MyD88 is essential to sustain mTOR activation necessary to promote T helper 17 cell proliferation by linking IL-1 and IL-23 signaling

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Myeloid differentiation primary response protein 88 (MyD88) is classically known as an adaptor, linking TLR and IL-1R to downstream signaling pathways in the innate immune system. In addition to its role in innate immune cells, MyD88 has been shown to play an important role in T cells. How MyD88 regulates helper T-cell differentiation remains largely unknown, however. Here we demonstrate that MyD88 is an important regulator of IL-17-producing CD4+ T helper cells (Th17) cell proliferation. MyD88-deficient CD4+ T cells showed a defect in Th17 cell differentiation, but not in Th1 cell or Th2 cell differentiation. The impaired IL-17 production from MyD88-deficient CD4+ T cells is not a result of defective RAR-related orphan receptor γt (RORγt) expression. Instead, MyD88 is essential for sustaining the mammalian target of rapamycin (mTOR) activation necessary to promote Th17 cell proliferation by linking IL-1 and IL-23 signaling. MyD88-deficient CD4+ T cells showed impaired mTOR activation and, consequently, reduced Th17 cell proliferation. Importantly, the absence of MyD88 in T cells ameliorated disease in the experimental autoimmune encephalomyelitis model. Taken together, our results demonstrate that MyD88 has a dual function in Th17 cells by delivering IL-1 signaling during the early differentiation stage and integrating IL-23 signaling to the mTOR complex to expand committed Th17 cells.

Myeloid differentiation primary response protein 88 (MyD88) was originally isolated as a cloned cDNA that was induced in M1 myeloblastic leukemia cells on activation with IL-6 (1). The function of MyD88 was then uncovered, because the C-terminal portion of MyD88 was found to be similar to the Drosophila Toll receptor and the mammalian IL-1 receptor (IL-1R) (2). This conserved region in the cytoplasmic tails of IL-1R and Toll-like receptor (TLR) is referred to as the Toll/IL-1R (TIR) domain. MyD88 is now known to play an essential role in the innate immune response by linking members of the TLR and IL-1R superfamily to the downstream activation of NF-κB and MAPKs (3).

Among cytokines produced by activated innate immune cells, IL-23 has been shown to promote production of the proinflammatory cytokine IL-17 in activated T cells (4). IL-17-producing CD4+ T helper (Th17) cells were identified after the discovery that IL-23 is linked to traditionally Th1-associated autoimmune disorders, such as experimental autoimmune encephalitis (EAE). II23a−/− mice, but not II12a−/− mice, were shown to be autoimmune-resistant (5). IL-23 is required for Th17 cell-mediated autoimmune disorders in vivo, but the role of IL-23 in Th17 cell differentiation remains controversial (6). Previous studies support the idea that IL-23 helps expand or maintain Th17 cells (7–9). In addition, a recent study reemphasized the importance of IL-23 in the generation of pathogenic Th17 cells, showing that they can be generated with IL-23, IL-6, and IL-1 (10).

In addition to IL-23, other cytokines, including TGF-β, IL-6, and IL-1, play roles in Th17 cell development. TGF-β with proinflammatory cytokines was shown to be critical in the support of Th17 cell differentiation (8, 11, 12). In particular, an inflammatory cytokine, IL-6, favors Th17 cell development by inhibiting regulatory T cells (13). The role of IL-1 in Th17 cell differentiation has been investigated as well. IL-1 receptor type 1-deficient (Il1r1−/−) mice showed a lower incidence of EAE and severe defects in the induction of IL-17–producing T cells (14). IL-1 signaling in T cells was further shown to be involved in early Th17 cell differentiation by regulating IFN regulatory factor 4 and RAR-related orphan receptor γt (RORγt) (15).

Although roles for MyD88 in the innate immune system are well established, little is known about their potential function in the adaptive immune system. Several studies have demonstrated important roles of MyD88 in T cells. For instance, T-cell expression of MyD88 is required for resistance to Toxoplasma gondii (16); the MyD88-dependent signaling pathway in CD4+ T cells has been shown to enhance proliferation and augment humoral immune responses (17); and MyD88 is required for T-cell effector function in the development of inflammatory bowel disease (18). Interestingly, although Myd88−/− T cells were found to exhibit decreased IL-17 production (18), how T-cell differentiation could be regulated by MyD88 was not clear in that study.

