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*J Immunol* 2004; 173:5852-5862; doi: 10.4049/jimmunol.173.9.5852

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Identification of a Human HLA-E-Restricted CD8\(^+\) T Cell Subset in Volunteers Immunized with *Salmonella enterica* Serovar Typhi Strain Ty21a Typhoid Vaccine\(^1\)

Rosângela Salerno-Gonçalves,* Marcelo Fernandez-Viña,† David M. Lewinsohn,‡ and Marcelo B. Sztein\(^2*\)

Our previous studies in volunteers immunized with *Salmonella enterica* serovar Typhi (S. Typhi) have suggested an important role for CD8\(^+\) T cells in host defense. In this study we describe a novel subset of nonclassical human HLA-E-restricted S. Typhi-specific CD8\(^+\) T cells derived from PBMC of Ty21a typhoid vaccinees. CD3\(^+\)CD8\(^+\)CD4\(^-\)CD56\(^-\) T cells effectively killed S. Typhi-infected targets regardless of whether they share classical HLA class I molecules with them, by a FAS-independent, granule-dependent mechanism, as evidenced by induction of granzyme B release and the blocking effects of concanamycin and strontium ions. The expression of HLA-E Ags, but not CD1-a, -b, or -c, on the membrane of S. Typhi-infected targets rendered them susceptible to lysis. Moreover, anti-HLA-E Abs partially blocked these responses. We also demonstrated that presentation of S. Typhi Ags via HLA-E could stimulate IFN-γ production. Increases in the net frequency of IFN-γ spot-forming cells were observed in the presence of targets coated with peptides that contain S. Typhi GroEL HLA-E binding motifs. These results demonstrate that HLA-E binds nonamer peptides derived from bacterial proteins and trigger CD8\(^+\)-mediated lysis and IFN-γ production when exposed to infected targets, raising the possibility that this novel effector mechanism might contribute to host defense against intracellular bacterial infections. *The Journal of Immunology*, 2004, 173: 5852–5862.

Typhoid fever remains an important public health priority, particularly in developing countries, with an estimated 16 million new cases annually and 600,000 deaths (1, 2). The appearance of antibiotic-resistant strains of *Salmonella enterica* serovar Typhi (S. Typhi),\(^3\) the causative agent of typhoid fever, has added new urgency for the development of improved typhoid vaccines. New generation attenuated S. Typhi vaccine strains have the potential to become the preferred public health tool to immunize against typhoid fever because of their ability to elicit long-lasting protective systemic and mucosal immune responses. In addition, significant effort has been focused in recent years on the use of attenuated S. Typhi vaccines as carriers of foreign Ags to deliver foreign genes because of their ability to gain access to DC (3, 4) and deliver Ags or DNA coding for the Ags (5–7). These characteristics make the use of *Salmonella* live vectors particularly attractive for mass immunization programs. Moreover, live attenuated S. Typhi vaccines are expected to have relatively low manufacture costs compared with many other types of vaccines.

Our previous studies in volunteers immunized orally with attenuated strains of S. Typhi, including Ty21a, the only licensed attenuated live typhoid vaccine, as well as with the novel attenuated typhoid vaccine candidates strains CVD 908 or CVD 908-htrA, have suggested an important role for CD8\(^+\) T cells in host defense (8–10). They are capable of secreting IFN-γ and to kill S. Typhi-infected cells (cytotoxicity) (8–10). Therefore, elucidating the mechanism(s) involved in S. Typhi-specific CD8\(^+\) T cell responses is of paramount importance in understanding the host response to S. Typhi and in the design of improved live oral vaccines.

CD8\(^+\) CTL generally recognize Ag peptides in association with classical MHC class Ia molecules (HLA-A, -B, and -C in humans). However, a growing body of literature demonstrates the capability of nonclassical MHC class Ib molecules (HLA-E, -F, and -G) to mediate T cell recognition (11). In this regard, the presence of Qa-1-restricted CTL recognizing a GroEL epitope was shown in mice infected with *Salmonella typhimurium* (12). The Qa-1 locus in mice is syntenic with the human HLA-E locus, and Qa-1 gene products share properties with HLA-E (13). The human MHC class Ib HLA-E locus shows very limited polymorphism, and HLA-E molecules are present on the cell surface of virtually all PBMC (PBMC; including B cells, T cells, NK cells, and macrophages) with levels varying over an order of magnitude depending on the cell type (14, 15). Moreover, HLA-E expression has been detected in several nonlymphoid tissues, including liver, skin, lung, and, notably, placental cells (16). HLA-E molecules have been shown to interact with two cell subsets: NK cells (11) by CD94/ NKG2A, -B, and -C receptors (17) and, in a single report in humans infected with *Mycobacterium tuberculosis*, a subpopulation of CD8\(^+\) T cells (18) by an unknown receptor. In this study we investigated the mechanisms involved in killing of S. Typhi-infected targets by specific-CD8\(^+\) T cells using cells obtained from volunteers immunized with S. Typhi vaccine strains CVD 908 and CVD 908-htrA.

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*Abbreviations used in this paper: S. Typhi, *Salmonella enterica* serovar Typhi; B-LCL, EBV-transformed lymphoblastoid B cell line; CMA, concanamycin A; CSA-1, *Salmonella* common structural Ag; MFI, mean fluorescence intensity; rHIL-2, recombinant human IL-2; SFC, spot-forming cell.

\(^1\)This work was supported by a grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (R01AI36525; to M.B.S.).

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Received for publication November 18, 2003. Accepted for publication August 19, 2004.

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with Ty21a as a prototype of live attenuated typhoid vaccine. We identified a novel subset of nonclassical HLA-E-restricted CD3\(^+\)CD8\(^+\)CD4\(^-\)CD56\(^-\) CTL, which specifically killed S. Typhi-infected cells bearing HLA-E by using a granule-dependent mechanism.

