Signaling through the Adaptor Molecule MyD88 in CD4+ T Cells Is Required to Overcome Suppression by Regulator T Cells

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SUMMARY

Innate immune recognition controls adaptive immune responses through multiple mechanisms. The MyD88 signaling adaptor operates in many cell types downstream of Toll-like receptors (TLRs) and interleukin-1 (IL-1) receptor family members. Cell-type-specific functions of MyD88 signaling remain poorly characterized. Here, we have shown that the T cell-specific ablation of MyD88 in mice impairs not only T helper 17 (Th17) cell responses, but also Th1 cell responses. MyD88 relayed signals of TLR-induced IL-1, which became dispensable for Th1 cell responses in the absence of T regulatory (Treg) cells. Treg cell-specific ablation of MyD88 had no effect, suggesting that IL-1 acts on naive CD4+ T cells instead of Treg cells themselves. Together, these findings demonstrate that IL-1 renders naive CD4+ T cells refractory to Treg cell-mediated suppression in order to allow their differentiation into Th1 cells. In addition, IL-1 was also important for the generation of functional CD4+ memory T cells.

INTRODUCTION

The innate instruction of adaptive immunity is controlled on multiple levels. The activation of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) in dendritic cells (DCs) leads to the upregulation of costimulatory molecules and secretion of proinflammatory cytokines. This activation program provides a critical layer in the discrimination between self and non-self and is essential for the activation of T cell responses (Iwasaki and Medzhitov, 2010; Schenten and Medzhitov, 2011). Despite the progress in the general understanding of the rules that govern the interaction between DCs and cognate CD4+ T cells following TLR activation, the specific roles of individual TLR-induced cytokines and T cell-specific TLR signals in shaping CD4+ T cell responses remain incompletely understood. CD4+ T cells express several TLRs, although the precise patterns of TLR expression in particular CD4+ T cell subsets are still subject to debate (Cairns et al., 2006; Caramalho et al., 2003; Fukata et al., 2008; Gelman et al., 2004; González-Navajas et al., 2010; Kabelitz, 2007). Multiple studies have demonstrated various effects of TLR stimulation in T cells. For example, the stimulation of CD4+ T cells with TLR9 agonists causes enhanced proliferation, survival, and secretion of interleukin-2 (IL-2) (Gelman et al., 2004). Similarly, T cell-specific TLR2 activation can enhance the generation of Th17 cell responses (Reynolds et al., 2010). Some TLRs also appear to influence naturally-occurring CD4+ CD25+ Treg cells directly by dampening their suppressive capabilities, in part by lowering the expression of FoxP3, the lineage-defining transcription factors of these cells (LaRosa et al., 2007; Liu et al., 2006; Sutmuller et al., 2006). Thus, TLRs seem to modulate both CD4+ effector T cell and Treg cell responses simultaneously in order to promote the generation of CD4+ T cell responses.

Members of the IL-1 family of cytokines are known to control several aspects of T cell responses directly (Dinarello, 2009; Sims and Smith, 2010). In recent years, IL-1 has received much attention because of its involvement in the differentiation of Th17 cells. These cells express the IL-1 receptor (IL-1R) and several studies have suggested that IL-1 enhances the differentiation of naive CD4+ T cells into Th17 cells in vitro (Acosta-Rodriguez et al., 2007; Chung et al., 2009; Kryczek et al., 2007; Wilson et al., 2007). IL-1 signaling in CD4+ T cells is also important for the induction of Th17 cells in vivo, which is illustrated by the observation that CD4+ T cells deficient in IL-1 signaling fail to induce the Th17 cell-dependent diseases experimental...
autoimmune encephalomyelitis (EAE) and colitis (Chung et al., 2009; Fukata et al., 2008; Sutton et al., 2006; Tomita et al., 2009). In addition to its role in Th17 cell biology, however, it has been suggested that IL-1 plays an important role in the generation of both primary as well as secondary Th1 cell responses (Ben-Sasson et al., 2009; Sims and Smith, 2010). Some studies detect the IL-1R on naive and Th1 cells, whereas others find no evidence for this (Chung et al., 2009; Guo et al., 2009; Taylor-Robinson and Phillips, 1994). Nonetheless, it seems clear that IL-1 signaling contributes to the generation of Th1 cell responses. For example, it has been suggested to promote the upregulation of the IL-2 receptor α chain (CD25), prevent apoptosis, and enhance Th1 cell expansion (Ben-Sasson et al., 2009; O’Neill, 2008). Moreover, bone marrow chimeras harboring IL-1R-deficient T cells have reduced numbers of Th1 cells during the course of EAE (Ben-Sasson et al., 2009; Chung et al., 2009). Therefore, IL-1 plays a role in the generation of both Th1 and Th17 cell responses. In contrast, the IL-1-related cytokine IL-18 seems to be more exclusively linked to the generation of Th1 cell responses because Th1 cells can express high amounts of the IL-18R in a T-bet-dependent fashion. In this regard, the function of IL-18 in Th1 cells might parallel that of IL-1 and IL-33 in Th17 and Th2 cells, respectively, namely to reinforce or stabilize CD4+ T cell lineage commitments (Guo et al., 2009). Thus, members of the IL-1 family appear to be involved in both the activation of CD4+ T cells and maintaining the subsequent lineage commitment decision. In addition to its effects on the development of specific CD4+ T effector subsets, IL-1 signaling also appears to regulate the interaction between effector T cells and Treg cells. Treg cells express the IL-1R, even though the roles of IL-1R on T cell function are not clearly understood (Chaudhry et al., 2009; Mercer et al., 2010). Nonetheless, IL-1 might influence the function of Treg cells directly by blocking the suppressive function of Treg cells (O’Sullivan et al., 2006). Treg cells are also thought to deprive CD4+ effector T cells of IL-1 (Chaudhry et al., 2009). Moreover, IL-14 has been implicated enabling the conversion of induced Treg cells into Th17 cells (Chung et al., 2009).

