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CUTTING EDGE

Cutting Edge: Protective Cell-Mediated Immunity to *Listeria monocytogenes* in the Absence of Myeloid Differentiation Factor 88[1]

Sing Sing Way, Tobias R. Kollmann,* Adeline M. Hajjar,† and Christopher B. Wilson[2]*†*

***In addition to their role in triggering innate immune responses, Toll-like receptors are proposed to play a key role in linking the innate and adaptive arms of the immune response. The majority of cellular responses downstream of Toll-like receptors are mediated through the adapter molecule myeloid differentiation factor 88 (MyD88), and mice with a targeted deletion of MyD88 are highly susceptible to bacterial infections, including primary infection with* Listeria monocytogenes *(LM). In contrast, herein we demonstrate that MyD88-deficient mice have only a modest impairment in their LM-specific CD4 T cell response, and no impairment in their CD8 T cell response following infection with ActA-deficient LM. Furthermore, CD8 T cells from immunized MyD88-deficient mice protected naive recipient mice following adoptive splenocyte transfer, and immunized MyD88-deficient mice were protected from infection with wild-type LM. These results indicate that adaptive immune responses can be generated and provide protective immunity in the absence of MyD88. The Journal of Immunology,* 2003, 171: 533–537.**

Toll-like receptors (TLRs)[3] recognize pathogen-associated molecular patterns (PAMPs) and transduce signals required for an effective innate immune response (1, 2). All TLRs identified to date and the cytokines IL-1 and IL-18, use the signaling molecule myeloid differentiation factor 88 (MyD88) to transduce activation signals leading to proinflammatory cytokine production. Although TLR3 and TLR4 can also use MyD88-independent pathways for cell activation, the response is delayed and cytokine production is less robust in the absence of MyD88 (3–5). Consequently, MyD88-deficient mice are more susceptible to primary infection with bacterial pathogens (6–8).

Despite major advances in the identification of PAMPs specific to each TLR and the TLR downstream signaling pathways that lead to cellular activation, the role of TLRs in the generation of adaptive immune responses following experimental infection remains largely undefined. Following immunization with either OVA or keyhole limpet hemocyanin in CFA (9, 10) or soluble *Toxoplasma gondii* extracts (11), which induce a robust Th1 response in control mice, lymphocytes from MyD88-deficient mice mount a preferential Th2 immune response, as demonstrated by the lack of IFN-γ production and by increased IL-4 and IL-13 production by Ag-stimulated CD4 T cells. However, to our knowledge, no studies have directly addressed the contribution of MyD88 in the development of CD4 T cell responses to an infectious pathogen or CD8 T cell responses to infection or immunization.

Listeria monocytogenes (LM) is a facultative intracellular Gram-positive bacterium responsible for enteric infections in immunocompetent humans and for severe systemic infections in immunocompromised individuals. The innate and adaptive immune responses to LM have been characterized in great detail (12). In infected cells, the bacterium escapes from the endocytic vacuole, gains access to the cell cytoplasm, and organizes host cell actin into unipolar tails that it uses to generate motile force facilitating intracellular and intercellular spread. In this manner, the bacterium is able to remain confined to the intracellular compartment of host cells, and thus evade humoral immunity. It has been demonstrated in experimental models of LM infection that CD8 T cells are the principal mediators of protective immunity, while CD4 T cells and Ab play only minor roles. In this report, we examine the requirement for MyD88 in the generation of adaptive immune responses following experimental LM infection.

Materials and Methods

Mice

MyD88-deficient mice (13) used in this study were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan) and backcrossed to C57BL/6 (H-2^b) mice for six generations or to C57B10.D2 (H-2^d) for five generations before use. Because LM class I immunodominant peptides have been described only in the murine H-2^d haplotype and class II immunodominant peptides only in the H-2^b haplotype, MyD88-deficient (*myd88*$^{-/-}$) and littermate control (*myd88*$^{+/-}$) mice used were F_1 (H-2^b × H-2^d) derived from matings between MyD88$^{-/-}$ (H-2^b) and MyD88$^{+/-}$ (H-2^d) mice. In preliminary experiments,

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; PAMP, pathogen-associated molecular pattern; MyD88, myeloid differentiation factor 88; LM, *Listeria monocytogenes*; wt, wild type; LLO, listeriolysin O.