**Materials and Methods**

**Subjects**

Seven healthy volunteers, 29–45 yr old, recruited from the Baltimore-Washington area and University of Maryland at Baltimore campus, participated in this study. They were immunized with four spaced doses of 2–6 × 10\(^5\) CFU of Ty21a at an interval of 48 h between doses (9, 19). PBMC were isolated by density gradient centrifugation (9). Volunteers were treated with antimicrobial treatment at the times of leukapheresis. Before the leukapheresis procedures, volunteers were explained the purpose of this study and signed informed consents.

**Metabolic inhibition of Ag presentation or recognition**

In some experiments, lactacystin (40 \(\mu\)M; Sigma-Aldrich, St. Louis, MO) or chloroquine (100 \(\mu\)M; Sigma-Aldrich) were added to target/stimulator cells 1 h before S. Typhi-infection (20). After 18 h, treated cells were used as described below. In other experiments, effectors were treated with strontium ions (Sr\(^{2+}\); Sigma-Aldrich) for 12 h before coculture with targets (21) or with matrix metalloproteinase inhibitor (KB8301, 10 \(\mu\)M; BD Immunocytometry Systems, San Jose, CA) (22) or concanamycin A (CMA; Sigma-Aldrich) (23) for 2 h before coculture with targets during the \(^{51}\)Cr release assay.

**Peptides**

Synthetic peptides used in this study were prepared by Synpep (Dublin, CA). These peptides included an HLA-E-restricted S. typhimurium GroEL CTL epitope identified in a mouse model (12) (GMQFDGRGYL) and six S. Typhi GroEL-derivated peptides selected based on the presence of anchor residues (underlined) shown to bind HLA-E molecules (AMLQDIATL, KMLRGVYNVL, VEGEALATL, AVEELAKL, AKVAKGKLP, KLQERVAKL) (14, 17, 24, 25). The M. tuberculosis GroEL peptide was used as negative control (GMRFDKGYI) (12). The purity (95%) of each peptide was confirmed by HPLC. For ELISPOT assays, 10\(^9\) target cells were coated with 30 \(\mu\)g of peptide for 2 h at 37°C before culture with effector cells.

**Blocking Abs and medium**

Anti-CD3 (clone OKT3) and anti-HLA class I (clone W6/32) mAbs were obtained from the American Type Culture Collection (Manassas, VA) (10, 26–28). The anti-HLA-E mAb was provided by Dr. D. Geraghty (3D12 clone; Fred Hutchinson Cancer Research Center, Seattle, WA) (14, 18) or purchased from Abcam (MEM-E06 and MEM-E02 clones; Cambridge, MA) (29). Anti-CD1d mAbs were obtained from commercial sources: anti-CD1d (clone NAI34) from Serotec (Oxford, U.K.), anti-CD1b (clone WM25) from Research Diagnostics (Flanders, NJ), and anti-CD1c (clone L161) from Serotec. Anti-CD95 (clone ZB4) and anti-CD8 (clone B9.11) were purchased from Immunotech (Marseilles, France). Anti-CD4 (clone RPA-T4) and anti-HLA-class II (clone L243) mAbs were purchased from BD Immunocytometry Systems. Culture medium consisted of RPMI 1640 (Invitrogen Life Technologies, Grand Island, NY) supplemented with 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 50 \(\mu\)g/ml gentamicin, 1 mmol/l-glutamine, 2.5 mM sodium pyruvate, 10 mM HEPES buffer, 1% nonessential amino acids, and 10% heat-inactivated FBS (R10).

**Target/stimulator cells**

EBV-transformed lymphoblastoid B cell lines (B-LCL), blasts, and macrophages were isolated from PBMC obtained from Ty21a vaccinees following standard procedures (8–10). Briefly, blasts were generated by incubating 5–10 \(\times\) 10\(^6\) PBMC with 1 \(\mu\)g/ml PHA-leucoagglutinin (PHA-L; Sigma-Aldrich) for 24 h in R10. PHA-activated PBMC were then washed three times with RPMI 1640 and cultured in complete RPMI supplemented with 20 IU/ml recombinant human IL-2 (rIL-2; Roche, Mannheim, Germany) for 5–6 days.

B-LCL were established by using B95-8 cell line (ATCC CRL1612; American Type Culture Collection) supernatants as the EBV source (10, 30). B-LCL were grown in culture in R10 or cryopreserved until used in the experiments.

The HLA class I-defective B cell line 721.221 as well its transfectants 721.221.AEH (which has been transfected with HLA-E fused to the HLA-A2 leader peptide, and therefore express the HLA-E*0101 allele on the cell surface), 721.221.F (which was transfected only with HLA-F), and 721.221.E6 (which was transfected with HLA-E without a leader peptide) were provided by Dr. D. Geraghty (14, 18). The 721.221 cell line is characterized by the lack of expression of HLA-class Ia A, B, and C due to gamma ray-induced mutation in the HLA complex. Thus, this cell line has complete absence of HLA-A, -B, and -C mRNA transcripts and \(\alpha\)-chains (31) and only express endogenous HLA-E (11, 14). Previous work has shown peptide-induced stabilization of HLA-E molecules on the surface of cold-treated 721.221 cells (14). 721.221 cells were cultured in R10, whereas the derivative transfected cells were cultured in R10 supplemented with 200 \(\mu\)M/ml hygromycin B (Sigma-Aldrich).

Macrophages were isolated as previously described (32). Briefly, PBMC (10\(^5\)/cm\(^2\)) were incubated for 2 h at 37°C in flasks coated with 2% gelatin (Sigma-Aldrich). Nonadherent cells were removed by washing four times with R10. Macrophages spontaneously detached after 60 h in culture. Detached cells were then washed three times with PBS/2.5 mM EDTA. Macrophages isolated by this technique were found to be >95% pure by flow cytometric analysis after staining with an mAb to CD14, a macrophage-specific membrane marker (clone MoP9; BD Immunocytometry Systems).