Despite this progress in the understanding of TLR and IL-1 biology, the T cell-specific function of these signals are still incompletely understood, in part due to the pleiotropic nature of these triggers. In this study, we sought to investigate the T cell-specific roles of TLRs, IL-1, and IL-18 on the generation of CD4+ T cell responses in vivo. We report on the generation and analysis of mice carrying a T cell-specific ablation of Myd88, the essential signaling adaptor of most TLRs and receptors of the IL-1 family. Our study identifies a function for IL-1 in rendering CD4+ T cells refractory to Treg cell-mediated suppression. In addition, we show that IL-1 is also necessary to generate functional Th1 memory cells.

RESULTS

T Cell-Specific MyD88 Signaling Is Required for the Induction of Both Th1 and Th17 Cell Responses

In order to investigate the T cell-specific function of signals induced by TLRs and IL-1 family members, we generated mice carrying a T cell-specific ablation of Myd88. We flanked exons 3–5 of the Myd88 gene with loxP sites to allow its deletion by Cre-mediated recombination. These exons encode the essential Toll-IL-1 receptor (TIR) domain of Myd88. Moreover, splicing from exon 2 to exon 6 resulted in a frameshift mutation. The targeting strategy and generation of the mice is outlined in Figures S1A–S1C available online. We intercrossed the resulting Myd88FL/FL mice with Cd4-cre mice in order to obtain Myd88FL/FL Cd4-cre mice, which ablated Myd88 in all T cells (Figure S1D).

We immunized Myd88FL/FL Cd4-cre and control mice with ovalbumin (OVA) in the presence of lipopolysaccharide (LPS) in incomplete Freund’s adjuvant (IFA) as a carrier and measured the ensuing CD4+ T cell response. To this end, we isolated CD4+ T cells from the draining lymph nodes 7 days after immunization, at which point the majority of cells displayed a phenotype of CXCR5+ PD-1+ T follicular helper (Tfh) cells (Figure S2A), and restimulated the cells with OVA in the presence of irradiated splenocytes as antigen presenting cells (APCs) in vitro. Wild-type (WT) CD4+ T cells proliferated robustly and secreted both interferon-γ (IFN-γ) and IL-17, whereas Myd88-deficient CD4+ T cells were significantly impaired in their ability to proliferate and secrete both IFN-γ and IL-17 (Figures 1A and 1B, Figures S4A and S4B). Because dying cells, such as irradiated splenocytes, might have been a source of IL-1 in the T cell cultures and thus might have affected the experimental outcome during the in vitro phase of the assay, we controlled for the presence of IL-1 in the cultures. We could detect IL-1α and IL-1β in cultured macrophages after stimulation with LPS and ATP, but failed to do so in the T cell assays after restimulation with OVA, even in the presence of a 4-fold higher number of irradiated splenocytes (Figure S2B). Indeed, CD4+ T cells from Myd88FL/FL Cd4-cre mice also failed to expand and secrete IFN-γ after restimulation in the presence of irradiated APCs from Caspase-1-deficient mice, which are impaired in their ability to secrete IL-1 (Figures S2C and S2D). These results suggest that IL-1 released by dying APCs after in vitro stimulation with antigen is not responsible for the defects observed in Myd88-deficient CD4+ T cells. The impairment of the CD4+ T cell response in Myd88FL/FL Cd4-cre mice was therefore due to defective IL-1 signaling in vivo.

In order to assess whether the activation of other TLRs results in a similar defect of the CD4+ T cell response in Myd88FL/FL Cd4-cre mice, we also immunized mice with OVA plus either polyIC or CpG DNA in IFA as a carrier. The CD4+ T cell response of Myd88FL/FL Cd4-cre mice was also defective with these additional stimuli (Figures S2E and S2F). We obtained similar results following immunization with OVA in the more complex complete Freund’s adjuvant (CFA) (Figure S2G). Next, we asked whether the impairment of the CD4+ T cell response was caused by a defect in the generation or function of the cells. We therefore immunized the mice with 2W peptide in the presence of LPS and IFA, because this system allows for the tracking of the antigen-specific CD4+ T cells with 2WI-A9 tetramers (Moon et al., 2007). Upon immunization with 2W peptide, the Th1 cell response in Myd88FL/FL Cd4-cre mice was also impaired, suggesting that the choice of antigen does not influence the outcome of the T cell response as measured by restimulation (Figure 1C). At the peak of the response, activated CD44+ CD4+ T cells specific for the 2W peptide in the draining lymph nodes of Myd88FL/FL Cd4-cre mice...