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there was no difference in the LM-specific T cell responses in MyD88$^{+/-}$ compared with MyD88$^{+/+}$ mice (data not shown).

Infections with LM

Wild-type (wt) LM strain 10403s and the ActA-deficient strain DPL1942 were kindly provided by Drs. D. Portnoy (University of California, Berkeley, CA) and N. Freitag (Seattle Biomedical Research Institute, Seattle, WA). Strains of LM were grown to mid log phase (OD_{600} 0.1) at 37°C before infection. LM were diluted in 200 μl of saline and injected i.v. into mice. The number of LM in lysates of infected spleens and livers were determined as described (7). All experiments were performed under Institutional Animal Care and Use Committee approved protocols.

Adoptive transfer

RBC-lysed single cell splenocyte suspensions were obtained from either MyD88-deficient or control mice 7 days after infection with 10^6 ActA-deficient LM or saline, adjusted to 6.0×10^7 cells/200 μl, and injected i.v. into recipient mice. Where indicated, CD4 or CD8 T cell-depleted splenocytes were prepared by negative selection using the appropriate mAb conjugated to biotin (BD PharMingen, San Diego, CA) and streptavidin-coated Dynabeads (Dynal Biotech, Great Neck, NY). The efficacy of both CD4 and CD8 T cell depletion was at least 93% in each experiment, as determined by flow cytometry.

Enumeration of LM-specific T cells by intracellular cytokine staining

Intracellular cytokine staining was performed as described (14), using reagents from BD PharMingen or Caltag Laboratories (Burlingame, CA). Briefly, 2.0×10^7 splenocytes were incubated in the presence of the indicated LM-specific peptide (10^{-6} M) and GolgiStop for 5 h. Cells were stained with either PE-labeled anti-CD8 or anti-CD4 Abs, permeabilized with Cytoperm solution, and stained with FITC-conjugated anti-IFN-γ Ab. Stained cells were then analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences).

Cytokine production

The concentration of IFN-γ and IL-4 in the supernatants of splenocyte cultures were determined 72 h after peptide stimulation by ELISA using reagents from R&D Systems (Minneapolis, MN).

Statistics

The differences in geometric mean CFUs, the percentages and numbers of activated splenocytes, and cytokine concentrations were evaluated by using the Student *t* test with $p < 0.05$ taken as statistically significant.

Results

Although MyD88-deficient mice are susceptible to infection with wt LM, they clear high-dose infection with ActA-deficient LM

The increased susceptibility of MyD88-deficient mice to primary infection with wt LM has been recently described (7, 8), and our initial experiments confirmed these findings (data not shown). By contrast, MyD88-deficient mice were resistant to infection with a mutant strain of LM deficient in intracellular motility as a result of targeted deletion in the *actA* gene (15). Following i.v. infection of MyD88-deficient mice with 1×10^6 CFUs of ActA-deficient LM, no mortality was observed. Furthermore, examination of spleen and liver homogenates of MyD88-deficient mice 7 days after i.v. infection with this same dose of ActA-deficient LM revealed no recoverable bacteria (limits of detection 20 and 30 CFUs for spleen and liver, respectively). Thus, although MyD88-deficient mice are highly susceptible to infection with wt LM, they are resistant to and efficiently clear ActA-deficient LM.

Specific CD8 and CD4 T cells are generated in MyD88-deficient mice following infection with ActA-deficient LM

The relative resistance of MyD88-deficient mice to infection with ActA-deficient LM allowed us to examine adaptive immune responses to LM in the absence of MyD88. Previous studies have demonstrated no defects in MyD88-deficient mice in the total numbers of CD4 or CD8 T cells, and in their ability to produce IFN-γ or IL-4 after nonspecific activation (9). Therefore, we examined the generation of LM-specific CD8 and CD4 T cells in MyD88-deficient mice. Seven days after infection with 10^6 CFUs of ActA-deficient LM, we enumerated LM-specific CD8 and CD4 T cells in MyD88-deficient and control mice by intracellular IFN-γ staining of splenocytes stimulated with LM-specific MHC class I (listeriolysin O (LLO) 91–99, P60 217–225, P60 449–457, metalloprotease 84–92) or class II (LLO 189–201) -restricted peptides (Fig. 1). There was a modest (~50%) reduction in the percentage and total numbers of IFN-γ producing LLO 189–201 peptide-specific CD4 T cells in MyD88-deficient compared with control mice ($p \leq 0.015$). Surprisingly, MyD88-deficient and control mice generated similar numbers and percentages of LM-specific CD8 T cells.

