Nonclassical MHC class Ib–restricted cytotoxic T cells monitor antigen processing in the endoplasmic reticulum

Niranjana A Nagarajan, Federico Gonzalez & Nilabh Shastri

The aminopeptidase ERAAP is essential for trimming peptides presented by major histocompatibility complex (MHC) class I molecules. Inhibition of ERAAP by cytomegalovirus results in evasion of the immune response by this virus, and polymorphisms in ERAAP are associated with autoimmune disorders. How normal ERAAP function is monitored is unknown. We found that inhibition of ERAAP rapidly induced presentation of the peptide FYAEATPML (FL9) by the MHC class Ib molecule Qa-1b. Antigen-experienced T cells specific for the Qa-1b–FL9 complex were frequent in naive mice. Wild-type mice immunized with ligands for circulating CD8+ T cells or natural killer (NK) cells1,3.

Peptide transport by TAP causes considerable loss of pMHCI from the cell surface3. The absence of pMHCI results in loss of recognition by CTLs. The role of ERAAP in generating peptides for presentation by MHC class Ib molecules was unclear. However, the pMHCI ligands expressed by ERAAP-deficient cells that activate CTLs remain undefined. Here we used T cells as probes to analyze the immunogenic pMHCI expression on the cell surface14–16. Nevertheless, loss of ERAAP function does not generally cause a large change in pMHCI expression on the cell surface14–16. However, the pMHCI ligands expressed by ERAAP-deficient cells that activate CTLs remain undefined. We found that cells lacking ERAAP elicited CD8+ T cells specific for both classical pMHCIa and nonclassical pMHCIb complexes. We identified the highly conserved peptide FYAEATPML (FL9) presented by the MHC class Ib molecule Qa-1b exclusively in ERAAP-deficient cells. Notably, we found that abundant antigen-experienced T cells specific for the
Qa-1β−FL9 complex existed even in naive wild-type mice and proliferated to yield cytotoxic effector cells that rapidly eliminated cells lacking ERAAP in vitro as well as in vivo. Thus, nonclassical MHC class Ib molecules are sensors for defects in peptide processing in the endoplasmic reticulum.

RESULTS

ERAAP-deficient cells present immunogenic MHC class Ib ligands

The immune system efficiently detects differences between self and non-self. Therefore, to identify immunologically important changes caused by ERAAP deficiency, we immunized wild-type (C57BL/6J) mice with MHC-matched spleen cells from ERAAP-deficient (ERAAP-KO) mice.14 We generated wild-type CD8+ T cells directed against ERAAP-KO spleen cells by restimulating host spleen cells with ERAAP-KO antigen-presenting cells (APCs) in vitro (which resulted in 'anti-ERAAP-KO' cells). Similar to published results15, CD8+ T cells generated from immunized mice responded strongly by producing more interferon-γ (IFN-γ) and tumor necrosis factor (TNF) when cultured with ERAAP-KO APCs than their background responses to self APCs (Fig. 1a,b and Supplementary Fig. 1). Similar fractions of the same CD8+ T cells responded to ERAAP-KO cells that also lacked any classical H-2Kb or H-2Db MHC class Ia molecules18 (ERAAP-MHC-Ia-TKO cells; 30% versus 25%, on average; Fig. 1a,b). MHC class I molecules were nevertheless required for the IFN-γ response, because APCs that lacked both ERAAP and β2-microglobulin, an essential subunit of all MHC class I molecules19,20, failed to stimulate wild-type anti-ERAAP-KO CD8+ T cells (Fig. 1b). Likewise, APCs deficient in both ERAAP and TAP19 also failed to stimulate CD8+ T cells (Fig. 1b), which demonstrated that their responses required peptide(s) transported into the endoplasmic reticulum. Therefore we concluded that loss of ERAAP function resulted in the presentation of immunologically distinct peptides by MHC class Ia and class Ib molecules to wild-type CD8+ T cells.

To further characterize those MHC class Ib ligands expressed by ERAAP-KO cells, we generated the monoclonal CD8+ T cell hybridoma BEko8Z, with inducible expression of β-galactosidase (LacZ) in response to triggering of the T cell antigen receptor (TCR), by fusing wild-type anti-ERAAP-KO CD8+ T cells with BWZ.36-CD8α cells (TCRαβ+ BW5147 thymoma cell line with TCR-inducible expression of lacZ that expresses the coreceptor CD8α)21. The BEko8Z hybridoma produced lacZ when cultured with ERAAP-KO cells but not when cultured with wild-type splenic APCs (Fig. 1c, left). Consistent with our earlier observations (Fig. 1a), the ligand for BEko8Z T cells was expressed by ERAAP-KO APCs and did not require expression of classical MHC class Ia molecules (Fig. 1c, middle). BEko8Z cells did not respond to ERAAP-KO APCs that lacked β2-microglobulin or TAP (Fig. 1c). Thus, the BEko8Z hybridoma was specific for a TAP-transported peptide that was presented by a nonclassical MHC class Ib molecule exclusively on the surface of ERAAP-KO cells.

