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MyD88 Intrinsically Regulates CD4 T-Cell Responses

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Myeloid differentiation factor 88 (MyD88) is an essential adaptor protein in the Toll-like receptor-mediated innate signaling pathway, as well as in interleukin-1 receptor (IL-1R) and IL-18R signaling. The importance of MyD88 in the regulation of innate immunity to viral pathogens and neuropathogenesis is not entirely clear. In the present study, we examine the role of MyD88 in the CD4+ T-cell response following lymphocytic choriomeningitis virus (LCMV) infection. We demonstrate that wild-type (WT) mice developed a CD4+ T-cell-mediated wasting disease after intracranial infection with LCMV. In contrast, MyD88 knockout (KO) mice did not develop wasting disease in response to the same infection. This effect was not the result of MyD88 regulation of IL-1 or IL-18 responses since IL-1RI KO and IL-18R KO mice were not protected from weight loss. In the absence of MyD88, naive CD4+ T cells failed to differentiate to LCMV-specific CD4+ T cells. We demonstrated that MyD88 KO antigen-presenting cells are capable of activating WT CD4+ T cells. Importantly, when MyD88 KO CD4+ T cells were reconstituted with an MyD88-expressing lentivirus, the rescued CD4+ T cells were able to respond to LCMV infection and support IgG2a antibody production. Overall, these studies reveal a previously unknown role of MyD88-dependent signaling in CD4+ T cells in the regulation of the virus-specific CD4+ T-cell response and in viral infection-induced immunopathology in the central nervous system.

Myeloid differentiation factor (MyD88) is an essential adaptor molecule in all Toll-like receptor (TLR) signaling pathways except TLR3. In addition, MyD88 is also important for interleukin-1 receptor (IL-1R)/IL18R-mediated signaling. Many studies have demonstrated the importance of MyD88-dependent signaling in the regulation of innate as well as acquired immunity, in particular, T-cell responses, to various microbial pathogens (1, 2, 37, 39). Although it is not entirely clear how innate MyD88-dependent signaling regulates the activation of T-cell responses, it has been suggested that MyD88 expression in antigen-presenting cells (APCs), including dendritic cells (DCs), plays a key role in the activation of T-cell responses (2, 15, 27, 39). Additionally, very recent studies have revealed an important role of MyD88 expression in T cells in regulating T-cell activation and pathogenesis in response to model antigens or parasites (11, 26). However, it is not known whether MyD88 expression in T cells plays a similar role in the activation of T cells and regulation of pathogenesis in response to a virus.

Lymphocytic choriomeningitis virus (LCMV) is a noncytolytic virus, and most of the diseases associated with LCMV infection in mice are mediated by either innate or acquired immune responses. It has been well demonstrated that both CD8+ and CD4+ T cells play a role in LCMV-associated diseases, i.e., CD8+ T-cell-mediated meningitis (3, 8) or CD4+ T-cell-mediated weight loss (14, 21). In the absence of both CD8+ and CD4+ T cells, mice do not develop symptoms following intracranial LCMV infection (29, 51). Thus, LCMV is a suitable model to evaluate the contribution of a variety of signaling molecules in the activation of CD4+ as well as CD8+ T-cell responses to virus infection and viral pathogenesis (3, 8).

Studies from our group and others have shown that MyD88 is critical for the induction of the LCMV-specific CD8+ T-cell response (18, 40, 49). Moreover, we have also shown that the defective CD8+ T-cell response to LCMV infection in MyD88 knockout (KO) mice is not due to a total failure of the APC system because adoptively transferred P14 T-cell receptor (TCR) transgenic CD8+ T cells (expressing a TCR transgene specific for the LCMV glycoprotein consisting of residues 33 to 41 [GP33-41] epitope) expand and function comparably in both MyD88 KO and wild-type (WT) mice (49). In the present study, we used LCMV infection as a model to test whether the MyD88 signaling pathway plays a role in the activation of CD4+ T cells in response to a natural viral pathogen. Surprisingly, we found that the MyD88 signaling pathway functions within the CD4+ T cells themselves and is essential for normal CD4+ T-cell function, and we further demonstrated that this MyD88 signaling is independent of IL-1R- and IL-18R-mediated signaling.

