

Impaired response to *Listeria* in H2-M3-deficient mice reveals a nonredundant role of MHC class Ib-specific T cells in host defense

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The major histocompatibility complex (MHC) class Ib molecule H2-M3 primes the rapid expansion of CD8⁺ T cells by presenting *N*-formylated bacterial peptides. However, the significance of H2-M3-restricted T cells in host defense against bacteria is unclear. We generated H2-M3-deficient mice to investigate the role of H2-M3 in immunity against *Listeria monocytogenes* (LM), a model intracellular bacterial pathogen. H2-M3-deficient mice are impaired in early bacterial clearance during primary infection, with diminished LM-specific CD8⁺ T cell responses and compromised innate immune functions. Although H2-M3-restricted CD8⁺ T cells constitute a significant proportion of the anti-listerial CD8⁺ T cell repertoire, the kinetics and magnitude of MHC class Ia-restricted T cell responses are not altered in H2-M3-deficient mice. The fact that MHC class Ia-restricted responses cannot compensate for the H2-M3-mediated immunity suggests a nonredundant role of H2-M3 in the protective immunity against LM. Thus, the early H2-M3-restricted response temporally bridges the gap between innate and adaptive immune responses, subsequently affecting the function of both branches of the immune system.

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Abbreviations used: HKLM, heat-killed *Listeria monocytogenes*; LM, *Listeria monocytogenes*; neo, neomycin; rLM-ova, recombinant LM strain expressing OVA protein.

The MHC contains a large number of class I genes, most of which belong to the MHC class Ib family and are distinguished from MHC class Ia molecules by their lack of polymorphism. The MHC class Ib molecules are thought to serve a more specialized function than MHC class Ia molecules, possibly through the presentation of structurally distinct, yet conserved, microbial antigens to T cells. H2-M3 is one of the best studied MHC class Ib molecules in the mouse. H2-M3 was originally shown to present a polymorphic *N*-formylated peptide, derived from the mitochondrially encoded ND1 protein, as a histocompatibility antigen to CD8⁺ T cells (1–3). Further studies have demonstrated that H2-M3 specifically binds *N*-formylated hydrophobic peptides, exhibiting 100–1,000-fold

lower affinity for nonformylated peptides (4, 5). Like mitochondrial-encoded proteins, bacterial proteins commonly possess a formylated NH₂ terminus. The unique binding specificity of H2-M3 makes it well suited to present bacterially derived NH₂-terminal peptides to CTLs (6). Indeed, H2-M3-restricted CTL epitopes have been identified in both *Listeria monocytogenes* (LM) and *Mycobacterium tuberculosis* (7–10).

The supply of endogenous *N*-formylated mitochondrial peptides is limited, likely explaining the relatively low surface expression of H2-M3 as compared with MHC class Ia molecules (11). A significant pool of H2-M3 exists intracellularly and can be rapidly mobilized to the surface when provided with appropriate *N*-formylated peptides (11). This observation suggests that a rapid and effective display of bacterial peptides by H2-M3 may occur during bacteria infection. Despite low levels of surface expression and a limited repertoire of self-ligands, H2-M3 plays an active role in thymic selection (12, 13). A recent study showed that H2-M3-restricted CD8⁺ T cells could be

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efficiently positively selected by H2-M3-expressing hematopoietic cells, unlike thymic epithelial cell-mediated selection of MHC class Ia-restricted CD8⁺ T cells (14, 15). The disparate thymic selection may, in part, account for the distinct behavior of MHC class Ia- and class Ib-restricted T cells in response to infection.

LM is a Gram-positive facultative intracellular bacterial pathogen that causes disseminated infection in immunocompromised human hosts. Experimental listeriosis of mice has been instrumental in analyzing cellular immunity against intracellular bacteria. Investigations of this model have demonstrated that the immune response to LM proceeds in two stages: an early innate immune response that controls the initial bacterial burden (16); and then a later “sterilizing” adaptive immune response that involves antigen-specific T cells, particularly CD8⁺ T cells, which clear the infection and are responsible for a protective memory response (17). In addition to an MHC class Ia-restricted CD8⁺ T cell response, mice infected with LM also develop a robust H2-M3-restricted CD8⁺ T cell response. To date, three listerial peptides presented by H2-M3 have been identified, including Fr38 (fMIVIL; reference 9), LemA (fMIGWII; reference 8), and AttM (fMIVTLF; reference 7). One unique feature of the H2-M3-restricted CD8⁺ T cell response to LM is that it occurs earlier than the MHC class Ia-restricted response during primary infection (peaking at days 5–7 vs. days 7–9 after infection; references 18 and 19). However, upon secondary LM infection H2-M3-restricted T cells fail to undergo significant expansion while MHC class Ia-restricted T cells undergo very vigorous expansion (18–20). The distinct kinetics of H2-M3-restricted and MHC class Ia-restricted T cells during LM infection suggest that these two CD8⁺ T cell populations may fulfill nonoverlapping functions in host defense against intracellular bacteria. A protective role of H2-M3-restricted T cells in both primary and secondary LM infection has been implicated in studying the function of residual CD8⁺ T cells in mice lacking MHC class Ia molecules ($K^b^{-/-}D^b^{-/-}$; references 19, 21, and 22). However, these experiments are complicated by the contribution of other MHC class Ib-restricted responses, such as the LM-specific Qa-1-restricted response (23–25).

In this study, we specifically investigated the role of H2-M3 in protective immunity against LM and examined the interrelationship between MHC class Ia- and class Ib-restricted T cells during LM infection using H2-M3-deficient ($M3^{-/-}$) mice. We showed that H2-M3 is important for the protective anti-listerial response, as demonstrated by higher bacterial burdens in the liver and the spleen, as well as increased susceptibility to infection in $M3^{-/-}$ mice. Although naive $M3^{-/-}$ mice do not display an apparent defect in the CD8⁺ T cell compartment, we observed significantly reduced numbers of effector CD8⁺ T cells, concurrent with lower levels of LM-specific IFN- γ production and cytotoxicity in $M3^{-/-}$ mice during primary LM infection. These results suggest that H2-M3-restricted T cells comprise a substantial proportion

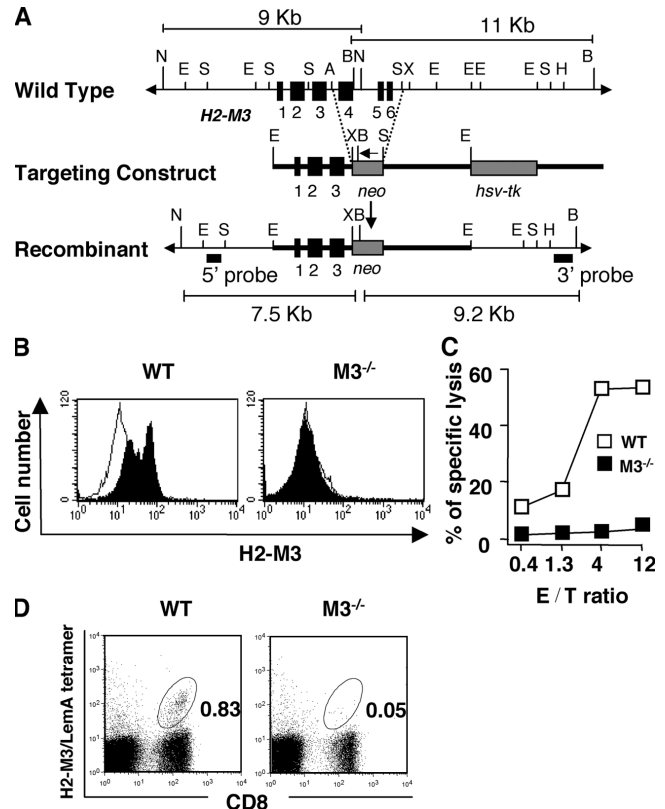


Figure 1. Generation and characterization of $M3^{-/-}$ mice.