Mice express a large number of nonclassical MHC class Ib molecules that serve as ligands for various innate as well as adaptive immune responses2. Among the three MHC class Ib molecules known to present peptides, we considered H-2M3 the least likely because this molecule presents amino-formylated peptides that should be refractory to the aminopeptidase activity of ERAAP. We tested the other two molecules, Qa-1 and Qa-2, as potential ligands for BEko8Z T cells. We used splenocytes from Qa-1β-deficient mice22 and Qa-2-deficient (B6.K1) mice23 as APCs, after treating them with leucinethiol, a potent inhibitor of aminopeptidases, including ERAAP23 (Fig. 1d). The BEko8Z hybridoma responded to leucinethiol-treated splenocytes from wild-type mice as well as those from Qa-2-deficient B6.K1 mice but failed to recognize untreated splenocytes or those from Qa-1β-deficient mice (Fig. 1d). We further confirmed the requirement for Qa-1β by showing that BEko8Z cells did not respond to APCs from mice with genetic deficiency in both ERAAP and Qa-1β (Fig. 1e). We concluded that BEko8Z T cells recognized a unique peptide presented by the MHC class Ib molecule Qa-1β on ERAAP-KO APCs.

Presentation of FL9 by Qa-1β to BEko8Z T cells

Qa-1β is best known for presenting the Qdm peptide derived from the endoplasmic reticulum–translocation signal sequence of classical MHC class Ia molecules24,25. The finding that the BEko8Z hybridoma recognized ERAAP-MHC-Ia-TKO APCs that lack H-2Db, which is the source
of the Qdm peptide in C57BL/6 mice, suggested that BEko8Z T cells were specific for a different peptide. Additionally, neither a synthetic Qdm peptide (AMAPRTLLL) nor its analogs with amino-terminal extension were able to stimulate BEko8Z T cells (data not shown).

Next we identified the naturally processed peptide presented by Qa-1b to BEko8Z cells by screening cDNA libraries prepared from ERAAP-KO cells. To increase the efficiency of the screen, we first determined that CD11c+ B220+ dendritic cells from ERAAP-KO spleens were better APCs for stimulating BEko8Z than were unfractionated spleen cells or other splenocyte subsets (Supplementary Fig. 2). We constructed a cDNA expression library with mRNA from splenic dendritic cells isolated from ERAAP-KO mice treated with B16 cells expressing the cytokine Flt3L. We fractionated the cDNA library into small pools and transfected those into Qa-1b-expressing L cells (H-2b mouse fibroblasts). Because the BEko8Z ligand was generated only in the absence of ERAAP, we treated the transfected cells briefly with leucinethiol to inhibit aminopeptidases and then assessed their ability to stimulate the BEko8Z hybridoma. One of the 2,880 pools screened stimulated the BEko8Z T cell hybridoma (Fig. 2a). After rescreening 24 individual cDNA clones from that cDNA pool, we identified three stimulatory clones and chose clone 23D9.15 for further analysis (Supplementary Fig. 3). BEko8Z T cells responded more strongly to APCs cotransfected with 23D9.15 cDNA and Qa-1b cDNA and treated with leucinethiol than to APCs treated with dithiothreitol alone (Supplementary Fig. 4), which suggested that the 23D9.15 plasmid encoded the antigenic peptide. All the stimulatory cDNAs encoded the ‘hypothetical protein’ Fam49b of the National Center for Biotechnology Information database (accession code NM_144846).

The motif of the peptides that binds Qa-1b is unknown. Therefore, to identify the minimal antigenic peptide in Fam49b, we cloned a series of deletion constructs of 23D9.15 cDNA into mammalian expression vectors and assessed their ability to generate the BEko8Z ligand (Fig. 2b). The antigenic activity was produced in cells transfected with cDNA fragments R1 and R4 of Fam49b. In the next series of truncations of DNA fragment R4, we found that the carboxyl-terminal leucine encoded by fragment R13 was absolutely essential for T cell–stimulatory activity, as truncated R14 DNA or R15 DNA, which lacked sequence encoding this leucine residue, was unable to stimulate the T cell hybridoma (Fig. 2c). The antigenic activity thus mapped to the carboxyl terminus of peptide encoded by DNA fragment R13.

**Natural processing and presentation of FL9 by Qa-1b**

To establish the identity of the antigenic peptide and define its precise boundary at the amino terminus, we assessed the ability panel of synthetic peptides of various lengths, which all terminated at the same essential leucine residue, to activate BEko8Z cells (Fig. 3a). The nine-residue peptide FYAEATPML (FL9) and the ten-residue peptide LFYAEATPML (LL10) were the most potent stimulators of BEko8Z T cells (Fig. 3a). In contrast, further addition to or deletion of one or two residues from the amino terminus of FL9 (11- and 12-residue peptides or 8- and 7-residue peptides, respectively) resulted in substantial loss of activity. Thus, the nonapeptide FL9 defined the minimum core sequence for the stimulation of BEko8Z T cells.

