKDKY KNIGAKLVQD VANNTNEEAG DGTTTATVLA
RSIAKEGFEK ISKGANPVEI RRGVMLAVDA VIAELKKQSK PVTTPEEIAQ VATISANGDK
EIGNIISDAM KKVGRKGVIT VKDGKTLNDE LEIIEGMKFD RGYISPYFIN TSKGQKCEFQ
DAYVLLSEKK ISSIQSIVPA LEIANAHRKP LVIIAEDVDG EALSTLVLNR LKVGLQVVAV
KAPGFGDNRK NQLKDMAIAT GGAVFGEEGL TLNLEDVQPH DLGKVGEVIV TKDDAMLLKG
KGDKAQIEKR IQEIIEQLDV TTSEYEKEKL NERLAKLSDG VAVLKVGGTS DVEVNEKKDR
VTDALNATRA AVEEGIVLGG GCALLRCIPA LDSLTPANED QKIGIEIIKR TLKIPAMTIA
KNAGVEGSLI VEKIMQSSSE VGYDAMAGDF VNMVEKGIID PTKVVRTALL DAAGVASLLT
TAEVVVTEIP KEEKDPGMGA MGGMGGGMGG GMF
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Figure 1. The protein sequence of human hsp60. The mitochondrial targeting signal is shown in gray. Boxed are the four peptide sequences displaying a methionine followed by leucine or isoleucine seven amino acids COOH-terminally, two important residues for binding to HLA-E pockets. Hsp60sp corresponds to residues 10–18 in the sequence (QMRPVSRVL).

Table I. *Peptide Sequence Comparisons between HLA Class I Molecules and hsp60*

Protein	(Residues)	Peptide sequence	Signal peptide (SP) Mature protein (P)
HLA-A★0201	(3–11)	VMAPRTLVL	SP
HLA-A★0301	(3–11)	VMAPRTLLL	SP
HLA-A★3401	(3–11)	IMAPRTLVL	SP
HLA-B★0701	(3–11)	VMAPRTVLL	SP
HLA-Cw★0102	(3–11)	VMAPRTLIL	SP
HLA-G★0101	(3–11)	VMAPRTLFL	SP
hsp60sp	(10–18)	QMRPVSRVL	SP
hsp60.2	(39–47)	LMLQGVDLL	P
hsp60.3	(144–152)	VMLAVDAVI	P
hsp60.4	(216–224)	GMKFDRGYI	P

Figure 2. Stabilization of HLA-E by hsp60sp and B7sp on K562 cells transfected either with HLA-E★0101 or HLA-E★01033. Cell surface expression of HLA-E★0101 (top panel) and HLA-E★01033 (bottom panel) after overnight incubation at 26°C with 300 μM of either hsp60sp (left panel, bold line) or B7sp (right panels, bold line). The dashed line represents HLA-E expression after incubation with 300 μM of a control peptide (P18I10). Cells were stained with anti-MHC class I mAb DX17, followed by RPE-conjugated goat anti–mouse IgG. The HLA-E expression was confirmed by staining with the anti–HLA-E mAb 3D12. Staining with isotype-matched control antibody is shown as shaded gray. 1 representative experiment out of more than 10 is shown.

chondrial targeting sequence consisting of 26 amino acids (i.e., hsp60L, see Fig. 1). Biochemical studies have established that cleavage of the hsp60L requires import of the precursor protein into the mitochondrial matrix, and that this cleavage is unlikely to occur in the cytosol, as no mitochondrial import of hsp60 is observed in the absence of the hsp60L (31). The final destination for the hsp60L after its cleavage is unknown. Upon stress, hsp60 is regulated by increased transcription as well as by posttranscriptional events affecting its intracellular levels and distribution (32–35). In an effort to follow the localization of the hsp60L, and particularly whether the hsp60sp can gain access to HLA-E molecules, we established a system based on K562 cells transfected with chimeric constructs containing either the wild-type hsp60L, or a mutated variant in which the methionine at position 11 was substituted by a glycine. This methionine corresponds to position 2 in hsp60sp nonamer, and is required for stable binding to HLA-E. The wild-type and mutated hsp60L were grafted in frame onto the NH$_2$ terminus of GFP to ensure that, upon transfection, green fluorescent cells also translate each of the individual hsp60 leader sequences. After transfection, we observed that GFP localized inside the mitochondria in the two hsp60L-GFP transfected cell lines (Fig. 3), indicating that the substitution of methionine at position 11 did not alter the transport into the mitochondria. GFP showed no obvious subcellular localization when the GFP gene was transfected alone (unpublished data).