**Infection of target/stimulator cells by S. Typhi**

Targets were infected by incubation in RPMI (without antibiotics) for 3 h at 37°C with wild-type S. Typhi strain ISP1820 (wt S. Typhi) (9). We have previously shown that S. Typhi readily infects B-LCL and blasts and that S. Typhi Ags, as determined by flow cytometry, are expressed on the cell membrane (8–10). In some experiments, the following day stimulator cells were gamma irradiated (blasts, 4,000 rad; B-LCL and 721.221 cell lines, 6000 rad) and used as stimulators. For CTL assays, infected and noninfected cells were labeled with 200 \(\mu\)Ci of sodium chromate (\(^{51}\)Cr; American Biosciences, Piscataway, NJ) for 1 h at 37°C, washed, and used as targets.

Aliquots of targets were surface or intracellularly stained with mAbs to CD3 (clone UCHT1; Beckman Coulter, Miami, FL), CD19 (clone SJ25-C1; BD Immunocytometry Systems), HLA-E (clones 3D12 or MEM-E/06), and W6/32 and analyzed by flow cytometry (9). To confirm that targets were infected with S. Typhi, cells were stained with anti-Salmonella common structural Ag (CSA-1)-FITC (Kierkegaard & Perry, Gaithersburg, MD) (9).

**Effector cells**

For cytotoxicity assays, both ex vivo and in vitro expanded PBMC were used as positive controls. In vitro expanded effectors were obtained using a modification of a previously described technique (8, 9). Briefly, PBMC were cocultured with stimulator cells at an effector to stimulator cell ratio of 7:1 in R10 containing 60 IU/ml rIL-2 for 8–10 days. Aliquots of effectors were stained with flowcytometer-labeled mAbs to CD3, CD4 (clone SFC1274011; Beckman Coulter), CD8 (clone SK1), CD56 (clone B159), CD94 (clone HP-3D9; BD Immunocytometry Systems), and FasL (CD95L; clone NOK-1; Caltag Laboratories, Burlingame, CA) in various combinations and analyzed by multicolor flow cytometry using an EPICS Elite ESP flow cytometer/cell sorter system (Beckman Coulter) or a MoFlow flow cytometer/cell sorter system (DakoCytomation, Fort Collins, CO). The flowcytochrome-conjugated mAbs used are indicated in the corresponding figures.

For IFN-\(\gamma\) ELISPOT assays, both ex vivo and in vitro expanded PBMC (as described above) were used as effectors. In some experiments PBMC were fractionated into NK-depleted subsets using anti-CD56 microbeads (Miltenyi Biotec, Auburn, CA) or sorted by flow cytometry. Cell populations were >90% pure as determined by flow cytometric analysis.

**Cytotoxicity (chromium release test) and competitive inhibition assays**

Cytotoxicity was determined in a 4- or 2.5-h \(^{51}\)Cr-release assay against B-LCL or the 721.221 cell line and its transfectants, respectively (8–10, 33). Specific cytotoxicity mediated by effector cells was calculated by subtracting the lysis of uninfected targets from the lysis of S. Typhi-infected targets. Lysis of noninfected targets was generally <10 and 20% for B-LCL and the 721.221 cell line and its transfectant, respectively. The cutoff for positive responses in CTL assays was defined as >10% specific cytotoxicity (9). Competitive inhibition studies were conducted as previously described (9).

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Granzyme B and IFN-γ ELISPOT assays

The human granzyme B ELISPOT assay was performed using a commercial kit (BD Immunocytometry Systems) following the manufacturer’s recommendations. IFN-γ ELISPOT assays were performed as previously described (8, 9). Peptide-coated or S. Typhi-infected cells were used as stimulator cells. Effector cells cultured without stimulator cells or with CD3/CD28 beads (0.6 μl/ml; Dynal Laboratories, Great Neck, NY), were used as negative and positive controls, respectively. Each sample was tested in triplicate. The data were read with an automated ELISPOT reader (Biorad 3000 PRO; Bio-Sys, Karben, Germany). Net frequencies of spot-forming cells (SFC) were calculated as previously described (8, 9). The cut-off for assays was established as the frequency of granzyme B or IFN-γ SFC per 10⁶ PBMC in coculture of effectors with uninfected targets + 3 SE.

Peptide binding assay

Peptide-induced stabilization of HLA-E molecules on 721.221 cells was conducted as previously described (16). Briefly, 721.221 cells were cultured at 25.5°C. After 32 h, peptides were added to the cultures at final concentrations of 100 μM (100 μg/ml) and incubated at the same temperature for an additional 15 h. Cells were then incubated for 5 h at 37°C, centrifuged, washed twice, and incubated with anti-HLA-E (clone MEM-185, BD Immunocytometry Systems). The HLA-E concentration was determined as described in Materials and Methods. Results are shown as the mean percentage of specific cytotoxicity ± SD. Shown in A and C are experiments representative of at least two with similar results using cells from different donors.

Results

S. Typhi-infected target killing by CTL is granule mediated and depends on proteosome processing in APCs

To uncover the mechanisms of lysis involved in the killing of S. Typhi-infected targets, we evaluated the relative importance of Fas-FasL interactions and the granule-dependent pathway. The involvement of Fas-FasL interactions was evaluated by blocking experiments using mAbs to Fas (anti-CD95; clone ZB4) (21) or by the addition of the matrix metalloproteinase inhibitor KB8301, previously shown to augment the level of FasL expression on the cell surface of CTL (therefore increasing effector CTL activity) (22, 34). To this end, 8-day restimulated PBMC were cocultured with ⁵¹Cr-labeled S. Typhi-infected autologous B-LCL. We observed that the cytotoxicity mediated by CTL was not affected by a blocking anti-CD95 mAb (10 μg/ml) or by the addition of KB8301 (10 μM; Fig. 1A). To assess whether KB8301 treatment was effective despite having no impact on the cytotoxicity, we evaluated FasL expression on the cell surface of effector cells. To this end, 8-day restimulated effectors from volunteer 3 were incubated for 2 h at 37°C and stained with FasL-PE mAb (clone NOK-1), and FasL expression was measured by flow cytometry. In agreement with previous observations (22, 34), FasL staining intensity was higher in KB8301-treated than untreated effectors (Fig. 1B).

