Response to *Listeria monocytogenes* in Mice Lacking MHC Class Ia Molecules

Michael S. Seaman, Béatrice Péranau, Kirsten Fischer Lindahl, Francois A. Lemonnier and James Forman

*J Immunol* 1999; 162:5429-5436; [http://www.jimmunol.org/content/162/9/5429](http://www.jimmunol.org/content/162/9/5429)
Response to *Listeria monocytogenes* in Mice Lacking MHC Class Ia Molecules

Michael S. Seaman, Béatrice Péarnau, Kirsten Fischer Lindahl, François A. Lemonnier, and James Forman

MHC class Ia-deficient mice (H2 Kb−/− Db−/−) inoculated with the intracellular pathogen *Listeria monocytogenes* (LM) displayed a three- to fourfold expansion of splenic CD8+ T cells 6 days following infection. Culture of these spleen cells in vitro gave rise to CTL that recognized LM-infected target cells and were restricted by the class Ib molecules, Qa1b and M3. Exposure of target cells to heat-killed LM (HKLM) rather than live bacteria did not result in CTL-mediated lysis. Target cells pulsed with three LM peptides known to bind M3, f-MIGWII, f-MIVTLF, and f-MIVIL, were recognized by effector cells from both B6 and Kb−/− Db−/− animals. In vivo analysis showed that B6 and Kb−/− Db−/− mice clear LM from the spleen and liver rapidly with similar kinetics, whereas TAP.1−/− mice, which are deficient in class Ia and Ib molecules, clear LM slowly upon infection. To establish the in vivo role of CD8+ T cells in Kb−/− Db−/− animals, we showed that depletion of such cells from the spleens of immune mice prevented the adoptive transfer of protective immunity to syngeneic recipients. Spleen cells from Kb−/− Db−/− mice were also capable of generating responses directed against syngeneic as well as allogeneic class Ia molecules in vitro. Thus, class Ia-deficient animals have a CD8+ T cell repertoire capable of recognizing both class Ia and class Ib molecules and can generate protective immunity to LM. *The Journal of Immunology*, 1999, 162: 5429–5436.

Infection of mice with the intracellular bacterium *Listeria monocytogenes* (LM) has been extensively used as a model system to study the mechanisms of antimicrobial immunity. Effector cells of both the innate and adaptive immune systems play critical, integrated roles in resolving infection. The early stages of listeriosis are marked by the rapid recruitment of neutrophils and macrophages to sites of bacterial growth (1, 2). Early production of IFN-γ by NK cells has been shown to be crucial for activating the bactericidal mechanisms of macrophages (3, 4). While the innate immune response is efficient at limiting the initial spread of infection, rapid clearance of *Listeria* depends on T cell-mediated immunity (5). Both CD4+ and CD8+ T cells specific for LM Ags expand during the course of infection (6). Although CD4+ T cells are capable of eliciting a *Listeria*-specific delayed-type hypersensitivity response, long term protective antilisterial immunity mainly resides within the CD8+ T cell population (6, 7).

In addition to the presentation of *Listeria* Ags by MHC class Ia proteins, nonclassical MHC class Ib molecules also present peptides to antilisterial CTL. For example, early studies demonstrated the generation of CD8+ T cells following LM infection that were capable of providing short term antilisterial immunity upon adoptive transfer into allogeneic strains of mice (8, 9). At first termed MHC unrestricted, it is now understood that these effector cells recognize Ags presented by nonclassical class I molecules. Class Ib MHC molecules, encoded by the Q, T, and M regions, exhibit limited polymorphism compared with their class Ia counterparts and are therefore often shared among various strains of inbred mice. Three LM-derived peptides have been identified that are presented to CD8+ αβ T cells by the class Ib molecule M3 (10–12). A unique feature of M3 is its higher binding affinity for peptides beginning with N-formyl methionine (13). Prokaryotic organisms initiate protein synthesis with formylated methionine, which suggests that M3 has evolved to provide a specialized role in antimicrobial immunity. The T-region-encoded class Ib molecule Qa1b has also been shown to function as a restriction element for LM-specific CTL (14, 15). While the LM-derived Ag(s) presented to CD8+ T cells by Qa1b has yet to be identified, a previous study demonstrated that this process is TAP dependent (15), and thus a peptide is most likely recognized.