Previously, Imani and Soloski have demonstrated that cell surface levels of mouse Qa-1^b molecules are substantially up-regulated during cellular stress (36). Based on the homology between Qa-1^b and HLA-E, both in terms of sequence, biological function, and peptide binding specificity, we set up experiments to test whether the nonameric peptide located inside the mitochondrial targeting sequence of hsp60 may ultimately gain access to HLA-E, particularly under conditions of cellular stress. To this end we cotrans-
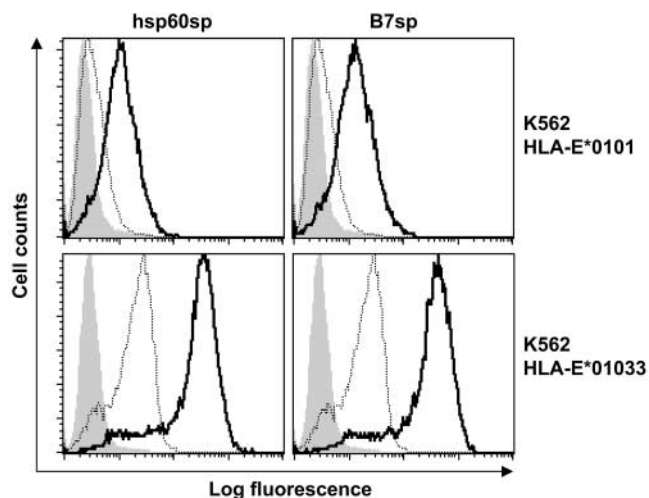
fected K562 cells with an HLA-E★01033-encoding plasmid together with either the wild-type hsp60L–GFP construct or with its mutated variant. We then monitored HLA-E cell surface expression of these transfectants as the cultures were subjected to stress by means of growth at increasing cellular density. Cells transfected with the wild-type hsp60L–GFP construct consistently expressed higher levels of HLA-E than cells cotransfected with the mutant construct (Fig. 4 a). It should be noted that this difference depended on the growth conditions; at day 1, the difference in HLA-E cell surface levels between cells expressing wild-type and mutated hsp60sp was marginal, while it was substantial at day 5. We also observed a certain increase of HLA-E levels in the cells transfected with the mutated hsp60L–GFP construct when grown under stress versus normal conditions (Fig. 4 a, day 1 vs. day 5). This could be due either to a residual capacity of the mutated peptide to bind HLA-E, or by an access of endogenously derived hsp60 peptides to HLA-E. Consistent with the latter possibility, we observed increased HLA-E levels as a consequence of culture-induced stress also in K562 cells that had been transfected with the HLA-E gene alone (Fig. 4 b, bottom panel), whereas untransfected K562 cells remained HLA-E negative (Fig. 4 b, top panel). However, at this point we cannot exclude an influence by other HLA-E binding peptides, nor posttranscriptional, but peptide independent, regulation of HLA-E in stressed cells. Note that the K562-E★01033 cell line and the cotransfected cell lines presented in Fig. 4 a were generated and selected independently, which may account for the higher HLA-E back-
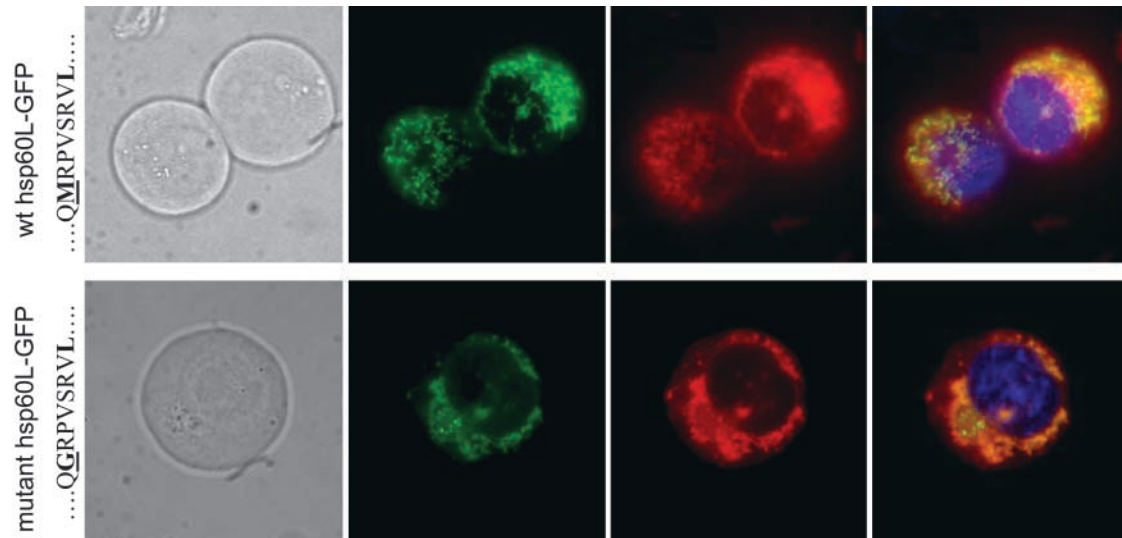
Figure 3. Hsp60 signal peptide targets GFP into mitochondria and the methionine residue at position 11 is not crucial for mitochondrial translocation. K562 cells were transfected with either a chimeric construct consisting of the wild-type full-length hsp60 signal peptide (residues 1–26) directly upstream and in frame of GFP (top panel, green), or a mutated variant of the full-length hsp60 signal peptide where the methionine at position 11 was replaced with a glycine (bottom panel, green). Both constructs target GFP into mitochondria. Cells were counter stained with the mitochondrial probe TMRE (bright red) and the nuclear stain Hoechst 33342 (blue). An overlay of all colors is shown to the right.

ground level observed at day 1. Therefore, the absolute levels of HLA-E should not be directly compared between Fig. 4, a and b.

Our interpretation of the transfectant studies is, that the stress response results in an increased accessibility of mitochondrial hsp60sp to HLA-E intracellularly, eventually causing up-regulated HLA-E/hsp60sp cell surface levels. This should at least in part be due to posttranscriptional control of hsp60sp during the stress response, as both the hsp60L–GFP and HLA-E constructs used were under control of the same CMV promoter, and the GFP expression level did not change with increased cell density (Fig. 4 a).

HLA-E–mediated Presentation of hsp60sp Abrogates Recognition by CD94/NKG2A and CD94/NKG2C Receptors. The inhibitory lectin-like receptor heterodimer CD94/NKG2A is present on ∼50% of all NK cells in the peripheral blood both in humans and mice. This HLA-E–specific receptor mediates a negative signal upon binding to HLA-E presenting various protective HLA class I signal peptides, which results in the inactivation of NK cell effector functions. In a similar fashion, Qa-1[b] in complex with a permissive MHC class I leader peptide is efficiently recognized by murine CD94/NKG2A receptors, suggesting evolutionary conservation in humans and mice at both receptor and ligand levels.