FIGURE 1. Identification of CTL cytotoxicity mechanisms. A, The ability of a blocking Ab to Fas (anti-CD95; ○) or a matrix metalloproteinase inhibitor (KB8301; ▼) to affect S. Typhi-specific cytotoxicity was determined. Effector cells from volunteer 3 expanded in vitro for 8 days were tested for lysis of autologous ⁵¹Cr-labeled S. Typhi-infected targets. Cells incubated with medium alone (●) were used as a control. B, Eight-day restimulated effectors from volunteer 5 were incubated in the presence or the absence of 10 μM KB8301 for 2 h at 37°C and stained with FasL-PE mAb. FasL expression on effector cells was measured by flow cytometry. Numbers correspond to the percentage of FasL-positive cells in the indicated quadrants in each histogram. C, CTL activity of volunteer 3 against autologous S. Typhi-infected targets after treatment of effectors with strontium ions (Sr²⁺; 2.5 mM; ○) or CMA (▼, 0.1 μM; ▲, 1 μM). Cells incubated with medium alone (●) were used as a control. D, Secretion of granzyme B in cocultures of effector cells from three different volunteers with S. Typhi-infected targets by ELISPOT. Results are shown as the mean net frequency of granzyme B SFC per 10⁶ PBMC ± SD. E, CTL activity of volunteers 1 and 3 against autologous S. Typhi-infected targets treated with lactacystin (□) or chloroquine (■). Cells incubated with medium alone (●) were used as a control. The dashed-dotted line represents the cut-off for positive ⁵¹Cr release assays and granzyme B ELISPOT determined as described in Materials and Methods. Results are shown as the mean percentage of specific cytotoxicity ± SD. Shown in A and C are experiments representative of at least two with similar results using cells from different donors.
The contribution of the granule-dependent pathway in the lysis of targets was evaluated by using strontium ions (Sr\(^{2+}\)) and CMA, which induce degranulation of CTL, transiently inhibiting lytic activity (23, 35). Preincubation with Sr\(^{2+}\) (2.5 mM) or CMA (0.1 to 1 µM) selectively inhibited the ability of CTL to kill infected targets (Fig. 1C). This inhibition was not the result of treatment toxicity. The Sr\(^{2+}\) or CMA treatment did not affect the viability of effector cells, as monitored by their ability to exclude trypan blue. Moreover, an ELISPOT assay detected the release of granzyme B, characteristic of cytotoxic granules, in cocultures of CTL with S. Typhi-infected targets (Fig. 1D). Taken together, these results indicate that the granule-dependent pathway is largely responsible for the killing of S. Typhi-infected targets by specific CTL.

To study whether S. Typhi Ags are processed and presented by target cells after a class I or class II MHC pathway, we evaluated the effects of two Ag presentation metabolic inhibitors: 1) lactacystin (40 µM), which affects HLA class I Ag processing by inhibition of proteasome processing (36); and 2) chloroquine (100 µM), a strong inhibitor of class II processing (20). Although cytotoxic activity was markedly blocked by lactacystin, chloroquine had no significant effect on S. Typhi-specific CTL activity (Fig. 1E). These results demonstrate that processing through the proteasome-dependent HLA class I pathway is required for the recognition of S. Typhi-infected targets by specific CTL.

Presence of HLA-class I unrestricted CTL against S. Typhi-infected targets

To characterize the HLA class I restriction of effector cells, PBMC were expanded for 8 days in vitro with S. Typhi-infected targets and screened against a panel of B-LCL targets either sharing or not sharing classical HLA-A, -B, or -C alleles. These results were not affected by NK cell depletion (data not shown). Of note, killing of autologous targets was found to be of equal or greater magnitude than that observed against allogeneic target cells. Moreover, as observed in the autologous system, preincubation of fully mismatched allogeneic targets with lactacystin, but not with chloroquine, was found to block CTL activity in volunteers 1 and 3 (data not shown).

FIGURE 2. Characterization of the HLA class I recognition pattern of effector cells. A, Specific lysis of S. Typhi-infected B-LCL by CTL. Effector CTL from volunteer S were tested on HLA autologous, class I-matched, or class I-mismatched (allogeneic) target cells. The percentage of specific lysis was calculated by subtracting the specific \(^{51}\)Cr release of uninfected target (controls). Each result is the mean percentage of specific cytotoxicity ± SD observed for each target at an E:T cell ratio of 10:1. B, CTL activity from additional volunteers was evaluated against HLA autologous (□) and allogeneic (■ and □) target cells. The dashed-dotted line represents the cut-off for positive \(^{51}\)Cr release assays.
Because 1) a previous study showed the presence of Qa-1-restricted CTL (the homologue of HLA-E in humans) in mice infected with S. typhimurium (12), and 2) the W6/32 mAb blocks both classical and nonclassical HLA class I molecules such as HLA-E (37), we explored the possibility that HLA-E molecules might play a role in mediating the CTL activity observed in Ty21a vaccinees. To this end, CTL were assayed for their ability to kill the S. Typhi-infected B lymphoblastoid cell line 721.221 (a cell line characterized by the lack of expression of HLA-A, -B, -C, or -G, which does not normally exhibit HLA-E at the surface (11), as well as its transfectants expressing in the cell membrane HLA-E (721.221.AEH) or HLA-F (721.221.F) (14, 18). Cells transfected with HLA-E that is expressed only intracellularly (E6) were also used as controls in these experiments. Surprisingly, CTL experiments indicated that although killing of 721.221.AEH cells was consistently higher, significant CTL activity was also observed against all cell lines (Fig. 4A). Specific killing of S. Typhi-infected 721.221.AEH cells was also observed using PBMC derived from three additional volunteers (Fig. 4B).