(A) Genomic configuration of the *H2-M3* locus before (top) and after (bottom) homologous recombination with the targeting construct (middle). Exons (black boxes), lengths of diagnostic restriction fragments, and the probes used for Southern analysis are shown. An arrow indicates the transcription orientation of the *neo* gene. B, BglII; N, NheI; E, EcoRI; S, SacI; A, ApaI; H, HindIII; X, XbaI. (B) Splenocytes from WT and $M3^{-/-}$ mice were incubated overnight with (filled histogram) or without (open histogram) 10 μ M LemA peptide. Cells were stained with an anti-H2-M3 mAb 130, followed by FITC-anti-hamster IgG. (C) H2-M3-specific CTL clone 4E3 was incubated with LPS blasts from WT and $M3^{-/-}$ during a ⁵¹Cr release assay. The effector-target ratio is indicated in the figure. (D) Identification of LM-specific H2-M3-restricted T cells after i.v. infection with LM. Peripheral blood lymphocytes were isolated from LM-infected $M3^{-/-}$ mice (N6) or WT controls on day 7 after infection. Cells were stained with FITC-anti-CD8 α and PE-H2-M3/LemA-tetramer. CD8⁺ tetramer⁺ T cells are highlighted by the gate, and the proportion of gated cells is indicated.

of the anti-listerial CD8⁺ T cell population. In addition, lack of early activation of H2-M3-restricted T cells resulted in reduced functions of macrophages and NK cells. In contrast, H2-M3 deficiency has no significant effect on anti-listeria immunity during secondary infection. Interestingly, the kinetics and magnitude of LM-specific MHC class Ia-restricted T cell responses were not significantly altered when the H2-M3-restricted responses were absent. Our finding that MHC class Ia-restricted T cells were not able to completely reconstitute the CD8⁺ T cell compartment in $M3^{-/-}$ mice during

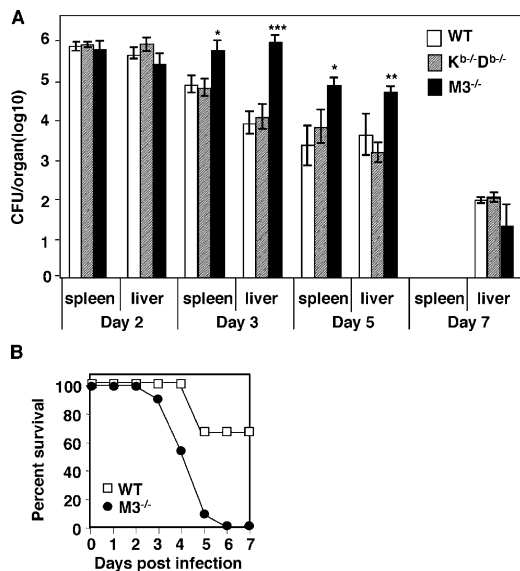


Figure 2. *M3^{-/-}* mice exhibit increased susceptibility to LM infection compared with WT and *K^{b-/-}D^{b-/-}* mice. (A) Increased bacterial burdens in the spleens and livers of LM-infected *M3^{-/-}* mice. *M3^{-/-}*, *K^{b-/-}D^{b-/-}*, and WT mice were infected i.v. with 5×10^3 CFU of LM. Bacterial burdens in the spleens and livers were determined at indicated time points after infection. Data are presented as the mean \pm SEM from six mice per group per time point. Statistically significant differences between WT and *M3^{-/-}* mice (N6) are indicated. At day 7 of infection, the bacterial burdens in the spleens of LM-infected mice were below the detection limit. (B) Enhanced susceptibility of *M3^{-/-}* mice to LM. *M3^{-/-}* mice (N10) and WT controls were infected with LM at the dose of 5×10^4 CFU. The survival of the mice was monitored daily up to 7 d after infection. Results are from 11 mice per group.

LM infection suggests a unique role of H2-M3–restricted T cells in host defense against bacteria infection.

RESULTS

Generation and characterization of H2-M3–deficient (*M3^{-/-}*) mice

To assess the function of H2-M3, we generated *M3^{-/-}* mice by a gene-targeting approach in which exons 4–6 of the *H2-M3* gene were replaced by a neomycin (*neo*)–resistance gene (Fig. 1 A). Flow cytometric analysis and CTL assays were performed to demonstrate that mice homozygous for the H2-M3 mutation do not express H2-M3 on the cell surface. As shown in Fig. 1 B, surface expression of H2-M3 cannot be induced in splenocytes from *M3^{-/-}* mice by incubating with H2-M3–restricted LemA peptide. Splenocytes from WT mice, but not from *M3^{-/-}* mice, can be recognized by a mitochondrial ND1 peptide–specific, H2-M3–restricted CTL clone (Fig. 1 C). To confirm that H2-M3 deficiency affects its restricted CTL response, we infected WT and *M3^{-/-}* mice with LM and measured reactivity with H2-M3/LemA tetramer at day 7 after infection. As shown in Fig. 1 D, a substantial population of tetramer⁺ CD8⁺ T cells was elicited in WT mice, but was not detectable in *M3^{-/-}* mice. Thus, M3 protein and its restricted CD8⁺ T cells were completely absent in *M3^{-/-}* mice.

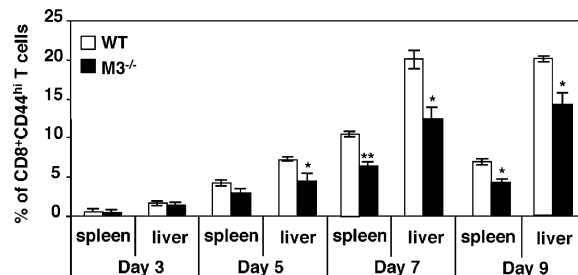


Figure 3. Decreased frequency of effector CD8⁺ T cells in *M3^{-/-}* mice during LM infection. Splenocytes and hepatic leukocytes were harvested from LM-infected *M3^{-/-}* and *M3^{+/+}* mice (N10) on the indicated days and stained with mAbs specific for CD8 α , CD4, and CD44. Percentages of CD44^{high} CD8⁺ T cells were determined by flow cytometric analysis. Data shown are the mean \pm SEM of three to eight animals per interval.