MATERIALS AND METHODS

Reagents. The Armstrong strain of LCMV was kindly provided by Lisa K. Selin (University of Massachusetts Medical School, Worcester, MA) and was propagated on BHK-21 cells (ATCC) at a low multiplicity of infection (MOI) 0.01. Viral titers were determined with an immunological focus assay (4). Rat anti-LCMV nucleoprotein antibody was kindly provided by Demetrios Moskophidis (Medical College of Georgia, Augusta, GA) (4). The DC line DC2.4 was kindly provided by Kenneth L. Rock (University of Massachusetts Medical School, Worcester, MA). The CD8a T-cell depletion antibody, clone 2.43 hybridoma (rat immunoglobulin G1 [IgG1]) (41), was obtained from ATCC. CD4+ CD25+ regulatory T (Treg) cell-depletion antibody, anti-mouse CD25 hybrid-
oma (clone PC61; rat IgG1 isotype), was obtained from ATCC. Culture supernatants and purified using protein G columns. To deplete Treg cells, 5 × 10^5 purified LCMV-immune (day 10 postinfection) CD4^+ T cells were cocultured with 5 × 10^5 cDCs. Both WT and MyD88 KO cells were subcutaneously injected with 2.5 × 10^5 B16-F10L melanoma cells in 200 μl of PBS to increase the frequencies of cDCs. On day 10 after infection, spleens were collected and CD11c^+ DCs were enriched using anti-CD11c magnetic beads (Miltenyi Biotec). Enriched CD11c^+ DCs, together with a control DC line, DC2.4, were infected with LCMV Armstrong (5 × 10^5 PFU) diluted with phosphate-buffered saline (PBS) and incubated for 5 days. To weight loss in intracellular infection, as follows: [weight after infection – weight before infection]/weight before infection] × 100. For intravenous infection, mice were infected with 200 μl of LCMV Armstrong (5 × 10^5 PFU) diluted with minimal essential medium–2% fetal calf serum (FCS). Animals were housed and experiments were performed in accordance with animal welfare guidelines.

**Viral peptide.** The LCMV-specific CD4^+ T-cell epitope peptide used in this study was mapped to MHC class II–restricted Gp^34-50 (34). The peptide was synthesized by the Tufts University peptide core facility and purified by high-performance liquid chromatography.

**Quantification of CD4^+ T-cell response by intracellular staining for IFN-γ and tumor necrosis factor alpha (TNF-α).** Methods used to isolate mononuclear cells (MNC) from the brain have been described previously (50). Briefly, mice were perfused through the right ventricle with 10 ml of PBS prior to tissue removal. Brain tissues were washed through a 70-μm pore-size strainer in RPMI medium with 10% FCS. The resulting cell suspensions were centrifuged, and the pellet was resuspended in a 38% Percoll solution (Pharmacia), layered on a 68% Percoll solution, and centrifuged at 400 × g for 20 min at 4°C. MNC were recovered from the gradient interface and washed twice with complete RPMI medium before use.

**Viral load.** The production of infectious particles (uDCs) (uDCs) CD11c^+ DCs, both WT and MyD88 KO mice were backcrossed with C57BL/6 mice at least six generations. The genotypes of the mice were determined by PCR of tail DNA. Mice were breed and maintained under specific-pathogen-free conditions. Age-matched C57BL/6 mice (WT control mice), gamma interferon (IFN-γ) KO mice, CD8^+ T-cell-deficient mice, IL-1R1 KO mice, IL-18 KO mice, and TCR-β/δ-deficient mice were purchased from the Jackson Laboratories (Bar Harbor, ME). All mice used were 6 to 10 weeks old.

