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Nonconventional CD8+ T Cell Responses to Listeria Infection in Mice Lacking MHC Class Ia and H2-M3

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CD8+ T cells restricted to MHC class Ib molecules other than H2-M3 have been shown to recognize bacterial Ags. However, the contribution of these T cells to immune responses against bacterial infection is not well defined. To investigate the immune potential of MHC class Ib-restricted CD8+ T cells, we have generated mice that lack both MHC class Ia and H2-M3 molecules (Kb2−/−D b−/−M3−/−). The CD8+ T cells present in Kb2−/−D b−/−M3−/− mice display an activated surface phenotype and are able to secrete IFN-γ rapidly upon anti-CD3 and anti-CD28 stimulation. Although the CD8+ T cell population is reduced in Kb2−/−D b−/−M3−/− mice compared with that in Kb2−/−D b−/− mice, this population retains the capacity to expand significantly in response to primary infection with the bacteria Listeria monocytogenes. However, Kb2−/−D b−/−M3−/− CD8+ T cells do not expand upon secondary infection, similar to what has been observed for H2-M3–restricted T cells. CD8+ T cells isolated from Listeria-infected Kb2−/−D b−/−M3−/− mice exhibit cytotoxicity and secrete proinflammatory cytokines in response to Listeria-infected APCs. These T cells are protective against primary Listeria infection, as Listeria-infected Kb2−/−D b−/−M3−/− mice exhibit reduced bacterial burden compared with that of infected β2-microglobulin–deficient mice that lack MHC class Ib-restricted CD8+ T cells altogether. In addition, adoptive transfer of Listeria-experienced Kb2−/−D b−/−M3−/− splenocytes protects recipient mice against subsequent Listeria infection in a CD8+ T cell–dependent manner. These data demonstrate that other MHC class Ib-restricted CD8+ T cells, in addition to H2-M3–restricted T cells, contribute to antilisterial immunity and may contribute to immune responses against other intracellular bacteria. The Journal of Immunology, 2011, 186: 489–498.

Effector CD8+ T cells restricted to the classical MHC class I (MHC class Ia) Ag-presenting molecules have been shown to play critical roles in the clearance of bacterial and viral infection. MHC class Ib molecules are structurally related to MHC class Ia and are likewise composed of three Ig-like domains that noncovalently associate with β2-microglobulin (β2m) (1). Although the mammalian genome encodes many more MHC class Ib molecules than MHC class Ia molecules, comparatively little is known regarding their immunological function. However, the conservation of these molecules in mammals indicates that they play important roles that are nonredundant to those of MHC class Ia (1–4). Genes encoding MHC class Ib molecules can be found linked to the MHC (e.g., H2-M3, Qa-1/HLA-E, Qa-2) on chromosome 6 in humans and on chromosome 17 in mice, as well as elsewhere in the genome (e.g., CD1, MR1) (1). In general, MHC class Ib molecules are significantly less polymorphic, are more restricted in their tissue distribution, and have lower cell surface expression than MHC class Ia (1, 5), although in some cases these expression levels can be increased in the presence of Ag (6, 7). Importantly, over the past decade, emerging studies have found that MHC class Ib molecules can contribute to host immune responses through the presentation of microbial Ags to T cells (1, 8, 9).

Some MHC class Ib molecules, such as CD1 and H2-M3, have Ag-binding regions specialized to accommodate Ags that are unique in structure, perhaps positioning them to recognize hallmark signs of microbial infection. The hydrophobic binding cleft of CD1 allows it to accommodate and present bacterial lipid Ags to T cells (10–17), whereas H2-M3 preferentially binds peptides that have N-terminal formylation, a signature of bacterial peptide synthesis, with up to a thousand-fold stronger affinity than that of nonformylated peptides (18, 19). H2-M3–restricted T cells have been shown to recognize peptides derived from many bacteria, including Listeria monocytogenes, Mycobacterium tuberculosis, Salmonella typhimurium, and Chlamydia pneumoniae (20–25). We have previously demonstrated that H2-M3–restricted CD8+ T cells play a nonredundant role in host responses against L. monocytogenes and that mice lacking H2-M3 (M3−/−) have an increased susceptibility to L. monocytogenes infection (26). In addition to H2-M3, there is some evidence that Qa-1 can present listerial Ags (27–30). Qa-1 and its human homologue, HLA-E, have been shown to present peptides derived from Salmonella to CD8+ T cells (8, 31, 32). HLA-E–restricted T cells can also respond to Ags derived from M. tuberculosis (33) and have been isolated from M. tuberculosis–infected patients (34). Recent studies have demonstrated that mucosal-associated invariant T (MAIT) cells can be activated by MR1-expressing APCs that have been cultured with various bacteria, indicating that they recognize bacterial Ags presented by MR1 (35, 36). In addition to bacterial peptides, both HLA-E and Qa-2 have been shown to present peptides of viral origin to CD8+ T cells, suggesting that MHC class Ib molecules are also involved in antiviral immune responses (9, 37).

Like MHC class Ia-restricted CD8+ T cells, most MHC class Ib-restricted T cells are cytotoxic and can secrete inflammatory cytokines. The expression of MHC class Ib molecules is also involved in innate immune responses (38–42). MHC class Ib molecules are structurally and functionally distinct from MHC class Ia molecules (43, 44). MHC class Ib molecules are significantly less polymorphic, are more restricted in their tissue distribution, and have lower cell surface expression than MHC class Ia (1, 5), although in some cases these expression levels can be increased in the presence of Ag (6, 7). Importantly, over the past decade, emerging studies have found that MHC class Ib molecules can contribute to host immune responses through the presentation of microbial Ags to T cells (1, 8, 9).

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cytokines such as IFN-γ upon stimulation with Ag (31, 38–40). However, other characteristics of MHC class Ib-restricted T cells distinguish them from conventional T cells. Whereas the majority of T cells restricted by the MHC-linked MHC class Ib molecules studied to date express the CD8 coreceptor (2, 9, 19, 20, 22, 24, 25, 33), the T cells restricted by MHC-unlinked MHC class Ib molecules are predominately CD8− (38, 41). Many MHC class Ib-restricted T cells, including H2-M3-restricted CD8+ T cells, CD1d-restricted NKT cells, and MAIT cells, display an activated cell surface phenotype in the absence of infection (41–45). This preactivated status may contribute to the unique kinetics of MHC class Ib-restricted T cell responses, as H2-M3–restricted T cells, NKts, and MAIT cells all respond more rapidly to antigenic stimulation than do conventional T cells (40, 42, 43, 45–47). Notably, although their responses to primary stimuli are rapid, H2-M3–restricted T cell and NKT cell responses to secondary stimulation lack the accelerated responses and significant expansion that characterize secondary conventional T cell responses (42, 43, 46–50). It is not clear whether this limited responsiveness is a general feature of MHC class Ib-restricted T cells upon secondary stimulation.