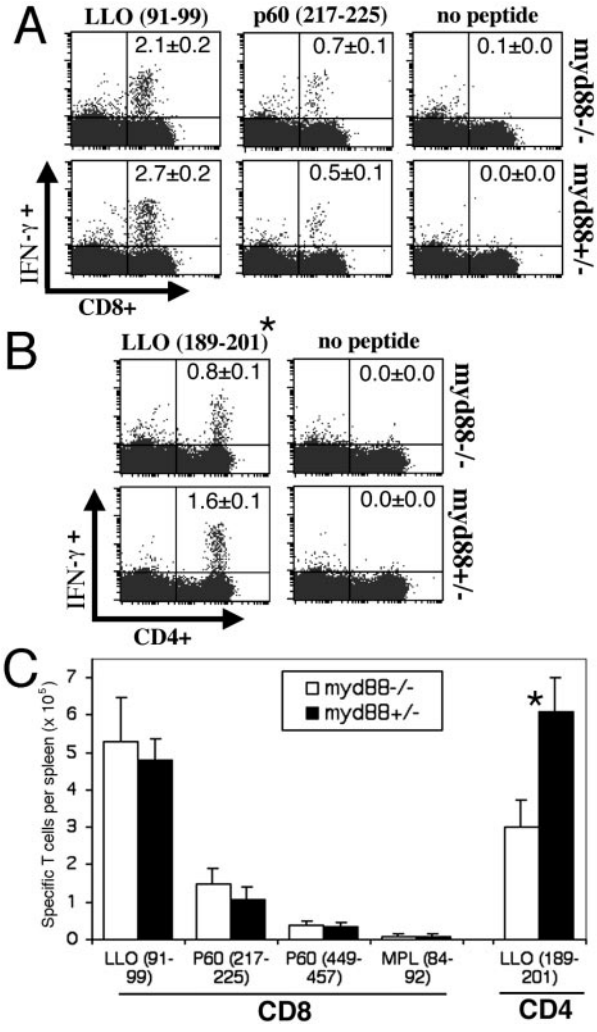

FIGURE 1. IFN-γ production by CD8 (*A*) and CD4 T cells (*B*) from MyD88-deficient (*myd88*$^{-/-}$) and control (*myd88*$^{+/-}$) mice following stimulation with the indicated LM-specific peptides or no peptide control. Cells were obtained from indicated mice 7 days after infection with ActA-deficient LM. The numbers in the *upper right quadrant* indicate the mean percentages (±SE) of IFN-γ-producing CD8 or CD4 T cells. *C*, Total numbers of IFN-γ-producing CD8 and CD4 T cells per mouse spleen following stimulation with MHC class I- or class II-restricted LM-specific peptides. These data represent seven mice per group from two combined experiments. Bar, SE; *, statistically significant difference.

Table I. *Cytokine production by LM-specific CD4 T cells*[a]

	myd88$^{-/-}$	*myd88*$^{+/-}$	*p* Value
IFN-γ, unstimulated cells	30.6 ± 30.6	91.2 ± 62.7	0.46
IFN-γ, stimulated cells	3085.6 ± 806.0	11589.4 ± 1427.6	<0.001
IL-4, unstimulated cells	n.d.	n.d.	n.a.
IL-4, stimulated cells	n.d.	n.d.	n.a.