To characterize possible NK cell receptors that interact with HLA-E in complex with hsp60sp or MHC class I signal peptides, we analyzed whether MHC tetrameric complexes could bind CD94/NKG2 receptors expressed on transfectants and NK cells. Recombinant soluble HLA-E molecules were refolded in vitro in the presence of human β_2-microglobulin and B7sp (VMAPRTVLL) or hsp60sp (QMRPVSRVL). The refolded MHC complexes were

used to create tetrameric HLA-E molecules, which enable analysis of HLA-E binding receptors. Both peptides permitted an effective refolding of HLA-E in vitro and were effectively biotinylated as analyzed by gel-shift assays (unpublished data). As expected, HLA-E/B7sp tetramers efficiently bound to mouse Ba/F3 pro-B cells cotransfected with CD94 and NKG2A or CD94, NKG2C, and DAP12 (Fig. 5, a and b). This result was confirmed by staining NK cell lines that express the inhibitory receptor CD94/NKG2A (Fig. 5 c), or freshly isolated NK cells expressing predominantly the CD94/NKG2A receptor (unpublished data). In contrast, the HLA-E/hsp60sp tetramers failed to bind Ba/F3 pro-B cells cotransfected with either CD94/NKG2A or CD94/NKG2C/DAP12, and all NK cells examined (Fig. 5, a–c). However, both HLA-E/B7sp and HLA-E/hsp60sp bound to a similar extent to a control B cell hybridoma, specific for HLA class I molecules (Fig. 5 d). Thus, although hsp60sp can efficiently gain access to HLA-E physiologically, this complex is no longer recognized by the CD94/NKG2A and CD94/NKG2C receptors, demonstrating that they are peptide selective.

HLA-E/hsp60sp Fails to Inhibit CD94/NKG2A+ NK Cells in Cytotoxic Assays; Critical Role for Position 5 in the Peptide. To address the functional significance of increased HLA-E/hsp60sp cell surface levels, we investigated whether cells expressing these MHC complexes were protected from killing by CD94/NKG2A+ NK cells. K562-E*01033 cells, incubated overnight at 26°C with either hsp60sp or B7sp peptides, were tested as targets in 2 h chromium release assays with the CD94/NKG2A+ NK cell lines Nishi and NKL as effectors. A clear protection from killing was observed when incubating the otherwise susceptible K562-E*01033 cells with B7sp, whereas incu-
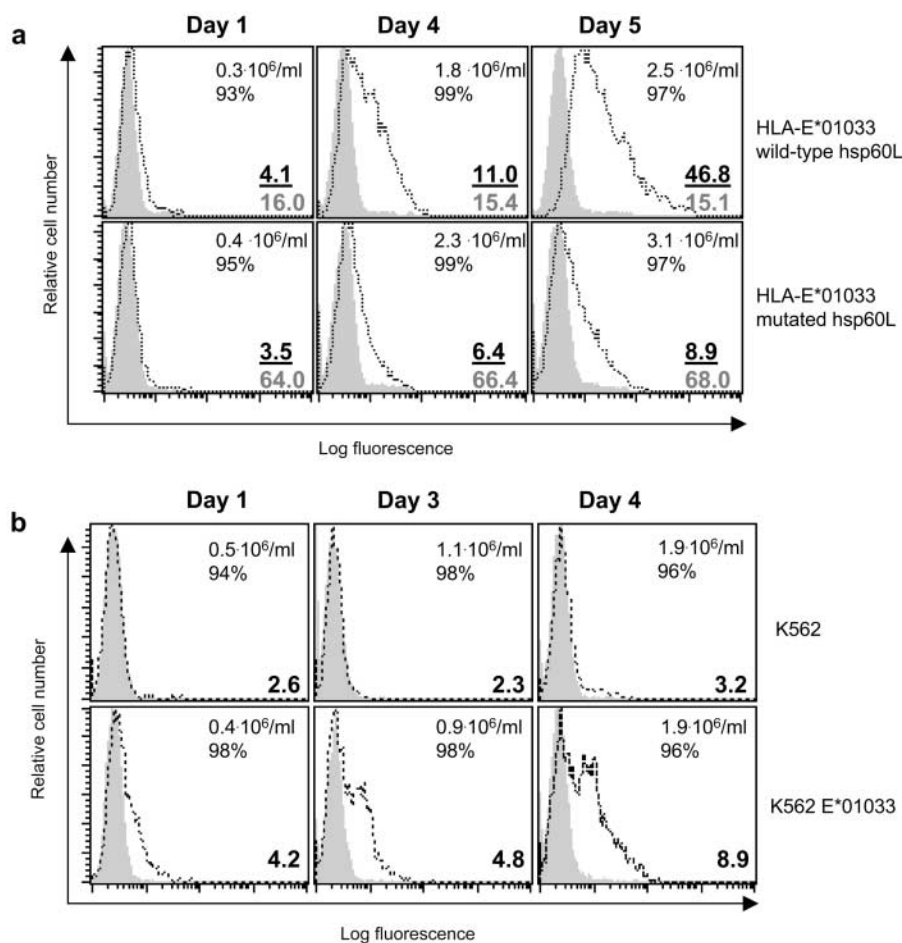
Figure 4. Up-regulation of HLA-E by overexpression of the full-length hsp60 signal peptide is enhanced by cellular stress. HLA-E surface expression was monitored on cells growing at increasing densities. Cells were collected and analyzed for HLA-E expression between day 1 and day 5 (as indicated on the top of the histograms). The numbers in the top right corner of each histogram indicate cell density (cells/ml) and percent viability at the time of analysis, respectively. The numbers in the bottom right corner of each histogram in panel a indicate the MFI of HLA-E expression (top, black) and the MFI of GFP (bottom, gray). The numbers in the lower right corner of each histogram in b indicate the MFI of HLA-E expression. All cells were stained with an HLA specific antibody (DX17, dashed line) or with control Ig (gray histogram), followed by RPE-conjugated goat anti–mouse IgG. (a) K562 cells cotransfected with HLA-E*01033 and the full-length (residues 1–26) wild-type hsp60 signal peptide-GFP construct (wild-type hsp60L, top panel) or mutant hsp60 signal peptide-GFP (mutated hsp60L, bottom panel), cultured at increasing cell density. A gate was set on GFP positive cells and 10 000 events were acquired within this gate. (b) K562 cells (top panel) and K562 transfected with HLA-E*01033 (K562 E*01033, bottom panel) cultured at increasing cell density. Note that the K562-E*01033 cell line in b and the cotransfected cell lines presented in panel a were generated and selected independently, which may account for the higher HLA-E background level observed at day 1. Therefore the absolute levels of HLA-E should not be directly compared between Fig. 4, a and b.