*L. monocytogenes* infection has long been used to study conventional CD8+ T cell responses to intracellular bacteria (51–54) but has also proved to be a useful model for studying MHC class Ib-restricted responses. Studies performed using MHC class Ia-deficient (Kb−/−Dd−/−) mice have demonstrated that MHC class Ib-restricted CD8+ T cells are protective against listerial infection and that antilisterial responses are not limited to H2-M3–restricted T cells (30, 42, 55). As listerial Ags that bind H2-M3 have been identified, and that antilisterial responses are few in number, they are cytotoxic, secrete proinflammatory cytokines, and can protect against *L. monocytogenes* infection. Given that MHC class Ib-restricted T cells display significantly less polymorphism than MHC class Ia, these new findings position MHC class Ib molecules and their bound bacterial Ags as attractive vaccine targets that could be widely recognized across the general population to protect against bacterial infection.

### Materials and Methods

#### Mouse

C57BL/6 and β2m−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). M3−/− and Kb−/−Dd−/− mice (backcrossed with C57BL/6) [B6 mice for at least 10 generations] were generated or maintained in house as previously described (26). Kb−/−Dd−/−M3−/− mice were generated by crossing Kβ−/−Dd−/− mice with M3−/−Dd−/− mice. F1 offspring were intercrossed, and all resulting F2 progeny that lacked surface expression of H2-Kb and Dd on PBLs were screened for an intra-H2 recombinant using PCR analysis using the following primer set: M3 forward (5′-CAGCGTGAACCATGACATGTGAC-3′), M3 reverse (5′-AGACTAAGCAAGATGACATGTGAC-3′), and Neo (5′-GATTTG-CAGCGATGACATGTGAC-3′) (26). Of 165 F2 offspring tested, one male mouse was found to carry the desired intra-H2 recombination (Kb−/−Dd−/−M3−/−). We bred this male with Kβ−/−Dd−/− females to produce Kb−/−Dd−/−M3−/− offspring, which were then intercrossed to generate Kβ−/−Dd−/−M3−/− mice. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD). The protocol was approved by the Animal Care and Use Committee of Northwestern University (Chicago, IL).

#### Primary cell preparation and dendritic cell generation

Single-cell suspensions were prepared from whole tissues by mechanical disruption in HBSS/2% FBS or as described (56). T cells were purified from splenic lymphocytes using a Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA). To enrich for CD8+ T cells, splenocytes were labeled with biotin-conjugated anti-B220, anti-CD4, and anti-CD11c, anti-CD49h, and anti-NK1.1 Abs (eBioscience, San Diego, CA) followed by anti-biotin-conjugated magnetic beads. CD8+ T cells were then isolated to a purity of ~95% by negative selection according to the manufacturer’s instruction (Miltenyi Biotec). Bone marrow–derived dendritic cells (BMDCs) were derived from mouse bone marrow progenitors using GM-CSF and IL-4 (PeproTech, Rocky Hill, NJ) as previously described (57).

#### Abs and flow cytometry

FITC-conjugated Abs specific for CD8β, CD44, CD62L, hamster IgG, H2-Kb, Vβ2, Vβ5.1/5.2, Vβ6, Vβ8.1/8.2, Vβ3, Vβ12, and Vβ13, FITC-conjugated streptavidin, PE-conjugated Abs specific for CD4 and TCRβ, and biotinylated Ab specific for H2-Dd were purchased from BD Pharmingen (San Diego, CA). Cells were incubated with 2.42D FcγRI/RII blocking mAb (hybridoma supernatant) for 15 min, then stained in HBSS containing 2% FBS for 30 min at 4°C. For detection of H2-M3 surface expression, splenocytes from B6 and Kb−/−Dd−/−M3−/− mice were cultured overnight at 37°C in RPMI 10 containing 10 μM LeuA peptide (t-MIGWII). Cells were stained first with the anti–H2-M3 Ab 130 (7) followed by staining with anti-hamster IgG. Flow cytometric analysis was performed using a FACSCan (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR).

#### Cytokine assay

For polyclonal TCR stimulation, enriched CD8+ T cells (5 × 105 cells/well) from naive wild-type (WT), Kβ−/−Dd−/−, and Kb−/−Dd−/−M3−/− mice were stimulated with anti-CD3 (39) followed by staining with anti-hamster IgG. Flow cytometric analysis was performed using a FACSCan. AB (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR).

#### Intracellular cytokine staining

Splenocytes or hepatic leukocytes from naive or *L. monocytogenes*-infected mice were cultured in 96-well plates and either stimulated or left unstimulated for 7 h at 37°C. For the last 2 h of stimulation, 10 μM monensin (Sigma, St. Louis, MO) was added to block cytokine secretion. Cells were washed and stained for the cell surface markers CD8β, CD4, and TCRβ. After fixation with 4% paraformaldehyde and permeabilization with 0.15% saponin (Sigma), cells were stained with an allophycocyanin-conjugated anti-IFN-γ Ab (eBioscience) for 30 min in PBS containing 1% BSA and 0.1% saponin. Flow cytometry was performed as described earlier.

#### Listeria infection and CFU assay

The *L. monocytogenes* EGD strain was provided by R. Kurlander (National Institutes of Health). Bacteria were grown in brain–heart infusion broth (Difco Laboratories, Detroit, MI) and virulent stocks were maintained by repeated passage through B6 mice. For primary infection, mice were i.v. injected with 2 × 10^7 CFU (one tenth LD₅₀) *L. monocytogenes*. For rechallenge with *L. monocytogenes*, mice were rested for 1 mo and then infected with 2 × 10^6 CFU.
infected with 5 × 10^7 CFU L. monocytogenes. Bacterial CFUs in the spleen and liver were determined at indicated time points postinfection. Briefly, organs were homogenized in sterile water with 0.2% Noned P-40, and serial dilutions were plated onto brain–heart infusion agar plates. CFUs were counted after incubation at 37°C for 24 h.