We analyzed cell extracts to define the naturally processed peptide recognized by the BEko8Z hybridoma. We fractionated the extracts by reverse-phase HPLC and assessed their antigenic activity. We took advantage of the ability of BEko8Z cells to detect FL9 as well as its 10-, 11- and 12-residue analogs with amino-terminal extension (Fig. 3a). Each peptide eluted with a different retention time and could be readily distinguished from the others (Fig. 3b). We used cDNA encoding the R3 fragment of Fam49b (Fig. 2b) to transfet Qa-1b-expressing COS-7 monkey kidney cells (Fig. 3c). Because generation of the final antigenic peptide from the Fam49b cDNA was much lower in transfected cells when ERAAP function was not inhibited (Supplementary Fig. 4), we did all subsequent experiments in the presence of ERAAP inhibition. We extracted peptides from transfected cells after leucinethiol treatment to inhibit ERAAP and fractionated the peptides by HPLC. We assessed the ability of the fractions to stimulate BEko8Z cells in the presence of Qa-1b-expressing fibroblasts as APCs. We detected a single peak of antigenic activity that corresponded to the synthetic peptide FL9 in HPLC-fractionated extracts of cells transfected with R3 cDNA (Fig. 3c). We detected a peak with the same retention time in extracts of Qa-1b-expressing COS-7 cells transfected with a minigene encoding FL9 peptide alone (Fig. 3d). Furthermore, we detected the antigenic activity only in extracts of cells that expressed the restricting MHC class Ib molecule Qa-1b (Fig. 3e). The requirement for the restricting MHC molecule for detection of antigenic activity is similar to that observed with naturally processed peptides presented by classical MHC class Ia molecules. We concluded that the nonapeptide FL9 corresponded to the naturally processed peptide presented by Qa-1b in the absence of ERAAP function.

**The Qa-1b-FL9 complex is an immunodominant T cell ligand**

The Qa-1b-Qdm complex is recognized by the receptor CD94-NKG2A expressed on NK cells and some activated T cells. To assess whether FL9 peptide presented by Qa-1b (Qa-1b–FL9) was also recognized by a subset of NK cells, we stained wild-type spleen cells with Qa-1b–FL9 tetramers obtained from the Tetramer Core Facility of
Abundant antigen-experienced QFL T cells in naive mice

We assessed the abundance of QFL T cells in the spleens of naive mice. We labeled spleen cells with Qa-1\(^b\)–FL9 tetramers conjugated to two different fluorophores to enhance specificity, followed by magnetic enrichment of cells positive for the Qa-1\(^b\)–FL9 tetramer as described before for MHC class Ia– and MHC class II–restricted T cells\(^{30-32}\) (Fig. 5a,b). On average, we detected 1,030 QFL T cells in a naive wild-type spleens (Fig. 5a,b), which suggested that QFL T cells were present at a frequency of \(1 \times 10^4\) in a naive wild-type spleen. Enrichment for CD8\(^+\) T cells with the Qa-1\(^b\)–FL9 tetramer was specific, as the number of CD4\(^+\) cells positive for the Qa-1\(^b\)–FL9 tetramer enriched from wild-type mice was 2% of the corresponding number of CD8\(^+\) cells (Fig. 5a,b and Supplementary Fig. 5). In contrast, the spleens of TAP-deficient mice contained less than 5% of the cells specific for Qa-1\(^b\)–FL9 present in wild-type spleens (Fig. 5a,b). Most notably, we also found cells specific for Qa-1\(^b\)–FL9 in mice that lacked Qa-1\(^b\) expression, but only at a frequency of about 30% of those in wild-type spleens (Fig. 5a,b). The frequency of cells specific for Qa-1\(^b\)–FL9 was higher than that of typical pMHC\(_{\text{II}}\)-specific precursors, which range from 1 in \(1 \times 10^4\) to 1 in \(1 \times 10^5\) total CD8\(^+\) T cells in combined spleens and lymph nodes of naive mice\(^{31,33}\), in contrast to the numbers we found here in spleens alone. We concluded that CD8\(^+\) QFL T cells were relatively abundant in normal wild-type mice. Furthermore, although the transport of antigenic peptides by TAP was crucial, the restricting Qa-1\(^b\) molecule was not absolutely essential for the development of QFL T cells, which indicated that other MHC class I molecules were also able to support the development of QFL T cells.

Figure 3 FL9 is the naturally processed peptide presented by Qa-1\(^b\) molecules. (a) Response of the BEko8Z hybridoma (below; assessed as in Fig. 1c) to Qa-1\(^b\)–expressing L cells loaded with synthetic peptides with progressive amino-terminal truncation or extension in the polypeptide encoded by R13 (above). (b) Response of the BEko8Z hybridoma (as in a) to peptides fractionated by reverse-phase HPLC (resulting in elution of synthetic FL9 and its analogs with amino-terminal extension) and loaded on Qa-1\(^b\)–expressing L cells. (c,d) Elution of antigenic activity from peptide extracts obtained from Qa-1\(^b\)–expressing COS-7 cells transfected with a minigene encoding R13 (Qa-1 + R13; c) or FL9 (Qa-1 + FL9; d) and fractionated as in b (assessed as in a). (e) Elution of antigenic activity from peptide extracts of COS-7 cells that do not express Qa-1\(^b\), transfected with the minigene encoding R13, analyzed as in c,d. Downward arrows indicate peak fractions. Data are from one experiment representative of three.