In order to determine whether the TLR-MyD88 pathway plays a role in CD4^+ T-cell-mediated central nervous system immunopathology, CD8^+ T cells were depleted before intracranial infection with LCMV by intraperitoneal injection with a rat monoclonal antibody against mouse Lyt2. antibody 2.43 (ATCC) (17, 21, 41), on day −5, −2, 0, +2, and +5 relative to intracranial LCMV infection and then at weekly intervals. The efficacy of CD8 T-cell depletion was confirmed by flow cytometry using the anti-CD8 antibody (BD Pharmingen). For intracranial infection, mice were lightly anesthetized with isoflurane and injected with 30 μl of LCMV Armstrong (200 PFU) diluted with phosphate-buffered saline (PBS) and incubated for 5 days. To weight loss in intracellular infection, as follows: [weight after infection – weight before infection]/weight before infection] × 100. For intravenous infection, mice were infected with 200 μl of LCMV Armstrong (5 × 10^5 PFU) diluted with minimal essential medium–2% fetal calf serum (FCS). Animals were housed and experiments were performed in accordance with animal welfare guidelines.

**Constitution of mouse MyD88 lentivirus.** The gene coding for mouse MyD88 was amplified by using reverse transcription-PCR. Total RNA was prepared from normal C57BL/6 mouse splenocytes using a Qiagen RNeasy Mini kit. The primers were designed according to the sequence deposited in the GenBank database (accession no. NM_010851): forward primer, 5′-AGACACCGGTTGCGCAGGGAGAC-3′ and reverse primer, 5′-ATTGCCCTTGGCTCTAGAGGGTCATCTGTAGGGCAGGG-3′. The expression of Thy1.1 was confirmed by restriction analysis and DNA sequencing (Tufts University core facility). Expression of MyD88 is under the control of ubiquitin-C promoter. Additional plasmids necessary for generating lentivirus including cytomegalovirus-Eco (rodent specific) and Rous sarcoma virus-Rev were the gift of Z. Chen. All plasmids were extracted using a Qiagen Hi-Speed Maxi kit.

**Production and quantification of MyD88 lentivirus.** Protocols provided by Chen and also described by Dull et al. (9) were followed, with some modifications. Briefly, 5 × 10^6 of the GP2-293 packaging cells (BD) were plated into 10-cm petri dishes. GP2-293 is an HEK-293-based packaging cell line that stably expresses the viral gag and pol genes. When cells were 90% to 95% confluent, they were transfected using a calcium chloride transfection protocol. The following amounts of plasmids were used per dish: 20 μg of pU6-MyD88/Thy1.1, or control pU6-EGFP/Thy1.1 and 10 μg of both Rous sarcoma virus-Rev and cytomegalovirus-Eco. At 4 h after transfection, medium was removed, and cells were washed with PBS, and 10 μl of Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum was added. At 36 to 48 h after transfection, culture supernatants were collected and spun at low speed (2,000 rpm for 10 min). The titers of the MyD88 lentivirus and control lentivirus were determined by transducing NIH 3T3 fibroblasts seeded in a 12-well plate (5 × 10^4 cells per well) with serially diluted lentivirus-containing supernatants supplemented with 6.0 μg/ml polybrene (Sigma). Thy1.1 was used as a marker to determine the concentration of the lentivirus. After incubation for 48 h, the expression of Thy1.1 was analyzed by FACs staining with anti-Thy1 antibody (BD). The concentration of lentivirus was calculated on the basis of the number of Thy1.1-positive cells and expressed as infectious virus particle (titer is defined as the number of infectious particles/ml).
RESULTS

LCMV-induced weight loss is MyD88 dependent but is IL-1R1/IL-18R independent. Intracranial infection of immuno-compotent mice with LCMV leads to fatal CD8+ T-cell-mediated LCM while intracranial infection of CD8+ T-cell-deficient mice with LCMV causes wasting disease, characterized by CD4+ T cell-mediated weight loss (7, 10, 14, 29). Our previous studies have demonstrated that CD8+ T cells are abnormal in MyD88 KO mice (49). Our initial experiments showed that intracranial LCMV infection in MyD88 KO mice induced a delayed lethal meningitis compared to WT mice (data not shown), suggesting that, although impaired, the CD8+ T-cell response could still be detrimental to the host in an intracranial infection model. In the present study, we eliminated CD8+ T cells and then determined the role of the MyD88 adaptor molecule in the CD4+ T-cell response to LCMV infection. Interestingly, after depletion of CD8+ T cells and intracranial infection with LCMV, WT mice started losing weight at about day 7 post-infection, and at peak they lost up to 30% of their body weight (Fig. 1A). In contrast, CD8+ T-cell-depleted MyD88 KO mice did not show any symptoms of disease.