Here we investigated the molecular mechanism by which MyD88 regulates CD4+ T-cell differentiation. Our results demonstrate that MyD88 contributes to Th17 cell differentiation, but not to Th1 or Th2 cell differentiation. Both IL-1 and IL-23 signaling depend on MyD88 and result in up-regulation of IL-23R. MyD88-deficient Th17 cells show reduced IL-23R expression and mTOR activation, leading to impaired Th17 cell proliferation. Furthermore, MyD88 is crucial for proper Th17 cell differentiation in vivo. Thus, our findings reveal a unique role for the innate adaptor MyD88 in the regulation of Th17 cell differentiation.

Results

Impaired IL-17A Production in MyD88-Deficient CD4+ T Cells. MyD88-deficient (Myd88−/−) T cells have been shown to exhibit decreased IL-17 production (18), but the underlying mechanism...
has been unclear. To study how MyD88 regulates Th17 cell differentiation, we enriched CD4+ T cells from MyD88-sufficient (i.e., WT) and Myd88−/− mice and performed in vitro T-cell differentiation. The Myd88−/− CD4+ T cells showed a substantial defect in IL-17A production under Th17 cell-polarizing conditions (Fig. 1A). MyD88 deficiency did not affect IFN-γ production under Th1 cell-polarizing conditions or IL-4 production under Th2 cell-polarizing conditions (Fig. 1A).

Given our observation of impaired IL-17A production after T-cell restimulation, we next asked whether the defect started during T-cell differentiation. Myd88−/− CD4+ T cells already showed a lower frequency of IL-17A+ cells after 3 d of Th17 cell differentiation (Fig. S1A), although the defect was less dramatic than that observed after restimulation. Again, Myd88−/− CD4+ T cells showed no defect in IFN-γ expression under Th1 cell-polarizing conditions (Fig. S1A). In addition, the induction of Il17a mRNA was poorer in Myd88−/− CD4+ T cells compared with WT under Th17 cell-polarizing conditions; however, Ifng mRNA levels were comparable in WT and Myd88−/− CD4+ T cells (Fig. S1B).

Along with IL-17A, the production of IL-17F, another IL-17 cytokine family member, was severely impaired from Myd88−/− CD4+ T cells after restimulation, and the defect in Il17f mRNA level was detected based on the early Th17 cell differentiation (Fig. S1C).

**RORγt Is Expressed Normally in Myd88−/− Th17 Cells.** IL-2 or IL-10 signaling has been shown to modulate Th17 cell generation (19, 20); however, in the present study, impaired IL-17 production in Myd88−/− CD4+ T cells was not related to increased IL-2 or IL-10 production (Fig. S2). Given that Myd88−/− CD4+ T cells are defective in IL-17 production under Th17 cell-polarizing conditions but not in IFN-γ or IL-4 production under Th1 or Th2 cell-polarizing conditions, respectively, we speculated that the defect might result from cytokine signaling specific to Th17 cell differentiation. Among Th17-skewing cytokines, including TGF-β, IL-6, IL-1p, and IL-23, we first focused on IL-1p because of the well-established role of MyD88 as an adaptor in IL-1R signaling (3). Indeed, the defect in IL-17A production from Myd88−/− CD4+ T cells was less marked in the absence of IL-1p (Fig. S3A).

However, the defect could not be explained solely by the lack of IL-1p signaling in Myd88−/− CD4+ T cells, because they still exhibited impaired IL-17A production without IL-1p (Fig. S3A). A similar defect in Il17a mRNA expression from Myd88−/− CD4+ T cells was observed in the absence of IL-1p (Fig. S3B).