Adoptive transfer study
Donor WT and K^b−/−D^b−/−M^3−/− mice were immunized with 2 × 10^3 CFU L. monocytogenes. Seven days later, splenocytes from L. monocytogenes-immune mice were isolated and were divided into two groups. One donor group was incubated with anti-Cd8a mAb (3,15) at 4°C for 30 min. Cells were then washed and incubated with 10% rabbit complement (Cedarlane Labs, Burlington, NC) at 37°C for 30 min to deplete CD8+ T cells. All donor cells from naïve spleen, CD8+ T cell-depleted immune spleen, and nondepleted immune spleen were washed twice with PBS. Naïve WT mice received an i.v. injection of 2 × 10^7 donor splenocytes and were then challenged with 5 × 10^7 CFU L. monocytogenes 30-60 min after cell transfer. Three days postinfusion, spleen and liver were removed from the recipients, and the bacterial CFUs per organ was determined as described earlier.

ELISPOT assay
Multiscreen-IP plates (Millipore, Bedford, MA) were coated with anti–IFN-γ mAb (eBioscience) at 5μg/ml in PBS. BMDCs were infected with L. monocytogenes at 37°C for 4 h before each assay as described earlier. For blocking experiments, L. monocytogenes-infected BMDCs were preincubated with mouse IgG or mAb against CD8α (31H3; in-house) (58), H2-M3 (130; in-house) (7), MR1 (26.5) (4), QA-1b (6A8.6F10.1A6; American Type Culture Collection, Manassas, VA) (31, 59), and Qa-2 (M46) (60) for 30 min at 37°C prior to assay set up (42). Enriched CD8+ T cells (10^2–10^4) were mixed with BMDC stimulator cells (5 × 10^5/well) in RPMI 10 medium and plated in triplicate wells. After 18 h incubation at 37°C, plates were washed free of cells using PBS–Tween (PBS and 0.05% Tween 20) and incubated overnight at 4°C with biotinylated anti–IFN-γ mAb (eBioscience) at 1μg/ml. Plates were washed and incubated with streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA). After 1 h incubation at room temperature, plates were developed with a BCIP/NBT substrate kit (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Spots were counted using an ImmunoSpot reader (Cellular Technology, Shaker Heights, OH).

CTL assay
To examine L. monocytogenes-specific CTL ex vivo, splenocytes from L. monocytogenes-infected mice (at day 7 postinfection) were enriched for CD8+ T cells and cultured in RPMI 10 with 1μg/ml Con A. After 3 d in culture, cells were used as effectors in a 51Cr-release assay. Mono-layers of the macrophage cell line J774A.1 were grown on bio-inert medium and infected with L. monocytogenes for 1 h at a multiplicity of infection 5:1. Cells were then washed with warm PBS and cultured in DMEM containing 40 μg/ml gentamicin for an additional 3 h. Both uninfected and L. monocytogenes-infected J774 target cells were labeled with 51Cr at 1 h at 37°C. To examine cytotoxicity in the T2 CTL line, BMDCs were labeled with 51Cr and used as target cells. BMDC targets were derived from B6 mice or K^b−/−D^b−/−M^3−/− mice and either left untreated or treated overnight with HKLM. Some target cells were additionally pretreated with blocking mAb against MR1, QA-1b, or Qa-2 before labeling as described earlier. Target cells (10^6) were added to a round-bottom 96-well plate containing varying concentrations of effector cells. Four hours after incubation, 100 μl supernatant was collected from each well, and the amount of 51Cr release was determined using a TopCount scintillation counter. The percentage of specific lysis was calculated as 100 × (experimental cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm).

Generation of K^b−/−D^b−/−M^3−/− CTL lines
To generate the T2 CTL line, K^b−/−D^b−/−M^3−/− mice were immunized with 1 × 10^6 HKLM-pulsed BMDCs. Seven days postinmunization, splenocytes were harvested and placed in culture for 1 wk in RPMI 10. Cells were subsequently cultured in supplemented Michiels Dutton medium with 20 μM IL-2 (partially purified from EL4.II2 cell supernatant) and 20 ng/ml IL-7 (PeproTech). Cells were restimulated weekly with HKLM-pulsed irradiated K^b−/−D^b−/−M^3−/− BMDCs.

Statistical analysis
Mean values were compared using unpaired Student t tests. All statistical analyses were performed with the Prism program (GraphPad, La Jolla, CA).

Results
Generation and characterization of K^b−/−D^b−/−M^3−/− mice
To investigate the relative contribution of H2-M3 and other MHC class Ib molecules to CD8+ T cell development and associated responses to bacterial infection, we generated K^b−/−D^b−/−M^3−/− mice by crossing K^b−/−D^b−/− and M^3−/− mice (26). F1 offspring were intercrossed, and the resulting F2 offspring were then screened for intra-H2 recombination using FACS and PCR analysis (Fig. 1A). The genetic distance between H2-D and H2-M3 is ~0.7 cM (61), necessitating extensive screening. Of 165 F2 offspring tested, one mouse was found to carry each targeted locus on the same chromosome (K^b−/−D^b−/−M^3−/−) and was selected for further breeding to establish K^b−/−D^b−/−M^3−/− mice. Flow cytometry confirmed that K^b−/−D^b−/−M^3−/− mice do not express H2-K^b, H2-D^b, or H2-M3 molecules on the cell surface (Fig. 1B). To determine the respective role of H2-M3 and other MHC class Ib molecules in the development of CD8+ T cells, we compared the CD8+ T cell populations in the spleen, liver, and lymph nodes of WT, K^b−/−D^b−/−, and K^b−/−D^b−/−M^3−/− mice. Total lymphocyte numbers were comparable between these three genotypes (data not shown). However, compared with WT mice, the percentage of CD8+ T cells was profoundly reduced in the spleens and lymph nodes of K^b−/−D^b−/− mice and was further reduced, albeit modestly, in K^b−/−D^b−/−M^3−/− mice (Fig. 2A, 2B). Notably, the reduction in CD8+ T cell percentage was less profound in the livers of K^b−/−D^b−/− mice compared with that of WT mice, whereas K^b−/−D^b−/−M^3−/− mice exhibited a 2- to 3-fold reduction in the percentage of hepatic CD8+ T cells compared with that of K^b−/−D^b−/− mice (Fig. 2A, 2B). Judging from the enumeration of CD8+ T cell populations in K^b−/−D^b−/− and K^b−/−D^b−/−M^3−/− mice, H2-M3–restricted CD8+ T cells constitute ~20–30% of the MHC class Ib–restricted CD8+ T cells found in the peripheral lymphoid tissues of naive animals but contribute 50–75% of the MHC class Ib–restricted CD8+ T cell population in the liver. These data indicate that CD8+ T cells restricted by different MHC class Ib molecules may have distinct tissue distributions, with H2-M3–restricted CD8+ T cells particularly enriched in the liver.

