

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 proteins: (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 K^d–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

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Abbreviations used: Act^R, actinonin–resistant; BHI, brain heart infusion; BMMΦ, bone marrow–derived macrophages; *fmt*, formyltransferase; ICCS, intracellular cytokine staining; MTF, methionyl–tRNA^{Met} formyltransferase; PDF, peptide deformylase.

The online version of this article contains supplemental material.

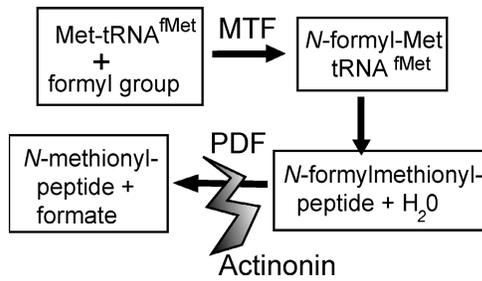


Figure 1. Actinonin inhibits bacterial peptide deformylase (PDF) activity. Bacterial methionyl-tRNA^{fMet} formyltransferase (MTF) adds a formyl group to methionines that are bound to initiator tRNAs. Formylation promotes binding of initiator factor 2 (IF-2) and impairs binding of elongation factor Tu and, therefore, provides a basis for the initiation of protein synthesis. The formyl group is cleaved from the mature protein by peptide deformylase (PDF), and in many cases the NH₂-terminal methionine is removed by methionine amino peptidase (not depicted). Actinonin is a naturally occurring inhibitor of metallohydrolases that blocks PDF activity in bacteria (reference 20).

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 K^d-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

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

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

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

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 (Act^R) 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 Act^R colonies arose at a frequency of 1.25 × 10⁻⁶. Three Act^R colonies were passaged 25 times on BHI plates lacking antibiotics 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 Act^R strains contained frameshift mutations in the *fmt* gene (Table I). Strains SD-A1, SD-A4, and SD-A8 were each predicted to encode methionyl-tRNA^{fMet} formyltransferase (MTF) proteins truncated at the COOH terminus. The COOH-terminal portion of MTF is thought to be necessary for binding to methionine-loaded tRNA^{fMet} (21); therefore, these truncated proteins should lack the ability to formylate nascent polypeptides.

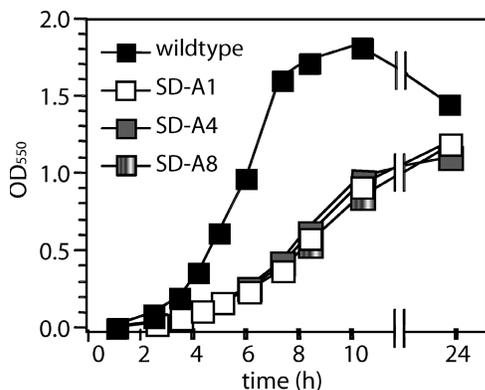


Figure 2. The doubling time of *fmt* mutant *Listeria* is increased compared with wild-type *Listeria* during growth in BHI broth. Freshly streaked wild-type (Lm 10403s) or *fmt* mutant (SD-A1, SD-A4, SD-A8) *L. monocytogenes* strains were incubated overnight at 37°C with aeration in BHI broth. The next day, each culture was diluted to an OD₅₅₀ of ~0.015 in fresh BHI broth and further incubated at 37°C with aeration. Bacterial growth was assessed by determining the OD₅₅₀ at various time points as indicated. Representative data from one of two different experiments are shown.

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*.

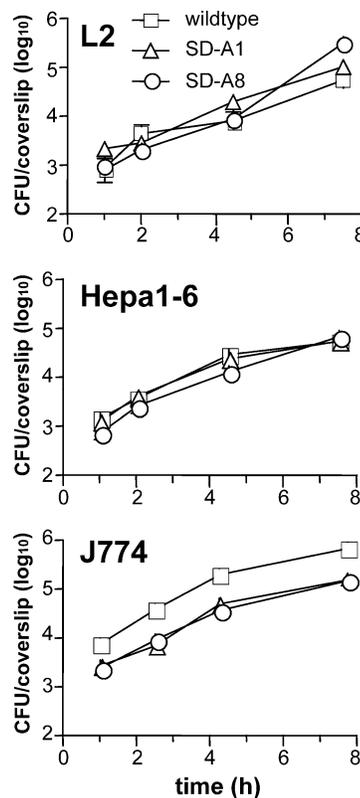


Figure 3. *fmt* mutant *Listeria* do not have a replication defect during intracellular growth. Monolayers of L2 fibroblasts, Hepa1-6 epithelial cells, or J774 macrophage-like cells plated on glass coverslips were infected with the *Listeria* strains indicated for 30 min, washed extensively, and incubated in media containing 25 µg/ml gentamicin to kill extracellular bacteria. At each of the time points indicated, the cells attached to each coverslip were lysed in sterile water to release intracellular bacteria. Serial dilutions of each lysate were plated on BHI agar to determine the total number of bacterial colony forming units (CFU) associated with each coverslip. Data shown represent the average values ± SD for triplicate coverslips from one of two separate experiments.

