H2-M3-Restricted CD8$^+$ T Cells are not Required for MHC Class Ib-Restricted Immunity Against *Listeria monocytogenes*

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H2-M3–restricted CD8+ T cells are not required for MHC class Ib–restricted immunity against Listeria monocytogenes

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Studies using major histocompatibility complex (MHC)-Ia–deficient mice have shown that MHC-Ib–restricted CD8+ T cells can clear infections caused by intracellular pathogens such as Listeria monocytogenes. M3–restricted CD8+ T cells, which recognize short hydrophobic N–formylated peptides, appear to comprise a substantial portion of the MHC-Ib–restricted T cell response in the mouse model of L. monocytogenes infection. In this study, we isolated formyltransferase (fmt) mutant strains of L. monocytogenes that lacked the ability to add formyl groups to nascent polypeptides. These fmt mutant Listeria strains did not produce antigens that could be recognized by M3–restricted T cells. We showed that immunization of MHC-Ia–deficient mice with fmt mutant Listeria resulted in stimulation of a protective memory response that cleared subsequent challenge with wild-type L. monocytogenes, despite the fact that M3–restricted CD8+ T cells did not proliferate in these mice. These data suggest that M3–restricted T cells are not required for protection against L. monocytogenes and underscore the importance of searching for new antigen–presenting molecules among the large MHC-Ib family of proteins.

Although innate immune responses are essential to limit the spread of intracellular bacterial infections, it is the adaptive immune response that allows the body to completely clear tremendous bacterial burdens. Memory CD8+ T cells that recognize antigens presented by MHC-I on the surface of infected cells are generally thought to be required for clearance of intracellular pathogens. Mice express two groups of MHC-I molecules: (a) the classically studied MHC-Ia molecules (K, D, and L) and (b) the nonclassical or MHC-Ib proteins, including Q, T, and M gene products. Much effort has been devoted to identifying peptide antigens within bacterial proteins that are predicted to bind to classical MHC-I molecules. For example, at least four dominant Kd-binding epitopes derived from secreted virulence factors are known to be presented during Listeria monocytogenes infection, and several other subdominant epitopes have also been identified (1, 2). However, in recent years, it has become increasingly clear that the nonclassical MHC-Ib molecules can also present antigens to T cells during bacterial infection (3).

Nonclassical MHC-I molecules are a widely diverse set of proteins encoded both within and outside the MHC locus that have been collectively referred to as MHC-Ib. It is difficult to make generalizations about MHC-Ib molecules because of the diversity of the members of this protein family; however, many differences from classical MHC-I have been observed. Cell surface levels of many MHC-Ib proteins are lower than MHC-Ia and some MHC-Ib molecules display a tissue-specific expression pattern, unlike MHC-Ia molecules that are ubiquitously expressed (4, 5). MHC-Ib molecules have also been shown to bind a more diverse set of antigens than MHC-Ia, which bind only 8–10 mer peptides. For example, during Mycobacterium tuberculosis infection, CD1 proteins present lipid molecules to T cells (6, 7). When mice are infected with L. monocytogenes, CD8+ T cells recognize formylated peptides bound to M3 (8).

Although our knowledge of most MHC-Ib molecules is limited, M3 has been studied in great detail. The peptide-binding groove of M3 is shallower than classical MHC-I molecules and it has been demonstrated to specifically
bind short (5–6 mer) hydrophobic peptides with an NH2-terminal formylated methionine (9–11). It has been suggested that M3 evolved specifically to present bacterial antigens to the mammalian immune system because N-formylation is used as a signal to initiate protein synthesis only in prokaryotes and for a limited set of mitochondrial and chloroplast proteins. At least three formylated peptides derived from L. monocytogenes (fMIVIL, fMIGWII, and fMIVTLF) have been shown to bind M3, and T cells that recognize each of these peptides are known to be activated during Listeria infection (12–14). In fact, in BALB/c mice, the magnitude of the fMIGWII-specific T cell response is nearly as great as the immunodominant Kβ-binding LLO91-99 T cell response (8).

Using MHC-Ia-deficient mice, we and others have shown that MHC-Ib–restricted CD8+ T cells alone are sufficient to clear infection with the intracellular bacterial pathogen L. monocytogenes (15, 16). It has been suggested that M3-restricted T cells are largely responsible for this protective immunity in mice (17). If this were true, use of the L. monocytogenes murine infection model would be more limited as a tool to study the role of MHC-Ib–restricted T cells in human immunity against intracellular pathogens because no human homologue for M3 has yet been discovered.