Figure 4 The Qa-1\(^b\)–FL9 complex is an immunodominant T cell ligand. (a) Flow cytometry of spleen cells from naive mice labeled with the Qa-1\(^b\)–Qdm or Qa-1\(^b\)–FL9 tetramer together with markers for other spleen cell populations, showing tetramer labeling on gated B220\(^-\)NK1.1\(^-\)TCR\(_{\beta}\)\(^-\) cells. (b) Staining of NK2G2ANK1.1\(^-\)TCR\(_{\beta}\)\(^-\) cells in a with the Qa-1\(^b\)–Qdm tetramer (Qdm) or Qa-1\(^b\)–FL9 tetramer (FL9), presented as mean fluorescence intensity (MFI). (c) IFN-\(\gamma\) production by anti-ERAAP-KO CTLs incubated with T cell–depleted splenic APC populations (above plots), then labeled with Qa-1\(^b\)–FL9 tetramers. Numbers above outlined areas indicate percent Qa-1\(^b\)–FL9 tetramer–positive IFN-\(\gamma\)\(^+\) cells in the CD8\(_{\beta}\)CD4\(^+\) population. (d) Frequency of Qa-1\(^b\)–FL9 tetramer–positive (Tet\(^+\)) cells among the CD4\(_{\beta}\)CD8\(^+\)IFN-\(\gamma\)\(^+\) cells in c. Data are representative of two experiments (a,b; mean and s.e.m. of six mice) or three experiments (c) or are pooled from three independent experiments with three mice per group (d; mean and s.e.m.).
Qa-1b–FL9 tetramer–positive cells did not have the antigen-experienced CD44hiCD122+ phenotype seen in wild-type mice (Fig. 6d). The activation and population expansion of these cells were specific to challenge with ERAAP-KO cells, as we did not detect QFL T cells at similar frequencies in naive mice (Fig. 6) or in mice challenged with wild-type cells (data not shown). We obtained these results (Fig. 6) with mice that were not rechallenged before measurement of memory responses, and they showed that memory QFL T cell populations had expanded and were maintained at a high frequency after a single encounter with ERAAP-KO cells. We concluded that in contrast to other pMHC–Ib–restricted T cells, QFL T cells specific for the ligand Qa–1b–FL9 formed functional memory cells after encountering ERAAP-KO cells.

**QFL T cells proliferate in response to ERAAP-KO cells**

MHC class Ib–restricted T cells participate in primary immune responses in a variety of situations but are not thought to generate strong secondary responses. To determine how QFL T cells responded to ERAAP deficiency, we immunized wild-type mice with ERAAP-KO cells and measured the frequency of QFL T cells ex vivo by tetramer staining. We analyzed mice either 10 d after immunization (during the effector phase of the response) or 8–12 weeks after immunization (during the memory phase). CD8+ QFL T cell populations expanded during the effector phase in wild-type mice immunized with ERAAP-KO cells (Fig. 6a,b). Notably, we also detected memory QFL T cells in wild-type mice 8–12 weeks after the initial immunization with ERAAP-KO cells (Fig. 6a,b). We restimulated spleen cells from the same mice with FL9 peptide immediately after isolation; intracellular cytokine staining for IFN-γ showed that both effector and memory QFL T cells were readily activated (Fig. 6c,d). The activation and population expansion of these cells were specific to challenge with ERAAP-KO cells, as we did not detect QFL T cells at similar frequencies in naive mice (Fig. 6) or in mice challenged with wild-type cells (data not shown). We obtained these results (Fig. 6) with mice that were not rechallenged before measurement of memory responses, and they showed that memory QFL T cell populations had expanded and were maintained at a high frequency after a single encounter with ERAAP-KO cells. We concluded that in contrast to other pMHC–Ib–restricted T cells, QFL T cells specific for the ligand Qa–1b–FL9 formed functional memory cells after encountering ERAAP-KO cells.

**Elimination of ERAAP-KO cells by MHC class Ib–specific T cells**

We hypothesized that CD8+ T cells specific for ERAAP-KO cells elicited in wild-type mice may carry out immunosurveillance of...
ERAAP deficiency. We assessed whether CD8+ T cells from wild-type mice immunized with ERAAP-KO cells were able to eliminate ERAAP-KO target cells. Indeed, wild-type anti-ERAAP-KO CTLs (generated as in Fig. 1a) were cytotoxic and efficiently killed both ERAAP-KO target cells and ERAAP-MHClα-TKO target cells in vitro (Fig. 7a). Furthermore, wild-type anti-ERAAP-KO CTLs also killed TAP-sufficient RMA mouse tumor cells that were treated for 5 h with leucinomethyl to inhibit ERAAP, but did not kill leucinomethyl-treated TAP-deficient RMA-S mouse tumor cells (Fig. 7b). Notably, B16 mouse melanoma cells transinfected with a melanoma encoding FL9 were also efficiently killed by wild-type anti-ERAAP-KO CTLs, despite the absence of ERAAP inhibition, but untransfected B16 cells were not (Fig. 7c). Thus, expression of FL9 was both necessary and sufficient for the elimination of target cells. We concluded that wild-type CTLs, elicited by ERAAP-KO cells, were able to detect and eliminate ERAAP-KO or FL9-presenting cells.

To assess whether ERAAP-KO cells could also be eliminated in vivo, we injected wild-type mice with a mixture of self and ERAAP-KO cells labeled with two different concentrations of the intracellular dye CFSE. The input cells could therefore be distinguished from each other and from unlabeled host cells by flow cytometry (Fig. 7d, left). We recovered self and ERAAP-KO fluorescent cells from naive mice at approximately the same ratio (1:1) as in the input cell mixture. In contrast, ERAAP-KO cells were specifically rejected without affecting the recovery of self cells by wild-type mice primed with ERAAP-KO cells 7 d earlier (Fig. 7d). As expected, ERAAP-MHClα-TKO cells were also rejected with similar efficiency in primed mice. Notably, the elimination or loss of target cells required CD8+ T cells rather than CD4+ T cells, as mice depleted of CD8+ cells no longer rejected the ERAAP-KO target cells, unlike mice depleted of CD4+ T cells, which did reject the ERAAP-KO target cells (Fig. 7e). In contrast to another study suggesting a role for NK cells in the rejection of ERAAP-KO cells, we did not detect a role for NK cells in our experiments with mice depleted of NK1.1+ cells (Fig. 7b). We concluded that ERAAP deficiency elicited a potent MHC class Ib–restricted CD8+ T cell response in wild-type mice that effectively eliminated ERAAP-KO cells in vitro as well as in vivo.