Previous reports showed that HLA-E expression on the cell membrane of 721.221 can be induced under certain conditions (11). To explore whether a similar phenomenon could explain our results, we investigated HLA-E expression in uninfected or S. Typhi-injected 721.221 cells targets cells upon incubation with effector cells. We reasoned that if HLA-E is indeed involved in mediating killing, the induction of its expression on the cell membrane should occur soon after exposure to targets. Because it takes at least 1 h at room temperature to prepare the cells for CTL assays, we evaluated HLA-E expression on targets after incubation with effectors for 30, 60, and 120 min at room temperature. After incubation, cells were stained on the surface and intracellularly with a polyclonal Ab recognizing CSA-1 and mAbs to CD3, CD19, and either HLA class I (clone W6/32) or anti-HLA-E (clones 3D12 (38) or MEM-E/06 (29)).

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Previous studies have

Presentation by HLA-E of S. Typhi-Ags to CD8+ T cells

The unexpected observations that effector cells can kill both autologous and allogeneic infected targets led us to investigate which Ag-presenting molecules are important in the generation of S. Typhi-specific CTL. In humans, there are several class I molecules other than classical HLA-A, -B, and -C that are involved in Ag presentation. These include CD1-a, -b, and -c, as well as HLA-E, -F, or -G (11). The results of experiments using anti-CD1-a (NA1/34 clone), -b (WM25 clone), and -c (L161 clone; 20 μg/ml) blocking mAbs (21) suggest that CD1-a, -b, and -c molecules are not involved in the killing of S. Typhi-infected targets by specific CTL (Fig. 3D).
shown that HLA-E expression can be detected in HLA-E-expressing cells (e.g., 721.221.AEH) using mAbs to either HLA class I (clone W6/32) or anti-HLA-E (clones 3D12 (38) or MEM-E/06 (29)), an observation that we confirmed in the present study (data not shown). Thus, based on availability, these mAbs were used as indicated to measure HLA-E expression on 721.221 cells or its derivatives. HLA-E expression was up-regulated on targets after 60 min of incubation (Fig. 5). Similar results were observed after 120 min (data not shown). In contrast, no significant HLA-E up-regulation was observed after 30 min of incubation (data not shown). Based on these findings, an incubation period of 1 h at room temperature was chosen for coculture assays in subsequent studies.

To determine whether an S. Typhi-specific T response is necessary for HLA-E up-regulation, uninfected and S. Typhi-infected cells were used as targets. As can be observed in Fig. 5, coculturing effector cells with either uninfected or infected targets induced high levels of surface expression of HLA-E in all targets (Fig. 5). Of note, the HLA-E up-regulation was more pronounced when cocultured with S. Typhi-infected cells than in the presence of uninfected targets. Intracellular staining also showed an up-regulation, albeit to a lesser extent than on the cell surface, of the expression of HLA-E upon exposure to effector cells in most cocultures, as evidenced by increases in the mean fluorescence intensity (MFI), particularly in cocultures of effector cells with infected targets (Fig. 5). To determine whether this phenomenon is also observed in cells susceptible to S. Typhi infection, we next evaluated the effects of exposure of purified macrophages isolated from human PBMC with effector cells. As shown in Fig. 6, HLA-E expression was up-regulated >2-fold on uninfected human macrophages after exposure to effector cells. Taken together, these data suggested that up-regulation of HLA-E could be induced by specific and nonspecific mechanisms upon coculture with effector cells.

To investigate whether the expression of HLA-E on the surface of nonexpressing cell lines upon exposure to effector cells is an active process, targets or effectors were treated with 4% paraformaldehyde before coculture. Targets failed to express HLA-E on their surface upon coculture with effectors when either population was treated with paraformaldehyde (Fig. 7A). The fact that CD8+ T cells are the effector cell population mediating specific CTL activity against allogeneic targets indicates that this phenomenon is restricted by class I molecules. In humans, in addition to classical HLA class Ia molecules, there are several nonclassical HLA class Ib molecules. Thus, to ensure that the observed effects are restricted by HLA-E rather than by other HLA class Ib molecules, we measured simultaneously the expression of HLA-E and other HLA-class Ib molecules (e.g., HLA-G and CD1-a, -b, and -c) on the cell surface of 721.221 targets after coculture with effector cells. Similar to our previous results described above, 48% of uninfected 721.221 targets cells and 75% of S. Typhi-infected 721.221 targets cells were found to express HLA-E Ags on their cell membrane after 1 h of incubation in the presence of effector cells. In contrast, no significant expression of HLA-G or CD1-a, -b, and -c molecules was observed on the same targets (data not shown).