Phenotypic and functional analysis of residual CD8+ T cells in naïve K^b−/−D^b−/−M^3−/− mice
Two unconventional subsets of MHC class Ib-restricted T cells, namely CD1d-restricted invariant NKT cells and MR1-restricted MAIT cells, exhibit restricted TCR usage (45, 62). To examine the diversity of the TCR Vb region expressed by H2-M3–restricted and non–H2-M3 MHC class Ib-restricted CD8+ T cells, splenocytes from WT, K^b−/−D^b−/−, and K^b−/−D^b−/−M^3−/− mice were stained with Abs against various TCR V regions. Although the CD8+ T cell population found in K^b−/−D^b−/−M^3−/− mice exhibited a decreased representation of Vb2, Vb5, Vb6, Vb8.3, Vb11, and Vb13 compared with that of the CD8+ T cell population found in WT mice, residual K^b−/−D^b−/−M^3−/− CD8+ T cells still exhibit a large diversity in TCRβ usage (Supplemental Fig. 1). This observation could indicate that the CD8+ T cell population found in K^b−/−D^b−/−M^3−/− mice does not exhibit restricted TCR Vβ-chains usage or could merely be reflective of a polyclonal population of CD8+ T cells recognizing diverse restriction elements. However, the residual CD8+ T cells found in K^b−/−D^b−/−M^3−/− mice exhibit an increase in the proportion of Vβ8.1/8.2 and Vb14 usage that is not observed in K^b−/−D^b−/−M^3−/− mice, suggesting that H2-M3–restricted CD8+ T cells may display a preferential usage for these Vβ-chains.

It has been shown that a large proportion of CD8+ T cells in naïve K^b−/−D^b−/− mice display an activated/memory-like pheno-
type (42–44). However, it is not clear whether H2-M3–restricted and/or other MHC class Ib-restricted CD8+ T cells contribute to this phenotype. To address this question, we compared the expression of various activation markers on CD8+ T cells isolated from WT, K b<sup>−/−</sup>Db<sup>−/−</sup>, and K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice. Similar to K b<sup>−/−</sup>Db<sup>−/−</sup> mice, the majority of CD8+ T cells in K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice exhibit an activated T cell phenotype that is CD44<sup>high</sup> and Ly6C<sup>high</sup> (Fig. 2C). In addition, the percentages of CD62L<sup>low</sup> cells are also increased in both K b<sup>−/−</sup>Db<sup>−/−</sup> and K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice compared with that of WT mice. These data indicate that in the absence of MHC class Ia-restricted CD8+ T cells, most H2-M3–restricted as well as other MHC class Ib-restricted CD8+ T cells are activated in naive mice.

To examine the functional properties of non–H2-M3 MHC class Ib-restricted CD8+ T cells, CD8+ T cells were enriched from WT, K b<sup>−/−</sup>Db<sup>−/−</sup>, and K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice and stimulated with anti-CD3 and anti-CD28 Abs. After in vitro stimulation, intracellular staining revealed that the majority of MHC class Ib-restricted K b<sup>−/−</sup>Db<sup>−/−</sup> and K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> CD8+ T cells robustly secreted IFN-γ, whereas a significantly smaller proportion of

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**FIGURE 1.** Generation of K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice. A, Schematic detailing the meiotic intra-H2 recombination required to generate K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice. B, Flow cytometric analysis of H2-K b, H2-D b, and H2-M3 cell surface expression on B220<sup>+</sup> splenocytes isolated from WT (thick line) and K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> (dotted line) mice. Isotype controls are shown for comparison as shaded histograms. To detect H2-M3 expression, splenocytes from indicated mice were incubated overnight with 10 μM LemA peptide.

**FIGURE 2.** Characterization of CD8+ T cells in naive K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice. A–C, Flow cytometric analysis of CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations in WT, K b<sup>−/−</sup>Db<sup>−/−</sup>, and K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice. Data shown are representative of three independent experiments. A, Lymphocytes were isolated from the spleen, liver, and lymph nodes. Numbers indicate the percentage of cells in each quadrant in the lymphocyte gate. B, Bar graphs indicate the percentage of CD8<sup>+</sup> T cells. Data are presented as the mean ± SEM using six mice per genotype. *p < 0.05; **p < 0.01; ***p < 0.001. C, Cell surface expression of activation markers on TCRβ<sup>+</sup>CD8<sup>+</sup> splenocytes in naive WT, K b<sup>−/−</sup>Db<sup>−/−</sup>, and K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice. D, Ex vivo anti-CD3 and anti-CD28 Ab stimulation of CD8+ T cells enriched from the spleens of WT, K b<sup>−/−</sup>Db<sup>−/−</sup>, and K b<sup>−/−</sup>Db<sup>−/−</sup>M3<sup>−/−</sup> mice. Intracellular staining for IFN-γ was performed at 12 h poststimulation. Data shown are representative of three experiments.
WT CD8+ T cells was able to do so (Fig. 2D). These data indicate that, consistent with their activated phenotype, MHC class Ib-restricted CD8+ T cells more readily produce proinflammatory cytokines compared with MHC class la-restricted CD8+ T cells.

Non–H2-M3 MHC class Ib-restricted CD8+ T cells expand upon primary infection with Listeria

To assess whether residual CD8+ T cells from Kb-b/- Db-b/- M3-/- mice expand in response to bacterial infection, WT, Kb-b/- Db-b/-, and Kb-b/- Db-b/- M3-/- mice were infected with a sublethal dose of *L. monocytogenes*. Seven days after infection, splenocytes and hepatic leukocytes were harvested, and the CD44+ and CD8+ T cell populations were analyzed by flow cytometry. Compared with naive mice, a significant increase in overall cellularity (2-fold in spleen and 3-fold in liver) was detected in all three *L. monocytogenes*-infected mouse strains (data not shown). In addition, the percentages of CD8+ T cells increased by 2- to 4-fold and 5- to 7-fold in *L. monocytogenes*-infected Kb-b/- Db-b/- M3-/- and Kb-b/- Db-b/- M3-/- mice, respectively (Fig. 3A). These data indicate that, similar to H2-M3-restricted CD8+ T cells, CD8+ T cells restricted to other MHC class Iib molecules are able to undergo extensive proliferation after primary infection with *L. monocytogenes*.