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mice were as frequent as in WT controls (Figure 1D). While the cellular composition of the lymph nodes did not change after immunization (data not shown), the number of cells in the draining lymph nodes of Myd88FL/FL Cd4-cre mice was reduced compared to that of control mice, leading to a reduction in the absolute number of 2W:I-Ab-positive CD4+ T cells in

Figure 1. Impaired CD4+ T Cells Response in Myd88FL/FL Cd4-cre Mice

(A and B) Proliferation of CD4+ T cells from Myd88FL/FL Cd4-cre mice and WT controls. CD4+ T cells were isolated from the draining lymph nodes 7 days after immunization in the feet with OVA + LPS in IFA and restimulated with OVA in the presence of irradiated splenocytes. Proliferation (A) and cytokine secretion (B) were measured 3 days later by 3H-thymidine incorporation and ELISA, respectively. Shown is the mean ± SD.

(C) Th1 cell response in Myd88FL/FL Cd4-cre mice and WT controls after immunization with 2W peptide + LPS in IFA and restimulation with 2W peptide. Shown is the mean ± SD.

(D) Frequency of 2W:I-Ab+ CD4+ T cells in Myd88FL/FL Cd4-cre and WT control mice T cells 7 days after footpad immunization with 2W peptide + LPS in IFA.

(E) Cytokine production of 2W:I-Ab+ CD4+ T cells in Myd88FL/FL Cd4-cre and WT control mice after restimulation with an αCD3ε antibody.

(F) Absolute numbers of all cells, CD4+ T cells, and 2W:I-Ab+ CD4+ T cells in the draining lymph nodes of Myd88FL/FL Cd4-cre and WT controls immunized with 2W peptide + LPS in IFA 7 days earlier. For all experiments, a representative out of at least three independent experiments is shown. See also Figure S1 and S2.

mice were as frequent as in WT controls (Figure 1D). While the cellular composition of the lymph nodes did not change after immunization (data not shown), the number of cells in the draining lymph nodes of Myd88FL/FL Cd4-cre mice was reduced compared to that of control mice, leading to a reduction in the absolute number of 2W:I-Ab+ positive CD4+ T cells in
MyD88 Signaling in CD4+ T Cells Blocks Treg Cells

Figure 2. Proliferation, Viability, and Differentiation of 2W:I-A(b)+ CD4+ T Cells from Myd88FL/FL Cd4-cre and WT Control Mice

(A) Proliferation of antigen-specific 2W:I-A(b)+ CD4+ T cells in the draining lymph nodes of Myd88FL/FL Cd4-cre and WT control mice between day 4–7 after immunization with 2W peptide + LPS in IFA as measured by incorporation of BrdU. Left panel shows a representative experiment. Right panel shows statistical representation of the combined data of three independent experiments, normalized to the level of BrdU incorporation of 2W:I-A(b)+ CD4+ T cells of Myd88FL/FL Cd4-cre mice. Each dot represents one mouse.

(B) Frequency of apoptotic antigen-specific 2W:I-A(b)+ CD4+ T cells in the draining lymph nodes of Myd88FL/FL Cd4-cre and WT control mice on day 7 after immunization with 2W peptide + LPS in IFA, measured by staining for active caspase-3. Shown is the mean ± SD of the combined data of two independent experiments using a total of four mice per genotype.

(C) T-bet expression by qPCR in immunized CD4+ T cells in the draining lymph nodes of Myd88FL/FL Cd4-cre and WT control mice. A representative experiment out of two independent experiments, each using the pooled samples from 5–10 mice per genotype, is shown. Error bars reflect the SD.

Myd88FL/FL Cd4-cre mice, which might have been caused by a failure of the antigen-specific T cells to secrete inflammatory cytokines (Figure 1E).

Consistent with normal frequency of antigen-specific CD4+ T cells in Myd88FL/FL Cd4-cre mice, these cells incorporated BrdU into their DNA as efficiently as WT controls, implying that the clonal expansion of antigen-specific CD4+ T cells is not impaired in Myd88FL/FL Cd4-cre mice (Figure 2A). CD4+ T cells from these mice also did not display an increased tendency to undergo apoptosis (Figure 2B). However, antigen-specific 2W+ CD4+ T cells of Myd88FL/FL Cd4-cre mice expressed lower amounts of T-bet, the lineage-defining transcription factor of Th1 cells, when compared to WT control cells (Figure 2C). Together, our findings therefore demonstrate that T cell-specific MyD88 signaling is required for the generation of functionally competent Th1 cells, even though the initial expansion of the antigen-specific CD4+ T cells is not compromised.

Impaired CD4+ T Cell Response in Myd88FL/FL Cd4-cre Mice Is Caused by Defective IL-1 Signaling

MyD88 relays signals from most TLRs and also from members of the IL-1 family. In order to distinguish between these two
types of signals, we first analyzed the expression of IL-1 family receptors on antigen-specific T cells 7 days after immunization with 2W peptide and LPS in IFA. We detected an upregulation of the IL-1R in antigen-specific CD4+ T cells from both Myd88FL/FL Cd4-cre mice and WT controls compared to naive CD4+ T cells. In contrast, the IL-18R was downregulated in all CD4+ T cells from both Myd88FL/FL Cd4-cre mice and WT controls compared to naive CD4+ T cells (Figures 3A and 3B).

The regulation of the IL-1R implicated IL-1 signaling as an important event in the generation of the CD4+ T cell response, but did not rule out an involvement of TLR or IL-18R signaling. We therefore generated mixed bone marrow chimeras, in which the T cell compartment consisted of T cells derived from TLR2- and TLR4-deficient compound mutant mice, IL-1R-deficient mice, IL-18R-deficient mice, or WT controls. Isolated CD4+ T cells were restimulated with OVA after the immunization of the mice with OVA + LPS in IFA. The proliferation was measured 3 days later by 3H-thymidine incorporation (C and E) and cytokine secretion was measured by ELISA (D and F). A representative experiment out of three independent experiments is shown. Error bars reflect SD. See also Figure S3.