We used CD8α+ T-cell-deficient mice to validate our observations. These mice lack CD8+ T cells but have normal CD4+ T cells. After intracranial infection with LCMV, CD8α+ T-cell KO mice steadily lost weight (Fig. 1A), which is consistent with previous findings that CD4+ T cells are responsible for weight loss (10). Taken together, these studies suggested that MyD88 is involved in LCMV-induced CD4+ T-cell responses and the CD4+ T-cell-dependent wasting disease.

MyD88 is an essential adaptor molecule for several receptors including all TLRs (except TLR3) and both the IL-1R and the IL-18R-mediated signaling pathways (1, 44). To determine whether IL-1R and IL-18R rather than TLRs were responsible for MyD88-dependent wasting disease, IL-1R1 KO and IL-18R KO mice were depleted of CD8+ T cells and infected intracranially with LCMV. CD8+ T-cell-depleted IL-1R1 KO and IL-18R KO mice exhibited weight loss compared to CD8+ T-cell-depleted MyD88 KO mice (Fig. 1B). Thus, these studies demonstrated that neither IL-1R1 nor IL-18R alone played a major role in LCMV-induced MyD88-dependent wasting disease.

Intracranial LCMV infection induced severe inflammation in the brains of WT and IL-1R1 KO mice but not in MyD88 KO mice. To determine whether the observed clinical symp-
gene were related to inflammation in the brain, histopathologic analysis was performed. After depletion of CD8+ T cells and intracranial infection with LCMV, the brains of WT and IL-1R1 KO mice demonstrated a prominent infiltration of MNC compared to the brains of uninfected WT mice. The severity of the meningitis was assessed based on the accumulation of the inflammatory cells in the meninges of the brains (two arrows in each image point to the thickness of the meninges, representing the amount of the accumulated inflammatory cells). Representative sections of cerebral cortex, with a focus on meninges/meningitis, are shown. The relative severity of the meningitis was as follows: uninfected WT mice (−), LCMV-infected WT mice (++), IL-1R1 KO mice (++), and MyD88 KO mice (+).

FIG. 2. LCMV-induced brain histological change is MyD88 dependent but IL-1R1 or IL-18R independent. Mice were depleted of CD8+ T cells with monoclonal antibody 2.43 and were intracranially infected with LCMV Armstrong as described in Materials and Methods. Two mice from each of WT, MyD88 KO, and IL-1R1 KO groups were sacrificed on day 12 postinfection; brains were sectioned and stained with hematoxylin and eosin. WT mouse brain injected intracranially with PBS was a negative control. The severity of the meningitis was assessed based on the accumulation of the inflammatory cells in the meninges of the brains (two arrows in each image point to the thickness of the meninges, representing the amount of the accumulated inflammatory cells). Representative sections of cerebral cortex, with a focus on meninges/meningitis, are shown. The relative severity of the meningitis was as follows: uninfected WT mice (−), LCMV-infected WT mice (++), IL-1R1 KO mice (++), and MyD88 KO mice (+).