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Modulation of HLA-E expression on human macrophages (Mφ) in the presence of effectors. Uninfected Mφ were cocultured with effector cells for 2 h at room temperature, stained with mAbs to CD14 and HLA-E (clone MEM-E/06), and analyzed by flow cytometry. Data are presented as HLA-E expression in Mφ (CD14+)-gated populations. Numbers in parentheses correspond to the MFI of HLA-E positive cells.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Modulation of HLA-E expression on the 721.221 cell line and its transfectants in the presence of effectors. Both uninfected and S. Typhi-infected cell lines were incubated with medium alone or with effector cells at an E:T cell ratio of 7:1. After 1 h, the cells were stained on the surface or intracellularly with anti-CSA-1, anti-CD3, anti-CD19, and anti-HLA-E mAbs and analyzed by flow cytometry. Data are presented as HLA-E expression in uninfected (CD19-CD3-CSA-1-) or S. Typhi-infected (CD19+CD3-CSA-1-) gated populations. Numbers correspond to the percentage of HLA-E-positive cells (clone MEM-E/06) in the indicated quadrants in each histogram, followed by the MFI in parentheses. Shown is an experiment representative of five performed with cells from different donors showing similar results.
Frequency of HLA-E-specific T cell effectors ex vivo or after an 8-day in vitro restimulation. A, Uninfected 721.221 cells were untreated (dotted line) or treated with 4% paraformaldehyde (dashed-dotted line) and cocultured with effectors at an E:T cell ratio of 7:1. Untreated (dashed line) or 4% paraformaldehyde-treated (full line) 721.221 cells cultured in medium alone or with 4% paraformaldehyde-treated effector cells (dashed-dotted-dotted line) were used as controls. After 1 h, cells were surface-stained with anti-CD3, -CD19, and -HLA class I (W6/32) mAbs and analyzed by flow cytometry. Data are presented as HLA class I-expressed cells in CD19−/CD3+−/gated populations. Values correspond to the percentage of positive cells in untreated 721.221 cells cocultured with untreated effectors. B, PBMC from five different volunteers were stimulated ex vivo with autologous blasts (■), 721.221 cells (□), 721.221.AEH cells (▲), or 4% paraformaldehyde-treated 721.221 cells (△) infected with S. Typhi. The frequency of effectors releasing granzyme B was determined by ELISPOT. Results are shown as the mean net frequency of granzyme B SFC per 10^6 PBMC ± SD. C, HLA-E expression on blasts and 721.221.AEH cells infected with S. Typhi. Cells were surface-stained with the anti-HLA-E 3D12 mAb (dashed-dotted-dotted line, blasts; full line, 721.221.AEH) or isotype control (dotted line, blasts; dashed line, 721.221.AEH), followed by a PE-labeled anti-mouse antiserum, and analyzed by flow cytometry.

Finally, it is unlikely that CD94, a molecule present on NK cells known to interact with HLA-E (16, 17, 39), is involved in killing by CD8^+^ T cells. Staining of effectors from two different volunteers showed that only 9−14% of CD8^+^ cells coexpressed CD94 after expansion. Taken together, these results provide strong evidence for the involvement of HLA-E molecules in killing of S. Typhi-infected targets by CD8^+^ T cells.

**Frequency of HLA-E-specific T cell effectors**

We next examined ex vivo the frequency of HLA-E-specific T effectors PBMC from different vaccinees. After S. Typhi infection, autologous blasts, 721.221 cells treated, or not, with 4% paraformaldehyde, and 721.221.AEH cells were cultured with unstimulated PBMC, and the frequency of specific T cells was measured by their release of granzyme B in an ELISPOT assay. Increases in the net frequency of granzyme B SFC were observed in coculture of autologous blasts with effectors from all five volunteers and in coculture with either untreated 721.221 cells or 721.221.AEH cells with effector cells from three of the five volunteers. Of note, in volunteers responsive to S. Typhi-infected 721.221, the frequency was in most cases lower than or equal to that against S. Typhi-infected blasts (Fig. 7B). However, it should be noted that the frequencies of effectors that release granzyme B to S. Typhi-infected autologous blasts or 721.221.AEH cannot be directly compared because of the differential expression of HLA-E on these cells. Considerably higher levels of HLA-E expression, as evidenced by increased MFI, were observed in the surface of S. Typhi-infected 721.221.AEH cells than on S. Typhi-infected blasts (Fig. 7C).

Coculture of effectors with either 4% paraformaldehyde-treated 721.221 targets (which fail to express HLA-E on their surface; Fig. 7, A and B) or with controls (medium only or uninfected targets; data not shown) showed no increases in granzyme B SFC. These results demonstrate that in addition to classical class I molecules, S. Typhi-specific T cell effectors can recognize bacteria-derived peptides presented by nonclassical HLA-E molecules in most volunteers.

**Blocking assays**

The involvement of HLA-E molecules in the killing of S. Typhi-infected targets and in the release of granzyme B by effector cells was further investigated by blocking experiments using mAbs to anti-HLA-E and anti-HLA class I. CTL activity against S. Typhi-infected 721.221.AEH targets (which only express the E*0101 allele) (16) was blocked to a considerable extent by an mAb to pan HLA class-I (10 μg/well; clone W6/32). Partial blocking, albeit to a lesser extent, was also observed using mAbs to anti-HLA-E (a mixture of clones MEM-E/06 and MEM-E/02; 10 μg/well of each one; Fig. 8A). Similar findings were observed using granzyme B as the functional measurement when S. Typhi-infected targets were preincubated with normal mouse Igs or with anti-HLA-E mAbs (clones MEM-E/06 and MEM-E/02; 33 μg/10^6 cells, 16.5 μg each) and cocultured with ex vivo PBMC from two separate volunteers (Fig. 8B).

**Peptide binding**

In an effort to identify HLA-E-restricted peptides that might be involved in mediating the killing of S. Typhi-infected targets, we initially evaluated the peptide-induced stabilization of HLA-E molecules on 721.221 cells by performing flow cytometric analysis with an anti-HLA-E mAb (clone MEM-E/06) on 721.221 cells. As shown in the Fig. 9, HLA-E expression was induced when 721.221 cells were incubated in the presence of the selected peptide, but not when cells were incubated in medium alone. The magnitude of the shift in fluorescence intensity of HLA-E surface expression in this experiment is similar to that observed in other HLA-E studies using the 721.221 cell line (14). Based on these results, we next examined the frequency of effector cells able to release granzyme B upon exposure to 721.221.AEH cells coated with these peptides known to contain HLA-E binding motifs. These peptides included an HLA-E-restricted S. typhimurium GroEL CTL epitope identified in a mouse model (12) (GMQFDRGYL) and six additional peptides selected based on the presence of anchor residues shown to bind HLA-E molecules (14, 17, 24, 25). Significant increases in the net frequency of granzyme B SFC were observed in presence of targets coated with four of the seven peptides used (Fig. 10). No increases were observed in the presence of
targets coated with an *M. tuberculosis* Gro-EL peptide bearing an HLA-E-binding motif, which was used as a control (KLQERVAKL). These results show that several peptides derived from GroEL are recognized in the context of HLA-E molecules in *S.* Typhi-infected targets.