However, IL-1 signaling was also required under these conditions (Figures S3C–S3F). Thus, our results collectively demonstrate that the defective CD4+ T cell response in Myd88FL/FL Cd4-cre mice is caused by the inability of the T cells to recognize IL-1 signals, whereas TLR-driven signals play a minor role.

**IL-1 Signaling Is Required to Render CD4+ T Cells Refractory to Treg Cell-Mediated Suppression**

We had previously shown that transient depletion of regulatory T (Treg) cells in complete MyD88-deficient mice is able to restore the Th1 cell response (Pasare and Medzhitov, 2004). However, neither the molecular signals nor the cell type responsible for this phenotype were known. In light of our current results, we asked whether IL-1 signaling regulates the interplay between the naive CD4+ T cell population and the Treg cell population.

In order to analyze the CD4+ T cell response in the absence of Treg cells, we depleted CD25+ Treg cells from Myd88FL/FL Cd4-cre and control mice with a monoclonal CD25 antibody three days prior to immunization with OVA and LPS in IFA. We obtained similar results for bone marrow chimeras that carried either a TLR9- or IL-1R-deficient T cell compartment, which we had immunized with OVA and CpG DNA in IFA. We noticed in some, but not all, experiments a moderate contribution of TLR9 signaling to the response, consistent with the suggested role of T cell-intrinsic TLR9 signaling in the generation of CD4+ T cell responses (Gelman et al., 2004).
verified the successful Treg cell depletion on the day of immunization by flow cytometry of blood leukocytes (data not shown). Treg cell depletion was unable to restore the Th17 cell response in *Myd88*<sup>FL/FL</sup> *Cd4-cre* mice, consistent with a requirement for IL-1 signaling in the differentiation of these cells. However, in the absence of Treg cells, proliferation and secretion of IFN-γ by restimulated CD4<sup>+</sup> T cells was restored in *Myd88*<sup>FL/FL</sup> *Cd4-cre* (Figures 4A and 4B, Figures S4A and S4B). We obtained similar results after immunization of Treg cell-depleted *Myd88*<sup>FL/FL</sup> *Foxp3-cre* mice and WT controls with OVA + CpG DNA (Figures S4C and S4F).

While these results suggested that IL-1 signaling in CD4<sup>+</sup> T cells might function to overcome Treg cell-mediated suppression, they did not distinguish between the effects of IL-1 on the various CD4<sup>+</sup> T cell populations. Because IL-1 might be acting on Treg cells directly (Mercer et al., 2010; O’Sullivan et al., 2006), we generated mice in which *Myd88* is specifically ablated in FoxP3<sup>+</sup> T cells by expression of the Foxp3-cre allele (*Myd88*<sup>FL/FL</sup> Foxp3-cre mice). Immunization of *Myd88*<sup>FL/FL</sup> Foxp3-cre mice with OVA and LPS in IFA revealed that these mice mounted CD4<sup>+</sup> T cell responses that were indistinguishable from WT controls (Figures 4C and 4D). Thus, these results indicate that IL-1 does not act on Treg cells, but instead is required in naive or effector CD4<sup>+</sup> T cells in order to render these cells refractory to Treg cell-mediated suppression.

**IL-1 Signaling Does Not Cause an Increased Frequency of Induced Treg Cells**

The results so far implied a requirement for IL-1 in naive or effector CD4<sup>+</sup> T cells to overcome suppression by “naturally-occurring” Treg cells (nTreg cells). However, TLR-induced cytokines such as IL-6 can affect the balance between the frequencies of Th17 cells and transforming growth factor-β (TGF-β)-induced (iTreg) cells, and this mechanism can result in an increased frequency of iTreg cells under conditions where the development of Th17 cells is genetically compromised. A similar function has recently been suggested for IL-1 (Chung et al., 2009). The absence of a Th17 cell response in *Myd88*<sup>FL/FL</sup> *Cd4-cre* mice might therefore lead to an increased frequency of iTreg cells, which could provide an alternative explanation for the impaired Th1 cell response in *Myd88*<sup>FL/FL</sup> *Cd4-cre* mice. Thus, we investigated whether the frequency of iTreg cells was increased in *Myd88*<sup>FL/FL</sup> *Cd4-cre* mice. We did not observe an increased frequency of FoxP3<sup>+</sup> Treg cells in unimmunized
Myd88FL/FL Cd4-cre mice (Figure 5A). Similarly, the frequency of Treg cells was also not altered in Myd88FL/FL Cd4-cre mice after immunization with OVA and LPS in IFA and subsequent restimulation in vitro (Figure 5B). In order to measure the frequency of Treg cells more directly, we also analyzed their presence in the draining lymph nodes of immunized mice. Although we noticed a modest tendency of a higher frequency of Treg cells in the draining lymph nodes of some immunized Myd88FL/FL Cd4-cre mice, we did not detect consistent differences between these mice and WT controls (Figure 5C). Because it remained possible that the lack of IL-1 signaling in the T cells of Myd88FL/FL Cd4-cre mice alters the frequency of antigen-specific Treg cells, we immunized Myd88FL/FL Cd4-cre mice and WT controls with 2W peptide and LPS in IFA and measured the frequency of 2W:I-A(b)+ FoxP3+ Treg cells in the draining lymph nodes 7 days later. Neither the frequency of these cells as a percentage of all CD4+ T cells, nor as percentage of all 2W:I-A(b)+ CD4+ T cells was changed in Myd88FL/FL Cd4-cre mice compared to WT mice (Figures 5D and 5E). Consistent with of the overall reduced size of the lymph nodes of immunized Myd88FL/FL Cd4-cre mice, we also observed a trend toward lower absolute numbers of 2W:I-A(b)+ FoxP3+ Treg cells in Myd88FL/FL Cd4-cre mice compared to WT mice (Figures 5D and 5F). Thus, we conclude that T cell-intrinsic MyD88 signaling does not affect the frequency of induced Treg cells, at least not under the conditions used in this study.