To compare the kinetics of H2-M3-restricted and non–H2-M3 MHC class Ib-restricted CD8+ effector T cells during primary *L. monocytogenes* infection, we infected K(–)/Db(–)/M3(–) and K(–)/Db(–)/M3(–) mice with *L. monocytogenes* and examined the CD44hi/CD8+ T cell populations in these mice at various time points after infection. Both M3-restricted infected K(–)/Db(–)/M3(–) and Kb-b/- Db-b/- M3-/- mice exhibited a steady increase in the total number of CD44hi/CD8+ T cells during the first week of infection even though the number of CD44hi/CD8+ T cells was significantly greater in K-b/- Db-b/- M3-/- mice than in K-b/- Db-b/- M3-/- mice (Fig. 3B). The number of CD44hi/CD8+ T cells in L. monocytogenes–infected K(–)/Db(–)/M3(–) and K(–)/Db(–)/M3(–) mice was significantly increased in the spleen (~5-fold) and liver (~7-fold) at day 5 postinfection compared with that at day 3 postinfection. At day 7 postinfection, the number of CD44hi/CD8+ T cells was further increased in both the spleen (~1.5-fold) and liver (~4.5-fold) of K-b/- Db-b/- and K-b/- Db-b/- M3-/- mice. These data suggest that the kinetics of non–H2-M3 MHC class Iib-restricted CD8+ T cells are similar to those of H2-M3-restricted CD8+ T cells in response to primary *L. monocytogenes* infection. Notably, as observed in L. monocytogenes–infected K-b/- Db-b/-, hepatic CD8+ effector T cells in L. monocytogenes–infected K-b/- Db-b/- M3-/- mice underwent a more vigorous expansion than did splenic CD8+ effector T cells. These data indicate that, similar to H2-M3-restricted T cells, non–H2-M3 class Ib-restricted effector CD8+ T cells may preferentially expand in the liver or may be preferentially recruited to the liver during *L. monocytogenes* infection.

CD8+ T cells in K(–)/Db(–)/M3-/- mice protect against Listeria infection

To examine the protective capacity of non–H2-M3 MHC class Ib-restricted CD8+ T cells during *L. monocytogenes* infection, we compared the bacterial burden in both the spleens and livers of infected K(–)/Db(–)/M3(–) and K-b/- Db-b/- M3-/- mice with that of infected β-m-/- mice that lack most MHC class Iib-restricted CD8+ T cells. At 5 and 7 d postinfection, the bacterial burden was significantly lower in both the spleens and livers of K-b/- Db-b/- M3-/- mice compared with that of β-m-/- mice (Fig. 4A), suggesting that non–H2-M3 MHC class Ib-restricted CD8+ T cells contribute to bacterial clearance. At day 7 postinfection, bacterial burdens were further reduced in K-b/- Db-b/- M3-/- mice compared with those in K-b/- Db-b/- M3-/- mice (Fig. 4A), confirming a protective role for H2-M3–restricted T cells against *L. monocytogenes* infection (26).

To demonstrate that the protective effect observed in *L. monocytogenes*-infected K-b/- Db-b/- M3-/- mice is mediated by CD8+ T cells, we adoptively transferred either splenocytes or CD8+ T cell-depleted splenocytes isolated from naive or *L. monocytogenes*-infected WT or K-b/- Db-b/- M3-/- mice into naive WT recipient mice. The recipient mice were then challenged with a lethal dose of *L. monocytogenes*, and protective immunity was evaluated on day 3 postinfection by determining the bacterial burden in the spleen and liver. Transfer of splenocytes from *L. monocytogenes*-vaccinated WT or K-b/- Db-b/- M3-/- mice provided significant protection to recipient mice against subsequent *L. monocytogenes* challenge compared with that provided by transfer of naive splenocytes (Fig. 4B). However, this protective effect was abolished when CD8+ T cells were depleted from WT or K-b/- Db-b/- M3-/- splenocytes mice prior to transfer. Collectively, these data indicate that residual CD8+ T cells in K-b/- Db-b/- M3-/- mice can contribute to protective immunity against *L. monocytogenes* infection.

Non–H2-M3 MHC class Iib-restricted CD8+ T cells are cytotoxic and secrete proinflammatory cytokines in response to Listeria infection

To determine the effector function of non–H2-M3 MHC class Ib-restricted CD8+ T cells during *L. monocytogenes* infection, we harvested splenocytes and hepatic leukocytes from *L. monocyto-

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**FIGURE 3.** Expansion of CD8+ T cells in K-b/- Db-b/- M3-/- mice during *L. monocytogenes* infection. A, WT, K-b/- Db-b/-, and K-b/- Db-b/- M3-/- mice were infected with 2 × 10^7 CFU *L. monocytogenes*. Seven days after infection, splenocytes and hepatic leukocytes were harvested and stained with Abs against CD8β and TCRβ. Bar graphs depict the mean ± SEM for the percentage of CD8+ T cells in the lymphocyte gate for uninfected and *L. monocytogenes* (LM)-infected WT, K-b/- Db-b/-, and K-b/- Db-b/- M3-/- mice. *p < 0.05; **p < 0.01; ***p < 0.001. B, Splenocytes and hepatic leukocytes were harvested from *L. monocytogenes*-infected K-b/- Db-b/- and K-b/- Db-b/- M3-/- mice at the indicated time points and stained with Abs against CD8α, TCRβ, and CD44. Bar graphs depict the mean ± SEM for the absolute number of CD44hi/CD8+ T cells for each indicated genotype. Results from three to nine mice per genotype are shown. *p < 0.05; **p < 0.01; ***p < 0.001.
cytogenes-infected K\textsuperscript{b-/-D-/-M3-/-} mice and stimulated them ex vivo with HKLM. Consistent with the kinetics of total CD8\textsuperscript{+} effector T cells upon L. monocytogenes infection, the number of L. monocytogenes-specific non–H2-M3 MHC class Ib-restricted CD8\textsuperscript{+} T cells was significantly increased at day 5 after infection and was further increased by day 7 (Supplemental Fig. 2). A significant proportion of K\textsuperscript{b-/-D-/-M3-/-} CD8\textsuperscript{+} T cells from L. monocytogenes-infected mice was able to produce IFN-\gamma when stimulated with HKLM, whereas unstimulated CD8\textsuperscript{+} T cells did not (Fig. 5A and Supplemental Fig. 3).