MHC class Ib-restricted CTL are induced in vivo following a sublethal infection with LM (15). The lytic activity of these effectors expands and contracts with kinetics similar to those observed for class Ia-restricted antilisterial CTL. Despite these observations, the relative significance of class Ia vs class Ib presentation in generating antilisterial immunity has not been defined. In this study we infected mice lacking MHC class Ia molecules (H2 Kb−/− Db−/−) with LM to assess whether MHC class Ib Ag presentation plays a significant role in generating a CD8+ T cell response to this pathogen.

Materials and Methods

Mice

C57BL/6, TAP.1−/−, BALB/c, and Kb−/− Db−/− mice were bred and maintained in animal colonies at the University of Texas Southwestern Medical Center (Dallas, TX). Kb−/− Db−/− double-knockout mice have genomic deletions of the MHC class Ia genes Kα and Dα and were generated from embryonic stem cells of strain 129/Ola (H2b) as previously described (16). The knockout haplotype has been backcrossed onto the C57BL/6 (B6) background three times, and homozygotes were selected.
after intercrossing. Because the strain thus derives at least 87.5% of its genes from B6, B6 was the most suitable wild-type control strain for the experiments reported here. The Mhc of 129, and thus of the knockout mice, is considered identical with H2b of B6 in the class Ia, class II, and class III regions (the classical H2 complex); although 129 mice do differ from B6 in the H2-T region (reviewed in Ref. 17), they express the same forms of Qa1 (17, 18) and M3 (19), and no difference was noted between 129 and B6 in the H2-M region (20).

**Bacteria**

LM 10403 serotype 1 was provided by H. G. A. Bouwer (Veterans Affairs Medical Center, Portland, OR). Bacteria were grown on brain-heart infusion agar plates (BHI; Difco Laboratories, Detroit, MI), and virulent stocks were maintained by repeated passage through C57BL/6 mice. The LD₅₀ dose for B6 mice is approximately 2 × 10⁵ bacteria. To generate HKLM, log phase cultures of bacteria grown in BHI broth were washed twice with PBS and incubated for 1 h at 75°C. Bacterial numbers were determined by plating dilutions of viable bacteria on BHI agar plates and incubating for 24 h at 37°C. Colony counts were used to calculate the CFU per milliliter.

**Cell lines and reagents**

The J774 macrophage line (H2d) was kindly provided by H. G. A. Bouwer (Veterans Affairs Medical Center, Portland, OR). Bacteria were grown in brain-heart infusion agar plates (BHI; Difco Laboratories, Detroit, MI), and virulent stocks were maintained by repeated passage through C57BL/6 mice. The LD₅₀ dose for B6 mice is approximately 2 × 10⁵ bacteria. To generate HKLM, log phase cultures of bacteria grown in BHI broth were washed twice with PBS and incubated for 1 h at 75°C. Bacterial numbers were determined by plating dilutions of viable bacteria on BHI agar plates and incubating for 24 h at 37°C. Colony counts were used to calculate the CFU per milliliter.

**Antibodies**

For flow cytometric analysis, anti-CD4-PE (RM4-5), anti-CD8-FITC (53-6.7), and anti-TCRβ-biotin (H57-597) Abs and streptavidin-PE were purchased from PharMingen (San Diego, CA). Spleen cells (5 × 10⁵) were stained for 20 min at 4°C and washed twice with PBS before analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

**Peptides**

Peptide synthesis was performed by automated solid phase techniques using standard F-moc chemistry on a Rainin Symphony peptide synthesizer (Rainin, Woburn, MA). The homogeneity of each peptide was determined by reverse phase HPLC and matrix-assisted laser desorption ionization-time of flight mass spectrometry. Stock solutions (2 mM) were prepared by dissolving lyophilized peptide in DMSO (Sigma, St. Louis, MO).

**Generation of LM-specific CTL**

For LM-specific CTL induction, mice were injected with approximately 2 × 10⁵ bacteria in 200 μl of PBS via the tail vein. Six days following infection, spleen cells from these animals were cultured at 2 × 10⁵/ml in a Corning T75 flask with 50 ml of complete RPMI medium containing 1 μg/ml Con A (Sigma). Cultures were maintained at 37°C in 7% CO₂ humidified air for 72 h. These cells were then used as effectors in a ³⁵Cr release CTL assay.