Impaired Memory CD4+ T Cell Response in Myd88FL/FL Cd4-cre Mice

The absence of Treg cells prior to the immunization enables a Th1 response in Myd88FL/FL Cd4-cre mice. Thus, we asked whether this response produces memory T cells at a frequency similar to WT mice. We immunized Myd88FL/FL Cd4-cre mice and WT controls with 2W peptide + LPS in IFA in the presence or absence of Treg cells and isolated the draining lymph nodes 30–60 days later. This time almost about half of 2W:I-A(b)+ CD4+ T cells of both Myd88FL/FL Cd4-cre mice and WT controls represented CD44+ CD62L+ memory T cells, irrespective of whether or not Treg cells were present during the immunization (Figure 6A). There was no difference between Myd88FL/FL Cd4-cre and WT control mice. Likewise, the lymph nodes of both Myd88FL/FL Cd4-cre mice and WT controls represented CD44+ CD62L+ memory T cells, irrespective of whether or not Treg cells were present during the immunization (Figure 6A). There was no difference between Myd88FL/FL Cd4-cre and WT control mice. Likewise, the lymph nodes of both Myd88FL/FL Cd4-cre mice and WT controls harbored equal frequencies of Ly-6C+ CXCR5− PD-1− Th1 effector memory cells, Ly-6C− CXCR5+ PD-1− central memory CD4+ T cells, and Ly-6C− CXCR5+ PD-1− Th17 cells (Figure 6B). These results suggest that in Myd88FL/FL Cd4-cre mice, antigen-specific CD4+ T cells become antigen-experienced and form memory cells at similar frequencies as WT mice, irrespective of whether or not Treg cells were present during the immunization.

We then asked whether the CD4+ memory T cell response is functionally impaired. We immunized Myd88FL/FL Cd4-cre mice and WT controls with OVA + LPS in IFA in either the presence or absence of Treg cells and reimmunized these mice 60 days...
later, again in either the presence of absence of Treg cells. We isolated CD4+ T cells from the draining lymph nodes 7 days later and restimulated the cells with OVA in vitro as described before. As expected, WT CD4+ T cells proliferated robustly after restimulation and secreted IFN-γ and IL-17, irrespective of whether or not Treg cells were present during both the primary and secondary immunization. In contrast, CD4+ T cells from Myd88<sup>FL/FL</sup> Cd4-cre mice failed to expand like WT cells and did not secrete large amounts of cytokines. Surprisingly, this was also the case under conditions in which Treg cells were absent during both the primary and secondary immunization (Figures 6C and 6D; Figure S5). Therefore, although memory CD4+ T cells do not appear to be dependent on IL-1 for their generation, they do require this cytokine in order to become functionally competent, suggesting a dependence on IL-1 that goes beyond a requirement for IL-1 to overcome Treg cell-mediated suppression.

**IL-1 and IL-6 Induce Different Gene-Expression Programs.**

The phenotype of Myd88<sup>FL/FL</sup> Cd4-cre mice resembled that of mice with a T cell-specific ablation of IL-6 signaling (Il6ra<sup>FL/FL</sup> Cd4-cre mice) as both IL-1 and IL-6 are essential for the generation of a Th1 cell response in the presence of Treg cells, but not essential in the absence of Treg cells (S.A.N., D.S., and R.M., unpublished data). It had been previously suggested that IL-6 induces the IL-1R and thus increases the sensitivity of CD4+ T cells to IL-1 (Chung et al., 2009). We therefore asked whether IL-1 regulates a subset of the IL-6-dependent gene-expression program
or whether these two cytokines regulate distinct gene-expression programs with little overlap. We isolated 2W:I-Ab+ CD4+ T cells from the draining lymph nodes of immunized Myd88FL/FL Cd4-cre, Il6raFL/FL Cd4-cre, and WT mice and analyzed polyA-containing RNAs by high-throughput sequencing (RNA-Seq). Comparison of the gene-expression profiles of MyD88-, and IL6Rα-deficient CD4+ T cells mice with that of WT controls revealed that IL-1 and IL-6 modified the expression levels of distinct sets of genes that have only a few genes in common with each other (Figure 7A). A principal component analysis (PCA) confirmed this further as the vectors for both Myd88FL/FL Cd4-cre, Il6raFL/FL Cd4-cre, and WT mice and analyzed polyA-containing RNAs by high-throughput sequencing (RNA-Seq). Comparison of the gene-expression profiles of MyD88-, and IL6Rα-deficient CD4+ T cells mice with that of WT controls revealed that IL-1 and IL-6 modified the expression levels of distinct sets of genes that have only a few genes in common with each other (Figure 7A). A principal component analysis (PCA) confirmed this further as the vectors for both Myd88FL/FL Cd4-cre and Il6raFL/FL Cd4-cre mice were not only distinct from that of WT controls but also different from each other (Figure 7B). We therefore conclude that IL-1 and IL-6 regulate different processes that are both required for an optimal CD4+ T cell response.