To characterize further the cytokines produced by the CD8\textsuperscript{+} T cells found in L. monocytogenes-infected K\textsuperscript{b-/-D-/-M3-/-} mice, CD8\textsuperscript{+} T cells were enriched from the spleens of K\textsuperscript{b-/-D-/-M3-/-} mice at 7 d post-L. monocytogenes infection and stimulated ex vivo with L. monocytogenes-infected BMDCs. We found that CD8\textsuperscript{+} T cells isolated from L. monocytogenes-infected K\textsuperscript{b-/-D-/-M3-/-} mice were able to produce significant amounts of TNF-\alpha, IFN-\gamma, IL-6, and IL-17A in response to stimulation with L. monocytogenes-infected BMDCs (Fig. 5B).

To investigate the cytolytic capacity of non–H2-M3 MHC class Ib-restricted CD8\textsuperscript{+} T cells during L. monocytogenes infection, we performed an in vitro CTL assay using Con A-activated CD8\textsuperscript{+} effector T cells enriched from L. monocytogenes-infected K\textsuperscript{b-/-D-/-M3-/-} mice. Effector T cells enriched from L. monocytogenes-infected K\textsuperscript{b-/-D-/-M3-/-} mice preferentially lysed L. monocytogenes-infected J774 target cells but not uninfected targets, indicating that the residual CD8\textsuperscript{+} T cell population found in K\textsuperscript{b-/-D-/-M3-/-} mice contains CTL specific to L. monocytogenes (Fig. 5C). The combined abilities of non–H2-M3 MHC class Ib-restricted L. monocytogenes-specific CD8\textsuperscript{+} cells to lyse L. monocytogenes-infected cells and to produce proinflammatory cytokines in response to listerial Ags likely contribute to their ability to protect against L. monocytogenes infection.

Antilisterial CD8\textsuperscript{+} T cells from K\textsuperscript{b-/-D-/-M3-/-} mice do not recognize previously characterized MHC class Ib molecules

Although more than 40 MHC class Ib molecules are encoded in the genome, only H2-M3 and Qa-1 have been shown to function...
as restriction elements for \( L.\) \textit{monocytogenes}-specific CTLs (20–22, 27–30). However, H2-M3– and Qa-1–restricted T cell responses can account for only a fraction of the MHC class Ib-restricted responses observed during \( L.\) \textit{monocytogenes} infection. To investigate which MHC class Ib molecules might serve as restriction elements for the CD8\(^+\) T cells found in \( L.\) \textit{monocytogenes}-infected K\textsubscript{b}–/–D\textsuperscript{b}–/– and K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice, we enriched splenic CD8\(^+\) T cells from these mice and cultured them with \( L.\) \textit{monocytogenes}-infected BMDCs that had been pretreated with blocking Abs against CD1d, H2-M3, Qa-1\(^\beta\), Qa-2, or MR1 in an ELISPOT assay. \( L.\) \textit{monocytogenes}-specific IFN-\( \gamma\) secretion by CD8\(^+\) T cells isolated from K\textsubscript{b}–/–D\textsuperscript{b}–/– mice was significantly reduced in the presence of anti-H2-M3 Ab, confirming that a substantial fraction of \( L.\) \textit{monocytogenes}-specific CD8\(^+\) T cells in K\textsubscript{b}–/–D\textsuperscript{b}–/– mice are restricted to H2-M3 (Fig. 6A and Supplemental Fig. 4). However, the presence of blocking Abs against H2-M3, Qa-1\(^\beta\), Qa-2, and MR1 had no effect on \( L.\) \textit{monocytogenes}-specific responses produced by CD8\(^+\) T cells isolated from K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice. These data suggest that neither Qa-1\(^\beta\), Qa-2, nor MR1 serve as major restriction elements for the MHC class Ib-restricted antilisterial CTLs found in K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice.

To attempt to identify novel MHC class Ib molecules that are capable of presenting bacterial Ags to CD8\(^+\) T cells, we established T cell lines from HKLM-immunized K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice. One of these CD8\(^+\) T cell lines, T2 CTL, preferentially lysed HKLM-pulsed BMDCs derived from K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice but not BMDCs derived from b\textsubscript{m}–/– mice, suggesting that T2 CTL recognizes a b\textsubscript{m}-associated MHC class Ib molecule (Fig. 6B). In addition, blocking Abs against Qa-1\(^\beta\), Qa-2, and MR1 did not inhibit the reactivity of T2 CTL, suggesting that this T cell line recognizes a novel MHC class Ib molecule (Fig. 6C).

**Non-H2-M3 MHC class Ib-restricted memory T cells do not exhibit enhanced recall responses to \( L.\) \textit{monocytogenes}**

Previous studies have shown that H2-M3–restricted CD8\(^+\) T cells do not undergo significant expansion after secondary infection with \( L.\) \textit{monocytogenes} (42, 43, 46, 47). To determine whether non-H2-M3 MHC class Ib-restricted CD8\(^+\) T cells can persist as long-lasting memory T cells and expand in response to secondary \( L.\) \textit{monocytogenes} infection, we compared the percentage of CD8\(^+\) T cells in the spleens of naive K\textsubscript{b}–/–D\textsuperscript{b}–/– and K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice with those in mice that had been infected 1 mo previously with \( L.\) \textit{monocytogenes} and either had been allowed to recover or had received a secondary lethal dose of \( L.\) \textit{monocytogenes}. The proportion of CD8\(^+\) T cells in K\textsubscript{b}–/–D\textsuperscript{b}–/– mice was increased 2-fold at 1 mo post-primary \( L.\) \textit{monocytogenes} infection, compared with that in naive mice, but did not increase further upon secondary challenge (Fig. 7A). Compared with naive K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice, K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice that had been infected 1 mo previously also exhibited a 2-fold increase in the proportion of CD8\(^+\) T cells (Fig. 7A). However, neither the percentage nor the total number of K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– CD8\(^+\) T cells changed significantly upon rechallenge with \( L.\) \textit{monocytogenes} (Fig. 7A; data not shown), suggesting that similar to H2-M3–restricted CD8\(^+\) T cells, non–H2-M3 MHC class Ib-restricted CD8\(^+\) T cells do not proliferate extensively during recall responses to \( L.\) \textit{monocytogenes} infection. Compared with the analogous CD8\(^+\) T cell population in rechallenged K\textsubscript{b}–/–D\textsuperscript{b}–/– mice, the percentage of CD8\(^+\) T cells in rechallenged K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice is substantially lower, suggesting that H2-M3–restricted CD8\(^+\) T cells remain the dominant MHC class Ib-restricted T cell population during secondary \( L.\) \textit{monocytogenes} infection (Fig. 7A). However, no differences in bacterial burden were observed between K\textsubscript{b}–/–D\textsuperscript{b}–/– and K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice upon rechallenge with \( L.\) \textit{monocytogenes} (Supplemental Fig. 5).