[a] Concentrations of IFN-γ and IL-4 in culture supernatants of splenocytes from MyD88-deficient (*myd88*$^{-/-}$) and control (*myd88*$^{+/-}$) mice following stimulation with the LM class II peptide LLO 189–201. Splenocytes were isolated from these mice 7 days after ActA-deficient LM infection. Data represent the mean (picograms per milliliter) ± SE from seven mice per group from two independent experiments. n.d., not detectable (≤ 37 pg/ml); n.a., not applicable.

To determine whether the reduction in IFN-γ-producing LM-specific CD4 T cells in MyD88-deficient mice was associated with a reciprocal increase in Th2 cytokine production, the production of IL-4 and IFN-γ by splenocytes following stimulation with the class II-restricted LM-specific peptide LLO 189–201 was examined. In agreement with results from intracellular cytokine staining, splenocytes from MyD88-deficient mice produced ~30% as much IFN-γ compared with splenocytes from control mice (Table I). However, there was no associated increase in production of IL-4 by splenocytes from MyD88-deficient mice, which was below the limit of detection (Table I), even by real-time RT-PCR (data not shown). These data indicate that the modest reduction in IFN-γ production by CD4 T cells from MyD88-deficient mice was not associated with increased Th2 cytokine production.

LM-specific CD8 T cells generated in MyD88-deficient mice transfer protective immunity

To determine whether LM-specific T cells generated in MyD88-deficient mice could confer protective immunity, we compared the ability of whole splenocytes derived from either MyD88-deficient or control mice to transfer protection. Naive C57BL/6 mice receiving 6.0×10^7 splenocytes from either MyD88-deficient or control mice that had been immunized by infection with 10^6 ActA-deficient LM 7 days previously had dramatically decreased numbers of LM in their spleens and livers compared with mice receiving naive splenocytes or saline alone (Fig. 2*A*). No significant differences were observed in the numbers of LM in either the spleen or liver between mice receiving splenocytes from immunized MyD88-deficient or immunized control mice. Protection was predominantly mediated by CD8 T cells in splenocytes from immunized MyD88-deficient mice, as CD8, but not CD4, T cell depletion abolished the protective effects of splenocytes from immunized MyD88-deficient mice (Fig. 2*B*). Taken together, these data indicate that CD8 T cells generated in MyD88-deficient mice efficiently transfer protective immunity to LM infection.

Protective immunity in MyD88-deficient mice

To determine whether prior infection of MyD88-deficient mice with ActA-deficient LM could protect these mice from subsequent challenge with wt LM, 28 days after immunization MyD88-deficient and control mice were i.v. challenged with an inoculum of wt LM, 5×10^3 CFUs, that is lethal for naive MyD88-deficient mice. When assessed 3 days postchallenge, the numbers of LM in the spleens and livers of immunized MyD88-deficient mice were dramatically reduced compared with naive MyD88-deficient mice (Fig. 3). Furthermore, comparable numbers of bacteria were recovered from immunized