bation with hsp60sp did not result in any significant protection (Fig. 6 a). As mentioned above, hsp60sp and B7sp have different dissociation rates from HLA-E, which could account for the difference in target susceptibility. Therefore, the HLA-E surface expression was monitored before and after the cytotoxic assays, to assure comparable levels of HLA-E on the targets throughout the assays.

To pinpoint the residues responsible for the loss of HLA-E recognition by CD94/NKG2A, we introduced mutations in the B7sp and hsp60sp. It has previously been demonstrated that a change at p5R in the Qa-1[b] binding peptide Qdm abrogated recognition by CD94/NKG2A in the
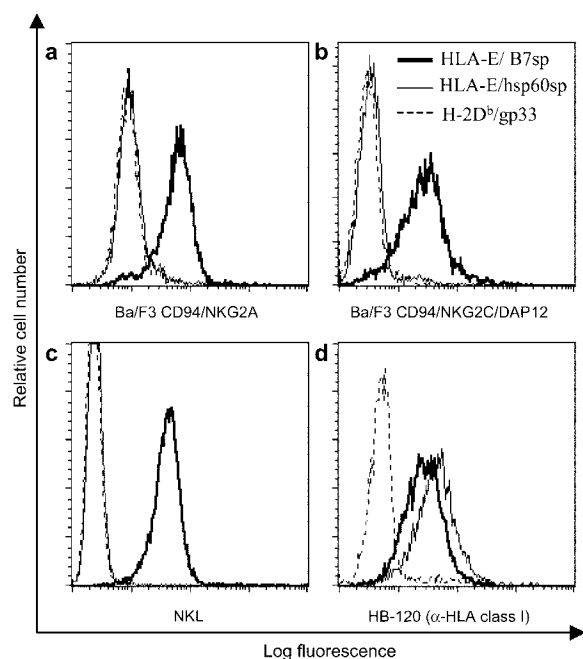