We then investigated whether TGF-β signaling was altered in Myd88−/− CD4+ T cells by assessing Foxp3 expression as a readout. Compared with WT, Myd88−/− CD4+ T cells expressed more Foxp3 after treatment with TGF-β alone (Fig. 1B, Upper). TGF-β-induced Foxp3 has been shown to inhibit Th17 cell differentiation by antagonizing RORγt (21). Thus, we evaluated whether

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**Fig. 1.** Impaired production of IL-17A in MyD88-deficient CD4+ T cells is independent of RORγt. (A) Purified Myd88−/− or WT CD4+ T cells were cultured under Th1-, Th2-, or Th17-polarizing conditions for 5 d, followed by restimulation with plate-bound anti-CD3 for 24 h, as described in Materials and Methods. ELISA was performed to detect the amounts of IFN-γ, IL-4, and IL-17A in culture supernatants. *P < 0.05. (B) Purified CD4+ cells were cultured in the presence of the indicated cytokines for 3 d, and Foxp3 and IL-17A expression was analyzed by flow cytometry. (C) RORγt protein levels were analyzed by flow cytometry during Th17 differentiation. Results are representative of three experiments (A and B) or two experiments (C).

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**Fig. 2.** IL-1p and IL-23 contribute synergistically to IL-17 production. (A) Purified CD4+ T cells were cultured under Th17-polarizing conditions for 5 d. Cells were harvested before restimulation (0 h) or after 6 h with plate-bound anti-CD3. The amounts of Il17a, Il17f, Il2, Il21, Il2, and Il10 mRNA were quantified by qRT-PCR. (B) CD4+ T cells were cultured with the indicated cytokines or media (Med) for 3 or 5 d. Culture supernatants were collected, and IL-17A production was assessed by ELISA. *P < 0.05. NS, not significant. (C and D) Cells were cultured with the indicated cytokines for 3 or 5 d. qRT-PCR was performed to measure the levels of Il17a and Il17f (C) and of Ahr, Batf, Socs3, Runx1, Rora, and Il23r (D). *P < 0.05, WT vs. Myd88−/− cells. After normalization to Actb, mRNA levels in fresh isolated CD4+ T cells (day 0) were used as a control to compare expression levels. Results are representative of three independent experiments.
enhanced TGF-β–induced Foxp3 expression in Myd88−/− CD4+ T cells results in reduced RORγt-directed IL-17 expression. After cells were cultured with various cytokines, both WT and Myd88−/− CD4+ T cells showed greatly reduced Foxp3 expression under IL-17–producing conditions (TGF-β + IL-6 or TGF-β + IL-6 + IL-1β + IL-23) (Fig. 1B). Importantly, under Th17-polarizing conditions, Myd88−/− CD4+ T cells showed no significant decrease in Rorc mRNA levels (Fig. S4) or RORγt expression (Fig. 1C). These data suggest that impaired IL-17 production from Myd88−/− CD4+ T cells is not related to reduced RORγt expression.

IL-1β and IL-23 Contribute Synergistically to IL-17 Production. Because we observed the greatest impairment in IL-17 production from Myd88−/− CD4+ T cells compared with WT CD4+ T cells, we examined the role of IL-1 and IL-23 signaling in this context. MyD88 is a downstream adapter molecule of IL-1 receptor and IL-17RA, which is essential for IL-17 production (23).

To understand how IL-1 and IL-23 signaling collaborates during Th17 cell differentiation, we first analyzed the temporal expression of their receptors. In line with the observation that IL-1 signaling is critical during early Th17 cell development (15), Il1r1 expression was increased during early Th17 cell differenti-
Phosphorylation of S6 ribosomal protein was sustained in WT at day 3 when IL-1β and IL-23 were present together with IL-6 and TGF-β (Fig. 4D). Furthermore, both IL-1β and IL-23 are required for activation of the mTOR signaling pathway (Fig. 4F). Because mTOR activation is linked to cell proliferation, we assessed whether IL-1 and IL-23 signaling promotes cell proliferation. WT and Myd88−/− CD4+ T cells proliferated at a similar rate during early stages of Th0 or Th17 cell differentiation (Fig. S7). However, during later Th17 cell differentiation (day 3–5), MyD88-dependent IL-1β and IL-23 signaling was required for the cell proliferation (Fig. 4F). Taken together, these findings indicate that MyD88 plays a critical role in integrating IL-1 and IL-23 signaling for Th17 cell proliferation and expansion.