**LM-specific CTL assay**

Target cells (1–1.5 × 10⁵) were plated in 48-well tissue culture plates 18 h before the assay in 0.5 ml of antibiotic-free medium. Log-phase cultures of LM grown in BHI broth were harvested and washed twice with PBS. Target cell monolayers were infected at a multiplicity of infection of 5:1 for J774 cells and 100:1 for HeLa, HeLa:Qa1b, BALB/c 3T3, and CM3 cells. Control cells received no bacteria. After 60–90 min, the target cell monolayers were washed twice with warm PBS, and 250 μl of medium containing 40 μg/ml gentamicin sulfate (Life Technologies) was added. Three hours after infection of 10 μl of ³⁵Cr was added to each well and incubated for 1 h at 37°C. Target cell monolayers were then washed twice with PBS and covered in 250 μl of medium with gentamicin. Effecter cells were added in 250 μl of medium. In certain experiments, J774 cells were incubated with HKLM (100:1) for 8 h at 37°C before effector addition. Spontaneous and maximum release wells received 250 μl of medium and 1% SDS, respectively. The assays were terminated 4–5 h later when 250 μl of culture supernatant was harvested and counted on a Micromedic ME plus gamma counter (Micromedic, Horsham, PA). The percent specific lysis was calculated as 100 × (experimental cpm – spontaneous cpm)/ (maximum cpm – spontaneous cpm). Data presented are the means of duplicate wells.

**In vivo LM CFU analysis**

Mice were infected with ~2 × 10⁵ LM in 200 μl of PBS via the tail vein. On days 3, 4, 7, 10, and 14 following infection, the spleens and livers were removed and homogenized in sterile water using a glass Dounce tissue grinder (Kontes, Vineland, NJ). Serial 10-fold dilutions of the homogenate were plated in triplicate on BHI agar plates and incubated for 24 h at 37°C. Colony counts were averaged and corrected for dilution to yield the LM CFU per organ. The CFU limit of detection is 50 for spleen and 100 for liver.

**Adoptive transfer of antilisterial immunity**

LM immune donor spleen cells were isolated from mice immunized 6 days previously with 2 × 10⁵ LM and were divided into three groups. Two donor spleen cell groups were incubated (1 × 10⁶/ml) for 30 min at 4°C with a 1/10 dilution of either 3.155 (anti-CD8) or 2B6 (anti-CD4) mAb. Cells were then washed twice and incubated (1 × 10⁷/ml) with 10% rabbit complement (Pel-Freez, Brown Deer, WI) in a 37°C water bath for 30 min. Nondepleted LM immune and naive donor spleen groups were treated with complement only. Viable cells were recovered by centrifugation over a ficoll gradient (Pharmacia, Piscataway, NJ) and subsequently washed twice with PBS. Naive syngeneic host animals received an i.v. injection of 5 × 10⁶ donor spleen cells in 200 μl. Thirty minutes following cell transfer, mice were challenged i.v. with 4 × 10³ LM. Three days after infection, spleens were removed from host animals, and the LM CFU per organ was calculated as previously described. Data are presented as the mean log₁₀ CFU per spleen of three mice per donor cell group.

**Generation of alloreactive CTL**

Standard 5-day mixed lymphocyte cultures were established for the generation of alloreactive CTL. Irradiated stimulator spleen cells were obtained from female C57BL/6 (H2b) and BALB/c (H2k) mice. Stimulators were cultured in complete RPMI medium at 5 × 10⁶/well in a 24-well tissue culture dish. Female K⁺⁻/⁻ D⁻⁻/⁻ or C57BL/6 responder spleen cells were added at 5 × 10⁵/well. Cultures were maintained at 37°C in 5% CO₂ for 3 days.

**Alloreactive and LM peptide-specific CTL assays**

RMA-S cells were cultured with 10 μM peptide or an equivalent volume of DMSO for 14 h at 25°C before assay. Peptide-sensitized RMA-S cells (2 × 10⁵) were labeled with ⁵¹Cr for 1.5 h at 25°C. All other target cells were labeled for 1 h at 37°C. Cells were then washed twice and added to a 96-well round-bottom plate at 5 × 10⁵/well in 100 μl of complete RPMI medium. Titrations of effector cells were added in 100 μl of medium, and the assays were incubated for 4 h at 37°C. The supernatants (100 μl) were harvested, and the percent specific lysis was determined as described above. Data presented are the means of triplicate wells.