**DISCUSSION**

Here, we have shown that T cell-intrinsic MyD88 activation delivers an important signal for the induction of CD4+ T cell responses in vivo. While T cell-specific TLR2 signaling can augment the generation of Th17 cell responses (Reynolds et al., 2010), we did not observe a major role for T cell-specific TLR4 or TLR9 activation in the generation of either Th1 or Th17 cell responses, at least not under the conditions used in our experiments. We also did not observe an impact of IL-18 signaling on the generation of the CD4+ T cell response. Consistent with this, we found that the expression of the IL-18 receptor is reduced in antigen-specific CD4+ T cells after immunization. IL-18 is usually seen as a cytokine that promotes Th1 cell responses. However, Th1 cell responses do not universally depend on this cytokine because Th1 cells can be generated in absence of IL-18 signaling in certain experimental settings (Haring and Harty, 2009; Su et al., 2005). Instead, we found that the impairment of the CD4+ T cell response in Myd88FL/FL Cd4-cre mice was due to the lack of IL-1 signaling. The role of IL-1 in the differentiation of Th17 cells has been appreciated in recent years and our results confirm the central role for this cytokine in the generation of this T cell subset. Importantly, however, we found that IL-1 also plays an important role in the induction of Th1 cell responses.

MyD88 and, by extension, TLR activation in T cells had been previously suggested to deliver a survival signal to CD4+ T cells, in part via the upregulation of BCLXL (Gelman et al., 2006; Rahman et al., 2008). Likewise, IL-1 had also been implicated in the provision of a survival signal to CD4+ T cells (Ben-Sasson et al., 2009). The absence of such a signal could have explained the phenotype that we observed in Myd88FL/FL Cd4-cre mice. However, antigen-specific MyD88-deficient CD4+ T cells proliferated normally and did not exhibit an increased tendency to undergo apoptosis. Consistent with this
observation, antigen-specific CD4+ T cells were present at a similar frequency in Myd88<sup>FL/FL</sup> Cd4-cre mice as in WT controls. Thus, the provision of a survival signal per se is unlikely to be a major function of IL-1 in this context.

Instead, we could restore the Th1 cell response in Myd88<sup>FL/FL</sup> Cd4-cre mice in the absence of Treg cells, while the Th17 cell response remained defective, presumably because of the additional requirement of IL-1 for the differentiation of Th17 cells. IL-1 signaling had been previously suggested to compromise the suppressive capability of Treg cells (Mercer et al., 2010; O’Sullivan et al., 2006). The normal CD4+ T cell response in Myd88<sup>FL/FL</sup> Foxp3-cre mice suggests that this was not the case and points toward a function for IL-1 in either the naive or effector CD4+ T cell compartment. The reciprocal relationship between developing Th17 cells and Treg cells can lead to an increase in iTreg cells in the absence of the former cells. This is the case in mice that are deficient in IL-6 signaling, at least under some conditions (Bettelli et al., 2006; Korn et al., 2008). A similar function has also been suggested for IL-1 (Chung et al., 2009). Because Myd88<sup>FL/FL</sup> Cd4-cre mice fail to generate Th17 cells, an increase of iTreg cells after immunization could have explained the impaired Th1 cell response in these animals. We used multiple different approaches in order to detect an increase in the frequency of iTreg cells in Myd88<sup>FL/FL</sup> Cd4-cre mice but were unable to find strong evidence for this. It is therefore likely that IL-1 acts on naive or effector CD4+ T cells in order to render them refractory to the suppressive effects of naturally-occurring nTreg cells.

The generation of a Th1 cell response in Myd88<sup>FL/FL</sup> Cd4-cre mice in the absence of Treg cells allowed us to determine the role of MyD88 signaling in memory T cells. Surprisingly, Myd88<sup>FL/FL</sup> Cd4-cre mice generated antigen-specific CD4+ memory T cells at WT frequencies. However, CD4+ memory T cells were impaired in their ability to differentiate into IFN-γ-secreting T cells after secondary immunization, even under conditions where Treg cells were absent during both the primary and secondary immunization. In the context of a Th1 cell response, the IL-1 signal seems therefore to carry additional information that goes beyond the release of naive or effector CD4+ T cells from Treg cell-mediated suppression. One possibility could be that during the primary immunization, CD4+ T cells fail to fully differentiate into memory CD4+ T cells and thus are present after the primary immunization in a functionally compromised state. Alternatively, the requirement for IL-1 signaling during a secondary Th1 cell response even in the absence of Treg cells might reflect activation requirements that are unique for memory CD4+ T cells. Indeed, it has been suggested that the expansion of memory CD4+ T cells is sensitive to IL-1 signaling after secondary challenge with antigen (Lucman et al., 1992). This aspect might be an intrinsic feature of memory CD4+ T cells that is independent of their control by Treg cells and might thus explain the lack of a memory CD4+ T cell response in Myd88<sup>FL/FL</sup> Cd4-cre mice even after transient depletion of Treg cells during the secondary challenge. Further experiments will be necessary to distinguish between these possibilities.