The purpose of this study was to determine whether MHC-Ib molecules other than M3 could mediate protective immunity against L. monocytogenes infection. We used an actinonin resistance selection strategy to generate mutant strains of L. monocytogenes that lacked the necessary enzyme systems to incorporate formylmethionine at the NH2 terminus of newly synthesized proteins. Mice infected with these mutant strains were therefore unable to present formylated peptide antigens derived from L. monocytogenes to naive M3-restricted CD8+ T cells. We found that activation and proliferation of M3-restricted CD8+ T cells was not required for protective immunity against Listeria in MHC-Ia-deficient mice, suggesting that other MHC-Ib must be capable of presenting antigens to T cells and stimulating a memory CD8+ T cell response. These data underscore the importance of discovering new classes of MHC-Ib molecules and identifying the types of antigens presented by these MHC-Ib family members.

RESULTS

Selection of L. monocytogenes strains containing formyltransferase (fmt) mutations

Previous studies have shown that formylation is not essential for initiation of protein synthesis in either Pseudomonas aeruginosa (18) or Staphylococcus aureus (19). We predicted that formylation would similarly not be required for protein synthesis in L. monocytogenes and that it would be possible to select for a mutant strain incapable of formylating nascent polypeptides. Such a strain could then be used as a tool to determine the role of M3-restricted T cells in MHC-Ib–mediated anti-Listeria immunity. We used actinonin, a naturally occurring peptide deformylase (PDF) inhibitor (20), to select for spontaneous fmt mutants in Listeria. Failure to remove formyl groups from the NH2-terminal methionine of newly synthesized proteins is toxic to most bacteria; however, if formyl groups are never added, then PDF activity would not be required, and bacteria should grow readily in the presence of actinonin (Fig. 1). Margolis et al. previously showed that growth of S. aureus in the presence of actinonin resulted in the appearance of actinonin-resistant (ActR) colonies that had spontaneous mutations in the S. aureus fmt gene (19). We used a similar selection strategy to isolate Listeria fmt mutants.

Wild-type L. monocytogenes 10403s was plated on brain-heart infusion (BHI) agar containing 100 μg/ml actinonin. Spontaneous ActR colonies arose at a frequency of 1.25 × 10−6. Three ActR colonies were passaged 25 times on BHI plates lacking actinonin and inoculated onto BHI agar with or without actinonin. No actinonin-sensitive revertants were observed and the resulting strains were named L. monocytogenes SD-A1, SD-A4, and SD-A8. DNA sequence analysis revealed that all three ActR strains contained frameshift mutations in the fmt gene (Table I). Strains SD-A1, SD-A4, and SD-A8 were each predicted to encode methionyl-tRNAfMet formyltransferase (MTF) proteins truncated at the COOH terminus. The COOH-terminal portion of MTF is thought to be necessary for binding to methionine-loaded tRNAfMet (21); therefore, these truncated proteins should lack the ability to formylate nascent polypeptides.

<table>
<thead>
<tr>
<th>Strain</th>
<th>fmt genotype</th>
<th>Composition of predicted MTF protein</th>
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<tbody>
<tr>
<td>10403s</td>
<td>Wild type</td>
<td>Complete coding sequence, 313 aa</td>
</tr>
<tr>
<td>SD-A1</td>
<td>Insertion (G) at bp 514</td>
<td>Frameshift after 171 aa</td>
</tr>
<tr>
<td>SD-A4</td>
<td>Deletion (A) at bp 267</td>
<td>Frameshift after 89 aa</td>
</tr>
<tr>
<td>SD-A8</td>
<td>Insertion (G) at bp 396</td>
<td>Frameshift after 132 aa</td>
</tr>
</tbody>
</table>

*bp refer to the nucleotide position within the 939 bp of the wt fmt ORF.

Table I. Characterization of actinonin-resistant L. monocytogenes strains

MTF MUTANT LISTERIA LACK M3-BINDING PEPTIDES | D’Orazio et al.
fmt mutant L. monocytogenes have an increased doubling time compared with wild-type L. monocytogenes

After 24–48 h of incubation at 37°C on BHI agar, colonies of the fmt mutant L. monocytogenes grew to only approximately half the size of wild-type Listeria colonies, suggesting that the fmt mutants had a slower growth rate. In fact, all three fmt mutant strains had a twofold increase in doubling time when grown in BHI broth (Fig. 2). Even after 48 h of incubation, growth of the fmt mutant strains did not reach the same density as wild-type L. monocytogenes (unpublished data). Slower growth rates have also been observed for fmt mutant strains of P. aeruginosa (18) and S. aureus (19). Collectively, these results suggest that using formylation to initiate protein synthesis enhances bacterial growth, but that formylation is not essential for in vitro growth of these bacteria.