Effect of H2-M3 on T cell development

To determine the effect of H2-M3 deficiency on T cell development, we performed flow cytometric analysis on lymphocytes isolated from the thymus, spleen, liver, bone marrow, and lymph nodes of *M3^{-/-}* and control mice. We found no significant differences in the number and proportions of CD4⁺, CD8⁺, CD4⁺CD8⁺, $\alpha\beta$, and $\gamma\delta$ T cells between the two groups (not depicted). It has been shown that the residual CD8⁺ T cells in *K^{b-/-}D^{b-/-}* mice are enriched for cells expressing activation markers, suggesting that MHC class Ib molecule(s) may be critical in the development of this unique subset of CD8⁺ T cells (19). We compared CD8⁺ cells from *M3^{-/-}* and WT mice for the expression of various activation markers, including CD44, CD69, CD122, and Ly6C. Although there is a trend toward the frequencies of CD8⁺CD44^{hi} T cells from the spleen and mesenteric lymph nodes being slightly decreased in *M3^{-/-}* mice, this difference is not statistically significant with the sample size analyzed (see Table S1, available at <http://www.jem.org/cgi/content/full/jem.20051866/DC1>). Our data suggests that H2-M3–restricted T cells may represent only a small fraction of the total CD8⁺ T cell population in naive animals.

H2-M3 deficiency increases susceptibility to *Listeria* infection

H2-M3–restricted T cells show a peak immune response that precedes their MHC class Ia–restricted counterparts after LM infection (18, 19). To investigate the relative significance of H2-M3–restricted and MHC class Ia–restricted CD8⁺ T cell responses in protective immunity against LM, we infected *M3^{-/-}*, *K^{b-/-}D^{b-/-}*, and WT mice with a sublethal dose of LM (5×10^3 CFU) and measured bacterial burden at various time points after infection. Compared with WT mice, *M3^{-/-}* mice showed significantly higher bacterial burdens (\sim 10–100-fold) in both the spleen and liver at early time points (days 3 and 5) after infection (Fig. 2 A). In contrast, *K^{b-/-}D^{b-/-}* and

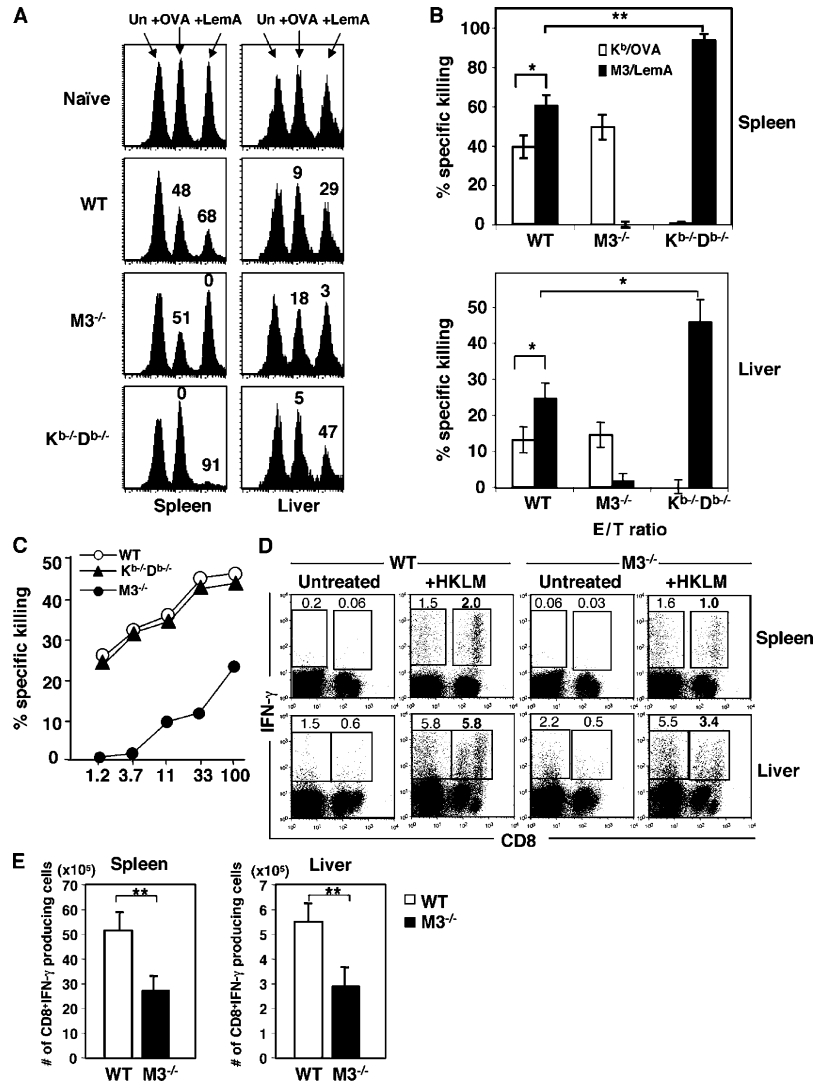


Figure 4. Impaired LM-specific cytolytic activity and the IFN- γ production in M3^{-/-} mice. (A) LM infection elicited a potent H2-M3-restricted CTL response during the early phase of infection. M3^{-/-}, K^{b-/-}D^{b-/-}, and WT mice were infected with rLM-ova. Fluorescence-labeled peptide-pulsed or unpulsed B6 splenocytes were coinjected i.v. into day 3 LM-infected or naive mice. In vivo cytotoxicity was assessed 15 h later by flow cytometry. Histograms are gated on PKH26⁺ target cells and the numbers above each peak represent the percent of specific killing. The data are representative of eight mice per group from two independent experiments. (B) The bar graph indicates the percentage of specific lysis for OVA- and LemA-coated targets from the indicated group of mice. Data shown are the mean \pm SEM of eight animals per group. (C) Diminished LM-specific cytolytic activity in M3^{-/-} mice. 5 d after LM infection, splenocytes from indicated mice were activated with Con A

and examined for CTL activity to ⁵¹Cr-labeled LM-infected J774-K^b target cells. Each data line represents the mean from two mice. Data are representative of results obtained from three independent experiments. Specific lysis for uninfected J774-K^b targets was below 5% from all effectors tested. (D and E) Decreased LM-specific, IFN- γ -producing CD8⁺ T cells in M3^{-/-} mice. Splenocytes and hepatic leukocytes from indicated mice were harvested on day 7 after LM infection and stimulated overnight with HKLM. Cells were stained for CD8 expression and intracellular IFN- γ , and analyzed by flow cytometry. The percentages of CD8⁺IFN- γ ⁺ and CD8⁻IFN- γ ⁺ cells are indicated (D). Results are representative from eight mice per group. Bar graph depicts mean \pm SEM for total number of LM-specific CD8⁺ cells in the spleens and livers of M3^{-/-} and WT mice (E). M3^{-/-} and WT controls used in these experiments are from N10 backcross.