Figure 5. Binding of soluble HLA-E tetrameric molecules to CD94/NKG2 receptors. (a) Ba/F3 cells transfected with CD94 and NKG2A were incubated with HLA-E/B7sp tetramers- (bold line), HLA-E/hsp60sp-tetramers (thin line), or control H-2D[b]/gp33-tetramers (dashed line). (b) Ba/F3 cells transfected with CD94, NKG2C, and DAP-12 were incubated with HLA-E/B7sp-tetramers (bold line), HLA-E/hsp60sp-tetramers (thin line), or control H-2D[b]/gp33-tetramers (dashed line). (c) The NK cell line NKL was incubated with HLA-E/B7sp-tetramers (bold line), HLA-E/hsp60sp-tetramers (thin line), or control H-2D[b]/gp33-tetramers (dashed line). (d) HB-120 B cell hybridoma (anti–MHC class I) was incubated with HLA-E/B7sp-tetramers (bold line), HLA-E/hsp60sp-tetramers (thin line), or control H-2D[b]/gp33-tetramers (dashed line). All incubations were done at 4°C for 45 min in PBS supplemented with 1% FCS. HLA-E/hsp60sp-tetramers failed to bind both CD94/NKG2A^{+} and CD94/NKG2C^{+} cells over a range of HLA-E/hsp60sp-tetramer concentrations (not shown). This is one representative experiment of more than five.
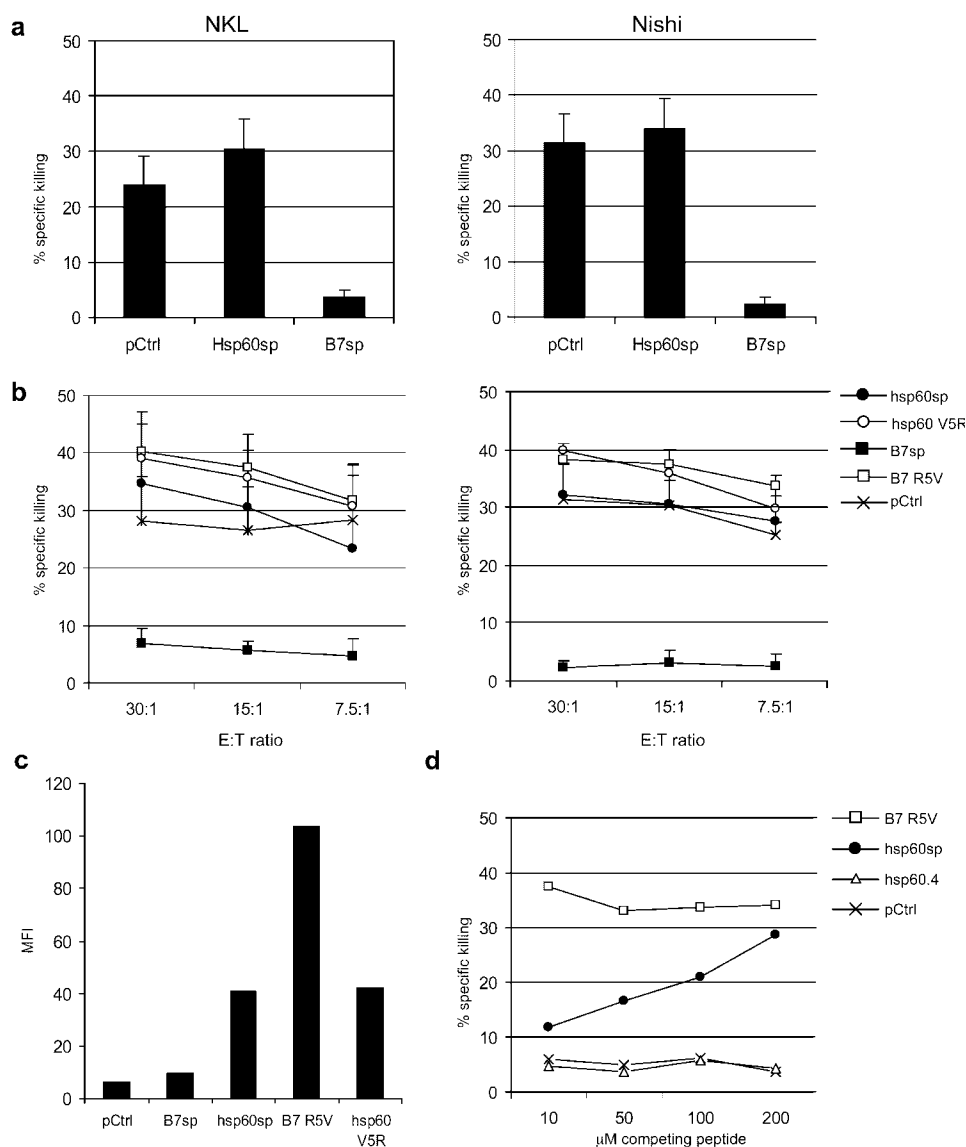
Figure 6. Hsp60sp fails to protect K562-E*01033 cells from killing by NK cells. K562-E*01033 cells were incubated with the different peptides at 26°C for 15–20 h, and then tested in 2h ^{51}Cr release assays. To ensure that the levels of HLA-E presenting a protective peptide, and not the HLA-E levels as such, provided the protective capacity we kept the nonprotective peptides, but omitted the B7sp, during the killing assays. (a) Killing of K562-E*01033 cells by NKL (left) or Nishi (right) after incubation with 300 μM P18I10 control peptide, 300 μM hsp60sp, or 30 μM B7sp. 50 μM of P18I10 control peptide and hsp60sp was also included during the assays. Data from an E:T ratio of 30:1 is shown. The figure represents the mean of at least three experiments. Error bars indicate standard error of the mean. (b) Killing of K562-E*01033 cells by NKL (left panel) or Nishi (right panel) incubated over night with 30 μM B7sp, 300 μM P18I10 (pCtrl), 300 μM B7 R5V, 300 μM hsp60sp, or 300 μM hsp60 V5R. 50 μM of all peptides, except B7sp, were included during the assay. Peptide concentrations were chosen according to (c). The figure represents the mean of at least three experiments. Error bars indicate standard error of the mean. (c) HLA-E cell surface expression by K562-E*01033 after the assay. A cold target preparation was prepared in parallel as in a and b, and was stained with DX17 mAb (anti–HLA class I), followed by RPE-conjugated goat anti–mouse IgG after the 2-h assay. One representative example out of more than five is shown. Note that, as in a and b, 50 μM of all peptides, except for B7sp, was present during the time of the assay, explaining the lower HLA-E expression with B7sp compared with Hsp60sp, Hsp60 V5R, and B7 R5V. (d) Killing of K562-E*01033 cells by Nishi after incubation for 30 min at room temperature with 0.1 μM B7sp and increasing amounts of competing peptides (hsp60sp, hsp60.4, B7 R5V, and P18I10). All the peptides were kept throughout the assay.

mouse (8). We therefore chose to mutate position 5 in both peptides, generating the peptides B7 R5V (VMAPVTVLL) and hsp60 V5R (QMRPRSRVL). The ability of these peptides to protect K562-E*01033 cells was tested in cytotoxic assays, as described above. K562-E*01033 cells incubated at 26°C with B7 R5V expressed high levels of HLA-E (Fig. 6 c), yet they were efficiently killed by CD94/NKG2A$^+$ NK cells (Fig. 6 b). This mutation is therefore sufficient to abrogate the protective capacity of B7sp. However, the V5R mutation introduced in hsp60sp was not sufficient to restore protection from killing using the same effector cells (Fig. 6 b).

Gays et al. recently reported that the hsp60.4 peptide (GMKFDRGYI) could bind to Qa-1^b, but did not induce protection from CD94/NKG2A$^+$ NK cells. However, this peptide failed to compete with the protective Qdm-peptide for binding to Qa-1^b, even when mixed in 100,000-fold excess with 1 nM Qdm (37). In contrast, we found that hsp60sp could interfere with HLA-E–mediated protection by competing with MHC class I signal peptides. K562-E*01033 cells were incubated with 0.1 μM B7sp together with increasing concentrations of competing peptides and tested in cytotoxic assays. Cells incubated with 0.1 μM B7sp and a control peptide remained protected from killing at all concentrations tested, whereas cells incubated with 0.1 μM B7sp and hsp60sp became more susceptible to killing with increasing concentrations of hsp60sp (Fig. 6 d). The B7 R5V peptide was an even stronger competitor than hsp60sp (Fig. 6 d). In line with the results on Qa-1^b peptide binding competition as reported by Gays et

al. (37), hsp60.4 was not able to compete with B7sp for binding to HLA-E (Fig. 6 d).