No intracellular growth defect is observed for fmt mutant L. monocytogenes

Wild-type L. monocytogenes encode several virulence factors that allow the bacteria to readily enter mammalian cells, escape from endocytic vesicles, and grow within the cytoplasm of infected cells. As shown in Fig. 3, we tested a variety of different cell types and found no difference in the ability of either fmt mutant or wild-type Listeria strains to replicate inside cells. In both L2 fibroblasts (Fig. 3 A) and Hepa1-6 liver epithelial cells (Fig. 3 B), the growth rate for strains SD-A1 and SD-A8 was indistinguishable from the wild-type strain 10403s. In J774 macrophage-like cells, we recovered fewer intracellular fmt mutant Listeria than wild-type Listeria 1 h after infection (Fig. 3 C). However, the growth rate over the next 7 h in J774 cells was the same for all three strains. These data demonstrate that intracellular growth of the fmt mutant Listeria strains was not impaired compared with wild-type Listeria.

N-formylated peptides do not accumulate in the supernatants of fmt mutant Listeria cultures

Formylated MIGWII (f-MIGWII), an M3-binding peptide derived from the L. monocytogenes LemA protein, has been shown to accumulate over time in the culture supernatant when L. monocytogenes is grown in broth (22). The mechanism for this accumulation of formylated peptide is not known, but it is thought that extracellular proteases could cleave the NH2-terminal portion of LemA, which is predicted to have an “Nout” topology. Presumably, this orientation protects the formyl group on the peptide from PDF activity in the bacterial cytoplasm. To ensure that formylation was not occurring in the fmt mutant strains, we used a T cell cytotoxicity assay to measure the amount of fMIGWII peptide found in either wild-type or fmt mutant Listeria culture supernatants. Target cells were pretreated with Listeria culture supernatant and exposed to the fMIGWII-specific CD8+ T cell line S172. Line S172 is able to lyse target cells coated
N

Figure 4. Formylated peptides do not accumulate in the culture supernatants of fmt mutant Listeria strains. T cell recognition of target cells was measured as a function of cytolysis (51Cr release into the supernatant) and is shown as percentage of specific lysis. (A) 51Cr-loaded EL-4 target cells were pretreated with media alone (BHI broth) or culture supernatant derived from either wild-type (wt Lm) or fmt mutant (SD-A1, SD-A8) L. monocytogenes strains. CD8+ T cells were added at various effector to target ratios and the cells were coincubated for 4 h. Line S172 T cells recognize N-formylated MIGWII peptide bound to M3; clone 2.5 is an M3-restricted CD8+ T clone of unknown specificity. (B) 51Cr-loaded EL-4 target cells were pretreated with synthetic peptide at the final concentrations indicated. Line S172 T cells were added at an effector:target ratio of 45:1.

with picomolar or greater concentrations of formylated MIGWII peptide but not unformylated peptide (Fig. 4 B). As shown in Fig. 4 A, line S172 CD8+ T cells lysed target cells treated with supernatant from wild-type Listeria, but did not lyse targets treated with either SD-A1 or SD-A8 supernatant. In addition, the CD8+ T cell clone 2.5, which recognizes an as yet unidentified Listeria antigen bound to M3 (unpublished data) was also not capable of lysing target cells treated with supernatant from fmt mutant Listeria. The lower limit of detection for the recognition of formylated peptides by CD8+ T cells using the cytotoxicity assay was 10^{-11}M (Fig. 4 B). These results suggested that N-formylated proteins were not present in the fmt mutant Listeria strains to provide a source of M3-binding peptides.

fmt mutant Listeria establish infection in mouse spleen, but have an impaired ability to replicate in mouse liver

Before characterizing the adaptive immune response against the fmt mutant Listeria in vivo, we first tested whether or not the fmt mutant strains were virulent enough to establish a sustained infection in mice that would allow for processing and presentation of bacterial antigens to the immune system. We used a competitive index assay to compare the virulence of the fmt mutant strains to wild-type L. monocytogenes DP-L3903. BALB/c mice were sublethally infected with a 1:1 mixture of wild-type (erythromycin-resistant) and fmt mutant L. monocytogenes (erythromycin-sensitive). Mice were killed 3 d later and the spleens and livers were homogenized and differentially plated to determine the total number of each bacterial strain present. Squares represent competitive indices for individual mice; thick black lines represent average indices for groups of mice. (B) A plaque assay was used to assess the degree of cell-to-spread achieved by fmt mutant Lm SD-A1 compared with wild-type Lm 10403s. Confluent monolayers of L2 fibroblasts were infected at low MOI (0.1) for 1 h and agarose overlays were added. After 3 d of incubation at 37°C in 7% CO2, neutral red overlays were added to visualize the plaques. Plaque size indicates the relative degree of cell-to-cell spread for each strain. Infections were performed in duplicate wells in two separate experiments.