WT mice clear LM from the spleen and liver with similar kinetics, consistent with the previous report (22). By day 7 after infection, LM was nearly absent from all three strains of mice. This data suggests that H2-M3-restricted responses may play an important and even more dominant role than MHC class Ia-restricted responses in protective

immunity against LM during the early phase of infection. In agreement with this notion, M3^{-/-} mice were more susceptible to a higher dose (5 \times 10⁴ CFU) of LM infection than WT controls (Fig. 2 B). Thus, H2-M3-restricted responses contribute significantly to host defense against LM infection.

The frequency of activated CD8⁺ T cells is reduced in LM-infected M3^{-/-} mice

To determine whether H2-M3–restricted CD8⁺ T cells constitute a significant proportion of the anti-listerial CD8⁺ T cell repertoire, we compared the CD8⁺ population in M3^{-/-} to WT mice during the course of LM infection. LM infection leads to expansion of LM-specific effector CD8⁺ T cells, which reach a peak number around day 7. On day 5 after LM infection, the frequency (Fig. 3) and total number (not depicted) of CD8⁺CD44⁺ T cells were slightly reduced in M3^{-/-} mice. On day 7 after infection, the percentage of CD8⁺CD44⁺ T cells in M3^{-/-} mice was decreased by nearly 50% compared with WT controls. The effect of H2-M3 deficiency is specific for CD8⁺ T cells, as no significant differences in the CD4⁺ population can be detected between LM-infected M3^{-/-} and control mice (not depicted).

The effector functions of CD8⁺ T cells are diminished in LM-infected M3^{-/-} mice

To investigate the mechanisms by which H2-M3–restricted T cells contribute to anti-listerial immunity, we compared the antigen-specific cytotoxic response and IFN- γ production between M3^{-/-} and WT mice. For comparison purposes, we also included K^b-D^b mice in our analysis. First, an *in vivo* cytotoxicity assay was used to assess the cytotoxic potential of H2-M3–restricted T cells during the early phase (day 3) of LM infection, the time point when the difference in bacterial clearance was already apparent. Because MHC class Ia–restricted listerial peptides are not well defined in the H-2^b background, we infected mice with a recombinant LM strain expressing OVA protein (rLM-ova) to compare H2-M3– and K^b–restricted CTL responses simultaneously. On day 3 after infection, mice were injected with B6 splenocytes pulsed with either H2-M3–restricted LemA peptide or K^b–restricted OVA peptide, and the clearance of these target cells was evaluated 15 h later by flow cytometry. H2-M3/LemA-specific and K^b/OVA-specific cytotoxic responses are readily detectable in WT mice (Fig. 4, A and B). However, the cytotoxic response to LemA-coated targets was significantly higher than that to OVA-bearing targets both in the spleens and livers, suggesting that H2-M3/LemA-specific CTLs may be more dominant than K^b/OVA-specific CTLs in WT mice at this time point (Fig. 4, A and B). In M3^{-/-} mice, no H2-M3/LemA-specific response was detected, confirming the specificity of the assay. The cytolytic activity to OVA-coated targets was comparable between M3^{-/-} mice and WT mice, suggesting that the generation/expansion of K^b–restricted CTLs was not affected in the absence of H2-M3–restricted responses. On the contrary, the H2-M3/LemA-specific cytotoxic response was significantly enhanced in K^b-D^b mice compared with WT mice (Fig. 4, A and B). Thus, the overall CTL activity is reduced in M3^{-/-} mice during the early phase of LM infection due to the lack of a compensatory increase of MHC class Ia–restricted CTL responses while remaining largely unchanged in K^b-D^b mice due to augmented H2-M3–restricted responses.

The use of *in vivo* cytotoxicity assays allowed us to detect relatively small numbers of LM-specific CTLs during early LM infection. However, the analysis was limited to a single immunodominant epitope for K^b and H2-M3. To further investigate the contribution of H2-M3–restricted T cells to LM-specific cytolytic response, we performed *in vitro* CTL assays using LM-infected J774-K^b transfectants as targets, which allowed us to measure overall LM-specific, H2-M3– and K^b–restricted CTL responses. Splenocytes from M3^{-/-}, K^b-D^b, or WT mice infected 5 d previously were isolated and stimulated with Con A for 3 d and then used as effectors in a ⁵¹Cr release assay. As shown in Fig. 4 C, effectors derived from WT and K^b-D^b mice lysed LM-infected J774-K^b targets to a similar extent, whereas effectors derived from M3^{-/-} mice displayed much lower lytic activity.

To determine whether H2-M3 deficiency affects the IFN- γ production by CD8⁺ T cells, intracellular IFN- γ staining was performed. Splenocytes and hepatic leukocytes harvested from M3^{-/-} or WT mice 7 d after infection were stimulated *in vitro* with heat-killed LM (HKLM). We found that the frequency and total numbers of LM-specific IFN- γ –producing CD8⁺ T cells were significantly reduced in the spleens and livers of M3^{-/-} mice compared with WT mice (Fig. 4, D and E). Collectively, these data demonstrate that cytolytic activity and IFN- γ production by CD8⁺ T cells are impaired in M3^{-/-} mice during primary LM infection.

H2-M3–deficient mice have compromised macrophage and NK cell functions

Innate immunity to LM is thought to limit the bacteria burden until the development of specific adaptive immunity. Although several studies have shown that innate immunity can influence the development of antigen-specific T cells (26, 27), the reciprocal effect of antigen-specific T cells on the innate response is less defined. As H2-M3–restricted T cells are functional before the receding of innate immune responses, we examined whether the lack of H2-M3–dependent responses can alter the intensity of the innate immune response to LM infection. At day 3 after LM infection, splenocytes and hepatic leukocytes from M3^{-/-} and littermate controls were assayed *in vitro* for nitric oxide production and cytotoxicity to YAC-1 cells to assess the function of macrophages and NK cells, respectively. As shown in Fig. 5 A, splenocytes and hepatic leukocytes from LM-infected M3^{-/-} mice produced less nitric oxide (as measured by nitrite formation) compared with that of M3^{+/-} littermate controls. Similar results were obtained from peritoneal exudate macrophages (not depicted). Although splenocytes and hepatic leukocytes from LM-infected M3^{-/-} mice were able to lyse NK targets, the percentage of killing was lower compared with WT mice (Fig. 5 B). Thus, H2-M3–restricted responses may potentiate the innate immune responses during the early phase of LM infection.