**DISCUSSION**

The pMHCI repertoire is the immune system’s window into the state of the cellular proteome. Mechanisms for evading the immune response often target the antigen-processing pathway to inhibit the presentation of pMHCI. Immune-surveillance mechanisms, in turn, detect defects in pMHCI presentation. Here we have described an MHC class Ib–restricted CD8+ T cell mechanism that sensed normal antigen processing in the endoplasmic reticulum.

Immune responses are influenced by alterations in ERAAP activity. A microRNA encoded by human cytomegalovirus specifically targets the predominant isoform of human ERAAP (hERAP1)11. Loss of hERAP1 function inhibits the presentation of viral epitopes and probably contributes to the chronic infections established by human cytomegalovirus. Likewise, changes in ERAAP expression in tumors might lead to the activation of NK cells37. Finally, polymorphisms in hERAP1 have been found to be associated with two autoimmune disorders, ankylosing spondylitis and psoriasis12,13. Thus, impaired ERAAP function can cause various immune disorders.

Analysis of the MHC class I–bound peptide pool in ERAAP-deficient cells has shown a distinct shift toward the presentation of peptides with amino-terminal extensions, as well as peptides unique to ERAAP-deficient cells, by MHC class Ia molecules14–17,38. Here we have shown that the changes in the pMHCI repertoire in ERAAP-KO cells also extended to MHC class Ib molecules. Furthermore, the unique immunogenic pMHCIib ligands on ERAAP-KO cells were immunodominant, inducing a large fraction of wild-type anti-ERAAP-KO CD8+ T cells. An MHC class Ib–specific response of this magnitude was not detected in an earlier study17, although a small fraction of wild-type anti-ERAAP-KO CTLs in that study were activated by MHC class Ia–deficient APCs. These differences in the relative abundance of MHC class Ia– and MHC class Ib–restricted CTLs may have arisen from differences in the culture conditions for in vitro restimulation. Also, in our study here, the Qa-1β–FL9 tetramer allowed direct measurement of ligand-specific CTLs.

We have identified Fam49b as the source of the antigenic peptide presented by Qa-1β in ERAAP-KO cells. Fam49b is highly conserved in vertebrates from zebrafish to humans and seems to have ubiquitous expression, as noted in the gene atlas of the European Bioinformatics Institute (http://www.ebi.ac.uk/gxa/gene/ENSMUSG00000022378), although its function is unknown. Notably, high expression of Fam49B has been detected in patients with relapsing multiple sclerosis and in non–small cell lung cancer tissues39,40. We suggest, therefore, that presentation of the peptide FL9 by Qa-1β could be a highly conserved mechanism for the detection of ERAAP deficiency.
We used the Qa-1β–FL9 tetramer to characterize lymphocytes specific for this ligand. We were unable to detect NK cells specific for the Qa-1β–FL9 tetramer, in contrast to the readily detectable subset of NKG2A+ NK cells specific for Qa-1β–Qdm. However, a large fraction of the CD8+ T cells elicited by ERAAP-KO cells was positive for the Qa-1β–FL9. Using Qa-1β–FL9 tetramers, we also enriched a sizeable number of CD8+ QFL T cells from naive wild-type mice, which suggested that these cells were present at a relatively high frequency. Notably, even in naive mice, QFL T cells had an antigen-experienced, CD44hiCD122hi phenotype, similar to that of other innate-like MHC class Ib–specific T cells, such as NK T cells and mucosa-associated invariant T cells44. Whether QFL T cells share other developmental and functional characteristics with these innate-like effector cells is not known.

Unexpectedly, we found QFL T cells in Qa-1β-deficient mice, albeit at a lower frequency than in wild-type mice. We did not, however, detect QFL T cells in TAP- or β2-microglobulin-deficient mice, which suggested that QFL T cells require the presentation of peptides by an MHC class Ib molecule other than Qa-1β for development. Notably, acquisition of the antigen-experienced phenotype did require Qa-1β expression, because QFL T cells in naive Qa-1β-deficient mice did not constitutively express CD44 or CD122. MHC class Ib–restricted T cells generally have an antigen-experienced phenotype in naive mice, possibly because they are selected by APCs of hematopoietic origin42,43. Our observations have shown that QFL T cells expressed markers of antigen experience only after encountering Qa-1β-presented peptides. One implication of these findings is that QFL T cells may have encountered their ligand even in naive mice. Because FL9 was produced exclusively in ERAAP-KO cells, the Qa-1β–FL9 complex or a cross-reactive ligand might have been generated during transient ERAAP deficiency in wild-type mice, caused, perhaps, by natural transformation events, endogenous viruses or commensal microbes.