**Results**

Increase of splenic CD⁸⁺ T cells in K⁺⁻/⁻ D⁻⁻/⁻ mice following LM infection

Naïve K⁺⁻/⁻ D⁻⁻/⁻ mice have ~2% CD8⁻αβ T cells in their spleens (Fig. 1). To assess whether this T cell population increases in response to an intracellular bacterial infection, we injected K⁺⁻/⁻ D⁻⁻/⁻ and B6 mice with a sublethal dose of LM. Six days following infection, spleen cells were harvested, and the CD4⁺ and CD8⁺ T cell populations were compared with those of naive animals. A three- to fourfold increase in splenic CD8⁺ T cells occurred in K⁺⁻/⁻ D⁻⁻/⁻ mice following infection, whereas no change in the CD4⁺ population was detected (Fig. 1). By contrast, no significant alteration in the percentage of CD4⁺ or CD8⁺ T cells was observed in B6 mice following LM infection.
Kb−/− Db−/− mice generate LM specific, MHC class Ib-restricted CTL.

To determine whether the CD8+ T cell population observed in Kb−/− Db−/− mice contained CTL specific for LM, spleen cells from mice infected 6 days previously were stimulated with Con A for 3 days and were used as effectors in a 51Cr release assay. The J774 macrophage line was specifically lysed by Kb−/− Db−/− effectors only upon infection with viable LM (Fig. 2A). The level of target cell killing was comparable to that by B6 antilisterial CTL, whereas no specific lysis was observed from naive B6 and Kb−/− Db−/− (data not shown) effectors. The depletion of CD8+ T cells from Con A cultured effectors before assay resulted in the loss of CTL activity (data not shown). Because J774 (H2d) expresses allogeneic MHC class Ia, this suggests that the CTL activity observed is MHC class Ib restricted.

M3 and Qa1b are restriction elements for Kb−/− Db−/− antilisterial CTL

The MHC class Ib molecules M3 and Qa1b have previously been shown to present LM Ags to CTL (15, 21). To examine whether Kb−/− Db−/− antilisterial CTL were restricted by either of these class Ib MHC molecules, transfected cell lines expressing M3 and Qa1b were used as targets in a CTL assay. Both B6 and Kb−/− Db−/− LM immune effectors specifically lysed CM3 targets (H2w17, M3wt) upon infection with LM, whereas the parental B10.CAS2 fibroblast line (H2w17, M3sw) was not lysed (Fig. 2B). These effector populations also lysed infected HeLa cells expressing Qa1b, whereas parental HeLa cells were not lysed (Fig. 2C). Effectors from a naive B6 or Kb−/− Db−/− (data not shown) mouse were incapable of lysing either target cell line upon LM infection. HeLa cells transfected to express the nonclassical class I molecules CD1 and Qa2 were also used as LM-infected targets in this assay. Neither cell line was recognized by B6 or Kb−/− Db−/− antilisterial CTL effectors (data not shown). These data demonstrate that Kb−/− Db−/− CTL can recognize LM Ags presented by the class Ib molecules M3 and Qa1b.

CTL recognition of Listeria Ags presented by M3

We examined the ability of B6 and Kb−/− Db−/− antilisterial CTL to recognize and lyse target cells sensitized with three LM-derived peptides previously demonstrated to bind M3 (10–12). TAP-deficient RMA-S cells were incubated in the presence or the absence of 10 μM peptide for 14 h at 25°C and used as targets in a 51Cr release assay. RMA-S cells pulsed with ND1, an N-formylated mitochondrion-derived peptide, were used as a negative control. Both B6 (Fig. 3A) and Kb−/− Db−/− (Fig. 3B) antilisterial CTL specifically lysed target cells pulsed with the three LM peptides, whereas RMA-S cells incubated without peptide or with the M3-restricted ND1 peptide were not recognized. Both effector populations exhibited the highest CTL activity against RMA-S cells pulsed with f-MIVTLF, a peptide derived from the LM LemA protein (11). B6 effectors lysed f-MIVTLF-pulsed target cells at a higher level than f-MIGWII-pulsed cells, while CTL activity against these peptides by Kb−/− Db−/− effectors was relatively equivalent. No target cell lysis was observed in the presence of naive B6 effectors (Fig. 3C).
Class Ib presentation of LM Ags requires infection with viable bacteria