MyD88 Is Required for IL-23 Signaling During Later Th17 Cell Differentiation. Although both IL-1β and IL-23 have been shown to be important for MyD88-mediated Th17 cell proliferation, whether MyD88 is required for IL-23 signaling is unclear. Because early IL-1 signaling is essential for MyD88-mediated Th17 cell differentiation, we addressed this question using inducible deletion of Myd88 during later Th17 cell differentiation. As shown in Fig. 5A, treatment of cells from Myd88fl/fl ERT2 mice with 4-hydroxytamoxifen (4-OHT) (27) reduced the level of Myd88. To test whether MyD88 is required for IL-23 signaling during later Th17 cell differentiation, CD4+ T cells from WT (Myd88fl/fl), Myd88fl/fl ERT2, or Myd88fl/fl CD4-Cre (Myd88ΔT) mice (27) were cultured initially with TGF-β, IL-6, and IL-1β. To avoid hindrance of early IL-1 signaling, 4-OHT was added at day 2, followed by IL-23 at day 3. As shown in previously (Fig. 4B), adding IL-23 to the cultures increased IL-17A expression and diminished after 3 d of culture (Fig. 4A and Fig. S5). Myd88−/− CD4+ T cells exhibited no defect in Il17a expression; in contrast, Il23r expression was low until day 3 and increased during later Th17 cell differentiation, which was not observed in Myd88−/− CD4+ T cells (Fig. 4A). Because these two receptors were expressed during different time periods, we asked whether IL-1 signaling and IL-23 signaling are sequentially involved in Th17 cell differentiation. To test this, we added IL-1 or IL-23 to the (designated −/−) or Myd88−/− (designated −/−) mice were activated and cultured with indicated cytokines. The whole-cell lysates were prepared on day 3 or day 5. P-S6, S6, and GAPDH were detected by immunoblot analysis. (E) Cells were activated and cultured with the indicated cytokines for 3 d. At the end of day 3, cells were washed, labeled with carboxyfluorescein diacetate succinimidyl ester, and cultured in T-cell medium for additional 3 d in the presence of same cytokines. Carboxyfluorescein diacetate succinimidyl ester dilution and IL-17A expression were analyzed by flow cytometry. Results are representative of two independent experiments (A and E) or three independent experiments (B, C, D, and F).

We next asked which signaling molecules are involved in the later Th17 cell differentiation. To test this, we added specific inhibitors for mTOR, IKK, JNK, and p38 at day 3 of Th17 cultures and assessed Il17a expression at day 5. Our results show that blocking NF-κB or MAPK signaling had little effect on Il17a expression (Fig. 4C). However, rapamycin treatment reduced Il17a expression, suggesting that the mammalian target of rapamycin (mTOR)-mediated signaling pathway is critical for the later Th17 cell differentiation. Moreover, Il23r expression was reduced by rapamycin treatment (Fig. S6). In fact, phosphorylation of S6 ribosomal protein was sustained in WT at day 5 when IL-1β and IL-23 were present together with IL-6 and TGF-β (Fig. 4D). Furthermore, both IL-1β and IL-23 are required for activation of the mTOR signaling pathway (Fig. 4F). Because mTOR activation is linked to cell proliferation, we assessed whether IL-1 and IL-23 signaling promotes cell proliferation. WT and Myd88−/− CD4+ T cells proliferated at a similar rate during early stages of Th0 or Th17 cell differentiation (Fig. S7). However, during later Th17 cell differentiation (day 3–5), MyD88-dependent IL-1β and IL-23 signaling was required for the cell proliferation (Fig. 4F). Taken together, these findings indicate that MyD88 plays a critical role in integrating IL-1 and IL-23 signaling for Th17 cell proliferation and expansion.

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production in WT, but not in Mydd88−/−, CD4+ T cells (Fig. 5B). Importantly, in the presence of 4-OHT, IL-17A production from Mydd88fl/fl ERCreT2 CD4+ T cells was significantly reduced compared with WT (Fig. 5 B and C). mRNA expression of Il17a and Il23r genes was also poorly induced in Mydd88fl/fl ERCreT2 CD4+ T cells compared with WT (Fig. 5D). In addition, Mydd88-dependent IL-23 signaling was required for activation of mTOR signaling pathway (Fig. S8). Taken together, these results indicate that Mydd88 is an important regulator not only of early IL-1, but also of later IL-23 signaling for Th17 cell proliferation.