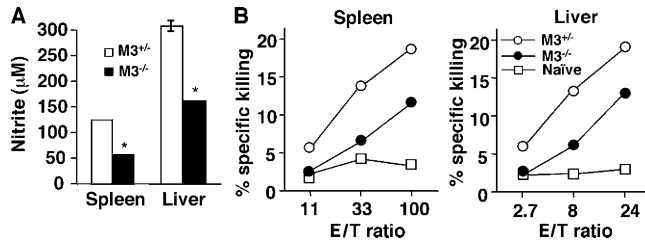


Figure 5. Defective nitrite production and NK cell activity in M3^{-/-} mice during the early phase of LM infection. M3^{-/-} and M3^{+/-} littermate controls (N6) were infected with 5×10^3 CFU of LM. (A) Measurement of nitrite production. 3 d after infection, splenocytes and hepatic leukocytes were isolated and stimulated with HKLM. After 48 h of incubation, the concentration of nitrite as a surrogate marker for the production of reactive nitrogen was determined with Greiss reagent. Bars represent mean \pm SEM of two mice per group and are representative of four independent experiments. (B) NK cell cytotoxicity assay. Splenocytes and hepatic lymphocytes were isolated from day 3 LM-infected mice and used as effectors. Varying numbers of effectors were combined with a constant number of ⁵¹Cr-labeled YAC-1 cells in a standard 4-h cytotoxicity assay. Each data line represents the mean from two mice and is representative of four independent experiments.

H2-M3 deficiency does not influence the secondary CD8⁺ T cell response to LM

Although H2-M3-restricted T cells exhibit limited expansion during secondary LM infection, a substantial number of H2-M3-restricted T cells are detectable 3 d after rechallenging with LM (18). To determine whether an H2-M3-restricted response plays a significant role in secondary LM infection, we immunized WT and M3^{-/-} mice with a sublethal dose of LM and rechallenged them 1 mo later with a high dose of LM. In contrast to the results seen in primary infection, M3^{-/-} and WT mice displayed equivalent numbers of bacteria in the liver at days 2 and 3 after secondary infection (Fig. 6 A). As early as day 2 after secondary LM infection, the bacterial burdens in the spleens approached the detection limits in both WT and M3^{-/-} mice (not depicted). In addition, the number of CD8⁺ T cells and anti-listerial CTL activity are comparable between M3^{-/-} and WT controls (Fig. 6, B and C). Thus, H2-M3 deficiency does not have a significant effect on the secondary immune response to LM.

Interrelationship between MHC class Ia- and H2-M3-restricted CD8⁺ T cells

The increased LM burden during the early phase of LM infection in M3^{-/-} mice could influence the antigenic load to which class Ia-restricted CD8⁺ T cells are exposed during infection, which consequently might affect the kinetics and magnitude of class Ia-restricted LM-specific CD8⁺ T responses. To address this question, we used K^b/OVA tetramer staining (not depicted) and intracellular IFN- γ staining to compare the K^b/OVA-specific response between WT and M3^{-/-} mice after primary and secondary infection with rLM-ova. We found no significant differences in the number of K^b/OVA-specific IFN- γ -producing CD8⁺ T

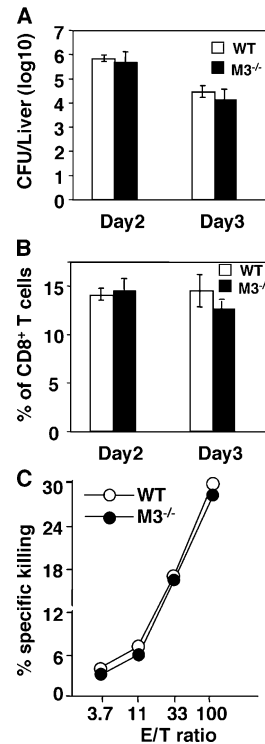


Figure 6. H2-M3 deficiency has no effect on secondary anti-listerial response. 1 mo after primary LM infection, M3^{-/-} (N6) and WT mice were rechallenged with 10^5 CFU of LM. (A) The bacterial burdens in the livers were determined on days 2 and 3 after secondary LM infection. (B) The percentage of CD8⁺ T cells in the spleens of LM-infected mice on the indicated days was determined by flow cytometric analysis. Data shown are mean \pm SEM of five to six mice per group per time point. (C) Comparable LM-specific cytolytic activity in M3^{-/-} and WT mice after secondary LM infection. 3 d after LM reinfection, splenocytes from indicated mice were activated with Con A and examined for CTL activity to ⁵¹Cr-labeled LM-infected J774-K^b target cells. Data are representative of results obtained from three mice per group.

cells between M3^{-/-} and WT mice either after primary or secondary LM infection (Fig. 7, A and B), indicating that H2-M3 deficiency does not affect the primary and secondary expansion of MHC class Ia-restricted T cells. In addition, comparable numbers of K^b/OVA-specific CD8⁺ T cells were found in WT control and H2-M3-deficient mice at day 30 after LM infection ($2.13 \pm 0.41 \times 10^5$ cells and $2.37 \pm 0.38 \times 10^5$ cells per spleen in WT and M3^{-/-} mice, respectively), suggesting that H2-M3 deficiency does not affect the development of the class Ia-restricted memory CD8⁺ T cell population. We also assessed the functional avidity of K^b/OVA-specific CD8⁺ T cells by incubation of splenocytes from LM-infected WT or M3^{-/-} mice with a serial dilution of OVA peptide and measured the amount of IFN- γ production by ELISA. No differences in functional avidity of K^b/OVA-specific T cells from WT or M3^{-/-} mice were observed (not depicted). A reciprocal analysis was performed to examine the effect of the lack of MHC class Ia-restricted responses on H2-M3/LemA-specific responses. We found

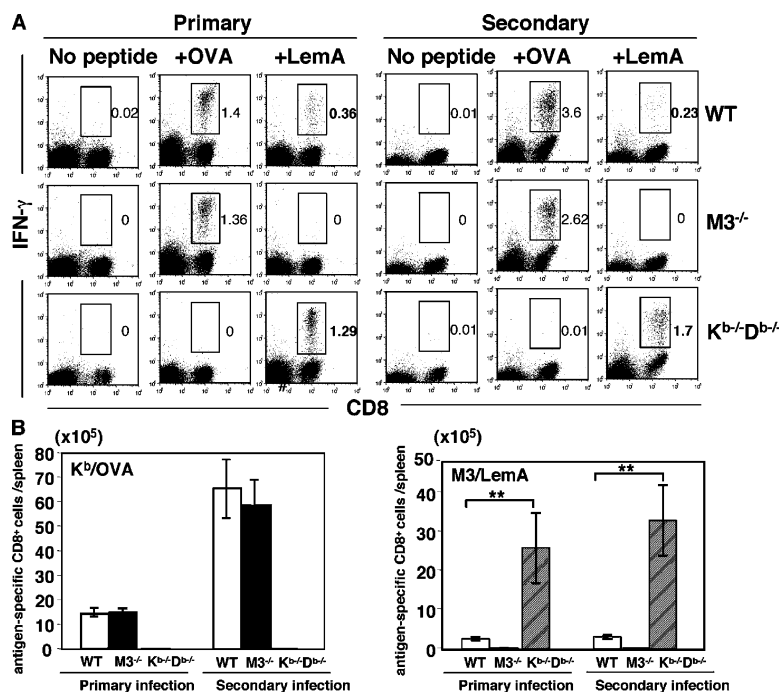


Figure 7. Comparison of MHC class Ia- and H2-M3-restricted CD8⁺ T cell responses in LM-infected M3^{-/-}, K^b-/-D^b-/-, and WT mice. M3^{-/-} (N10), K^b-/-D^b-/-, and WT mice were infected i.v. with 5×10^4 CFU of rLM-ova. Some of the mice were rechallenged 1 mo later with 10^6 CFU of the same bacteria. (A) The frequencies of K^b/OVA-specific and H2-M3/LemA-specific CD8⁺ T cells were determined by intracellular IFN- γ staining at day 7 after primary infection or day 3 after secondary infection. In brief, splenocytes isolated

from LM-infected mice were stimulated in vitro with either LemA or OVA peptide. 5 h later, intracellular cytokine staining for IFN- γ were performed. Antigen-specific CD8⁺IFN- γ ⁺ T cells are highlighted by the gate, and the percentage of gated cells is indicated. Data are representative of results obtained from five to eight mice per group. (B) Bar graphs depict mean \pm SEM for the total number of K^b/OVA-specific or H2-M3/LemA-specific CD8⁺ T cells in the spleens of WT, M3^{-/-}, and K^b-/-D^b-/- mice.