Entry of LM Ags into the class I presentation pathway is believed to require viable LM that are capable of lysing the phagosome and entering the host cell cytosol (22, 23). Yet previous studies have demonstrated that phagocytic cells pulsed with preparations of HKLM, which do not gain access to the host cell cytosol, are capable of presenting Ag to M3 restricted CTL clones in vitro (24, 25). Having demonstrated the presence of polyclonal M3-restricted antilisterial CTL in B6 and Kb
2/2 D b
2/2 effector populations, we wanted to determine whether target cell recognition could occur in the presence of HKLM or required infection with viable bacteria. J774 macrophage cells incubated with HKLM for 8 h at 37°C or infected with virulent LM for 4 h were used as targets in a 51Cr release assay. Both B6 (Fig. 4A) and Kb
2/2 D b
2/2 (Fig. 4B) antilisterial CTL, but not naive B6 effectors (Fig. 4C), efficiently recognized and lysed target cells infected with viable LM. CTL activity against J774 targets pulsed with HKLM was not any greater than that observed against untreated J774 cells, demonstrating that this is not a relevant pathway for class Ib presentation of LM Ags. Extending the time of target cell incubation with HKLM to 18 h did not enhance CTL recognition (data not shown).

Kb
2/2 D b
2/2 mice rapidly clear an in vivo LM infection

We next examined whether the immune response elicited in Kb
2/2 D b
2/2 animals was efficient in clearing an in vivo LM infection from spleen and liver. For these experiments we also used TAP.1
2/2 mice, which express very low levels of class Ia and, presumably, class Ib molecules and have approximately 1% CD8
1 T cells in the spleen (26). B6, Kb
2/2 D b
2/2, and TAP.1
2/2 mice were infected with a sublethal dose of LM. At various times following infection, spleens and livers were harvested from these animals, and the LM CFU per organ was measured. Kb
2/2 D b
2/2 mice clear LM from the spleen with kinetics similar to those observed in B6 mice, which induce both class Ia- and class Ib-restricted CTL (Fig. 5). No B6 mice had detectable LM on day 10, and only one of seven Kb
2/2 D b
2/2 mice showed CFU in its spleen at that time. By contrast, TAP.1
2/2 mice still showed evidence of infection on day 14. A similar bacterial clearance rate was observed in the livers of these animals (data not shown).

Role of CD8
+ T cells in antilisterial protection of Kb
2/2 D b
2/2 mice

We conducted adoptive transfer experiments to further demonstrate that CD8
+ T cells in Kb
2/2 D b
2/2 mice were involved in
FIGURE 5. In vivo clearance of a LM infection. B6 (open squares), K b /−/ D b /−/ (○), and TAP.1−/− (□) mice were challenged i.v. with 2 × 10^7 LM. On days 3, 4, 7, 10, and 14 following infection, spleens were harvested, and LM CFU were measured. Results are compiled from two different experiments, with each time point representing the mean CFU from seven animals ± SEM. The dashed line represents the CFU limit of detection.

Protection against LM infection. LM immune spleen cells from B6 or K b /−/ D b /−/ mice infected 6 days previously with a low dose of LM were left untreated or were depleted of CD4^+ T cells or CD8^+ T cells in vitro. Donor cells (5 × 10^7) were infused into naive syngeneic recipients, and 30 min later these animals were challenged with 4 × 10^7 LM. Three days following LM infection the spleens were harvested, and the LM CFU per organ was determined. As shown in Table I, transfer of LM immune spleen cells resulted in 5.6 log_{10} protection in B6 recipient mice and 3.4 log_{10} protection in K b /−/ D b /−/ mice when compared with control animals that received naive donor spleen cells. Both B6 and K b /−/ D b /−/ LM immune donor cells depleted of CD4^+ T cells were still capable of conferring protection in naive host animals, whereas almost all protection was lost when donor cells were depleted of CD8^+ T cells. These data demonstrate that, as in B6 mice, CD8^+ T cells in K b /−/ D b /−/ animals play an important role in the protective immune response against LM infection.