We have previously shown in volunteers immunized with the Ty21a typhoid vaccine as well as with the attenuated typhoid vaccine candidate strains CVD 908 or CVD 908-htrA that *S.* Typhi-specific CD8+ T lymphocytes can secrete IFN-γ in response to stimulation with autologous *S.* Typhi-infected targets (8–10). To evaluate whether HLA-E-restricted presentation of peptides derived from *S.* Typhi GroEL could also stimulate IFN-γ production, we cocultured effector cells with either autologous B-LCL or 721.221.AEH cells coated with four selected HLA-E binding peptides and evaluated IFN-γ production by ELISPOT assay. An increase in the net frequency of IFN-γ SFC was observed in the presence of either B-LCL or 721.221.AEH targets coated with three of the peptides studied (Fig. 11). Of note, in control experiments using peptide-coated 4% paraformaldehyde-treated 721.221.AEH cells as targets showed that although their Ag-presenting capacity was slightly reduced compared with that of non-treated targets, they still retained Ag-presenting capacity after paraformaldehyde fixation (data not shown). In conclusion, as observed in autologous systems, HLA-E-restricted *S.* Typhi-derived peptides.

**Discussion**

In this study we observed two specific modes of recognition of *S.* Typhi-infected target cells by CD3+CD8+CD4+CD56+ CTL effectors derived from PBMC of healthy adults orally immunized with the Ty21a typhoid vaccine: 1) classical HLA class-Ia restriction, and 2) a novel nonclassical HLA-E-restriction mechanism. These nonclassical HLA-restricted CD3+CD8+CD4+CD56+ T cells are able to produce IFN-γ in response to targets coated with *S.* Typhi GroEL-derived peptides.

**FIGURE 8.** Ability of anti-HLA-E and anti-class I mAbs to block *S.* Typhi-specific CD8+ responses. A, mAb to HLA class I (clone W6/32) were used to block CTL activity in a 51Cr release assay. Eight-day in vitro expanded effector cells from volunteer 5 were tested against *S.* Typhi-infected 721.221.AEH cells that were preincubated with normal mouse Ig (nmIg; ), a mix of MEM-E/06 and MEM-E/02 anti-HLA-E mAbs ( ), and W6/32 anti-class I mAb ( ). B, mAb to HLA-E (mixture of clones MEM-E/06 and MEM-E/02) were used to block the release of granzyme B by effectors, as determined in an ELISPOT assay. Ex vivo effector cells from two different volunteers were tested against autologous blasts infected with *S.* Typhi that were preincubated with normal mouse Ig (mouse IgG, control; ) or anti-HLA-E mAb ( ). Numbers represent the percent inhibition of granzyme B released in the presence of anti-HLA-E mAbs ± SD.

**FIGURE 9.** Ability of HLA-E binding peptides to stabilize surface expression of HLA-E molecules. Peptide-induced stabilization of HLA-E molecules on 721.221 cells was evaluated by flow cytometric analysis using an anti-HLA-E mAb (clone MEM-E/06). 721.221 cells were cultured at 25.5°C, and peptide was added as described in Materials and Methods. The expression of HLA-E on 721.221.AEH cells was included for comparison.
CTL specifically killed S. Typhi-infected cells bearing HLA-E using a FAS-independent, granule-dependent mechanism. It is unclear why the immune system exhibits this dichotomy of recognition of S. Typhi-infected targets. Because the nonclassical class Ib molecules (i.e., HLA-E) are less polymorphic than classical class Ia HLA molecules, they are likely to present a more conserved set of peptides present in many microorganisms. Thus, it is reasonable to speculate that HLA-E-restricted CTL might bridge the innate and adaptive immune responses and represent a mechanism complementary to classical class Ia-restricted responses in protection of volunteers from typhoid infection. This contention is supported by the observation that coculturing effector cells with either uninfected or infected targets induced up-regulation of HLA-E, although this up-regulation was always more pronounced in S. Typhi-infected than in uninfected targets. However, more studies are necessary to confirm whether HLA-E up-regulation involves both specific and nonspecific mechanisms. Of note, it has previously been shown that kinetic responses of class Ia and class Ib effector populations differ. For example, the peak response of class Ia-restricted CD8⁰ T cells in mice infected with Listeria monocytogenes has been reported to occur a few days later than the peak of class Ib-restricted CD8⁰ T cells (40).