CD8+ T cells elicited by immunization with ERAAP-KO cells eliminated both MHC class Ia– and MHC class Ib–expressing targets cells that lacked ERAAP. Wild-type anti-ERAAP-KO T cell lines also killed tumor cells in which ERAAP was inhibited and ERAAP-sufficient cells that expressed FL9, which suggested that expression of this pMHCib complex was sufficient for the elimination of target cells. QFL T cell populations expanded robustly in response to a single challenge with ERAAP-KO cells and, unusually for a pMHCIb–specific response, also formed stable memory T cells that retained their effector function without rechallenge. QFL T cells might therefore be able to mediate long-term immunosurveillance of ERAAP deficiency. Qa-1β–FL9 complexes were rapidly presented by APCs treated for 5 h with an ERAAP inhibitor. Qa-1β–FL9 complexes were also a specific sign of ERAAP dysfunction and were not presented by TAP-deficient cells, unlike the Qa-1–associated peptides isolated from TAP-deficient cells described before44. Thus, the peptides presented by Qa-1β reflect the state of the antigen-processing pathway.

In conclusion, ERAAP deficiency resulted in the presentation of a highly conserved pMHCIb complex, Qa-1β–FL9, on the cell surface. Abundant antigen–experienced CD8+ T cells specific for Qa-1β–FL9 existed in naive mice, proliferated and effectively eliminated ERAAP-KO T cells. This response seems to combine the best of both worlds: the exquisite specificity of adaptive immunity, and the rapid responsiveness of the innate immune system.

METHODS

Methods and any associated references are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank the Tetramer Core Facility of the US National Institutes of Health for tetramer reagents; K. Söderstrom and E. Engleman (Stanford University) for Qa-1β-deficient mice generated by H. Cantor and colleagues (Harvard University); H. Cantor (Harvard University) for lentiviral vectors expressing Qa-1β; D. King for peptide synthesis; H. Nolla for assistance with cell sorting; K.C. Lind and A.H. Bakker for discussions and comments on the manuscript; and H. Dani for technical assistance. Supported by Irvington Institute Fellowship Program of the Cancer Research Institute (N.A.N.) and the US National Institutes of Health (N.S.).

AUTHOR CONTRIBUTIONS

N.A.N. and N.S. designed the study and wrote the manuscript; and N.A.N. and F.G. did the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
22. Hu, D. et al. Analysis of regulatory CD8+ T cells in Qa-1-deficient mice. Nat. Immunol. 5, 516–523 (2004).
23. Serwold, T., Gaw, S. & Shastri, N. ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. Nat. Immunol. 2, 644–651 (2001).
24. Lu, L., Werneck, M.B. & Cantor, H. The immunoregulatory effects of Qa-1. Immunol. Rev. 212, 51–59 (2006).
25. Aldrich, C.J. et al. Identification of a TAP-dependent leader peptide recognized by alloreactive T cells specific for a class 1b antigen. Cell 79, 649–658 (1994).
26. Karttunen, J., Sanderson, S. & Shastri, N. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. Proc. Natl. Acad. Sci. USA 89, 6020–6024 (1992).
27. Mach, N. et al. Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. Cancer Res. 60, 3239–3246 (2000).
28. Falk, K., Rötzschke, O. & Rammensee, H.G. Cellular peptide composition governed by major histocompatibility complex class I molecules. Nature 348, 248–251 (1990).
29. Malarkannan, S., Goth, S., Buchholz, D.R. & Shastri, N. The role of MHC class I molecules in the generation of endogenous peptide/MHC complexes. J. Immunol. 154, 585–598 (1995).
30. Moon, J.J. et al. Naive CD4+ T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity 27, 203–213 (2007).
31. Urdahl, K.B., Sun, J.C. & Bevan, M.J. Positive selection of MHC class Ib-restricted CD8+ T cells on hematopoietic cells. Nat. Immunol. 3, 772–779 (2002).
32. Cho, H., Choi, H.J., Xu, H., Felio, K. & Wang, C.R. Nonconventional CD8+ T cell responses to Listeria infection in mice lacking MHC class Ia and H2-M3. J. Immunol. 186, 489–498 (2011).
33. Bouwer, H.G., Barry, R.A. & Hinrichs, D.J. Lack of expansion of major histocompatibility complex class Ib-restricted effector cells following recovery from secondary infection with the intracellular pathogen Listeria monocytogenes. Infect. Immun. 69, 2286–2292 (2001).
34. Petroziello, J. et al. Suppression subtractive hybridization and expression profiling identifies a unique set of genes overexpressed in non-small-cell lung cancer. Oncogene 23, 7734–7745 (2004).
35. Gilli, F. et al. Learning from nature: pregnancy changes the expression of inflammation-related genes in patients with multiple sclerosis. PLoS ONE 5, e8962 (2010).
36. Yamagata, T., Benoist, C. & Mathis, D. A shared gene-expression signature in innate-like lymphocytes. Immune. Rev. 210, 52–66 (2006).
37. Urdahl, K.B., Sun, J.C. & Bevan, M.J. Positive selection of MHC class Ib-restricted CD8+ T cells on hematopoietic cells. Nat. Immunol. 3, 772–779 (2002).
38. Cho, H., Bediako, Y., Xu, H., Choi, H.J. & Wang, C.R. Positive selecting cell type determines the phenotype of MHC class Ib-restricted CD8+ T cells. Proc. Natl. Acad. Sci. USA 108, 13241–13246 (2011).
39. Oliveira, C.C. et al. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. J. Exp. Med. 207, 207–221 (2010).
ONLINE METHODS