Spleen cells from K b /−/ D b /−/ mice generate a response against MHC class Ia molecules

We tested whether spleen cells from K b /−/ D b /−/ mice could generate a CD8^+ T cell response against MHC class Ia Ags in an in vitro primary MLC. K b /−/ D b /−/ and B6 responder spleen cells were cultured in the presence of irradiated stimulator cells from BALB/c (H2^d) or B6 (H2^b) mice. After 5 days of culture, we observed a >20-fold increase in CD8^+ T cells in both B6 and K b /−/ D b /−/ anti-BALB/c MLC and K b /−/ D b /−/ anti-B6 MLC, but not in B6 anti-B6 MLC (data not shown). These effector populations were then tested for their cytolytic activity against P815 (H2^b) and RMA (H2^a) targets to determine whether alloreactive CTL were present. Spleen cells from B6 and K b /−/ D b /−/ mice were cultured with Con A and used as negative control effectors. Both B6 and K b /−/ D b /−/ anti-BALB/c MLC generate CTL capable of lysing P815 target cells (Fig. 6A). K b /−/ D b /−/ anti-B6 MLC effectors lyse RMA cells, whereas B6 anti-B6 MLC effectors fail to lyse the syngeneic target (Fig. 6B). No CTL activity was observed in these experiments using Con A-cultured effectors. To assess which MHC class Ia proteins were targets for K b /−/ D b /−/ anti-B6 CTL, Ltk (H2^k) cells expressing K b or D b were used as targets. L cells expressing both K b (Fig. 6C) and D b (Fig. 5D) were specifically lysed by these CTL, whereas parental L cells were not recognized (Fig. 6E). In contrast to a previous report (27), these experiments demonstrate that unprimed CD8^+ T cells from K b /−/ D b /−/ mice are capable of generating a response against MHC class Ia Ags from the H2^k, H2^b, and H2^a haplotypes following primary in vitro culture.

Discussion

Studies using β2m−/− (MHC class I-negative) and Αβ−/− (MHC class II-negative) mice have demonstrated that while both CD4^+ and CD8^+ T cells are required for optimal protection against LM, the absence of MHC class I molecules more severely impairs the ability to elicit sterilizing immunity in both primary and secondary infections (28). Adoptive transfer and in vivo depletion studies have further supported these findings by showing that long term protective immunity is provided by CD8^+ T cell effectors (6, 7). While antilisterial CTL recognize Ags presented by both MHC class Ia and class Ib proteins, the relative contribution of nonclassical class I Ag presentation in generating antilisterial immunity has not been well defined. In this study we have infected mice lacking class Ia molecules with LM to assess the potential of class Ib Ag presentation in generating a protective CD8^+ T cell response.

Following a sublethal LM infection, we observe an increase of CD8^+ αβ T cells in the spleen of K b /−/ D b /−/ mice, which are capable of lysing infected target cells in vitro. We demonstrate that these antilisterial CTL recognize LM Ags presented by the class Ib molecules, Qia^b and M3. Previous studies that have analyzed the three LM-derived antigenic peptides presented by M3 used CTL lines or clones propagated in vitro, while the in vivo response to these Ags has remained undefined. In this study we have compared the CTL activity against the M3-restricted peptides using polyclonal stimulated spleen cells from B6 and K b /−/ D b /−/ mice infected with LM. While all three LM peptides were recognized by these effector cells, both B6

Table I. CD8^+ T cells from LM immune K b /−/ D b /−/ mice are involved in antilisterial protection upon adoptive transfer into naive recipients

<table>
<thead>
<tr>
<th>Donor Cell Group</th>
<th>Phenotype (%)</th>
<th>Log_{10} Listeria(^{(}\text{CFU/Spleen}))</th>
<th>Log_{10} Protection(^{(}\dagger))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4^+</td>
<td>CD8^+</td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>18</td>
<td>9</td>
<td>7.5 (0.5)</td>
</tr>
<tr>
<td>LM immune</td>
<td>14</td>
<td>14</td>
<td>1.9 (0.2)*</td>
</tr>
<tr>
<td>CD8 depleted</td>
<td>0.6</td>
<td>8</td>
<td>3.9 (0.3)*</td>
</tr>
<tr>
<td>CD8 depleted</td>
<td>14</td>
<td>0.1</td>
<td>6.7 (0.2)</td>
</tr>
<tr>
<td>K b /−/ D b /−/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>19</td>
<td>2</td>
<td>7.3 (0.2)</td>
</tr>
<tr>
<td>LM immune</td>
<td>21</td>
<td>5</td>
<td>5.1 (0.3)*</td>
</tr>
<tr>
<td>CD8 depleted</td>
<td>0.1</td>
<td>6</td>
<td>6.4 (0.1)</td>
</tr>
</tbody>
</table>

\(\dagger\) Log_{10} protection was determined by subtracting the mean Log_{10} CFU/spleen values of the test group from the mean Log_{10} CFU/spleen values of mice receiving naive donor cells.