Wild Type Uninfected

Wild Type + LCMV

MyD88 KO + LCMV

IL-1R1 KO + LCMV

CD4+ T-cell activation in response to intracranial LCMV infection is MyD88 dependent but IL-1R1- or IL-18R independent. It has been demonstrated that the intracranially LCMV-induced wasting disease is mediated by LCMV-specific CD4+ T cells that infiltrate the central nervous system (7, 14, 21). LCMV-specific CD4+ T cells express both IFN-γ and TNF-α (17, 36), which we used as functional indicators to assess the CD4+ T-cell response. To determine whether the functionality of CD4+ T cells correlates with the clinical symptoms (Fig. 1A and B) and the pathological changes in the brain (Fig. 2), WT, MyD88 KO, IL-1R1 KO, and IL-18R KO mice were depleted of CD8+ T cells and infected intracranially with LCMV. The state of CD4+ T-cell activation in the brain as well as in the spleen and peripheral blood was determined by intracellular staining of IFN-γ and FACS analysis after restimulation in vitro with the LCMV-specific dominant CD4 epitope peptide, GP61-80 (5, 46). In MyD88 KO mice, the LCMV-specific CD4+ T-cell IFN-γ response in all the tissues tested was severely impaired compared to WT mice (Fig. 3A to D). In contrast, LCMV infection induced comparable CD4+ T-cell IFN-γ and TNF-α responses in WT, IL-1R1 KO, and IL-18R KO mice (Fig. 3A to D and data not shown). Taken together, these results correlated with the clinical symptoms and demonstrated that an IL-1R/IL-18R-independent but MyD88-dependent signaling pathway is essential for activation of the CD4+ T cells in response to LCMV infection.

To determine if the MyD88 molecule is involved in the development of LCMV-specific CD4+ T-cell responses, LCMV-specific CD4+ T cells were directly visualized using LCMV-specific MHC class II-restricted tetramer reagent (NIH Tetramer Core Facility). LCMV infection in WT mice induced a robust CD4+ T-cell response (Fig. 3E and F). Surprisingly, LCMV infection in MyD88 KO mice did not induce LCMV-specific CD4+ T-cell responses. Therefore, these results demonstrated that the MyD88 protein is essential for the development of LCMV-specific CD4+ T-cell responses.

MyD88 KO mice have normal APC function in response to LCMV infection. After being engaged by antigen-presenting cells (APCs), naïve CD4+ T cells undergo clonal expansion...
and then differentiate into functional subsets of either Th1 or Th2 cells (15, 42). Several studies have demonstrated that the MyD88 adaptor protein is involved in the activation of APCs (15). To determine how MyD88 is involved in the regulation of CD4+ T-cell responses to LCMV, the following experiments were conducted. First, we wanted to determine whether the impaired CD4+ T-cell response in MyD88 KO mice to LCMV infection is due to the failure of the APC system. Purified naive

FIG. 3. LCMV-induced CD4+ T-cell activation is MyD88 dependent but IL-1R1 and IL-18R independent. WT (n = 9), MyD88 KO (n = 9), IL-1R1 KO (n = 6), and IL-18R KO (n = 5) mice were intracranially infected with LCMV as described in Materials and Methods. Between days 13 to 15 postinfection, CD4+ T-cell responses in the brain, spleen, and peripheral blood lymphocytes were examined by restimulation in vitro with LCMV CD4 T-cell epitope peptide GP61–80 and intracellular staining for IFN-γ (A to C) or TNF-α (D). The average percentages of the total CD4+ T cells and CD4+ T cells expressing IFN-γ (CD4+ IFN-γ+ T cells) in brain, spleen, and peripheral blood lymphocytes are shown in panels A to C. Results are representative of at least four experiments for WT and MyD88 KO mice and two experiments for IL-1R1 and IL-18R KO mice. (E and F) WT (n = 3) and MyD88 KO mice (n = 3) were intracranially infected with LCMV. At day 9 postinfection, spleen and brain were collected. LCMV-specific CD4 T cells in spleen and brain were directly visualized using I-Aβ-restricted CD4 tetramer complexed with LCMV GP66–77 peptide or a control peptide. Results shown are the average from three mice per group. *, P < 0.05.
B6/Thy1.1 CD4+ T cells were transferred into CD8− T-cell-depleted WT and MyD88 KO recipients (n = 3 for each group), followed by intravenous infection with LCMV. At day 10 postinfection, CD4+ T-cell responses (IFN-γ expression) in the spleens were analyzed by ICS after restimulation with LCMV CD4 epitope peptide GP61–80 or phorbol myristate acetate (PMA) and ionomycin. Cells were gated on CD4+ Thy1.1+ T cells (donor origin for both MyD88 KO and WT recipients). (B) LCMV-infected CD11c+ cDCs or DC2.4 cells were cocultured with purified LCMV-immune (day 10 postinfection) CD4+ T cells. The expression of IFN-γ was analyzed using ICS. (C and D) A total of 5 × 10^6 purified naive WT or MyD88 KO CD4+ T cells were adoptively transferred into TCRβ−/δ− KO mice, followed by intracranial LCMV infection. At day 20 postinfection, the function of CD4+ T cells in spleen was examined by restimulating CD4+ T cells with LCMV GP61–80. Peptide for 5 h (C) or with immobilized anti-CD3 antibody for 72 h (D). The expression of IFN-γ was measured by intracellular IFN-γ staining. Results shown are the average from three mice per group. (E) Both MyD88 KO and WT mice were depleted of CD8+ T cells with monoclonal antibody 2.43 as described above. CD4+ CD25+ regulatory T cells in MyD88 KO mice were depleted with PC61 antibody or an equivalent amount of a rat IgG isotype antibody. Mice were intracranially infected with LCMV Armstrong as described above. Body weight loss was compared for up to 9 days postinfection. (F) CD8-depleted mice failed to clear LCMV Armstrong infection. Mice were depleted of CD8+ T cells as described in Material and Methods and intracranially infected with LCMV Armstrong. At day 30 postinfection, virus titers in spleens and brains were determined. Results shown are the average from three to five mice per group.
using both in vivo and ex vivo methods, we demonstrated that APCs in MyD88 KO mice are comparable to APCs from WT mice in their ability to activate WT CD4+ T cells. Thus, these studies demonstrated that the absence of MyD88 signaling in APCs does not account for the impaired CD4+ T-cell function in MyD88 KO mice in response to LCMV infection. These results suggested that MyD88 might be intrinsically involved in CD4+ T-cell functional maturation.