Mice. ERAAP-KO mice (B6.129.Arts1tm11(1)lcvb) have been described. ERAAP-MHCIIa-TKO mice and ERAAP-KO mice also deficient in TAP-β2-microglobulin or Qa-1 were generated in our facility by the crossing of ERAAP-KO mice with mice deficient in both H-2Kβ and H-2Dβ, TAP-1, β2-microglobulin or Qa-1, respectively. Wild-type C57BL/6J, Qa-2-deficient B6.K1 and β2-microglobulin-deficient mice were from the Jackson Laboratory or were bred in our facility. H-2T23-deficient Qa-1β-deficient mice were generated in the laboratory of H. Cantor and were a gift from E. Engleman. Mice were housed and all procedures were done with the approval of and in accordance with the institutional animal care guidelines of the University of California Berkeley.

Antibodies, cell lines and peptides. Antibodies for flow cytometry were from BD Biosciences (antibody to CD8α (anti-CD8α, 53-6.7), anti-CD4 (RM4-5), anti-B200 (RA362B), anti-TCRβ (H57-597), anti-CD44 (IM7), anti-CD3ε (PC5), anti-CD11c (HL3), anti-H-2Kβ (AF6-88.5), anti-H-2Dβ (H9K5), anti-CD8α (25-9-17), anti-IFN-γ (XMG1.2), anti-CD4 (RM4-5), anti-TCRβ and anti-TNF (MP6-XP22)) and eBioscience (anti-NKG2A (20D5)). Antibodies used for depletion of cells in vitro were from the Cell Culture Facility of the University of California, San Francisco. All peptides were synthesized by D. King (University of California Berkeley). Qa-1β-expressing L cells and COS cells were made by stable transduction of L cells (mouse fibroblasts lacking thymidine kinase (Ltk− cells)) and COS-7 cells, respectively, with lentivector expressing Qa-1β (H. Cantor). B16.FL9 cells were made by transfection of B16.BL6 cells with a plasmid containing the minigene encoding FL9 and sequence neo-resistant resistance. Stable cell lines were derived from single cells by selection with limiting dilution of the aminoglycoside G418.

CTL- and T cell–activation assays. Wild-type CTL lines that recognize ERAAP-KO cells were generated by immunization of C57BL/6J mice once intraperitoneally with 2 × 107 ERAAP-KO spleen cells. Spleens were collected from immunized mice 10 days after immunization. Responder spleen cells (5 × 106 cells per well in 24-well plates) were restimulated in vitro with an equal number of irradiated ERAAP-KO spleen cells and 20 U/ml of recombinant human interferon interleukin 2 (BD Biosciences). T cells were used for IFN-γ assays 6 days after restimulation and for in vitro cytotoxicity assays 5 days after restimulation. For measurement of intracellular IFN-γ, CTL lines were collected and stimulated for 5 h with lipopolysaccharide-stimulated spleen APC populations that were depleted of CD4+ cells and CD8+ cells. GolgiPlug (BD Biosciences) was added for the final 4 h of the incubation period. Cells were then stained first for surface markers or with tetramers, followed by other surface markers, then were fixed, permeabilized and stained for intracellular IFN-γ. For ex vivo analysis of IFN-γ production, spleen cells were collected from immunized mice and plated for 5 h in 96-well plates with 1 μM peptide in the presence of GolgiPlug. Cells were then stained as described above.

Hybridomas and expression cloning. Wild-type anti-ERAAP-KO CTL lines collected 4 d after in vitro restimulation were fused to the DB1 “TCRβ” cell line BWZ.36 that expresses a TCR-inducible gene encoding lacZ. Putative positive clones were screened with lipopolysaccharide-stimulated ERAAP-KO spleen cells as APCs, and positive wells were subcloned by limiting dilution to obtain clones derived from a single cell. Once a stable hybridoma BEko8Z was established, it was used for screening of cDNA libraries as described. ERAAP-KO mice were immunized subcutaneously with 3 × 106 Flt3L-secreting B16 melanoma cells as described. Two weeks later, their spleens were collected and ‘blasted’ overnight with 200 ng/ml lipopolysaccharide (Sigma Aldrich). Samples were enriched for CD11c+ cells through the use of CD11c microbeads followed by magnetic enrichment with LS columns (Miltenyi Biotec). The resultant cells were pelleted and frozen, then homogenized with a rotor-stator homogenizer. An Oligotex mRNA isolation kit (Qiagen) was used for isolation of mRNA. Then, cDNA was synthesized and cloned into pCMV-SPORT6 with the SuperScript Plasmid kit from Invitrogen, and transduced into Qa-1β-expressing L cells. Leucinethiol (15 μM in 500 μM dithiothreitol) was added 42 h later, then BEko8Z hybridoma cells were added 6 h after that, followed by overnight incubation. T cell activation was detected by colorimetric cleavage of CPRG (chlorophenylindol-β-n-galactopyranoside; Roche) by TCR-induced lacZ. When a positive well was detected, 1 μl cDNA from that well was transfected in bacteria and plasmid DNA was isolated from multiple individual colonies. Each individual cdNA was transfected into Qa-1β-expressing L cells. Each positive cDNA was sequenced and identified. Progressive truncations were made in the coding region of the gene encoding Fam49b with nested PCR primers; the resultant fragments were cloned into pcDNA1 and their activity was assayed by transfection into Qa-1β-expressing L cells. Once the candidate region was narrowed to R13 and the final leucine residue was determined to be essential, peptides with amino-terminal extension were synthesized and tested. Minigenes were cloned in the pcDNA1 expression vector and were designed to encode the desired amino acid sequence with an ATG initiation codon. The minigene encoding FL9, for example, encodes the amino acid sequence Met–FL9 (MFYAEATPMFL).