The precise frequency of classical HLA class Ia-restricted and nonclassical HLA-E-restricted effectors in vivo is currently unknown. Due to the invariant nature of HLA-E molecules, a striking difference between the classical and nonclassical CTL is the ability of HLA-E-restricted effectors to kill both autologous and allogeneic S. Typhi-infected targets, whereas classical CTL killing is restricted to killing targets sharing the same HLA class Ia specificities. Thus, the ⁵¹Cr release assays using autologous and allogeneic blasts in the same experiments provided an opportunity to preliminarily assess the relative magnitude of HLA classical and nonclassical restricted T cell responses in immunity to S. Typhi infection. In these experiments CTL activity was generally greater against autologous than against allogeneic S. Typhi-infected blasts, suggesting that the frequency of nonclassical CTL might be lower than that of classical class Ia-restricted T cells at the time points examined. Additional evidence supporting this conclusion can be derived from blocking experiments. Anti-HLA-E mAbs were able to partially block CTL activity, whereas the pan anti-class I mAb W6/32, which blocks classical and nonclassical restricted responses, almost completely abrogated CTL against S. Typhi-infected targets (10) (Fig. 3). Even though the possibility that the anti-HLA-E mAbs used in our studies were weak blockers cannot be ruled out, our results are supported by those reported by Heinzel et al. (18), who, in unpublished data, found that mAb to pan-HLA I (clone W6/32) were capable of inhibiting CD8⁰ T cell-dependent recognition in an HLA-E-restricted system. Unfortunately, the frequencies of effectors that release granzyme B to S. Typhi-infected autologous blasts or S. Typhi-infected 21A.AEH cannot be directly compared because of the differential expression of HLA-E between these cells (i.e., 721.221.AEH cells express considerably higher numbers of HLA-E molecules than blasts; Fig. 7C). It has been reported that CD8⁰ T cell responses against infected targets is dependent on the level of expression of HLA class I molecules. For example, a known mechanism of evasion of the host’s immune system involves both specific and nonspecific mechanisms. Of note, it has previously been shown that kinetic responses of class Ia and class Ib effector populations differ. For example, the peak response of class Ia-restricted CD8⁰ T cells in mice infected with Listeria monocytogenes has been reported to occur a few days later than the peak of class Ib-restricted CD8⁰ T cells (40).

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**FIGURE 10.** Identification of HLA-E binding peptides. After 8 days of expansion, effectors from volunteer 3 were cocultured with 721.221.AEH cells loaded with peptides whose sequences were derived from the S. Typhi GroEL sequence based on known HLA-E motifs. Peptides sequences are given as the single-letter code for amino acids, and the predicted anchor residues are shown in bold. The net frequencies of granzyme B SFC were assessed by a granzyme B ELISPOT assay, and results were calculated as described in Materials and Methods. *, M. tuberculosis GroEL peptide was used as a control. The dashed line represents the cut-off for positive ELISPOT assays, determined as described in Materials and Methods. Results are shown as the mean net frequency of granzyme B SFC per 10⁶ PBMC ± SD. Included is an experiment representative of at least two performed with similar results using cells from different donors.

**FIGURE 11.** Frequency of IFN-γ-producing cells in the presence of targets coated with HLA-E-binding peptides. After 8 days of expansion, effectors from volunteer 1 were cocultured with (A) B-LCL cells or (B) 721.221.AEH cells loaded with peptides whose sequences were derived from the S. Typhi GroEL sequence based on known HLA-E motifs. Peptides sequences are given as the single-letter code for amino acids, and the predicted anchor residues are shown in bold type. The net frequencies of IFN-γ SFC were assessed by an IFN-γ ELISPOT assay, and results were calculated as described in Materials and Methods. *, M. tuberculosis GroEL peptide was used as a control. The dashed line represents the cut-off for positive ELISPOT assays determined as described in Materials and Methods. Results are shown as the mean net frequency of IFN-γ SFC per 10⁶ PBMC ± SD. Included is an experiment representative of at least two performed with similar results with cells from the same donor using different target cells.
system through suppression of CD8+ responses is the down-regulation of expression of HLA class I molecules during viral infections and with tumors (41–44). Of note, down-regulation of the expression of HLA class I molecules by enterobacterial infection, including Salmonella, Yersinia, and Klebsiella infection, has also been reported (45). In this regard, it is important to note that exposure of human primary macrophages to effector cells resulted in enhanced expression of HLA-E on their cell surface. A phenomenon that might lead to enhanced killing of S Typhi-infected macrophages by HLA-E specific T cells.

Flow cytometry results of HLA-E expression before and after exposure of targets to effector cells demonstrates that only the expression of HLA-E, not that of other HLA class Ib molecules (e.g., HLA-G, CD1-a, -b, and -c), is up-regulated on the cell surface of 721.221 targets after coculture with effector cells. Moreover, the fact that pretreatment of 721.221 target cells with 4% paraformaldehyde precluded the expression of HLA-E on their surface indicates that this is an active mechanism. However, it is unknown whether CD8+ T cells induce HLA-E up-regulation by cell-cell contact and/or by the release of soluble factors, leading to the expression of endogenous HLA-E molecules on the cell membrane. We are currently investigating this phenomenon in detail.

Our results also demonstrate that HLA-E molecules are not only ligands for the NK inhibitory receptors CD94/NKG2A, -B, and -C, as previously reported (16, 17, 39), but they also bind nonamer peptides derived from bacterial proteins and mediate the lysis of targets and IFN-γ production by CD8+ T cells. These results raise the possibility that these effectors might contribute to host defense against S Typhi infection by directly eliminating infected targets as well as producing IFN-γ, both of which are key effector mechanisms against intracellular pathogens (46). This proposition is supported by a recent report demonstrating that class Ia MHC-deficient mice are able to generate class Ib MHC-restricted CD8+ T cell-mediated protective immunity against L. monocytogenes infection (47). Even a limited number of class Ib MHC-restricted T cells were sufficient to generate the rapid recall response required for protection against secondary infection with L. monocytogenes. Moreover, because we observed that only a small subset of CD8+ effector cells express CD94, it is unlikely that this molecule, which plays a key role in NK cell recognition, is involved in T cell recognition of HLA-E peptide on APCs infected with S Typhi.

Finally, it is important to highlight that our results together with a recent study in patients infected with M. tuberculosis (18) constitute the only evidence to date in humans of the presence of HLA-E restricted, CD8+ T cells during intracellular bacterial infections, a novel effector mechanism that might contribute to host defense.

Acknowledgments
We are indebted to the volunteers who allowed us to perform this study. We also thank Dr. Daniel E. Geraghty (Fred Hutchinson Cancer Research Center) for his generous gift of anti-HLA-E mAb and cell lines, Dr. Myron M. Levine for critical review of the manuscript, Dr. Bernadette McConnell and the staff from the Blood Bank of Maryland University hospital for their help in collecting leukapheresis specimens, and Regina Harley for excellent technical assistance with flow cytometric determinations and sorting.

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