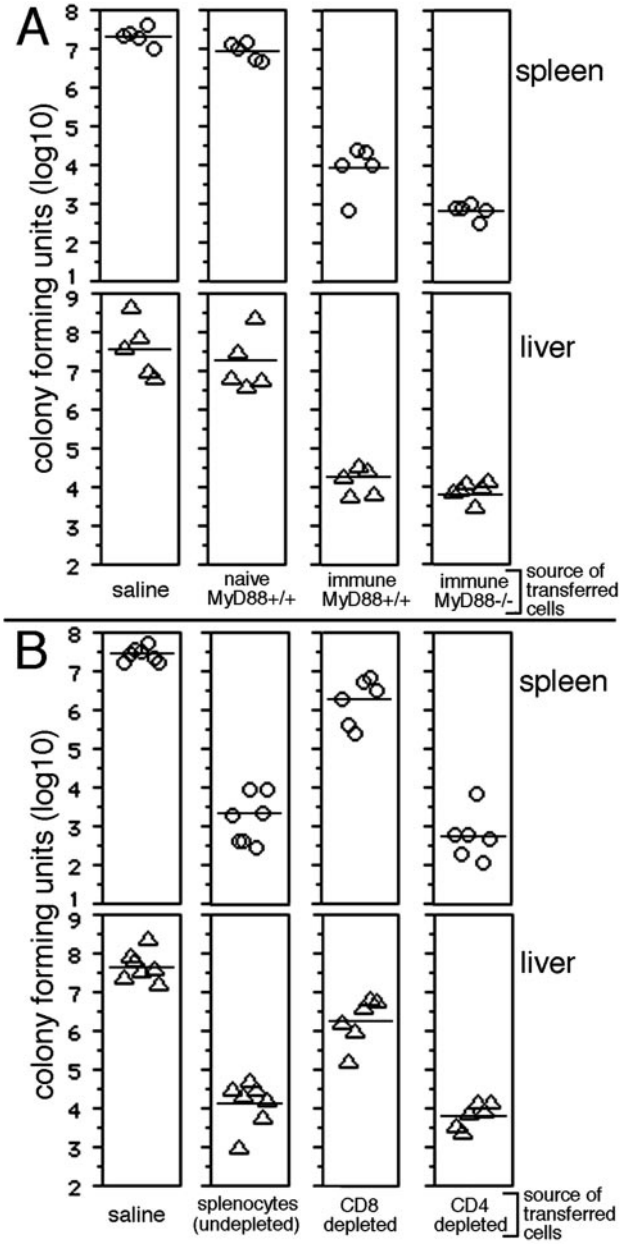

FIGURE 2. *A*, Splenocytes from either MyD88-deficient (*myd88*$^{-/-}$) or control (*myd88*$^{+/+}$) mice immunized with ActA-deficient LM transfer protection. *B*, CD8 T cells mediate the protective effects of splenocytes from immunized MyD88-deficient mice. Saline or 6.0×10^7 of the indicated splenocytes were injected into naive C57BL/6 mice 1 h before i.v. infection with 1.0×10^5 CFUs of wt LM 10403s. CFUs of LM in either the spleen (○) or the liver (△) of mice 3 days after infection were determined. The plot represents the data from five to seven mice per group combined from two separate experiments. Bar, geometric mean.

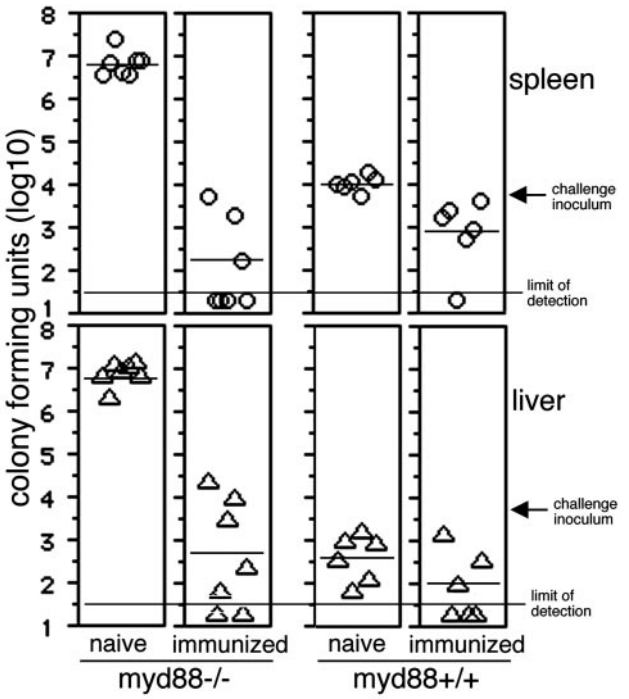

FIGURE 3. Immunization with ActA-deficient LM protects MyD88-deficient (*myd88*$^{-/-}$) and control (*myd88*$^{+/-}$) mice from infection with wt LM. CFUs of LM in either the spleen (○) or the liver (△) of MyD88-deficient or control mice 3 days after i.v. challenge with 5.0×10^3 CFUs of wt LM 10403s. The plot represents the data from six to seven mice per group combined from two separate experiments. Bar, geometric mean.