Figure 5. fmt mutant Listeria are less virulent in the liver than in the spleen. (A) A competitive index assay was used to determine the relative virulence of fmt mutant Listeria strains SD-A1 and SD-A8 compared with wild-type Listeria. BALB/c mice were sublethally infected intravenously with a 1:1 mixture of wild-type (erythromycin-resistant) and mutant Listeria (erythromycin-sensitive). Mice were killed 3 d later and the spleens and livers were homogenized and differentially plated to determine the total number of each bacterial strain present. Squares represent competitive indices for individual mice; thick black lines represent average indices for groups of mice. (B) A plaque assay was used to assess the degree of cell-to-spread achieved by fmt mutant Lm SD-A1 compared with wild-type Lm 10403s. Confluent monolayers of L2 fibroblasts were infected at low MOI (0.1) for 1 h and agarose overlays were added. After 3 d of incubation at 37°C in 7% CO2, neutral red overlays were added to visualize the plaques. Plaque size indicates the relative degree of cell-to-cell spread for each strain. Infections were performed in duplicate wells in two separate experiments.

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plasmas in cell monolayers that were only half the size of plasmas formed by wild-type *Listeria monocytogenes* (Fig. 5 B). In L2 fibroblasts, strains SD-A1 and SD-A8 formed plasmas that were 63% and 54% of wild-type size, respectively. Similar results were observed when monolayers of Hepa1-6 epithelial cells were infected with *Listeria monocytogenes* fmt mutants (55% for SD-A1 and 61% for SD-A8). Collectively, these results suggest that fmt mutant *Listeria* are readily able to infect single cells but that their ability to spread to adjacent cells is impaired compared with wild-type *Listeria monocytogenes*.

**M3-restricted CD8+ T cells are not activated in mice immunized with fmt mutant *Listeria monocytogenes***

To confirm that M3-restricted CD8+ T cells were not activated in mice infected with fmt mutant *Listeria*, we harvested splenocytes from mice 7 d after infection and determined the number of MIGWII-specific CD8+ T cells present. In naive BALB/c mice, ~0.3% of all CD8+ T cells secreted IFNγ after incubation with MIGWII, the immunodominant M3-binding peptide epitope derived from *Listeria monocytogenes* (Fig. 6 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20052256/DC1). 7 d after infection with wild-type *Listeria*, those T cells had expanded ~10-fold. However, no increase in the number of MIGWII-specific T cells was observed in spleens of BALB/c mice infected with either SD-A1 or SD-A8. Similar results were observed when MHC-Ia−/− mice were used (unpublished data). As a control, we also counted the number of LLO91−99−specific CD8+ T cells found in the spleens of BALB/c mice infected with either wild-type or fmt mutant *Listeria*. A significant increase in the number of LLO91−99−specific CD8+ T cells was observed after infection with all three *Listeria* strains (Fig. 6 A). Therefore, the fmt mutant *Listeria* were able to infect enough cells to allow for sufficient processing and presentation of a MHC-Ia (Kb)−binding peptide to naive CD8+ T cells.

Although the fmt mutant *Listeria* did not appear to be producing formylated MIGWII peptide antigen, significant quantities of unformylated MIGWII peptide could be present in the cytoplasm of cells infected with either strain SD-A1 or SD-A8. Unformylated peptides bind to M3 with a much lower affinity than formylated peptides, and it has been thought that unformylated peptide bound to M3 would not stimulate naive CD8+ T cells in vivo. To verify that infection with fmt mutant *Listeria* did not result in activation of any M3–restricted MIGWII–specific CD8+ T cells, we also counted the number of T cells present that could recognize unformylated MIGWII peptide. Splenocytes harvested from mice infected with wild-type or fmt mutant *Listeria* 7 d earlier were exposed to high concentrations of either MIGWII or MIGWII and the number of CD8+ T cells that produced IFNγ in response to these stimuli was assessed by intracellular cytokine staining (ICCS).