Mydd88 Is Crucial for Th17 Cell Differentiation in Vivo. To determine whether the proposed role for Mydd88 in Th17 cell differentiation is relevant in vivo, EAE was induced in WT and Mydd88ΔT mice. All WT mice (16 of 16) developed EAE after immunization with myelin oligodendrocyte glycoprotein (MOG), compared with only 6 of 13 Mydd88ΔT mice (Table S1). EAE was less prevalent in Mydd88ΔT mice compared with WT mice (Fig. 5E); however, the disease was of similar onset and severity in the two groups (Table S1). Taken together, these data demonstrate that Mydd88 has a crucial role for proper Th17 cell differentiation in vivo.

Discussion

Many molecules classically known to be critical for the innate immune system also have important functions in adaptive immune responses (28, 29). The present study shows that Mydd88 plays an important role in Th17 cell differentiation. In addition, it demonstrates the underlying molecular mechanism by which Mydd88-mediated IL-1 signaling sequentially synergizes with IL-23 to promote Th17 cell proliferation.

IL-23 has a unique function in the maintenance of a T-cell subset producing IL-17 (6). IL-23R was shown to be essential for the terminal differentiation of Th17 cells in vivo (9); however, the molecular mechanism governing expression of the IL-23R remains unclear. IL-23R expression was abolished in the absence of Th17-inducing proinflammatory cytokines such as IL-6 (13). Importantly, it has been suggested that IL-23 induces IL-23R expression via positive feedback regulation (30, 31). Our results further show that Mydd88-mediated IL-1 signaling in CD4+ T cells is a prerequisite for the up-regulation of IL-23R by IL-23. IL-1 and IL-23 are produced mainly by activated antigen-presenting cells. Of interest, IL-1 also up-regulates IL-23 subunit p19 gene expression in antigen-presenting cells (32). Thus, IL-1 and IL-23 signaling seems to be closely regulated through Mydd88 in both innate and adaptive immune cells.

IL-1 signaling on T cells is known to be important (14, 15). The incidence of EAE is significantly lower in Il1r1−/− mice compared with WT mice, correlated with a failure to induce Th17 cells (14). The molecular mechanism by which IL-1R contributes to Th17 cell differentiation remains unclear, however. Chung et al. (15) reported that IL-1 signaling is required for early Th17 cell differentiation by up-regulating RORyt and IFN regulatory factor 4 expression; however, as shown in the present study, RORyt expression is not altered in Mydd88−/− CD4+ T cells. This discrepancy might be related to the fact that Mydd88 is an adaptor not only for IL-1R, but also for TLR. Chung et al. (15) used a coculture system in which dendritic cells and T cells were cultured in the presence of LPS. Several previous studies have uncovered the critical role of TLR in the adaptive immune system (17, 33, 34). Of note, Reynolds et al. (35) recently showed that TLR2 signaling in CD4+ T cells promotes Th17 responses. Thus, in the experimental setup of Chung et al. (15), the Mydd88-dependent signaling pathway was likely activated by TLR even in the absence of IL-1 signaling on T cells. Whether RORyt functions, such as transcriptional activity, are altered in Mydd88−/− CD4+ T cells remains elusive.

SIGIRR, a negative regulator for TLR and IL-1R signaling, has been shown to suppress Th17 cell proliferation (36). Importantly, this study demonstrated that Th17 cell effector function is linked to mTOR-mediated cell proliferation. When fully differentiated WT and Sigirr−/− Th17 cells were treated with IL-1, IL-1–induced mTOR kinase activation was increased in Sigirr−/− Th17 cells compared with WT cells (36). In line with this finding, our results show that Mydd88-dependent IL-1 and IL-23 signaling is required for the later stage of Th17 cell proliferation by activating the mTOR signaling pathway.

There is growing interest in the interplay between host metabolism and the immune system. In particular, mTOR plays an essential role in integrating environmental cues. Several previous studies have shown that mTOR critically regulates the differentiation of T cells (37–40). mTOR in T cells acts to integrate signal 1 (antigen recognition through T-cell receptor) and signal 2, the integrated sum of environmental cues, including CD28, IL-2R, amino acids, and cytokines (41). In the present study, Mydd88−/− CD4+ T cells demonstrated no global defect in mTOR activation; however, Mydd88 deficiency in T cells could
not integrate IL-1 and IL-23 signaling (signal 2) and failed to generate sustained mTOR activation. This may explain why MyD88 deficiency affects only IL-17 expression, even though mTORC1 was shown to be involved in both Th1 and Th17 responses (40).