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 “N_{out}” 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

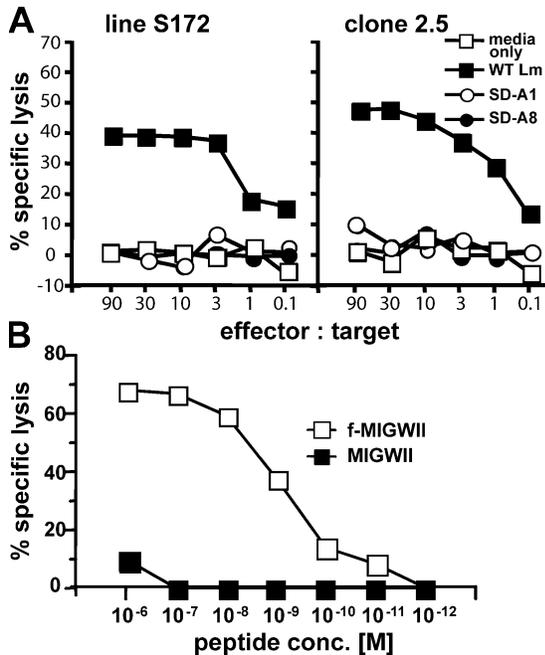


Figure 4. Formylated peptides do not accumulate in the culture supernatants of *fmt* mutant *Listeria* strains. T cell recognition of target cells was measured as a function of cytotoxicity (⁵¹Cr release into the supernatant) and is shown as percentage of specific lysis. (A) ⁵¹Cr-loaded EL-4 target cells 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 10⁻¹¹M (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

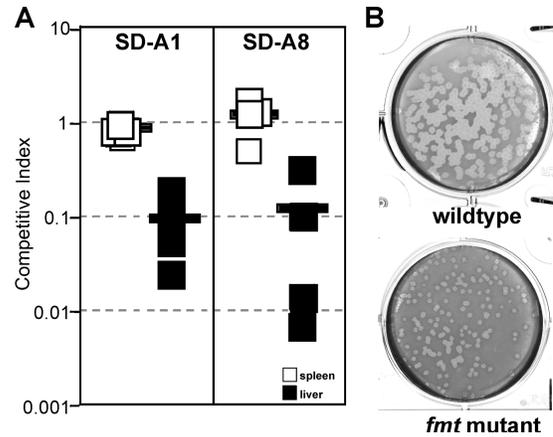


Figure 5. *fmt* mutant *Listeria* are less virulent in the liver than in the spleen. (A) A competitive index assay was used to determine the relative virulence of *fmt* mutant *Listeria* strains SD-A1 and SD-A8 compared with wild-type *Listeria*. BALB/c mice were sublethally infected intravenously with 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-cell 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.

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 infected with a 1:1 mixture of wild-type (erythromycin-resistant) and *fmt* mutant *L. monocytogenes* (erythromycin-sensitive). 3 d later, liver and spleen homogenates from each mouse were differentially plated on media with or without erythromycin to determine the total number of CFU of each *Listeria* strain present. As shown in Fig. 5 A, approximately equal numbers of wild-type and *fmt* mutant *L. monocytogenes* were recovered from the spleen of each mouse, indicating that *fmt* mutant *Listeria* were fully able to compete with wild-type *Listeria* for growth in the spleen. However, at least 10-fold fewer SD-A1 or SD-A8 *L. monocytogenes* were recovered from mouse livers compared with wild-type *Listeria* (Fig. 5 A), suggesting that the *fmt* mutant strains were less virulent in the liver and not able to compete with wild-type *L. monocytogenes* for growth.

A growth defect in the liver, but not the spleen, has also been observed for *L. monocytogenes* strains with mutations in the *actA* gene (23). ActA mutants are unable to spread efficiently from cell to cell and form smaller plaques than wild-type *L. monocytogenes* when assayed on cell monolayers. Similarly, we have observed that *fmt* mutant *Listeria* formed

plaques in cell monolayers that were only half the size of plaques formed by wild-type *L. monocytogenes* (Fig. 5 B). In L2 fibroblasts, strains SD-A1 and SD-A8 formed plaques that were 63% and 54% of wild-type size, respectively. Similar results were observed when monolayers of Hepa1-6 epithelial cells were infected with *L. 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 *L. monocytogenes*.