Our study has addressed the function of CD4+ T cell-specific MyD88 signaling in the context of immunizations by using TLR ligands as adjuvants. IL-1 induced under these conditions was required for the induction of a Th1 cell response. However, it is certainly possible that other cytokines or membrane-bound signals carry similar information and are also releasing CD4+ T cell from Treg cell-mediated suppression, particularly as a consequence of the activation of other PRR systems. In this regard, it is interesting to note that IL-4 and IL-15 have been proposed to operate in this manner (Ben Ahmed et al., 2009; Thornton et al., 2004). Thus, cytokines induced by different types of infections might function in a way analogous to IL-1 by making naive or effector T cells refractory to Treg cell-mediated suppression. Therefore, it remains to be seen to what extent IL-1 is required to overcome Treg cell-mediated suppression upon live infections.

IL-6 had been previously implicated in the release of CD4+ T cells from Treg cell-mediated suppression (Pasare and Medzhitov, 2003). The observation that Myd88<sup>FL/FL</sup> Cd4-cre and Il6ra<sup>FL/FL</sup> Cd4-cre mice share a similar overall phenotype supports the notion that that IL-1 and IL-6 both cooperate in this process in vivo (S.A.N., D.S., and R.M., unpublished data). IL-6 has been reported to induce the expression of the IL-1R on CD4+ T cells (Chung et al., 2009). However, several lines of evidence point to a distinct function of these cytokines in the CD4+ T cell response. First, the phenotype of Il6ra<sup>FL/FL</sup> Cd4-cre mice tends to be stronger than that of Myd88<sup>FL/FL</sup> Cd4-cre mice and we would expect the opposite to be true if IL-6 was merely a permissive signal. Moreover, we noticed subtle but important differences in the CD4+ T cell response between these strains as the frequency of antigen-specific CD4+ T cells was reduced in Il6ra<sup>FL/FL</sup> Cd4-cre mice, but not in Myd88<sup>FL/FL</sup> Cd4-cre mice. Finally, the comparison of the gene-expression profiles of antigen-specific CD4+ T cells from Il6ra<sup>FL/FL</sup> Cd4-cre mice and Myd88<sup>FL/FL</sup> Cd4-cre mice revealed little overlap in the differentially expressed genes. It therefore seems more likely that IL-1 and IL-6 integrate different aspects of the immunological challenge. This notion is particularly interesting in light of the unique mechanism involved in IL-1 secretion, namely the requirement for inflammasome activation (Strowig et al., 2012). Inflammasome activation requires both a TLR signal and a second signal that can be triggered by microbial virulence activities (Broder and Monack, 2009). Thus, IL-1 can “report” microbial virulence activity to CD4+ T cells (Brodsky et al., 2010). IL-1 also signals the presence of live infections, at least under some circumstances (Sander et al., 2011). The critical role of IL-1 in CD4+ T cell responses might therefore reflect its unique expression requirements: while TLR activation reports on microbial origin of antigens, IL-1 expression might indicate the nature or the status of microbial threat (live versus dead, innocuous versus virulent). While antigen origin can be either microbial or not, virulence is a graded function (pathogen can be more or less virulent) as is the viability at the level of a microbial population. Interestingly, CD4+ T cell susceptibility to Treg cell-mediated suppression also appears to be a graded phenomenon and can be shifted in either direction by varying the conditions of T cell activation (Shevach, 2009). Therefore, the amount of IL-1 production might calibrate CD4+ T cell responses by controlling susceptibility of CD4+ T cells to suppression by Treg cells. Notably in this regard, the commonly used adjuvants alum and mineral oil (in Freund’s adjuvants), might function as mimics of microbial virulence or viability signals that normally induce inflammasome activation, whereas microbial TLR and other PRR ligands instruct the...
microbial origin of the admixed antigens. Consistent with this notion, at least some adjuvants are known to activate inflammasomes (Lambrecht et al., 2003). Both types of signals must be present in vaccine formulations for the efficient induction of CD4+ T cell responses.

**EXPERIMENTAL PROCEDURES**

**Generation of the Conditional Myd88 Allele**

The targeting vector was based on a derivative of the pEZ-flox vector that contained a FRT-flanked neo cassette and a diphtheria toxin gene for negative selection (Schenten et al., 2002). PCR fragments containing the appropriate restriction sites were amplified from a C57BL/6-derived BAC encoding MyD88 and inserted into the targeting vector. The culture and transfections of C57BL/6-derived Bruce4 embryonic stem cells (ESCs) has been published previously. Targeted ESC clones were identified by Southern hybridization of EcoRI-digested genomic DNA with probe A, which was PCR amplified from a MyD88-encoding BAC using primers 5’-ACTAAACCCCGG GATGCGGGTG and 5’-CCCTCTTTTAATTCTTGCACCC. Cointegration of the second loxP site was confirmed by Southern hybridization of HindIII-digested ES cell DNA with probe C. That probe was amplified with primers 5’-GACAAAAACGCGGAAACCTTGGTG and 5’-AGAAGATCAAGATACAA CACGAGCCC. The neo cassette was deleted in vitro by transfection of a FLIP recombine encoding plasmid and confirmed by Southern blotting of EcoRI-digested ESC DNA with probe A. A correctly targeted ESC clone was injected C57BL/6 albino-derived blastocysts, which were implanted into pseudopregnant C57BL/6 foster mice in order to generate chimeric mice and achieve germline transmission. Mice carrying the floxed Myd88 allele (Myd88tm1cre) mice were and born at Mendelian ratios.