Expression of MyD88 in CD4+ T cells is critical to their functional maturation and to inducing weight loss. To determine how MyD88 is involved in the regulation of CD4+ T cells in response to LCMV, naïve CD4+ T cells were purified from both MyD88 KO and WT mice. A total of 5 x 10^6 purified naïve CD4+ T cells from both strains were adoptively transferred into TCR-β/δ KO recipients (deficient of both CD4+ and CD8+ T cells) via tail vein injection (Fig. 4C). The following day, TCR-β/δ KO recipients were challenged by intracranial LCMV infection. Consistent with results from the LCMV-infected WT mice (Fig. 1A and B), CD4+ T cells from WT naïve mice mediated weight loss (Fig. 1C). The function of transferred CD4+ T cells was examined by ICS. WT CD4+ T cells had normal function; i.e., they responded to stimulation with LCMV GP61-80 peptide by producing IFN-γ (Fig. 4C). In contrast, MyD88 KO CD4+ T cells failed to produce IFN-γ in response to LCMV GP61-80 peptide restimulation. Both transferred WT and MyD88 KO CD4+ T cells responded to anti-CD3 stimulation (Fig. 4D), suggesting that MyD88 KO CD4+ T cells were capable of IFN-γ expression when their TCR was cross-linked ex vivo although these cells failed to respond to LCMV infection in vivo. Thus, we conclude that the incompetent CD4+ T-cell response in MyD88 KO mice to LCMV is not due to the defective antigen presentation capacity of MyD88 KO APCs. Their defect is the inability to produce IFN-γ and TNF-α in response to LCMV.

In addition, it has been reported that CD4+ CD25+ Treg cells play a role in the regulation of MyD88-dependent CD4+ T-cell activation to model antigens (38, 39). To further determine whether Treg cells are responsible for the defective CD4+ T-cell response in MyD88 KO mice following acute LCMV infection, Treg cells in MyD88 KO mice were depleted before LCMV infection with the commonly used monoclonal antibody PC61 (12, 38, 39, 43). Treg-cell depletion was confirmed by flow cytometry. The percentage of CD25+ CD4+ T cells significantly decreased compared to their counterpart in the isotype control antibody-treated MyD88 KO mice (0.76% ± 0.03% versus 8.19% ± 1.27%, respectively). Depletion of CD4+ CD25+ Treg cells did not induce the wasting disease in MyD88 KO mice following intracranial LCMV infection (Fig. 4E), suggesting that Treg cells do not play a decisive role in the defective CD4+ T-cell response in MyD88 KO mice.