that the number of H2-M3/LemA-specific CD8⁺ T cells is drastically increased in K^b-/-D^b-/- mice during primary LM infection compared with WT mice (Fig. 7, A and B). A similar trend was also observed during secondary LM infection. Thus, these data suggest that H2-M3 deficiency has no quantitative or qualitative effect on MHC class Ia-restricted T cell responses. In stark contrast, MHC class Ia-restricted responses limit the expansion of H2-M3-restricted T cells during primary as well as secondary LM infection.

DISCUSSION

Emerging evidence suggests that MHC class Ib molecules can contribute to the host immune response by presenting conserved microbial antigens to T cells. However, the relative contributions and dynamic interactions between the MHC class Ia- and class Ib-restricted responses to infections have yet to be defined. In this study, we investigated the role of H2-M3 in T cell development and protective immunity against intracellular bacteria using M3^{-/-} mice in a *Listeria* infection model. We found that significantly higher numbers of bacteria were recovered from both the spleens and livers of M3^{-/-} mice than from WT and K^b-/-D^b-/- mice at days 3 and 5 after LM infection, suggesting that H2-M3-restricted CD8⁺ T cells may play a dominant role in protective immu-

nity against LM during the early phase of infection. The increased susceptibility of M3^{-/-} mice to LM infection is associated with a decreased number of LM-specific IFN- γ -producing CD8⁺ T cells and reduced cytolytic activity. In addition, innate immunity to *Listeria* infection is lessened in the absence of H2-M3-restricted responses. Lack of H2-M3-restricted T cells, however, does not alter the kinetics and magnitude of MHC class Ia-restricted T cell responses during LM infection. Conversely, lack of MHC class Ia-restricted T cells results in an increased number of LM-specific H2-M3-restricted T cells after LM infection. This differential ability in compensating for the loss of anti-listerial CD8⁺ T cell responses could be one of the factors underlying the increased susceptibility of M3^{-/-} mice, but not K^b-/-D^b-/- mice, to LM infection. Collectively, our results suggest that H2-M3-restricted responses play a unique role in early host defense against listerial infection by contributing to adaptive immunity as well as by modulating the innate response to *Listeria* in infected mice.

As the number of CD8⁺ T cells is reduced by 90% in the periphery of naive K^b-/-D^b-/- mice (28), one may expect that only a small fraction of the CD8⁺ T cell repertoire is selected by MHC class Ib molecules. Thus, it is not surprising that we were not able to detect significant differences in the

proportions of T cell subsets between $M3^{-/-}$ and WT mice. Although H2-M3-restricted T cells only represent a small proportion of the total pool of $CD8^+$ T cells in naive animals, we found that H2-M3-restricted T cells contribute substantially to the overall anti-listerial $CD8^+$ T cell responses during primary infection. The fact that the number of $CD8^+CD44^{hi}$ cells and LM-specific IFN- γ -producing $CD8^+$ T cells is reduced by 50% in LM-infected $M3^{-/-}$ mice suggests that the small pool of naive M3-restricted T cells are quite capable of expanding to a magnitude similar to their MHC class Ia-restricted counterparts after LM infection. This could be attributed to the accelerated expansion of H2-M3-restricted T cells in response to LM infection as a result of their “memory-like” phenotype (14, 19). Another possible explanation could be that the precursor frequency of LM-specific T cells within the H2-M3-restricted T cell repertoire is relatively high. It has been shown that some H2-M3-restricted LM-specific CTLs are cross-reactive with antigens derived from a variety of Gram-positive and Gram-negative bacteria (29). In addition, deletion of H2-M3-restricted epitopes from LM does not prevent the priming of H2-M3-restricted T cells with specificity for the deleted epitope, suggesting that promiscuous antigen recognition may be a feature common to many H2-M3-restricted T cells (30–32). The promiscuous recognition and cross-reactivity to common environmental bacteria may lead to the priming and expansion of LM-reactive H2-M3-restricted T cells in “naive” animals, resulting in increased precursor frequency as well as a “primed” state.

$CD8^+$ CTLs exert their protective functions against intracellular pathogens through cytolytic activity and secretion of cytokines, such as IFN- γ . Both activities are diminished in H2-M3-deficient mice. To address whether IFN- γ or cytotoxicity or both are important for M3-dependent immunity to *Listeria*, we treated LM-infected $M3^{-/-}$ and WT mice with neutralizing antibody against IFN- γ . CFU data obtained from both the spleens and livers at day 3 after infection showed that anti-IFN- γ -treated $M3^{-/-}$ mice are still more susceptible to LM infection when compared with anti-IFN- γ -treated WT mice (see Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051866/DC1>). Thus, neutralization of IFN- γ does not significantly affect the protection mediated by H2-M3-restricted response, suggesting that cytolytic activity may play an important role in H2-M3-mediated early protection during LM infection.

Our results have shown that the LM-specific $CD8^+$ T cell population is significantly reduced in $M3^{-/-}$ mice, suggesting that MHC class Ia-restricted LM-specific $CD8^+$ T cells did not undergo compensatory expansion during LM infection. This is further supported by the observations that $CD8^+$ T cell responses to two K^b -restricted epitopes, OVA_{257–264} and LLO_{296–304}, despite the difference in magnitude, were not affected by the H2-M3 deficiency (Fig. 7 and not depicted). One possible explanation is that the majority of naive LM-specific MHC class Ia-restricted $CD8^+$ T cell precursors have been recruited in our experimental settings. Thus, MHC class Ia-restricted LM-specific

$CD8^+$ T cells were not able to contribute further, even when a sizable amount of H2-M3-restricted T cells were absent. Recent studies have shown that the magnitude of LM-specific $CD8^+$ T cell responses is determined within the first 24 h of an infection and is independent of the severity and duration of in vivo bacterial infection (33). This notion may further explain the lack of a concordant increase in LM-specific class Ia-restricted T cells in $M3^{-/-}$ mice despite the presence of higher bacterial titers in the spleens and livers of $M3^{-/-}$ mice after 3–5 d of LM infection. However, it is conceivable that in an infection model where not all naive antigen-specific MHC class Ia-restricted T cells were recruited, either due to the presence of a large pool of antigen-specific MHC class Ia-restricted T cell precursors or due to the limited supplies of antigens/inflammatory cytokines, the presence or absence of an H2-M3-restricted response may affect the magnitude of MHC class Ia-restricted T cell responses.