As shown in Fig. 6 B, the number of MIGWII–specific T cells present in the spleens of mice infected with either strain SD-A1 or SD-A8 did not change over background levels. These results suggested that M3–restricted naive CD8+ T cells are not activated in mice infected with fmt mutant *Listeria monocytogenes*.

To determine whether CD8+ T cells restricted by other MHC-Ib molecules were being activated in the absence of an M3–restricted T cell response, we infected MHC-Ia−/− mice with a sublethal dose of *Listeria monocytogenes* SD-A1. 7 d later, splenocytes from these mice were incubated with either uninfected bone marrow–derived macrophages (BMMΦ) or cells that had been infected with fmt mutant *Listeria* strain SD-A1. As shown in Fig. 7 A and Fig. S2 (available at http://www.jem.org/cgi/content/full/jem.20052256/DC1), a significant number of CD8+ T cells capable of recognizing antigens derived from *Listeria monocytogenes* SD-A1 were found in the spleens of MHC-Ia−/− mice. Recognition of these antigens was not blocked by the addition of either anti-M3 or anti-Qa-1b antibody. Control groups of mice infected with wild-type *Listeria* also displayed significant T cell recognition of infected BMMΦ. However, as expected, pretreatment with anti-M3 antibody blocked recognition and subsequent IFNγ expression for a large fraction of the CD8+ T cells found in these mice (Fig. 7 B). These results suggest that other antigens besides formylated peptides were presented.
2,000 CFU of either (A) mice were harvested and exposed to MHC-Ia–deficient BMMΦs (either uninfected or infected for 3 h with the same strain of Listeria used to infect the mice). As indicated, some samples of Listeria-infected BMMΦs were pretreated with either anti-M3 or anti-Qa-1b monoclonal antibody before the addition of immune splenocytes. Data shown represent the average number of TCRβ+ CD8+ cells that stained IFNy+ within each group of mice ± SE from one out of two experiments.

Class Ia MHC-deficient mice immunized with fmt mutant Listeria develop protective immunity against infection with wild-type Listeria

MHC-Ia–deficient mice are able to clear secondary lethal challenge with L. monocytogenes as rapidly as wild-type mice (15, 16) and it has been suggested that M3-restricted CD8+ T cells are largely responsible for this protective immunity (17). We used the fmt mutant strains of Listeria to determine whether activation of M3-restricted T cells was required for protection against secondary Listeria infection in MHC-Ia–deficient mice. Groups of MHC-Ia–deficient mice were immunized with a sublethal dose of either wild-type or fmt mutant L. monocytogenes; a control group (naive mice) received PBS. 3 wk later, all groups of mice were challenged with 5 LD50 of wild-type L. monocytogenes 10403s. 3 d later, the total number of Listeria CFU present in the spleen or liver was determined. Mice immunized with wild-type Listeria had at least two logs fewer bacteria in the spleen or liver compared with naive mice (Fig. 8). Surprisingly, mice immunized with either SD-A1 or SD-A8 showed the same degree of protection against high dose secondary infection as was observed after immunization with wild-type L. monocytogenes. This result indicates that activation of M3-restricted CD8+ T cells was not essential for protective immunity against L. monocytogenes in these mice and suggests that other MHC-Ib–restricted T cells may have a significant role in the clearance of Listeria infection.

**DISCUSSION**

We isolated ActR+ fmt mutant strains of L. monocytogenes to use as a tool to address the role of M3-restricted CD8+ T cells in MHC-Ib–mediated protective immunity against intracellular bacterial pathogens. While performing assays to characterize these fmt mutant strains, we discovered several interesting phenotypes. For example, although the fmt mutant strains were able to compete with wild-type L. monocytogenes for growth in the spleen, significantly fewer fmt mutant Listeria were isolated from the liver. This difference in virulence can be at least partially explained by the observation that fmt mutant Listeria have a reduced ability to spread from cell-to-cell. One mechanism for Listeria to prolong survival in the host is thought to be the ability of the bacteria to spread to a neighboring cell without encountering the extracellular environment, where the bacteria are susceptible to phagocytosis by activated macrophages or neutrophils. Hepatocytes, which are readily infected by L. monocytogenes, are connected by cell junctions within the tissue, and this cell-to-cell contact presumably facilitates spread of the bacteria within the liver. In contrast, L. monocytogenes infects primarily circulating immune cells (monocytes, macrophages, and dendritic cells) in the spleen. Thus, the apparent increased virulence of the fmt mutant L. monocytogenes in the spleen may simply reflect the fact that efficient cell-to-cell spread of bacteria is not an important feature for growth in the spleen.