Enrichment for tetramer-positive cells. Qa-1β–FL9 tetramers were synthesized by the Tetramer Core Facility of the US National Institutes of Health. Samples were enriched for tetramer-positive cells as described. Splenocytes were collected from mice and homogenized with nylon filters. Red blood cells were lysed and the cells were resuspended in 200 μl sortor buffer (0.1% sodium azide and 5% FCS in PBS). Phycocyanin- or allophycocyanin-labeled Qa-1β–FL9 tetramers were added at a final dilution of 1:200. Spleens were incubated for 30 min at 23 °C, then were washed and resuspended in 0.45 ml sortor buffer. Anti-PE microbeads (50 μL; Miltenyi Biotec) were added to each sample, followed by incubation for 15 min at 4–8 °C. Cells were washed, and phycocyanin-labeled cells were isolated by passage over an LS magnetic column (Miltenyi Biotec). The entire positively selected fraction was collected and stained with anti-B200, anti-CD4, anti-CD8α, anti-TCRβ and anti-CD44. Tetramer-positive cells were gated as B200+CD4+CD8α+TCRβ+ phycocyanin-tetramer-positive and allophycocyanin-tetramer–negative cells. A fixed number of CountBright beads (Invitrogen) was added to each sample to allow counting of cells, and samples were acquired on a BD LSR II.

Reverse-phase HPLC. COS-7 or Qa-1β-expressing COS cells were transfected with cDNA encoding either Fam49b R13, or a minigene encoding the FL9 peptide. Leucinethiol (15 μM in 500 μM dithiothreitol) was added 42 h later. After 5 h of leucinethiol treatment, cells were collected and peptides were extracted with 10% acetic acid. Extracts were filtered through a 10-kilodalton exclusion filter, and the material less than 10 kilodaltons in size was fractionated with a C18 reversed-phase HPLC column (Grace Vydac). The fractionated material was collected in 96-well plates and lyophilized. Qa-1β-expressing L cells (5 × 106) and BEko8Z cells (1 × 106) were added to each well, followed by incubation overnight at 37 °C. The presence of activating peptide was detected the next day by colorimetric conversion of CPRG. Synthetic peptides (1 pmol each) were also assessed during the same experiment and detected in the same way.

Killing assays. For in vitro cytotoxicity assays, cells used as targets were labeled with the dye PKH26 (Sigma Aldrich), counted and plated. Effector CTL lines were collected and added at the appropriate ratio of effector cell to target cell, followed by incubation for 1 h and 45 min at 37 °C, after which the dye YoPRO-1 (Invitrogen) was added to a final concentration of 1 μM for visualization of apoptotic cells. Cells were washed and visualized on a flow cytometer. Cells that were both PKH26+ and YoPRO-1+ were considered apoptotic target cells. The frequency of apoptotic target cells was calculated as follows: 100 × (percent PKH26+YoPRO-1− E:T−X− percent PKH26+YoPRO-1− E:T−X−) / (100 − percent PKH26+YoPRO-1− E:T−X−), where ‘E:T’ = ‘X’ indicates the ratio of effector to target cells at target size ‘X’ and ‘E:T = 0’ indicates that ratio at time ‘0’. For in vivo cytotoxicity assays, wild-type C57BL/6 and ERAAP-KO spleen cells were collected and labeled with 0.25 μM and 2.5 μM of carboxyfluorescein-succinimidyl ester (CFSE, Invitrogen), respectively. Wild-type (CFSEβ) cells were mixed with ERAAP-KO or ERAAP-MHCIIa-TKO (CFSEαβ) cells (5 × 106 cells of each genotype) and their ratio was measured immediately before injection (input ratio). Target cells were injected intravenously into naive mice or mice that had been primed 7 days before by intraperitoneal injection of ERAAP-KO spleen cells. Splenocytes from mice injected with target cells were collected 24 h later and the ratio of CFSEβ cells to CFSEαβ cells was measured.
was measured by flow cytometry. Targets lost in a sample were calculated as follows: $100 - 100 \times (\text{fraction CFSE}^{\text{hi}} \text{cells/input fraction CFSE}^{\text{hi}} \text{cells})$, where ‘fraction CFSE$^{\text{hi}}$ cells’ was calculated as percent CFSE$^{\text{hi}}$ cells / (percent CFSE$^{\text{hi}}$ cells plus percent CFSE$^{\text{lo}}$ cells). Mice that received ERAAP-MHCII-TKO cells as targets were depleted of NK cells with 200 µg anti-NK1.1 36 h before challenge. In experiments in which the role of CD4$^+$ cells and CD8$^+$ cells was assessed, samples were depleted of CD4$^+$ cells and CD8$^+$ cells with 200 µg anti-CD8 (YTS169.4) or anti-CD4 (GK1.5) 24 h before challenge.

Statistical analyses. GraphPad Prism was used for all statistical analyses. A nonparametric Mann-Whitney U-test was used for estimation of statistical significance.