M3-restricted CD8⁺ T cells are not activated in mice immunized with *fmt* mutant *L. 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 fMIGWII-specific CD8⁺ T cells present. In naive BALB/c mice, ~0.3% of all CD8⁺ T cells secreted IFN γ after incubation with fMIGWII, the immunodominant M3-binding peptide epitope derived from *L. 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 fMIGWII-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-deficient 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 (K^d)-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 fMIGWII or MIGWII and the number of CD8⁺ T cells that produced IFN γ 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 *L. monocytogenes*.

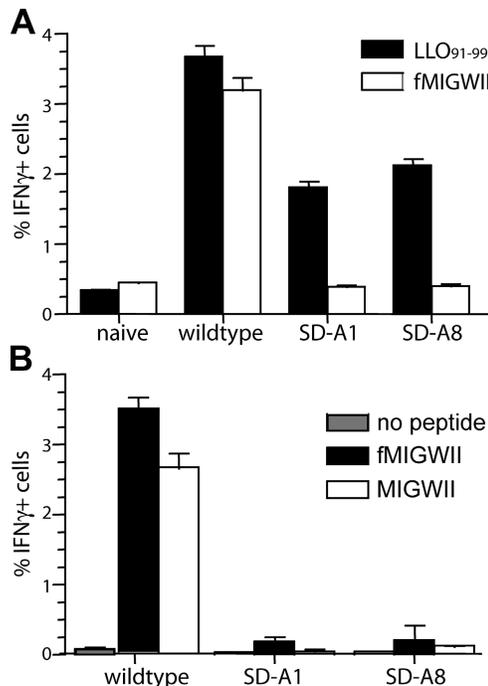


Figure 6. M3-restricted CD8⁺ T cells are not activated during infection with *fmt* mutant *Listeria*. Mice were immunized with PBS (naive group) or a sublethal dose of either wild-type (10403s) or *fmt* mutant (SD-A1, SD-A8) *L. monocytogenes*. 7 d later, the mice were killed and the number of antigen-specific CD8⁺ T cells present in the spleen was determined by IFN γ ICCS. (A) BALB/c splenocytes were exposed to either 1 μ M LLO₉₁₋₉₉ peptide, which binds to K^d (black bars) or 1 μ M fMIGWII peptide, which binds to M3 (white bars). (B) MHC-Ia-deficient splenocytes were incubated with either no peptide (gray bars), 1 μ M formylated fMIGWII peptide (black bars), or 1 μ M unformylated MIGWII peptide (white bars). Data shown represent the average number of CD8 _{β} ⁺ cells that were IFN γ ⁺ within each group of mice \pm SE from one of three separate experiments.

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-deficient mice with a sublethal dose of *L. monocytogenes* SD-A1. 7 d later, splenocytes from these mice were incubated with either uninfected bone marrow-derived macrophages (BMM Φ) 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 *L. monocytogenes* SD-A1 were found in the spleens of MHC-Ia-deficient mice. Recognition of these antigens was not blocked by the addition of either anti-M3 or anti-Qa-1^b antibody. Control groups of mice infected with wild-type *Listeria* also displayed significant T cell recognition of infected BMM Φ . However, as expected, pretreatment with anti-M3 antibody blocked recognition and subsequent IFN γ 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

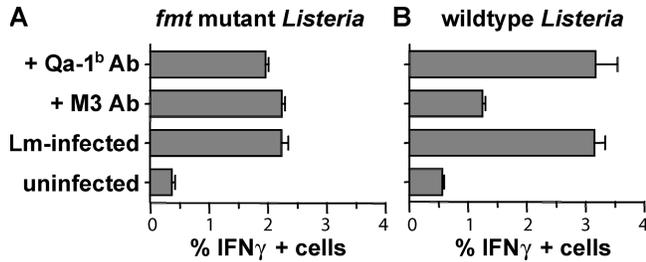


Figure 7. CD8⁺ T cells from *fmt* mutant *Listeria*-immune mice recognize antigens presented by an MHC-Ib molecule other than M3 or Qa-1^b. Groups of MHC-Ia-deficient mice were infected with 2,000 CFU of either (A) *fmt* mutant (Lm SD-A1) or (B) wild-type (Lm 10403s) *L. monocytogenes*. 7 d after infection, splenocytes from these mice were harvested and exposed to MHC-Ia-deficient BMM Φ (either uninfected or cells that had been infected for 3 h with the same strain of *Listeria* used to infect the mice). As indicated, some samples of *Listeria*-infected BMM Φ were pretreated with either anti-M3 or anti-Qa-1^b monoclonal antibody before the addition of immune splenocytes. Data shown represent the average number of TCR β ⁺ CD8⁺ cells that stained IFN γ ⁺ within each group of mice \pm SE from one out of two experiments.

to CD8⁺ T cells by novel MHC-Ib molecules during both wild-type and *fmt* mutant *Listeria* infection of mice.