In conclusion, our findings show that MyD88 has a dual function in Th17 cell differentiation. MyD88 delivers IL-1 signaling during the initial commitment stage and links it to IL-23 signaling, which expands committed Th17 cells via mTOR activation. Given that MyD88 also plays an important role in activating innate immune responses, MyD88 serves as a good example linking two arms of the immune systems. Our results have important implications for targeting MyD88-dependent signaling in both innate and adaptive immune cells to treat Th17 cell-associated diseases.

Materials and Methods

MyD88-deficient, MyD88+/fl/fl, MyD88+/+lox/lox Cre-D, MyD88+/+lox/lox CreT2, and WT mice were used for the experiments. Purified CD4+ T cells were activated by plate-bound anti-CD3 and soluble anti-CD28. Tumor-xenified MyD88 deletion was monitored by immunoblot analysis after treating the cells with 4-OHT at different time points. Mice were injected with MOG peptide and also pertussis toxin on days 0 and 2 after immunization. Full materials and methods with associated references, eight figures and one table can be found at the extended online supporting information.

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Antibodies and Reagents. Antibodies used for flow cytometry included anti-IL-17A (TC11-18H10), anti–IFN-γ (XM1G12), anti–IL-4 (1B11), anti–CD4 (RM4-5), anti–CD69 (H1.2F3), anti–CD132 (4G3), anti–CD25 (PC61), anti–CD44 (IM7), anti–CD62L (MEL-14), anti–CD45RB (16A), anti–CD90.1 (Thy1.1, OX-7), and anti–CD271 (NGFR; C40-1457) (all from BD Biosciences). The Foxp3 Fixation/Permeabilization Kit and anti–RAR-related orphan receptor γt (ROSYT) (AKFSK-9) and anti–Foxp3 (FJK-16s) antibodies were purchased from eBioscience. Anti–CD3 (2C11), anti–CD28 (37.51), anti–CD16/CD32 (2.4G2), neutralizing anti–IFN-γ (XM1G12), and anti–IL-4 (1B11) were puriﬁed from hybridoma supernatants. Anti–IL-2 and anti–IL-10 were purchased from BioLegend. IL-12, IL-4, IL-6, TGF-β, IFN-γ, IL-1β, and IL-23 were purchased from Peprotech. Plat E cells were purchased from Cell Biosciences. Rapamycin, SB202190, BMS-345541, and SP600125 were puriﬁed from Calbiochem, and 4-hydroxytamoxifen (4-OHT) was purchased from Sigma-Aldrich. Purified cytokine-speciﬁc ELISA antibodies were obtained from R&D Systems or eBioscience. Antibodies used for immunoblot analysis included phospho-mTOR (Ser248), phospho-p70 S6 kinase (Thr389), phospho-S6 ribosomal protein (Ser240/244), S6 ribosomal protein (SG10), phospho-Stat5 (Ytr705), Stat3, MyD88 (all from Cell Signaling Technology), GAPDH (6C5; from Abcam), and β-actin (from Sigma-Aldrich).

Cell Culture and T-Cell Differentiation in Vitro. Spleens and lymph nodes were isolated from Myd88<sup>−/−</sup> and C57BL/6 mice, and total CD4<sup>+</sup> T cells or CD4<sup>+</sup>CD26<sup>+</sup> T cells were puriﬁed using MACS beads (Miltenyi Biotec). Cells were grown in complete culture medium composed of RPMI supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 10 mM Hepes, 1 nM sodium pyruvate, and 55 μM β-mercaptoethanol. Then 0.7 × 10<sup>6</sup> cells/well were activated by plate-bound anti–CD3 (5 μg/mL) and soluble anti–CD28 (1 μg/mL) on 48-well tissue culture plates. The skewing conditions for different T-cell subsets were as follows: 10 μg/mL anti–IFN-γ and 10 μg/mL anti–IL-4 for Th0, 10 ng/mL IL-12 and 10 μg/mL anti–IL-4 for Th1, 10 ng/mL IL-4 and 10 μg/mL anti–IFN-γ for Th2, 5 ng/mL TGF-β, 50 ng/mL IL-6, 10 ng/mL IL-1β, 20 ng/mL IL-23, 10 μg/mL anti–IFN-γ, and 10 μg/mL anti–IL-4 for Th17.