MyD88-deficient and immunized control mice. As expected, the numbers of bacteria were significantly increased in naive MyD88-deficient compared with naive control mice, due to the previously described defects in innate immunity for MyD88-deficient mice. Lastly, immunized MyD88-deficient mice were also protected from challenge with an inoculum of LM that is lethal for immunocompetent mice, 1×10^5 CFUs. By day 3 postchallenge, MyD88-deficient mice also cleared this higher inoculum efficiently; LM was undetectable in the spleens in three of five, and in the livers of two of five, immunized MyD88-deficient mice. In the remaining few mice in which LM could still be detected, a substantial reduction from the challenge inoculum was observed in both the spleen (geometric mean CFU ± SE, 2.1 ± 0.4) and the liver (2.7 ± 0.6), indicating bacterial clearance. By contrast, bacterial replication occurred in naive control mice (10^7–10^8 CFUs in both the spleen and liver).

Discussion

In this study, we have demonstrated that LM-specific, IFN-γ-producing CD8 and CD4 T cells are generated in response to infection with the intracellular bacterium LM in the absence of MyD88. Although there was a modest reduction in the LM-specific Th1 CD4 T cell response in MyD88-deficient mice, the CD8 T cell response was not affected. The LM-specific CD8 T cells generated in MyD88-deficient mice conferred protective immunity to subsequent lethal challenge upon transfer into naive immunocompetent mice. Furthermore, immunization of MyD88-deficient mice conferred protective immunity to challenge with wt LM. Thus, the MyD88 pathway is not required either for the generation or for the effector phase of adaptive cell-mediated immune responses to LM infection.

Regarding the linkage between innate and Ag-specific T cell responses, these results suggest either that 1) TLRs that recognize PAMPs from LM can signal through MyD88-independent pathways, or 2) Ag-specific cellular immunity can develop without TLR-mediated signaling. The two TLRs that have been demonstrated to recognize PAMPs from LM in vitro are TLR2 for heat-killed LM (16) and TLR5 for LM flagellin (17). It is also likely that CpG DNA from LM can activate cells via TLR9 (18). To date, there have been no studies describing MyD88-independent cellular activation through TLR2, TLR5, or TLR9. Furthermore, the increased susceptibility of MyD88-deficient mice to primary infection with LM implies that if TLRs recognize PAMPs on LM and signal through MyD88-independent pathways, these pathways function minimally in innate immunity to LM infection.

Not only did we find that LM-specific cellular immune responses were generated in the absence of MyD88, but we also found that these immune responses were protective. Following immunization of MyD88-deficient mice with ActA-deficient LM, these mice were resistant to infection with wt LM challenge, and splenocytes from these mice transferred protective immunity to naive mice. This is in contrast to other published studies addressing the protective effects of Ag-specific immune responses generated in the absence of specific TLRs. Following infection with *Borrelia burgdorferi* in TLR2- deficient mice, spirochetes persisted at elevated levels despite normal production of specific Ab (19). These findings suggest that the cellular mediators of adaptive immunity that work in concert with Ab for *Borrelia* clearance also require TLR2.

LM-specific Th1 CD4 T cells were generated in MyD88-deficient mice, although the percentage and total number of IFN-γ-producing CD4 cells was reduced ~50% compared with control mice. This decrease did not reflect a compensatory increase in Th2 compared with Th1 responses in MyD88-deficient mice, because LM peptide-stimulated CD4 T cells neither from MyD88-deficient mice nor from control mice produced IL-4. These results contrast with the lack of IFN-γ, and increased IL-4 production observed for lymphoid cells in MyD88-deficient mice following immunization with either OVA or keyhole limpet hemocyanin in CFA (9, 10), or *T. gondii* extracts (11). Thus, live-attenuated LM infection induced Th1 differentiation of T cells through MyD88-independent pathways not shared by immunization with these other Ags. Whether this is unique to LM, to other cytoplasmic intracellular pathogens, or is a more general property of infection vs immunization is uncertain. Perhaps this is the same pathway that accounts for MyD88-independent production of IL-18 observed following LM infection in mice (20), and production of IFN-β in macrophages when LM gains access to the cytoplasm (21, 22). It is tantalizing to hypothesize that the key difference in induction of specific immunity between LM infection and the administration of soluble Ag lies in the ability of LM to gain access to the intracytoplasmic cellular compartment, and thereby activate classes of Ag sensing molecules unique to this compartment.

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