We also investigated whether the stress-induced HLA-E cell surface up-regulation observed in K562-E*01033 cells resulted in protection from NK cell–mediated lysis. K562-E*01033 cells grown at different densities were tested as targets in a 2 h chromium release assay with NKL and Nishi as effector cells. In spite of increased HLA-E levels, the killing increased rather than decreased, indicating that the HLA-E molecules induced on these cells were not protective. All target cells had a viability >90%, as measured by Annexin V staining and trypan blue (unpublished data). Moreover, and importantly, the cells grown at high density could be rescued from killing by addition of B7sp peptide (Fig. 7 b). This demonstrates that the increased killing was not terminally decided by the cell culture conditions, and that the HLA-E levels were sufficient for protection provided that an appropriate peptide was present. We conclude that HLA-E expression induced by stress is not sufficient to protect from NK cell–mediated killing. It should be noted that although K562 constitutively express MIC-A and MIC-B, ligands for the activating receptor NKG2D, these are not further up-regulated by the cellular stress imposed in these assays (unpublished data). Therefore, it is unlikely that the increased killing after cellular stress observed in some of our experiments is due to an increased expression of MIC-A or MIC-B. We cannot however exclude that up-regulation of other activating ligands, e.g. ULBP's (38), are responsible for the increase in killing.

Discussion

Our experiments using synthetic peptides demonstrate that HLA-E is capable of binding a peptide derived from the signal sequence of hsp60, and that such complexes cannot efficiently be recognized by inhibitory CD94/NKG2A receptors. This was shown by lack of binding of HLA-E/hsp60sp tetramers to CD94/NKG2A expressing cells and by NK cell–mediated killing of cells expressing such HLA-E/peptide complexes. Furthermore, our studies based on transfected cells suggest that hsp60sp can gain access to HLA-E molecules in vivo, particularly during conditions of cellular stress. We propose that the proportion of HLA-E in complex with this peptide is increased during stress, leading to a gradual shift in the HLA-E peptide repertoire from NK cell protective to nonprotective complexes. According to this model, NK cells can detect stressed cells during infectious and inflammatory responses, through surveillance of HLA-E/peptide complexes in a peptide selective manner. This could be of particular importance for the subset of NK cells uniformly expressing CD94/NKG2A as
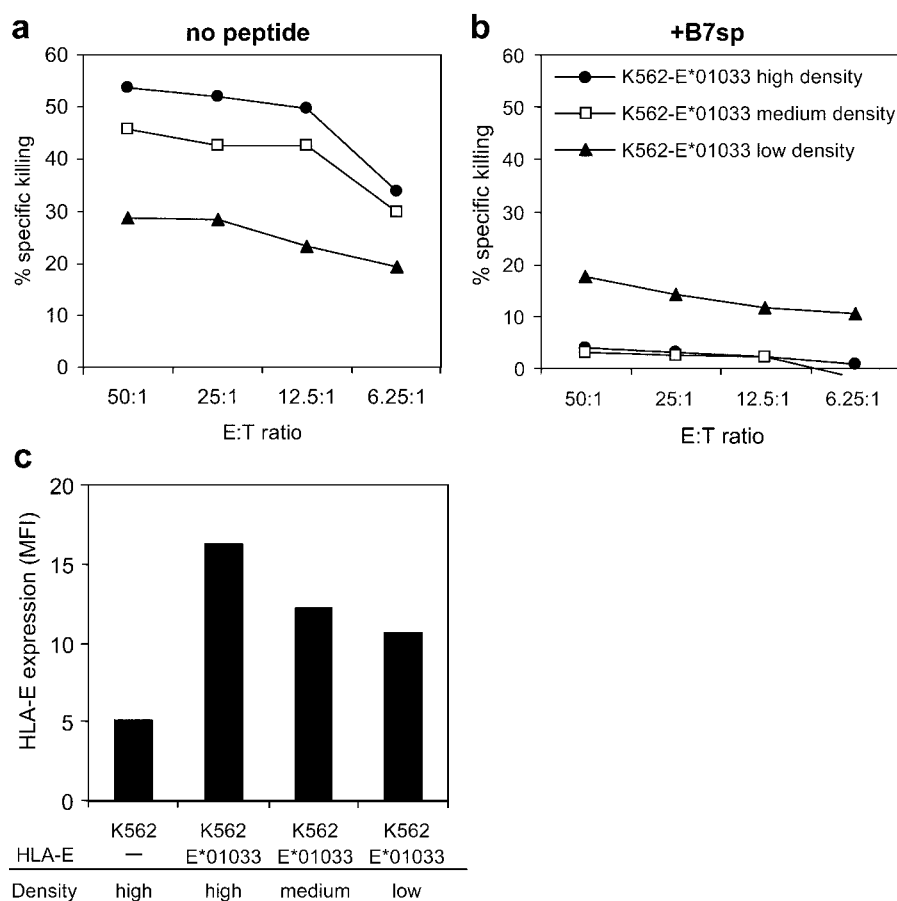

Figure 7. Increased HLA-E cell surface levels on K562-E*01033 after cellular stress does not protect from NK cell mediated killing. (a) Killing of K562-E*01033 cells (grown at increasing cell densities as in Fig. 4 b) by NKL in a 2-h ^{51}Cr release assay. (b) Same experimental setting as above, in the presence of 100 μM B7sp. Closed circles, high density; open squares, medium density; closed triangles, low density. (c) HLA-E expression on the K562-E*l01033 cells after culture at increasing cell density.

their main inhibitory receptor, and also for the subset of activated T cells that expresses this receptor.