\(\dagger\) In all experiments, spleens were harvested; the LM CFU/spleen was determined as described in Materials and Methods.

Downloaded from http://www.jimmunol.org/ by guest on October 26, 2017
and K<sup>b</sup>−/- D<sup>b</sup>−/- antilisterial CTL exhibited the highest lytic activity against target cells pulsed with f-MIGWII. The fact that CTL recognition of these epitopes titrated further using K<sup>b</sup>−/- D<sup>b</sup>−/- effector cells (6:1), compared with B6 effector cells (50:1), may indicate that a higher frequency of antilisterial CTL are restricted by M3 in these animals. Previous evidence has suggested that the M3 class Ib molecule can present LM Ags in a nonconventional manner. Data demonstrating that J774 macrophage cells pulsed with HKLM can present Ag to M3-restricted CTL clones has led to the hypothesis that M3 can associate with peptides outside the endoplasmic reticulum, perhaps binding Ag within phagocytic vacuoles analogous to the class I-like molecule CD1 (24). Our results, however, demonstrate that J774 cells infected with viable LM, and not cells pulsed with HKLM, are capable of presenting LM Ags to class Ib-restricted antilisterial CTL from B6 and K<sup>b</sup>−/- D<sup>b</sup>−/- mice. These conflicting results can be attributed to the effector population used for analysis. Previous studies demonstrating recognition of HKLM Ags have used peptide-specific CTL clones or purified CD8<sup>+</sup> T cell lines that were maintained in vitro with high concentrations of HKLM, most likely resulting in the selection of high-affinity CTL that were capable of responding to low levels of Ag (8, 24, 25). Our experiments are conducted using polyclonal stimulated T cells from an immunized animal, thus more accurately reflecting the CTL specificities generated during an in vivo infection. While one report has demonstrated that IL-2-cultured CD8<sup>+</sup> T cells from LM-immune animals could lyse macrophages pulsed with HKLM in a class Ib-restricted manner (9), we did not observe such recognition. Our findings demonstrate that, as for class Ia molecules, efficient presentation of LM Ags by M3 and other class Ib molecules requires infection with viable bacteria.

While the antigenic peptide presented by Qa1<sup>b</sup> is unknown, it is most likely derived from a protein secreted by LM in the host cell cytosol, like all other LM class I epitopes characterized to date (29). Qa1 and its human homologue HLA-E are unique class I molecules in that they preferentially bind conserved peptides derived from the leader sequence of MHC class Ia molecules (30, 31). In fact, the peptide binding groove of Qa1<sup>b</sup> is almost exclusively occupied by a single high affinity peptide derived from the signal sequence of D region molecules (32, 33). While it has been hypothesized that Qa1 functions in the regulation of NK cells (30), the possibility exists that the restrictive peptide binding groove has evolved for the binding and presentation of conserved pathogen-derived peptides, perhaps from signal sequences. Whether the absence of the high affinity D<sup>b</sup> leader peptide in K<sup>b</sup>−/- D<sup>b</sup>−/- mice facilitates loading of the LM-derived epitope, therefore eliciting a stronger Qa1<sup>b</sup>-restricted CTL response in these animals, is not currently known. The ratio of M3- vs Qa1<sup>b</sup>-restricted antilisterial CTL in K<sup>b</sup>−/- D<sup>b</sup>−/- mice and whether other class Ib proteins play a role in generating immunity to LM are also questions of interest. Our studies to date have excluded the presentation of LM Ags by CD1 and Qa2.