Together, these observations suggested that the MyD88 signaling pathway is intrinsically involved in the functional maturation of CD4+ T cells and independent of Treg cells.

Expression of MyD88 in MyD88 KO CD4+ T cells restores CD4+ T-cell function. Having shown that MyD88 could be intrinsically involved in the responsiveness of CD4+ T cells, we next determined if reconstitution of MyD88 KO CD4+ T cells with the MyD88 gene could restore function. We constructed an MyD88-expressing lentivirus, pUb-MyD88/Thy1.1 (Fig. 5A). MyD88 and control lentivirus efficiently transduced both NIH 3T3 cells and MyD88 KO CD4+ T cells (Fig. 5B and C). When MyD88 KO CD4+ T cells were transduced with the MyD88-expressing lentivirus to rescue MyD88 activity, the CD4+ T cells were able to respond to LCMV infection by expression of IFN-γ (Fig. 5D). In contrast, when MyD88 KO CD4+ T cells were transduced with a control lentivirus, MyD88 KO CD4+ T cells remained unable to respond to LCMV infection (Fig. 5D). Furthermore, when TCR-β/δ KO recipients received MyD88-reconstituted (lentiviral rescue) MyD88 KO CD4+ T cells, they produced significantly more IgG2a anti-LCMV antibody (Fig. 5E) than TCR-β/δ KO recipients that received control lentivirus-transduced KO CD4+ T cells in response to LCMV. Therefore, lentiviral gene transfer of mouse MyD88 into MyD88 KO CD4+ T cells was sufficient to restore function for both IFN-γ production and T helper function for the antibody response, demonstrating the critical role of MyD88 in T cells in the response to a viral infection.

**DISCUSSION**

A number of recent studies have highlighted the importance of MyD88 in the regulation of the inflammatory responses in innate immune cells, but its role in the regulation of the adaptive immune response and immunopathology is poorly defined (6, 23, 24). How do MyD88-dependent signals affect T-cell responses? The current paradigm for explaining the regulation of T cells by MyD88 is that TLR-dependent activation of MyD88 in DCs leads to their maturation as APCs with the secretion of immune-modulated chemokines and cytokines and upregulation of the MHC and other costimulatory molecules and, in turn, plays a central role in stimulation of the T-cell responses (15, 16). Our present study reveals an unappreciated role of MyD88 signaling in the regulation of the CD4+ T-cell response to virus infection. We demonstrate that the MyD88 molecule expressed in the CD4+ T cells themselves controls the development of virus-specific CD4+ T cells following challenge with a natural murine viral pathogen, LCMV.

The MyD88 adaptor protein is not only essential for TLR signaling (with the exception of TLR3) but is also a critical adaptor protein in IL-1R- and IL-18R-dependent signaling (1, 2, 44). By directly comparing the impact of the MyD88-, IL-1R-, and IL-18R-mediated signaling pathways on the activation of a virus-specific CD4+ T-cell response, we demonstrated that the defect is likely TLR specific since the IL-1R- and IL-18R-dependent signaling pathways do not play a major role in the activation and differentiation of CD4+ T cells to LCMV infection. A recent publication also indicated that the IL-18-dependent signaling pathway is not involved in the LCMV-specific CD4+ T-cell response (30). Thus, our study outlines the importance of the MyD88 signaling pathway in the regulation of CD4+ T-cell responses to a natural murine viral pathogen. Our studies are consistent with a recent publication by Larosa et al. (26), in which the authors demonstrated that the expression of MyD88 in CD4+ T cells is essential for the production of IFN-γ in CD4+ T cells and for the protection of mice from Toxoplasma gondii infection.

Although MyD88 is critically involved in the induction of chemokines and cytokines from APCs or other cells (2, 15, 37,
44, 49), our study has demonstrated that naïve WT CD4 T cells have comparable function when transferred into MyD88 KO recipients compared to those in WT recipients (Fig. 4A). Furthermore, our ex vivo study has shown that MyD88 KO APCs (cDCs) are comparable to WT APCs in their ability to present LCMV-specific CD4 epitopes to activate LCMV-immune WT CD4 T cells. Collectively, these results suggest that the defective CD4 T-cell responses to LCMV infection in MyD88 KO mice are not due to a failure of the APC system (Fig. 4A to C).