It has previously been discussed whether missing self-recognition could be based on peptide-specific recognition, in the sense that normal self peptides in complex with MHC class I would be permissive for binding of inhibitory receptors, while viral and other nonself peptides would be nonpermissive. There is good evidence that some receptors are strongly influenced by the bound peptide. This applies to immunoglobulin-like (39–43) as well C-type lectin-like receptors, including CD94/NKG2A (8–11, 44, 45). However, the protective capacity does not correlate with the origin of the peptide, i.e., whether it represents self versus nonself, or healthy versus sick. The balance between different HLA-E complexes may, however, represent a situation where cells can signal "normal" versus "abnormal" via peptides competing for MHC dependent presentation. The HLA-E–mediated protection would thus not only rely on whether sufficient permissive signal peptides (mainly from various MHC class I molecules) are produced, but also on how these are balanced by nonpermissive, stress-induced peptides. Although KIR recognition of MHC class I can be influenced by the bound peptides, a mechanism based on peptide selective surveillance of stressed cells may be primarily associated with the CD94/NKG2 receptors, as these are specifically designed to recognize the oligomorphic HLA-E molecules in complex with a restricted set of protective peptides. The KIRs, on the other hand, have primarily evolved to recognize a highly diverse repertoire of polymorphic HLA-A, -B, and -C molecules. A similar surveillance mechanism of stressed cells, if operating via KIRs, would require the presence of a vast array of stress–induced peptides capable of being loaded onto each HLA class I allele.

A first question on such stress-induced peptide interference (SPI) with inhibitory recognition relates to the structural aspects of different HLA-E peptide complexes. The crystal structure of HLA-E/B7sp reveals that five peptide residues lie within well-defined pockets of the HLA-E molecule (17), constraining the conformation of the peptide throughout the binding groove. Comparison between hsp60sp and MHC class I signal peptide sequences (Table I) reveals differences at five positions: p1, p3, p5, p6, and p7. Of these, p3, p6, and p7 are buried in pockets D, C and E, respectively, while p1 and p5 are exposed to the surface. Based on the HLA-E/B7sp structure, O'Callaghan et al. proposed that p5R in B7sp acts as an HLA-E contact residue for an HLA-E binding receptor (17). Indeed, a change in B7sp from arginine to valine (corresponding residue in the hsp60sp) at position 5 was sufficient to completely abrogate HLA-E–mediated protection from killing by CD94/NKG2A expressing NK cells. However, the reciprocal change in hsp60sp (valine to arginine at p5) was not sufficient to gain protection, suggesting that additional amino acids in this peptide are important. This has drawn our attention to the arginines at positions 3 and 7, which appear difficult to fit in the shallow and hydrophobic D-

and E-pockets. We propose that the altered positioning of these side chains also may interfere with receptor binding, either directly or indirectly by changing the overall conformation of the peptide in the HLA-E groove.

The second important question concerns the biological relevance of HLA-E/hsp60sp complexes. Our evidence suggests that the increase of HLA-E levels observed during stress results from an influx of hsp60-derived peptides into the HLA-E presentation pathway. To critically investigate this, we cotransfected K562 cells with HLA-E*01033 and the full-length hsp60 signal sequence coupled to GFP (hsp60L–GFP). This resulted in mitochondrial expression of GFP, while HLA-E was expressed at high levels intracellularly but only at low levels at the cell surface. As predicted from the model, the cell surface HLA-E levels were increased in such cells when they were subjected to culture induced stress, as compared with controls transfected with HLA-E*01033 and a mutated hsp60L–GFP construct where a critical HLA-E anchor residue had been substituted. Furthermore, our model predicted an up-regulation of HLA-E also as a consequence of higher levels and altered distribution of endogenous hsp60sp during stress. In line with this, K562 cells transfected with HLA-E*01033 alone also displayed increased levels of cell surface HLA-E upon stress. Moreover, the up-regulation of HLA-E at the cell surface, as a result of stress, did not protect from NK cell–mediated killing in any of these experiments, as predicted. It should however be possible to regain HLA-E–mediated protection by adding a protective peptide, e.g. the B7sp peptide. Indeed, we could protect the stressed cells, simply by adding the protective B7sp peptide in the assay. This indicates, but does not prove, that endogenous hsp60sp can be presented by HLA-E during stress. Therefore, it remains to be formally proven that HLA-E is important as a presenter of stress-induced peptides for NK cells and T cells during infection and inflammation. The elution and sequencing of peptides from isolated HLA-E molecules of cells growing under normal conditions and cells exposed to various stress stimuli would be required to assess whether hsp60sp is indeed predominantly presented by stressed cells.

Our results further indicate that at least a part of the stress-induced increased accessibility of hsp60sp to HLA-E must be due to posttranscriptional factors. Such factors could involve changes in protease activities, a more efficient peptide transport from mitochondria to the ER, an altered distribution of hsp60, or changes in the permeability of the mitochondrial membrane. A majority (80–90%) of the hsp60 pool is localized in the mitochondrial matrix in healthy cells (35). There are, however, reports on increased levels of extra-mitochondrial hsp60 after bacterial infection (32) as well as after cellular stress and proapoptotic events (33, 34). These observations have been made with the mature hsp60, but at least the effects on mitochondrial permeability would also apply to the cleaved signal peptide.