Despite their lack of expansion, CD8\(^+\) T cells isolated from both K\textsubscript{b}–/–D\textsuperscript{b}–/– and K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice at 3 d post-secondary \( L.\) \textit{monocytogenes} infection are capable of secreting IFN-\( \gamma\) upon ex vivo stimulation with listerial Ags (Fig. 7B), suggesting that both H2-M3–restricted and non–H2-M3 MHC class Ib-restricted CD8\(^+\) T cells can survive for extended periods of time postinfection and maintain effector function. Although secondary \( L.\) \textit{monocytogenes} infection led to an increase in total numbers of \( L.\) \textit{monocytogenes}-specific IFN-\( \gamma\)-producing CD8\(^+\) T cells in both K\textsubscript{b}–/–D\textsuperscript{b}–/– and K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice, the magnitude of \( L.\) \textit{monocytogenes}-specific CD8\(^+\) T cell responses to secondary infection was significantly lower compared with CD8\(^+\) T cell responses in K\textsubscript{b}–/–D\textsuperscript{b}–/– and K\textsubscript{b}–/–D\textsuperscript{b}–/–M\textsubscript{3}–/– mice after primary infection (Fig. 7C). These data indicate that the lack of ability to undergo memory cell expansion may be a common feature of MHC class Ib-restricted CD8\(^+\) T cell responses to \( L.\) \textit{monocytogenes} infection.

**Discussion**

Emerging evidence suggests that MHC class Ib molecules can contribute to host immune responses through the presentation of
CD8+ T cell responses in Kb-/-Db-/-M3-/- mice. The majority of L. monocytogenes-specific MHC class Ib-restricted CD8+ T cells isolated to date are restricted to H2-M3, which appears to play a dominant role in antilisterial immunity (26, 42). However, the L. monocytogenes-specific CD8+ T cell responses observed in infected Kb-/-Db-/-M3-/- mice were not noticeably inhibited by Abs specific to several known MHC class Ib molecules, including Qa-1b, Qa-2, MR1, and CD1d (Fig. 6A and Supplemental Fig. 4), suggesting that these L. monocytogenes-specific CD8+ T cells are restricted by a previously uncharacterized MHC class Ib molecule(s). Indeed, CD8+ T cells isolated from infected Kb-/-Db-/-M3-/- mice failed to lyse Qa-1b-transfectants (Supplemental Fig. 6). Of the more than 40 MHC-linked MHC class Ib genes present in the mouse, only three definitively present microbial Ags to CD8+ T cells while little is known regarding the expression status and function of the remaining MHC class Ib genes. Thus, T cell lines derived from L. monocytogenes-infected Kb-/-Db-/-M3-/- mice would be useful tools for identifying novel Ag-presenting MHC class Ib molecules.

Analysis of the surface phenotype and functional properties of the residual CD8+ T cell populations found in the peripheral lymphoid organs of Kb-/-Db-/- and Kb-/-Db-/-M3-/- mice indicated that most H2-M3-restricted and non-H2-M3 MHC class Ib-restricted CD8+ T cells display an activated/memory phenotype. However, most of the CD8+ T cells in the thymus of Kb-/-Db-/-M3-/- mice are CD44low (data not shown), suggesting that the acquisition of a memory phenotype by non-H2-M3 MHC class Ib-restricted CD8+ T cells is mainly a postthymic event. It is possible that these non-H2-M3 MHC class Ib-restricted CD8+ T cells recognize commensal Ags and thereby become primed once they enter the periphery. The residual CD8+ T cells in Kb-/-Db-/-M3-/- mice can rapidly produce IFN-γ after TCR stimulation. In addition, culture of Kb-/-Db-/-M3-/- CD8+ T cells with IL-12 and IL-18, cytokines induced by L. monocytogenes infection as well as by infection with other pathogens, can induce IFN-γ secretion (data not shown). These data suggest that non-H2-
M3 MHC class Ib-restricted CD8+ T cells may respond to L. monocytogenes infection in an Ag- and cytokine-dependent manner to contribute to the early phase of acquired immune responses against infection. Indeed, Kbβ2–/–D2–/–M3–/– mice are more resistant to primary L. monocytogenes infection compared with β2m–/– mice that lack all MHC class Ib-restricted CD8+ T cells. We cannot eliminate the possibility that the observed differences in susceptibility to L. monocytogenes infection between β2m–/– and Kbβ2–/–D2–/–M3–/– mice may in part be due to differential NK cell activity, as it has been shown that ligand expression during NK cell development can affect their functional activity (64, 65). However, adoptive transfer of splenocytes isolated from L. monocytogenes–immunized Kbβ2–/–D2–/–M3–/– mice confers significant protection upon recipient mice against lethal L. monocytogenes infection in a CD8+ T cell-dependent manner. These data provide direct evidence that CD8+ T cells restricted by MHC class Ib molecules other than H2-M3 can contribute to protective immunity against bacterial pathogens. Although H2-M3 appears to be the dominant restriction element for MHC class Ib-restricted responses during L. monocytogenes infection, our preliminary studies suggest that other MHC class Ib-restricted CD8+ T cells may play a more prominent role during M. tuberculosis infection, as we do not detect significant differences in CD8+ T cell expansion when comparing M. tuberculosis–infected Kbβ2–/–D2–/– and Kbβ2–/–D2β2–/–M3–/– mice (data not shown). Thus, CD8+ T cells restricted by distinct MHC class Ib molecules may play differential roles in host defense against different bacterial infections. A recent study has shown that HLA-E can present a panel of M. tuberculosis–derived peptides to CD8+ T cells that have both cytotoxic and immunoregulatory activity (34). In addition, M. tuberculosis–reactive MR1-restricted CD8+ T cells were found to be enriched in the lungs of patients with active tuberculosis and responded to M. tuberculosis–infected MR1-expressing lung epithelial cells (35). These studies suggest that MHC class Ib-restricted CD8+ T cells may participate in immune responses against bacterial infection in humans. Further studies of the in vivo function of MHC class Ib-restricted CD8+ T cells during microbial infection using mice deficient in MHC class Ia and various MHC class Ib molecules would provide insight into the relative contribution of different MHC class Ib-restricted T cell populations to antimicrobial immunity. In addition, they may lead to the identification of novel Ag-presenting MHC class Ib molecules, which could be further explored as targets for vaccines against intracellular bacteria. Whereas the highly polymorphic nature of MHC Ia molecules complicates vaccine design, vaccines that induce MHC class Ib-restricted T cell responses by targeting the relatively non-polymorphic MHC class Ib molecules would likely be effective in most human individuals.