Our finding that the number of LM-specific H2-M3-restricted T cells is significantly higher in $K^b^{-/-}D^b^{-/-}$ mice than in that of WT controls after primary infection is in accordance with previous observations using an in vitro cytotoxicity assay (22). It is possible that the precursor frequency of H2-M3-restricted $CD8^+$ T cells is increased in $K^b^{-/-}D^b^{-/-}$ mice. Another possible explanation, although not mutually exclusive, is that lack of MHC class Ia-restricted T cells may allow for maximal expansion of LM-specific H2-M3-restricted T cells during infection by providing a favorable environment with more access to APCs, cytokines, and growth factors. A recent study has shown that the expansion of LM-specific H2-M3-restricted memory T cells can be prevented by LM-specific MHC class Ia-restricted memory T cells, possibly through rapid removal of antigen-presenting DCs (34). Comparing the numbers of H2-M3/LemA-specific T cells elicited during primary and secondary LM infection, we found that H2-M3-restricted T cells did not experience a similar degree of secondary expansion as observed for MHC class Ia-restricted T cells even in $K^b^{-/-}D^b^{-/-}$ mice, consistent with the previous finding using IFN- γ ELISPOT assays (19). Thus, these data suggest that lack of memory expansion of H2-M3-restricted response during infection may be, at least in part, due to intrinsic differences between MHC class Ia- and H2-M3-restricted T cells. Alternatively, the presence of other LM-specific non-MHC class Ia-restricted memory T cells might also be able to limit the secondary expansion of H2-M3-restricted T cells. Similar to H2-M3-restricted T cells, lack of memory expansion is also evident for Qa-1-restricted T cells during LM infection (23). Thus, MHC class Ib-restricted responses against infectious organisms may play a unique and important role during the early stages of infection, but participate only minimally in the protective memory immune response.

In addition to H2-M3-restricted T cells, LM infection has been shown to activate/induce T cells restricted by other MHC class Ib molecules, such as CD1d and Qa-1^b (22, 24, 35). To see whether lack of H2-M3-restricted responses has an effect on other MHC class Ib-restricted responses, we

have examined the CD1d- and Qa-1^b-restricted T cell responses in LM-infected WT and M3^{-/-} mice. We found no significant differences in the number of CD1d-restricted NKT cells or the magnitude of Qa-1^b-specific cytolytic activity between LM-infected M3^{-/-} and control mice at all time points examined (not depicted). This finding further supports the nonredundant role of H2-M3-restricted T cells in anti-listerial immunity.

Previous studies have shown that H2-M3-restricted T cells share some characteristics with CD1d-restricted NKT cells, such as the capacity to elicit rapid effector function and ability to be positively selected by hematopoietic cells (36, 37). However, unlike CD1d-restricted NKT cells that express a limited TCR repertoire (38–41), the H2-M3-restricted T cells studied to date have diverse TCR usage (42). Consistent with this notion, analysis of TCR V α and V β chains in M3^{-/-} mice does not reveal significant alterations in V α and V β usage in CD8⁺ T cell populations compared with control (not depicted). In addition to the differences in TCR V α and V β repertoire diversity, the kinetics of H2-M3-restricted T cell responses are also different from that of CD1d-restricted NKT cells in LM infection. Although we can detect H2-M3-restricted cytotoxic responses as early as day 2 after LM infection using an in vivo CTL assay (not depicted), H2-M3-restricted T cell response peaks at days 5–7 after infection (18, 19). In contrast, the majority of CD1d-restricted NKT cells are activated within 24 h after LM infection (35 and not depicted). Thus, the distinct kinetics of H2-M3-restricted T cells positions them to fill the temporal gap between “innate” T cells and conventional adaptive T cells.

In summary, our study demonstrates that H2-M3-restricted responses play a significant and nonredundant role in protective immunity against intracellular bacterial infection. Similar to H2-M3, other MHC class Ib molecules may serve a specialized role in antimicrobial immunity by selective presentation of conserved molecular signatures of microbial antigens and/or stress-induced ligands. The recognition of specific molecular patterns and rapid acquisition of effector functions may provide the unique niche for the MHC class Ib-restricted T cells in host defense against microbial pathogens.

MATERIALS AND METHODS

Generation of H2-M3-deficient (M3^{-/-}) mice. A DNA fragment containing H2-M3 was isolated from a 129/sv genomic DNA phage library (Stratagene). The targeting construct was generated by insertion of a 3.8-kb fragment containing exons 1–3 of the H2-M3 and a 5.5-kb fragment containing the 3' end of H2-M3 into the EcoRI–BamHI and NotI–XhoI sites of pPNT, respectively (43). The targeting construct was designed to delete a 2.5-kb ApaI–SacI fragment containing exons 4–6 of the H2-M3 and replace these coding regions with the neo-resistance gene (Fig. 1 A). Strain 129/sv-derived R-1 embryonic stem cells were electroporated with 30 μ g of NotI-linearized targeting construct, and G418-resistant colonies were screened for homologous recombination by Southern blot hybridization. Embryonic stem clones were injected into blastocysts of B6 mice, and chimeras were mated to B6 females. Offspring heterozygous for the mutation were intercrossed to produce M3^{-/-} mice. Most of the M3^{-/-} mice used in this study

were backcrossed on to a B6 background (between 6 to 10 generations, N6–N10). The H2-M3 genotype was determined with PCR using the following three primers: M3-forward-CAGCGATGGACCACCCACAATGA, M3-reverse-CAGACTAGCAACGATGACCATGATGAC, and neo-GATTCGCAGCGCATCGCCTTCTA, which yield a 550-bp fragment for the mutant allele and a 700-bp fragment for WT allele.

Mice. K^b^{-/-}D^b^{-/-} mice were provided by J. Forman (Texas Southwestern Medical Center, Dallas, TX) and were backcrossed up to 10 generations onto a B6 background. B6 mice were purchased from The Jackson Laboratory. All experiments involving animals were performed in compliance with institutional guidelines and have been approved by the Institute Animal Care Use Committee.