Our in vitro data suggest that class Ib presentation of LM Ags is relevant for eliciting CD8<sup>+</sup> T cell immunity in K<sup>b</sup>−/- D<sup>b</sup>−/- animals. These data are further supported by our in vivo analysis, which demonstrates that K<sup>b</sup>−/- D<sup>b</sup>−/- mice clear LM from spleen and liver with the same kinetics as wild-type B6 mice and more rapidly than TAP<sup>−/-</sup> animals, which exhibit low expression of class I molecules. Rapid reduction of LM CFU in the liver of K<sup>b</sup>−/- D<sup>b</sup>−/- mice also corroborates prior evidence that infected hepatocytes can be lysed in a class Ib-restricted manner (34). It is not currently understood whether the lack of MHC class Ia proteins alters the innate immune response to LM infection. However, it is of interest to note that in the early phase of infection (day 3), K<sup>b</sup>−/- D<sup>b</sup>−/- mice consistently have lower LM CFU in the spleen than B6 or TAP<sup>−/-</sup> animals (Fig. 5). The activities of macrophages, NK cells, neutrophils, and γδ T cells following infection of K<sup>b</sup>−/- D<sup>b</sup>−/- mice are currently being investigated. We have further defined the role of CD8<sup>+</sup> T cells in K<sup>b</sup>−/- D<sup>b</sup>−/- mice by examining the ability of LM immune spleen cells to transfer protection to naive hosts. The adoptive transfer of K<sup>b</sup>−/- D<sup>b</sup>−/- spleen cells depleted of CD4<sup>+</sup> T cells protected recipients from subsequent LM challenge, whereas the transfer of CD8<sup>+</sup>-depleted cells did not. Together with our in vitro demonstration of M3- and Qa1<sup>b</sup>-restricted LM-specific CTL, these data indicate that class Ib molecules present LM Ags to CD8<sup>+</sup> T cells, which are probably
 responsible for generating sterilizing immunity following in vivo infection. A study comparing the in vivo responses of class Ia- and class Ib-restricted CD8 T cells has demonstrated that the CTL activities of the two populations expand and contract with similar kinetics, correlating with the clearance of bacterial infection (15). Despite these findings, the relative frequency of antilisterial CTL restricted by class Ia vs class Ib in a wild-type animal has not been defined. A recent report has analyzed four different class Ia-restricted CTL populations from BALB/c mice that recognize LM Ags presented by K* (35). At the peak antilisterial CTL response 7 days postinfection, these CTL groups together represented only approximately 2% of all splenic CD8 T cells. The three- to fourfold increase in CD8 T cells in K b 2/ D b 2/ mice 6 days after LM challenge suggests that class Ib-restricted CTL are capable of expanding in response to bacterial infection, although the possibility that these cells are recruited from another site within these animals cannot be excluded. Whether the class Ib-restricted CTL population exhibits the same expansion in wild-type mice remains to be determined.

How the peripheral CD8 T cell repertoire is generated in K b 2/ D b 2/ mice is not known. Our data demonstrate that in the absence of MHC class Ia molecules, these mice exhibit a substantial reduction in peripheral CD8 lymphocytes compared with wild-type animals. The ability of splenic CD8 T cells from K b 2/ D b 2/ mice to mount a vigorous alloresponse against poly-morphic MHC class Ia molecules following primary in vitro culture suggests that this population has undergone selection on structurally similar class Ib MHC. However, it is also possible that recognition of MHC class I molecules is inherent in the germline of TCRs independent of positive and negative selection (36). If the former possibility is correct, the drastic reduction in peripheral CD8 T lymphocytes indicates that only a minor population of thymocytes is capable of being selected on class Ib molecules. If the repertoire of mature CD8 T cells in K b 2/ D b 2/ mice is limited, it is surprising that we observed a three- to fourfold increase in splenic CD8 T cells 6 days following LM inoculation, but found no significant change in B6 mice. This difference may reflect that the percentage of naive precursor CD8 T lymphocytes in the T cell pool capable of recognizing LM antigens is substantially higher in these animals than in wild-type mice.

We have also conducted experiments to determine whether K b 2/ D b 2/ mice are capable of generating a CTL response against viral pathogens. Our results demonstrate that infection of these animals with vaccinia virus, vescular stomatitis virus (data not shown), and lymphocytic choriomeningitis virus (16) fails to elicit CD8 T cell immunity. The failure to detect viral Ags due to a limited repertoire of TCRs expressed by the peripheral CD8 population could explain this lack of CTL activation. Whether any virus-derived peptides are capable of binding to class Ib molecules for cell surface presentation is also not known. While M3 can bind a nonformylated peptide derived from the influenza hemagglutinin protein for presentation to CD8 T cells in vitro (37), the presentation of this peptide in vivo for activation of a CTL response has yet to be demonstrated.

In summary, we have shown that in the absence of MHC class Ia molecules, antilisterial CTL immunity can be efficiently generated through MHC class Ib Ag presentation. It will be of interest to determine whether the limited polymorphic nature of class Ib proteins can be targeted for peptide vaccination strategies, eliciting CD8 T cell immunity to bacterial Ags in various strains of inbred mice that express disparate MHC class Ia proteins.

References