Flow Cytometry. For intracellular staining, cells from culture were restimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich), 500 ng/mL ionomycin (Sigma-Aldrich) and GolgiPlug (BD Biosciences) for 4 h at 37°C. Cells were then incubated with anti–CD16/CD32 for 15 min at 4°C to block nonspeciﬁc antibody binding, and stained for appropriate surface markers for 20 min at 4°C. Cells were ﬁxed and permeabilized, followed by intracellular cytokine staining. For Foxp3 and ROsyT staining, cells were harvested without phorbol 12-myristate 13-acetate/ionomycin restimulation using the Foxp3 Fixation/Permeabilization Kit according to the manufacturer’s instructions. For carboxyﬂuorescein diacetate succinimidyl ester (CFSE) labeling, naive (at day 0 culture) or activated (at the end of day 3 culture) T cells were washed twice with PBS/0.1% BSA and labeled with the CellTrace CFSE Cell Proliferation Kit (Invitrogen) according to the manufacturer’s instructions. Events were acquired on a FACSCanto (BD Biosciences), and data were analyzed using FlowJo software (TreeStar).

Retroviral Transduction. Myd88 retroviral vectors have been described previously (2). The Il23r cDNA was cloned into murine stem cell virus vector IL-23R-IRES-Thy1.1 (a generous gift from Dan R. Littman, New York University, New York, NY). Retroviruses were produced by transfecting Plat E cells with the plasmids according to the manufacturer’s protocols (Cell Biolabs). For viral transduction, T cells were plated under Th0 conditions on day 0. On day 1, cells were infected with retroviral supernatants by centrifugation for 1 h at 650 × g (Eppendorf5810R) in the presence of 8 μg/mL polybrene (Sigma-Aldrich) and then cultured further in the presence of additional cytokines. Cells were harvested on day 6 for intracellular cytokine staining. CD4<sup>+</sup>NFGFR<sup>+</sup> cells were also sorted using FACSDiva software (BD Biosciences) for quantitative RT-PCR (qRT-PCR) analysis.

In Vitro Tamoxifen Treatment. First, 4-OHT was prepared in ethanol. Tamoxifen-induced Myd88 deletion was monitored at day 3 and day 5 by immunoblot analysis after the cells were treated in culture with 4-OHT at concentrations ranging from 0.01 to 1 μM at different time points (day 0 through day 3). In the experiment shown in Fig. 5, 1 μM 4-OHT was added to the cultures at day 2, IL-23 was added at day 3, and Th17 cell differentiation was tested at day 5.

ELISA, Real-Time qRT-PCR, and Immunoblot Analysis. Cytokine concentrations in supernatants were detected by ELISA, and mRNA accumulation of cytokines was analyzed by qRT-PCR as described previously (3). The primers used for Actb, Il17a, Il17f, Ifng, Il21, Il22, Rora, Il23r, Il1r1 (4), Rorc, Ahr, Batf, Socs3, and Rarα1 have been described previously (5). Immunoblot analysis was performed as described previously (3).


Fig. S1. Myd88−/− CD4+ T cells are defective in IL-17A and IL-17F production. (A) Purified Myd88−/− or WT CD4+ T cells were cultured under Th1 or Th17 conditions for 3 d, analyzed by flow cytometry for intracellular cytokines. (B) The amounts of Il17a and Ifng mRNA were quantified by qRT-PCR during Th17 and Th1 cell differentiation, respectively. Cytokine mRNA levels were normalized with the amounts of Actb. Values are presented as mean ± SD. *P < 0.05. (C) Cells were cultured under Th17 cell-polarizing conditions, then restimulated with plate-bound anti-CD3 for 24 h. ELISA was performed to detect IL-17F production in culture supernatants (Left). The amount of Il17f mRNA during Th17 cell differentiation was quantified by qRT-PCR. Cytokine mRNA levels were normalized with expression amounts of Actb (Right). *P < 0.05. Results are representative of at least three independent experiments.
Fig. S2. Impaired IL-17 production in Myd88−/− CD4+ T cells is not related to increased IL-2 or IL-10 production. (A and B) Myd88−/− or WT CD4+ T cells were cultured with the indicated cytokines for 5 d, with 10 μg/mL anti–IL-2 or 10 μg/mL anti–IL-10 added at day 0, as indicated. The amount of Il17a mRNA was quantified at day 5 by qRT-PCR. (−), TGF-β+IL-6+IL-1β+IL-23 culture without blocking antibodies. (C) The amounts of IL-2 in the culture supernatants and of Il10 mRNA were quantified at day 3 by ELISA and qRT-PCR, respectively. After normalization to Actb, mRNA levels in fresh isolated CD4+ T cells (day 0) were used as a control to compare expression levels. *P < 0.05. NS, not significant. Results are representative of three independent experiments (A) or two independent experiments (B and C).