Peptides, antibodies, and flow cytometry. LemA (f-MIGWII), OVA_{257–264} (SIINFEKL), and LLO_{296–304} (VAYGRQVYL; reference 44) peptides were synthesized at the University of Chicago Peptide Core facility. The following antibodies were used in this study: FITC-anti-Ly6C, FITC-anti-TCR- β , FITC-anti-CD3, FITC-anti-CD8 α , FITC-anti-CD122, FITC-anti-hamster IgG, PE-anti-CD8 β , PE-anti-CD44, PE-anti-CD69, PE-anti-NK1.1, PE-anti-F4/80, PE-anti-CD11c, PE-anti-Gr1, PE-anti-IFN- γ , CyChrome-anti-CD8 α , and CyChrome-anti-CD4 (BD Biosciences). H2-M3/LemA tetramers were prepared as described previously (18). For flow cytometric analysis, single cell suspensions from the thymus, spleen, lymph node, and liver were prepared by standard procedures. For detecting H2-M3 surface expression, splenocytes from M3^{-/-} and WT mice were incubated in RPMI 10 (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 20 mM Hepes, 50 μ M 2-mercaptoethanol, penicillin, and streptomycin) with or without 10 μ M LemA peptide overnight at 37°C. Cells were harvested and stained with anti-H2-M3 antibody (mAb130), followed by FITC-anti-hamster IgG (11). To examine T cell subsets and various immune cell types, cells were stained with combinations of fluorescent-conjugated antibodies for 30 min on ice in HBSS (Life Technologies) containing 2% FBS and 0.1% sodium azide (Sigma-Aldrich), followed by washing with the same buffer. The stained cells were analyzed by flow cytometry using a FACSCaliber with CELLQuest (Becton Dickinson) or Flowjo (Tree Star) software.

Bacteria, listeria infections, and CFU assays. LM strain EGD was provided by R. Kurlander (National Institutes of Health, Bethesda, MD) and was grown in brain–heart infusion broth (Difco Laboratories). The recombinant LM strain rLM-ova, which secretes OVA and contains erythromycin marker, was provided by L. Lefrançois (University of Connecticut Health Center, Farmington, CT; reference 45). rLM-ova was grown in brain–heart infusion broth supplemented with 5 μ g/ml erythromycin. For quantitation of bacterial titers from tissues, 12–20-wk-old mice were infected i.v. with 5×10^5 CFU LM (EGD) for primary infection. Recall infection with 10^5 CFU LM was performed 1 mo after primary infection. The bacterial dose was verified by plating dilutions of the inoculum on BH agar plates. At the indicated times after infection, mice were killed and specimens of liver and spleen were examined for bacterial titers. In brief, organs were homogenized and lysed in sterile water with 0.2% NP-40, serial dilutions of homogenates were plated on BH agar plates, and colonies were counted after incubation at 37°C for 24 h. For comparing the MHC class Ia-restricted and H2-M3-restricted T cell responses, mice were infected i.v. with 5×10^4 CFU rLM-ova for the primary infection and 10^6 CFU rLM-ova for the secondary infection.

⁵¹Cr release CTL assays. ND1 α -specific, H2-M3-restricted CTL clone (4E3; provided by K.F. Lindahl, Texas Southwestern Medical Center, Dallas, TX) was used as effector in CTL assays to determine the expression of H2-M3 on LPS blast from M3^{-/-} and WT mice. To measure LM-specific CTL activity, splenocytes from LM-infected mice (either 5 d after primary infection or 3 d after secondary infection) were cultured in RPMI 10 with 1 μ g/ml Con A. After 3 d, cells were harvested and used as effectors in a ⁵¹Cr

release CTL assay. LM-infected and noninfected J774 transfectant-expressing H2-K^b (J774-K^b; provided by J. Forman) were used as targets. In brief, monolayers of J774-K^b targets grown in antibiotic-free medium were infected with LM for 1 h at a multiplicity of infection of 5:1. Cells were washed three times with warm PBS and cultured in DMEM containing 40 µg/ml gentamicin for an additional 3 h. All target cells used in CTL assays were labeled with ⁵¹Cr for 1 h at 37°C. Target cells (10⁴) were added to round-bottom microtiter wells containing serial dilutions of effector cells. After 4 h of incubation at 37°C, 100 µl of supernatant from each well was collected to determine the amount of ⁵¹Cr release. The percent-specific lysis was calculated as 100 × (experimental cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm). Data presented are the mean of triplicate wells.

In vivo cytotoxicity assay. The in vivo cytotoxicity assay was performed as described by Barber et al. (46). In brief, splenocytes from B6 mice were labeled with red membrane dye PKH26 and either 5 µM, 500 nM, or 50 nM of cytosolic dye CFSE. The labeled cells were then pulsed with 1 µM LemA or OVA peptide for 4 h and transferred retro-orbitally (1:1:1 ratio, 5–10 million cells for each population) into the indicated groups of recipient mice. After 15 h of in vivo killing, lymphocytes were isolated from the spleens and livers and analyzed by flow cytometry for target cell clearance. Target cells were differentiated from recipient cells based on PKH26 staining and from each other based on different intensity of CFSE staining. Gated on PKH26⁺ cells, the percentage of specific killing was calculated as the following: 100 – [(% peptide pulsed in LM-infected/% unpulsed in LM-infected) / (% peptide pulsed in uninfected/% unpulsed in uninfected)] × 100.

Intracellular IFN-γ staining. Splenocytes or hepatic leukocytes from rLM-ova-infected mice were cultured in 96-well plates at a concentration of 10⁶ cells/well and stimulated with either 5 µM LemA or 1 µM OVA peptide for 5 h, or HKLM overnight. For the last 2 h of stimulation, 10 µM monensin was added to block the secretion of cytokines. Cells were washed and stained for cell surface markers CD8 and CD4. After fixation with 4% paraformaldehyde and permeabilization with 0.15% saponin, cells were then stained with PE-anti-IFN-γ antibody. Labeled cells were analyzed on a FACSCalibur.

NK cell cytotoxicity assays. Splenocytes and hepatic leukocytes from uninfected mice and mice infected for 3 d with LM were prepared and used as effectors. YAC-1, a mouse lymphoma cell line, was used as a target in this assay. Cells were harvested and labeled with 100 µCi of ⁵¹Cr for 1 h at 37°C. Target cells (10⁴) were added to round-bottom microtiter wells containing serial dilutions of effectors. After 4 h of incubation at 37°C, 100 µl of supernatant from each well was collected and assayed for ⁵¹Cr release. Percent specific lysis = (experimental cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm) × 100. Data presented are the means of triplicates.

Nitrite assay. 3 d after LM infection, hepatic leukocytes and splenocytes from M3^{-/-} and WT mice were isolated and stimulated with HKLM for 48 h at 4 × 10⁵–10⁶ cells/well in 96-well plates. Equivalent population of macrophages and DCs were assessed using PE-anti-F4/80 and PE-anti-CD11c by FACS analysis. Accumulated nitrite in the culture supernatant was measured by a colorimetric assay based on the Griess reaction with NaNO₂ as the standard (47).

Statistical analysis. Mean values were compared using the unpaired Student's *t* test. All statistical analyses were performed with the Prism program (GraphPad Software, Inc.). Statistically significant differences *P* < 0.05, *P* < 0.01, and *P* < 0.001 are noted with *, **, and ***, respectively.

Online supplemental material. Table S1 shows the expression of various activation markers on CD8⁺ T cells from M3^{-/-} and WT mice. Fig. S1 shows the effect of a neutralizing anti-IFN-γ mAb on M3-mediated early protection to LM infection. Table S1 and Fig. S1 are available at <http://www.jem.org/cgi/content/full/jem.20051866/DC1>.

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