Our adoptive transfer experiments with purified CD4 T cells indicate that the defective CD4 T-cell response is related to an intrinsic defect in CD4 T cells. When WT or MyD88 KO naïve CD4 T cells were transferred into TCR-β/δ KO recipients, only WT CD4 T cells had the capacity to mediate weight loss and to express IFN-γ (Fig. 1C, 4D). Furthermore, TCR-β/δ KO recipients that received WT CD4 T cells produced significantly more IgG2a isotype anti-LCMV antibody, a characteristic of a Th1-type immune response, in comparison to TCR-β/δ KO recipients receiving MyD88 KO CD4 T cells. Importantly, we provided evidence that the function of MyD88 KO CD4 T cells can be restored by...
transducing MyD88 KO CD4+ T cells with an MyD88-expressing lentivirus (Fig. 5). Therefore, our studies demonstrate that MyD88 in T cells is responsible for activation of CD4+ T cells in response to LCMV infection.

How MyD88 in CD4+ T cells regulates virus-specific CD4+ T-cell responses is currently not clear. TLRs are expressed in T cells, including conventional CD4+ T cells (CD4+ oT cells), at least at the mRNA level (19, 22). Certain types of TLR ligands can directly activate T cells in the absence of APCs, suggesting that TLRs, including TLR2, play a role in the activation of T cells (11, 19). A recent publication has provided direct evidence that MyD88 is responsible for CpG DNA-induced direct activation of naïve CD4+ T cells and that phosphatidylinositol 3-kinase is involved in this MyD88-dependent activation pathway (11). While our observations have some similarities to these studies, there are some fundamental differences between our model and other models that make our findings regarding the TLR adaptor protein, MyD88, and its role in the regulation of the virus-specific CD4+ T-cell responses distinct. Previous studies have used nonreplicating antigens or TLR ligands to evaluate the activation of CD4+ T cells. In our study, we used a natural murine viral pathogen, LCMV. After infection of the target cells with LCMV, endogenously synthesized LCMV GP (which contains the dominant CD4+ T-cell epitope GP$_{61-70}$) needs to be processed by the APCs to form a complex with MHC class II I-$\alpha$ molecules and to be presented on the APC surface in order to stimulate a CD4+ T-cell response (35). Moreover, virus-induced activation of T cells involves the dynamic interaction of APCs and T cells in the immunological synapse, which plays a major role in the activation of CD4+ T cells. In contrast, other TLR ligands, including tripalmitoyl-Cys-Ser-Lys-Lys-Lys-Lys (TLR1/TLR2 ligand) and CpG (TLR9 ligand), activate CD4+ T cells in a ligand-receptor-specific but antigen-unspecific interaction manner (11, 19). Thus, viruses like LCMV could use a distinctive mechanism to activate MyD88-dependent CD4+ T-cell activation.

The role of the Treg cells in a virus-induced immune response has recently attracted increased attention (38, 39). Pasare et al. showed that depletion of Treg cells with anti-CD25-depleting monoclonal antibody (clone PC61) restored CD4+ T-cell responses to ovalbumin in MyD88 KO mice, suggesting that Treg cells contribute to the defective CD4+ T-cell response to model antigens in MyD88 KO mice (39). In the present study, depletion of Treg cells had little effect on LCMV-induced CD4+ T-cell responses and weight loss in MyD88 KO mice (Fig. 4E). Thus, our study suggests that the impaired CD4+ T-cell responses in MyD88 KO mice in response to LCMV infection are unlikely to be due to the action of Treg cells.

In summary, these studies reveal for the first time that the expression of MyD88 in CD4+ T cells is essential for the activation of CD4+ T cells in response to a natural murine viral pathogen. Our studies and studies of others demonstrated that the effects of MyD88 on T-cell responses are independent of APCs and Treg cells (11, 26). Furthermore, we demonstrated that MyD88 expression in CD4+ T cells is required for the production of both IFN-γ and TNF-α as well as helper cell activity for antibody production in response to virus.

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