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Disclosures

The authors have no financial conflicts of interest.

References

FIGURE LEGENDS FOR SUPPLEMENTARY FIGURES

Figure S1. TCR Vβ usage of CD8+ T cells in WT, K\textsuperscript{b/-}D\textsuperscript{b/-} and K\textsuperscript{b/-}D\textsuperscript{b/-}M3\textsuperscript{1/-} mice. Splenocytes isolated from WT, K\textsuperscript{b/-}D\textsuperscript{b/-} and K\textsuperscript{b/-}D\textsuperscript{b/-}M3\textsuperscript{1/-} mice were stained with antibodies against TCRβ, CD8, and indicated TCR Vβ chains. Bar graphs depict mean ± SEM for the percentage of TCRβ+CD8+ cells expressing particular TCR Vβ chains in WT (open bars), K\textsuperscript{b/-}D\textsuperscript{b/-} (hatched bars) and K\textsuperscript{b/-}D\textsuperscript{b/-}M3\textsuperscript{1/-} (filled bars) mice. Results shown are means from four mice per genotype. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure S2. Kinetics of LM-specific CD8+ T cell responses in K\textsuperscript{b/-}D\textsuperscript{b/-}M3\textsuperscript{1/-} mice. Splenocytes were harvested from LM-infected K\textsuperscript{b/-}D\textsuperscript{b/-} and K\textsuperscript{b/-}D\textsuperscript{b/-}M3\textsuperscript{1/-} mice at various time points and stimulated ex vivo with HKLM for 7 h. Bar graph depicts mean ± SEM for the total number of IFN-γ-producing CD8+ T cells from 9 mice per experimental group. *, p < 0.05; ***, p < 0.001.

Figure S3. LM-specific CD8+ T cells isolated from LM-infected K\textsuperscript{b/-}D\textsuperscript{b/-}M3\textsuperscript{1/-} mice produce IFN-γ. Splenocytes and hepatic leukocytes were harvested from K\textsuperscript{b/-}D\textsuperscript{b/-}M3\textsuperscript{1/-} mice 7 days post LM infection. Cells were stimulated with HKLM for 7 h and stained with antibodies against CD8 and TCRβ. Cells were then intracellularly stained for IFN-γ and analyzed by flow cytometry. Bar graphs depict the mean ± SEM for the percentage of CD8+IFN-γ+ cells within the TCRβ+ gate. N=13 for untreated splenocytes as well as for HKLM-treated splenocytes.

Figure S4. LM-specific K\textsuperscript{b/-}D\textsuperscript{b/-}M3\textsuperscript{1/-} CD8+ T cells are not CD1d-restricted. Splenocytes were harvested from LM-infected K\textsuperscript{b/-}D\textsuperscript{b/-} and K\textsuperscript{b/-}D\textsuperscript{b/-}M3\textsuperscript{1/-} mice at 7 days post-infection and enriched for CD8+ T cells. These T cells were then used as effectors in an IFN-γ ELISPOT assay. Uninfected or LM-infected BMDC were used as stimulators.
and were incubated with CD8\(^+\) T cells in medium alone or in the presence of mAb against CD1d. Results are presented as the mean ± SEM of the number of IFN-\(\gamma\) spot-forming units from two pooled animals per genotype and are representative of two independent experiments.

**Figure S5.** H2-M3-restricted CD8\(^+\) T cell responses do not protect against secondary LM infection. \(K^b/-D^b/-\) and \(K^b/-D^b/-M3^-/-\) mice were infected with 2x10\(^3\) CFU of LM, then subsequently rechallenged with 5x10\(^4\) CFU of LM 1 mo following initial infection. Bacterial burden in the liver was determined at three days post secondary infection. Bar graphs depict the mean ± SEM for 7 mice per genotype.

**Figure S6.** LM-specific \(K^b/-D^b/-M3^-/-\) CD8\(^+\) T cells do not lyse Qa-1\(^b\)-expressing cells. Splenocytes were isolated from \(K^b/-D^b/-M3^-/-\) mice 7 days post LM infection, enriched for CD8\(^+\) T cells, and activated with ConA. After 3 days of ConA stimulation, splenocytes were used as effectors in a \(^{51}\)Cr release CTL assay at an effector:target cell ratio of 30:1. Uninfected or LM-infected J774 cells, HeLa cells, or HeLa Qa-1\(^b\)-transfectants were labeled with \(^{51}\)Cr and used as targets. Graph depicts the percentage of LM-specific killing pooling two mice per genotype. Data are representative of two independent experiments.
Figure S1.
Figure S2.

The figure shows the IFN-γ producing CD8+ T cells (x10^5) over different days (Day 3, Day 5, Day 7) for different conditions.

- K0-D-D
- K0-D-M3

The bars indicate the trend with asterisks (*) and triple asterisks (***), representing statistical significance.
Figure S3.

![Graph showing the percentage of IFN-γ producing CD8+ T cells gated on TCRβ+ cells in spleen and liver. The graph compares untreated and +HKLM conditions with statistical significance indicated by asterisks.](image)
Figure S4.

[Legend for the figure is not provided in the image.]
Figure S5.
Figure S6.