Interestingly, the fmt mutant L. monocytogenes had a slight growth defect in vitro but not in vivo. It has long been thought that formylation of initiator Met-tRNAs is essential for protein synthesis in bacteria and in eukaryotic organelles such as chloroplasts and mitochondria. This belief is based largely on studies that showed a marked (>10-fold) decrease in growth of Escherichia coli containing mutations in fmt (24) and the identification of formylmethionine at the NH2 terminus of several mitochondrial proteins. However, we show here that mutations in the L. monocytogenes fmt gene have
only a modest effect on bacterial growth in BHI broth. Other groups have shown a similar growth phenotype in *P. aeruginosa* (18) and *S. aureus* (19). These observations suggest that formylation may confer a slight advantage for in vitro growth of most bacterial species, but that only in *E. coli* has a strict requirement for fmt activity been demonstrated. Li et al. showed that protein synthesis could also be initiated in yeast mitochondria in the absence of formylated methionine (25).

A comparative analysis of the structural requirements for binding of IF-2 to fMet-tRNA vs. Met-tRNA in each of these organisms may elucidate the mechanism whereby protein synthesis can readily initiate in the absence of formylmethionine in prokaryotes or in mitochondria.

It is possible that during infection of mice, secondary mutations occurred that allowed the fmt mutant *L. monocytogenes* to replicate faster. However, we found that the fmt mutant strains were replicating at the same rate as wild-type *L. monocytogenes* after just 1–2 h of infection of tissue culture cells. It does not seem likely that selective pressures could have resulted in secondary mutations during this short time period. These data suggest that the cytoplasm of host cells may be an environment in which the bacterial enzymes involved in formylation are not necessary. Even if secondary mutations did occur in vivo that resulted in a low level of formylation in the fmt mutant bacteria, insufficient antigen was presented to activate the immunodominant M3-restricted *Listeria*-specific CD8+ T cell population.

During primary *Listeria* infection, M3-restricted T cells expand faster than K4-restricted T cells, with peak numbers occurring by 5–6 d after infection (8) and it has been shown that M3-restricted CD8+ T cells alone can clear sublethal *Listeria* infections within 5 d (26). The role of M3-restricted T cells during secondary infection, however, has been less clear. Kerkies et al. showed that M3-restricted memory CD8+ T cell populations are generated and maintained after primary infection with *L. monocytogenes*. However, despite the fact that these memory CD8+ T cells were activated in the same manner as K4-restricted T cells, the M3-restricted CD8+ cells failed to undergo a dramatic proliferative burst upon rechallenge with *Listeria* (27). It has been difficult to determine the precise role of M3-restricted CD8+ T cells during secondary *Listeria* infections because of the apparent paradox that M3-restricted T cells are a dominant fraction of the MHC-Ib–restricted T cell population (17), yet the recall expansion of these cells is small and perhaps not significant enough to clear large bacterial burdens. Hamilton et al. recently suggested that it is the MHC-Ia–restricted T cell response that limits the expansion of M3-restricted memory CD8+ T cells during secondary challenge (26).

In this report, we show that immunization with an fmt-mutant *Listeria* strain that lacks formylated peptides still results in the generation of protective immunity against secondary *Listeria* infection. These data argue that activation of M3-restricted CD8+ T cells is not essential for protective immunity against *Listeria* and suggests that presentation of antigen in the context of other MHC-Ib molecules is sufficient to elicit a protective memory CD8+ T cell response. Bouwer et al. previously showed that Qa-1b–restricted *Listeria*-specific cytotoxic T lymphocytes increase in number during *L. monocytogenes* infection (28). However, like M3-restricted CD8+ T cells, Qa-1b–restricted T cells do not expand significantly during secondary infection (29). We have previously shown that in MHC-Ia–deficient mice, the residual population of MHC-Ib–restricted CD8+ T cells does expand significantly after both primary and secondary *Listeria* infection, and that protective immunity is mediated by CD8+ T cells in these mice (15). Because neither M3-restricted nor Qa-1b–restricted CD8+ T cells expand significantly during secondary *Listeria* infection, the activated population of CD8+ T cells must recognize *L. monocytogenes* antigens presented by novel MHC-Ib molecules. These MHC-Ib–restricted CD8+ T cells appear to be necessary for clearance of *Listeria* in the absence of MHC-Ia–restricted and M3-restricted T cell responses because adoptive transfer of *L. monocytogenes* SD-A1 immune splenocytes depleted of CD8+ T cells failed to protect naive mice from challenge with *L. monocytogenes* (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20052256/DC1). Further studies will be required to determine whether these additional MHC-Ib–restricted CD8+ T cell specificities are involved in protective immunity in wild-type mice.