Fig. S3. Impaired IL-17 production from Myd88−/− CD4+ T cells in the absence of IL-1 signaling. (A) CD4+ T cells were cultured for 5 d with TGF-β, IL-6, IL-23 and in the presence or absence of IL-1β. Cells were then restimulated with anti-CD3 for overnight. Culture supernatants were collected and IL-17A production was assessed by ELISA. *P < 0.05; NS, not significant. (B) Cells were differentiated with TGF-β, IL-6 and IL-23. The amount of Il17a mRNA was quantified by qRT-PCR. *P < 0.05. Results are representative of at least three independent experiments.
Fig. S4. Impaired IL-17 production from Myd88−/− CD4+ T cells was not related to reduced RORγt expression. Rorc mRNA expression during Th17 differentiation was quantified by qRT-PCR. Results are representative of three experiments.

Fig. S5. Il1r1 expression during Th17 cell differentiation. Cells were cultured with the indicated cytokines for 3 or 5 d, then qRT-PCR was performed to measure Il1r1 levels. After normalization to Actb, mRNA levels in fresh isolated CD4+ T cells (day 0) were used as a control to compare expression levels. Results are representative of two experiments.

Fig. S6. The mTOR-mediated signaling pathway is critical for Il23r expression. CD4+ T cells were cultured under Th17-polarizing conditions for 3 d. Rapamycin (0.1 μM) or DMSO was added at the end of day 3. On day 5, mRNA expression of Il17a and Il23r was quantified by qRT-PCR. After normalization to Actb, mRNA levels in fresh isolated CD4+ T cells (day 0) were used as a control to compare expression levels. *P < 0.05. NS, not significant. Results are representative of three independent experiments.
**Fig. S7.** Cell proliferation was comparable between WT and *Myd88*−/− CD4+ T cells during early T-cell activation. (A) CFSE-labeled cells were cultured under Th0 or Th17 cell-polarizing conditions for 4 d. CFSE dilution and IL-17A expression were analyzed by flow cytometry. (B) Myd88−/− or WT CD4+ T cells were cultured with indicated cytokines for 2 d. Cell proliferation was measured by in vitro BrdU labeling according to the manufacturer’s instructions (BD Biosciences). In brief, cells activated for 24 h were pulsed with 10 μM BrdU for 90 min. After treatment of fixed cells with DNase, cells were stained with allophycocyanin-conjugated anti-BrdU. BrdU incorporation was analyzed by flow cytometry. Results are representative of two independent experiments.

**Fig. S8.** MyD88-dependent IL-23 signaling is required for mTOR activation. CD4+ T cells were activated and cultured initially with TGF-β + IL-6 + IL-1β. Then 1 μM 4-OHT was added to the cultures at day 2, and 20 ng/mL IL-23 was added into the indicated culture at day 3. On day 5, the whole-cell lysates were prepared, and P-S6, β-actin, and MyD88 were detected by immunoblot analysis.
Table S1. Decreased incidence of experimental autoimmune encephalitis in *Myd88ΔT* mice

<table>
<thead>
<tr>
<th></th>
<th>Incidence</th>
<th>Mean day of onset</th>
<th>Mean maximum score of all mice</th>
<th>Mean maximum score of clinically ill mice</th>
<th>Days at a score ≥2 for clinically ill mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>16/16</td>
<td>11.9 ± 5.5</td>
<td>2.6 ± 0.6</td>
<td>2.6 ± 0.6</td>
<td>17.4 ± 5.4</td>
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<tr>
<td><em>Myd88ΔT</em></td>
<td>6/13</td>
<td>10.7 ± 1.5</td>
<td>1.3 ± 1.5</td>
<td>2.8 ± 0.3</td>
<td>17 ± 5.33</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.