**Mice**

In order to ablate MyD88 function specifically in T cells, Myd88tm1cre mice were intercrossed with mice carrying a Cd4cre allele (Lee et al., 2001). For some experiments, Cd4tm1cre mice were used as controls in order to monitor for negative effects of Cre activity. FoxP3+ Treg cell-specific deletion was achieved by intercrossing Myd88tm1cre mice with Foxp3tm1cre mice (Rubtsov et al., 2009), Il1r–/– mice, Il18r–/– mice, and Tcrd–/– and Tcrd–/– compound mutant mice were purchased from the Jackson Laboratory (Giaccum et al., 1997; Hoshino et al., 1999), Tlr9–/– mice and Tlr2–/– and Tlr4–/– compound mutant mice were maintained at the Yale School of Medicine. All mice were born on a C57BL/6 genetic background. The mice were housed in the Yale Animal Resources Center (YARC) and the experiments were done with approval by the Yale University Institutional Animal Care and Use Committee (IACUC).

**Immunizations**

The mice were immunized in both feet with either 100 µg/mouse OVA (Sigma) or 100 µg/mouse 2W15 peptide (EAGLALANVAVDVA, Genscript) plus 10 µg/mouse LPS (Sigma) emulsified in incomplete Freund’s adjuvant (Sigma). For some experiments, LPS was replaced with either 20 µg/mouse CpG DNA 1826 (Keck Biotechnology Resource Laboratory, Yale Medical School), 50 µg/mouse poly(I:C) (Inovirgen), 20 µg/mouse Peptidoglycan (Inovirgen), or 150 µg/mouse heat-inactivated M. tuberculosis (Sigma). The effects of contaminating endotoxin in the OVA preparations were controlled by using endotoxin-free OVA (Hyglos/Biovendor) in key experiments. CD4+ memory responses were induced similarly with the exception that one foot was used for the primary immunization, while the other foot was used for the secondary immunization 30–60 days later.

**Antibodies and Other Reagents**

All standard antibodies for flow cytometry were purchased from either BD Biosciences or Ebiolosiences. FITC-PNA was purchased from Biovector, and the α-activated caspase-3 antibody was obtained from R&D Systems. PE-conjugated 2W1-A6 tetramers were a gift from Marc Jenkins (University of Minnesota). Bromodeoxyuridine (BrdU) incorporation was measured with the BrdU staining kit from BD Biosciences. The α-CD25 antibody PC61 was purified from hybridoma cells adapted to serum-free medium and grown in Cellline 2-compartment bioreactors (BD Biosciences). MyD88 protein was detected by immunoblotting with a goat α-MyD88 antibody (R&D Systems).

**T Cell Assay**

CD4+ T cells were isolated from the popliteal and inguinal lymph nodes 7 days after immunization by magnetic cell sorting with αCD4 beads (Miltenyi Biotech). The cells were seeded in round-bottom 96-well plates at a concentration of 100,000 cells per well in the presence of 300,000 irradiated splenocytes in the presence of serial dilutions of OVA, starting at 900 µg/ml. Proliferation was measured on day 4 following the addition of 3H-thymidine for the last 24 hr and the secretion of IFN-γ and IL-17 was measured by ELISA or intracellular flow cytometry.

**Treg Cell Depletion**

Treg cells were depleted in vivo with 150 µg/mouse of an αCD25 antibody (clone PC61) by intravenous injection 3 days prior to immunization. Depletion of Treg cells was confirmed on the day of immunization by flow cytometry upon staining of CD4+ T cells from the blood with an αCD25 antibody (clone 7D4).

**Bone Marrow Chimeras**

Bone marrow from Tcrd–/– and Tcrd–/– compound mutant mice was mixed in a ratio of 3:1 with bone marrow isolated from C57BL/6 WT mice, Il1r–/– mice, Il18r–/– mice, Tlr9–/– mice, or Tlr2–/– and Tlr4–/– compound mutant mice. We injected 10–105 total cells per mouse into Rag2–/– mice that had been lethally irradiated with 900 rad 1 day prior to the bone marrow transfer. The reconstitution of the hematopoetic system was confirmed after 6 weeks by flow cytometry.

**RNA-Seq**

Antigen-specific CD4+ T cells were isolated by flow cytometry from a total of 15–20 immunized mice per genotype. RNA was isolated from approximately 100,000 cells per genotype with the RNeasy isolation kit (QIAGEN) and processed and sequenced by the Yale Center for Genome Analysis (YCGA).

**Statistical Analysis**

Data were analyzed with a Student’s t test with Prism4 (Graphpad Software). In case with abnormal distribution, the data were also analyzed with a Mann-Whitney test using the same software. Unless not mentioned specifically, data are shown as mean ± SD. p values: *, p ≤ 0.05; **, p ≤ 0.005; ***, p ≤ 0.0005; n.s., not significant. PCA analysis of gene-expression data was performed with R (2.15.3).

**ACCESSION NUMBERS**

The accession number for the sequence reported in this paper is X.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.10.025.

**AUTHOR CONTRIBUTIONS**

D.S. generated the conditional Myd88 allele, designed and performed experiments, and wrote the manuscript. S.A.N. designed and performed experiments and wrote the manuscript; S.Y. did the genotyping and purification of antibodies; X.Y. and H.Z. helped with the bioinformatics analysis; H.K.L., I.B., L.P., and B.Y. provided overall intellectual direction and wrote the manuscript.

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