In the past decade, the majority of studies examining MHC-Ib–restricted T cells have focused on the recognition of formylated peptides presented by M3 molecules. The results presented here make a strong argument that a search for new MHC-Ib molecules capable of presenting antigen to CD8+ T cells would result in the identification of new antigenic specificities able to protect against secondary *Listeria* infection. Further characterization of the unique types of antigens that can be recognized by protective CD8+ T cells will greatly facilitate the rational design of effective therapeutic strategies to protect against other intracellular bacteria, including prevalent pathogens such as *Mycobacterium tuberculosis* and *Chlamydia trachomatis*.

**MATERIALS AND METHODS**

**Bacteria.** *L. monocytogenes* fmt mutant strains SD-A1, SD-A4, and SD-A8 were derived from wild-type *L. monocytogenes* 10403s. *L. monocytogenes* 10403s has a LD50 of ~10⁵ CFU in BALB/c mice. Bacteria were grown in BHI broth (Difco) supplemented with 100 μg/ml actinomycin (Sigma-Aldrich) as indicated. DNA fragments encoding fmt were amplified from each *Listeria* strain using Platinum PCR Supermix (Invitrogen) with the following primers: 3′–GACATGACGGACAGGGGAAGT–5′ and 3′–CACCCTGTTGACGAATTGTTAC–5′. The DNA sequence of each PCR product was directly determined using internal primers. Bacterial culture supernatants were prepared by inoculating BHI broth and incubating with aeration at 37°C for 24 h. Bacteria were centrifuged at 13,000 revolutions/min for 20 min and the supernatant was collected. Aliquots were stored at −20°C and thawed before use.

**Cell culture.** EL-4 thymoma cells (H-2₄), J774 macrophage-like cells, L2 fibroblasts, and Hepa1-6 hepatoma (epithelial) cells were obtained from the American Type Culture Collection and were maintained at 37°C in 5% CO₂. EL-4 and J774 cells were grown in RPMI 1640 (GIBCO BRL) supplemented with 1-glutamine, Hepes, 50 μM 2-ME, and 10% FCS; L2 and Hepa1-6 cells were grown in DMEM (GIBCO
BRL) plus 10% FCS. T cell lines were maintained by weekly restimulation on antigen-coated syngeneic splenocytes as described previously (15).

Mice. BALB/c/BByJ mice obtained from Jackson Immunoresearch Laboratories and eleventh backcross generation C.B10.H-2b/LiMcl/J K1−/−D10−/− mice (15) were housed in specific pathogen-free animal facilities at Harvard Medical School (HMS) and the University of Kentucky (UK). The experimental procedures used in this study were approved by IACUC committees at both HMS and UK. For primary infections, mice were given ~10^5 CFU of L. monocytogenes i.v. in the tail vein. For secondary challenges, mice were allowed to recover for 3 wk and infected i.v. with ~6 × 10^5 CFU L. monocytogenes. 3 d after infection, spleen and livers were harvested aseptically, diluted in 0.2% NP-40, and plated on BHI agar to determine the total number of CFU per organ.

**Intracellular growth assay.** Cells were seeded on 12-mm round coverslips and incubated overnight in RPMI-10 without antibiotics to reach confluence. Bacteria were grown to early log phase in BHI broth, washed once with PBS, and used to infect cell monolayers. Cells were infected (on coverslips in triplicate) at a multiplicity of infection of 0.1 (for J774 cells) or 5.0 (for L2 and Hepa-1-6 cells), incubated for 30 min, washed three times with warm PBS, and RP-10 containing 25 μg/ml gentamicin sulfate was added. At indicated time points, the number of bacteria associated with each coverslip were determined by placing the coverslip in sterile dH2O, vortexing vigorously for 30 s, and plating dilutions on BHI agar.