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 Act^R *fmt* mutant strains of *L. monocytogenes* to use as a tool to address the role of M3-restricted CD8⁺ T cells in

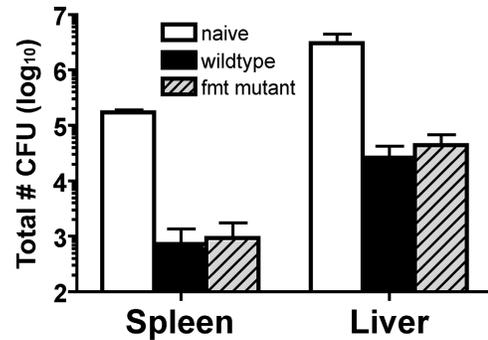


Figure 8. Immunization with *fmt* mutant *Listeria* results in full protective immunity against secondary challenge with wild-type *Listeria*. Groups of MHC-Ia-deficient mice were immunized with PBS (naive) or 10³ CFU of either 10403s (wild type) or SD-A1 (*fmt* mutant) *L. monocytogenes*. 3 wk later, all groups of mice were challenged intravenously with 5 \times 10⁴ wild-type *Listeria*. The total number of CFU per organ was determined 3 d after infection. Similar results were observed for groups of mice immunized with strain SD-A8 (not depicted). Data shown represent average values \pm SD for groups of mice from one of two independent experiments.

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 formyl-methionine 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 K^d-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. Kerksiek 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 K^d-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-1^b-restricted *Listeria*-specific cytotoxic T lymphocytes increase in number during *L. monocytogenes* infection (28). However, like M3-restricted CD8⁺ T cells, Qa-1^b-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-1^b-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 ~10⁴ CFU in BALB/c mice. Bacteria were grown in BHI broth (Difco) supplemented with 100 μg/ml actinonin (Sigma-Aldrich) as indicated. DNA fragments encoding *fmt* were amplified from each *Listeria* strain using Platinum PCR Supermix (Invitrogen) with the following primers: 3'-GACATGAGCGGACAGGGGAAGT-5' and 3'-CACCCCGTTG-ACGAATTTAGTTAC-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-2^b), 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 7% CO₂. EL-4 and J774 cells were grown in RP-10 media consisting of RPMI 1640 (GIBCO BRL) supplemented with L-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/By/J mice obtained from Jackson ImmunoResearch Laboratories and eleventh backcross generation C.B10-H-2^b/LiJMcld/J K^b^{-/-}D^b^{-/-} 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 $\sim 10^3$ 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 $\sim 6 \times 10^4$ 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 RP-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 Hepa1-6 cells), incubated for 30 min, washed three times with warm PBS, and RP-10 containing 25 μ g/ml gentamicin sulfate was added. At indicated time points, the number of bacteria associated with each coverslip was 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 coincubated with either 50 μ l of *L. monocytogenes* supernatant or synthetic peptide (Bio-Synthesis) and 100 μ Ci sodium ⁵¹chromate for 1 h at 37°C in 7% CO₂. The cells were washed three times with RPMI 1640, suspended in RP-10 at 10⁵ cells/ml and 100 μ l of cells per well was added to 96-well plates. Serial dilutions of T cells were added in a final volume of 200 μ l/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 = $100 \times [(release\ by\ T\ cells - spontaneous\ release) \div (maximum\ release - spontaneous\ release)]$.

Competitive index assay. Growth of *fnt* 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.v.) with a 1:1 mixture of wild-type strain DP-L3903 (Erm^R) and one of the *fnt* mutant strains. Mice were given a total of $\sim 10^3$ 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 μ g/ml erythromycin. Competitive indices were calculated by dividing the total number of test strain CFU (SD-A1 or SD-A8; erythromycin-sensitive) by the total number of reference strain CFU (DP-L3903; erythromycin-resistant).

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 μ g/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. IFN γ ICCS was performed using the Cytofix/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 μ M. 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 μ l) 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-CD8 α (53–6.7), anti-CD8 β (53–5.8), and anti-TCR β (H57–597) antibodies. The cells were fixed, permeabilized, and stained with anti-IFN γ (XMG1.2) 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 BMM Φ were pretreated with anti-M3 (clone 130) or anti-Qa-1^b (6A8.6F10.1A6) monoclonal antibodies (final concentration 500 μ g/ml) for 30 min at room temperature. The antibodies were maintained at a concentration of 20 μ g/ml for 5–6 h during the incubation of CD8-enriched splenocytes with infected or uninfected macrophages. The number of IFN γ -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 *fnt* 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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