In addition to altered mechanisms of peptide loading and increased expression of hsp60sp, other peptides capable of binding HLA-E may be up-regulated during stress. 10 yr

ago, Imani and Soloski reported that a brief heat treatment of L-cells increased the cell surface levels of Qa-1^b (36). Lo and coworkers recently reported that an hsp60-derived peptide (GMQFDRGYL in Salmonella, and GMK-FDRGYI in mouse) binds to Qa-1^b (21). In addition, we have confirmed that the peptide GMKFDRGYI (hsp60.4 in Table I) also can bind to HLA-E (unpublished data). In contrast to the hsp60sp, this peptide could not compete with the B7sp for binding to HLA-E (Fig. 6 d), nor could it compete for binding to Qa-1^b (37). It has also been demonstrated that Qa-1^b presenting hsp60.4 fail to protect the cells from NK cell–mediated lysis (37). These ligands are thus incapable of engaging CD94/NKG2A receptors, but can instead be detected by clonotypic T cell receptors during Salmonella infection in mice (21). It is therefore likely that also other hsp60-derived peptides may become HLA-E accessible during cellular stress provoked by an intracellular infection. Hypothetically, these peptides would divert the functional role of the HLA-E molecules as ligands for CD94/NKG2 receptors toward complexes being able to be recognized by certain T cells via their antigen-specific TCR during an infection.

It should be noted that T cells can also express CD94/NKG2A inhibitory receptors, and the balance between HLA-E molecules with hsp60sp and MHC class I signal peptide may therefore also modulate T cells in inflammatory responses. In this context our present findings provide an interesting extension to the recently published observations by Moser et al. (46), demonstrating that effector cytotoxic T lymphocytes directed against viral antigens may become restrained through expression of CD94/NKG2A. Recognition of Qa-1^b via this receptor inhibited proliferation and effector function of the T cells, with a dramatic influence on acute infection as well as oncogenesis by polyoma virus. The authors speculated that the peptide loading of Qa-1^b could be affected under pathological conditions, possibly influencing the interaction with restrained T cells. Our results demonstrate the loading of HLA-E with a peptide that is not only induced in stressed cells, but also interferes with the protection against CD94/NKG2A$^+$ NK cells normally conferred by HLA-E. This provides a potential explanation for the role of CD94/NKG2A expression during the regulation of T cells responses. The coexpression of this receptor would complement the TCR pathway in the discrimination between healthy and sick cells not only by sensing reduced production of MHC class I molecules but also increased accessibility to HLA-E of stress-induced peptides. Further studies to address this issue should test whether CD94/NKG2 expressing human T cells can be influenced by stressed induced changes in target cells. So far, and as expected, analysis of peripheral blood from healthy donors verified that the subset of CD94/NKG2A$^+$ T cells also binds to HLA-E/B7sp tetramers. Furthermore we have been unable to detect binding of HLA-E/hsp60sp tetramers to either CD94/NKG2A$^+$ or CD94/NKG2A$^-$ T cells (unpublished data), suggesting that T cells discriminate between different HLA-E complexes in the same way

as NK cells, and that T cells expressing a TCR specific for HLA-E/hsp60sp are not abundant in healthy individuals.

HLA-E molecules are recognized by CD94/NKG2A inhibitory and CD94/NKG2C activating complexes. The role of the activating forms has not yet been clearly defined. The possibility that HLA-E/hsp60sp complexes are recognized by CD94/NKG2C or another, unknown activating NK receptor is appealing. This could explain why stressed K562-E*01033 cells were killed more efficiently by NK cells, despite the increased HLA-E levels. However, the NKL cell line does not express the activating NKG2C receptor (unpublished data), and HLA-E/hsp60sp tetramers did not bind to CD94/NKG2C transfectants, nor to any NK cells examined. Thus, other ligands that trigger NK cell activating receptors may be involved. Our data show that neither MIC-A, or MIC-B, ligands for NKG2D, are up-regulated on culture stressed K562 or K562-E*01033 cells. However, additional ligands for NKG2D (e.g., ULBPs [38]), or other activating receptors, may influence the sensitivity of K562 and K562-E*01033 cells. Further experiments using reagents that specifically block activating NK cell receptors, may help to clarify the mechanism behind the increased NK cell sensitivity upon culture stress.

NK cells can be divided in two major subsets based on the level of CD56 cell surface expression (CD56^{dim} and CD56^{bright}; reference 47). Cells belonging to the minor CD56^{bright} subset all express high levels of CD94/NKG2A, and only a small fraction express KIRs. In contrast, a large majority of the CD56^{dim} NK cells express KIRs and display a lower cell surface level of CD94/NKG2A (48), although there are CD94/NKG2A$^+$, KIR$^-$ cells also in the CD56^{dim} subset. The phenotypical division between CD56^{dim} and CD56^{bright} NK cells is associated with different effector functions (49). When stimulated, CD56^{bright} NK cells are less cytototoxic, and more prone to cytokine production and have therefore been proposed to be immunoregulatory (49). These cells are potentially responsive to pro-inflammatory signals (based on their expression profile of chemokine receptors and adhesion molecules), and are largely over-represented at sites of inflammation (unpublished data). Moreover, macrophages have been reported to respond to human hsp60 with increased production of IL-12 and IL-15 (50), which are important activators of this NK cell subset. The findings presented here, and the fact that hsp60 is up-regulated during inflammation, suggest that HLA-E–mediated presentation of hsp60sp would primarily have an effect on CD94/NKG2A$^+$, KIR$^-$ NK cells in both major NK cell subsets described above, and in particular on cytokine production by the CD56^{bright} NK cells.

In summary, HLA-E could gradually change its peptide repertoire from NK cell protective to nonprotective complexes during infections and inflammatory responses. Such a mechanism could be important to decrease the threshold for NK cell activation during an immune response against infections, without necessarily involving a specific pathogen-induced cellular change. Thus, HLA-E plays a dual role as a marker for cellular health and integrity.

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