**T cell cytotoxicity.** Target cells were cocultured with either 50 μl of L. monocytogenes supernatant or synthetic peptide (Bio-Synthesis) and 100 μg/ml sodium chromate for 1 h at 37°C in 7% CO2. The cells were washed three times with RPMI 1640, suspended in RP-10 at 10^6 cells/ml and 100 μl of cells per well was added to 96-well plates. Serial dilutions of T cells were added in a final volume of 200 μl/well and the plates were incubated for 4 h at 37°C in 7% CO2. Spontaneous release was determined in wells containing target cells with no T cells; maximum release was determined by the addition of 1% Triton X-100. The cytotoxic activity of the T cells was evaluated by measuring 51Cr release in the supernatant on a Wallac 1470 Wizard γ counter. Percent specific lysis was calculated using the following formula: % specific lysis = 100 × [release by T cells − spontaneous release] / [maximum release − spontaneous release].

**Competitive index assay.** Growth of fmt mutant Listeria strains was compared with growth of wild-type Listeria using a competitive index assay as described previously (23). In brief, BALB/c mice were infected i.p. with a 1:1 mixture of wild-type strain DP-L3903 (ErmR) and one of the fmt mutant strains. Mice were given a total of ~10^5 CFU and were killed 72 h later. Spleens and livers were harvested aseptically, homogenized, diluted in 0.2% NP-40, and plated on BHI agar. At least 100 colonies per organ were replica plated or patched onto BHI agar containing 2 μg/ml gentamicin. The cytotoxic activity of the T cells was evaluated by measuring 51Cr release in the supernatant on a Wallac 1470 Wizard γ counter. Percent specific lysis was calculated using the following formula: % specific lysis = 100 × [release by T cells − spontaneous release] / [maximum release − spontaneous release].

**Plaque assay.** Cells were grown to confluence in six-well dishes, infected with L. monocytogenes for 1 h at various multiplicities of infection, and washed three times with warm PBS. overlays consisting of MEM (GIBCO BRL) containing 2.5% FBS, 1% agarose, and 10 μg/ml gentamicin sulfate were added and the cells were incubated for an additional 3 d. Plaques were visualized by the addition of neutral red overlays (1% agarose, 0.2% neutral red in MEM-2.5). Plaque size was measured by analyzing digital images of the overlays.

**ICCS.** IFNy ICCS was performed using the CytoFACS Cytoperm Plus (with GolgiPlug) kit (BD Biosciences). In brief, splenocytes were harvested from Listeria-infected mice and 5 × 10^6 cells/well were added to 24-well dishes. Synthetic peptides (Bio-Synthesis) corresponding to antigenic epitopes were added to some wells at a final concentration of 1 μM. In experiments where J774 cells were used as targets, 4 × 10^5 J774 cells per well were infected with L. monocytogenes SD-A1 at a multiplicity of infection of 1.0 for 2 h before the addition of splenocytes. Listeria culture supernatant (50 μl) was added directly to some wells. After 5–6 h of incubation in the presence of GolgiPlug at 37°C in 7% CO2, cells were stained with anti-CD8α (53-6.7), anti-CD8β (53-5.8), and anti–TCRγ (H57-597) antibodies. The cells were fixed, permeabilized, and stained with anti-IFNy (XM1G1.2) antibody according to the manufacturer’s instructions. Cells were suspended in FACS buffer (0.5% BSA, 0.1% NaN3 in HBSS) before flow cytometric analysis. Fluorescence intensities were measured using a FACSCalibur flow cytometer (Becton Dickinson) and analysis was performed using CELLQuest software. Dead cells and monocytes were excluded using forward and side scatter gating. Typically, 100,000 gated events were collected for each analysis.

**Antibody-blocking experiments.** CD8+ cells were enriched from whole splenocytes by negative selection using antibody-conjugated IMag magnetic beads (BD Biosciences). Typically, the enrichment protocol removed 90–95% of the splenocytes from MHC-Ia-deficient mice. Listeria-infected or uninfected MHC-Ia-deficient B6.B12/1.Syng slider mice were pretreated with anti-M3 (clone 130) and anti–Qa-1b (6A8.6F10.1A6) monoclonal antibodies (final concentration 500 μg/ml) for 30 min at room temperature. The antibodies were maintained at a concentration of 20 μg/ml for 5–6 h during the incubation of CD8+ enriched splenocytes with infected or uninfected macrophages. The number of IFNy-secreting CD8+ T cells was determined by ICCS as described in the previous paragraph.

**Online supplemental material.** Fig. S1 displays primary dot plots from individual mice representative of the data shown in Fig. 6 A. Fig. S2 includes primary FACS plots for individual mice representative of the data shown in Fig. 7. Fig. S3 shows that adoptive transfer of fmt mutant Listeria-immune spleen depleted of CD8+ T cells does not protect mice as well as whole immune spleen. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052256/DC1.

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