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Critical Regulation of Early Th17 Cell Differentiation by Interleukin-1 Signaling

Yeonseok Chung,[1] Seon Hee Chang,[1] Gustavo J. Martinez,[1] Xuexian O. Yang,[1] Roza Nurieva,[1] Hong Soon Kang,[2] Li Ma,[3] Stephanie S. Watowich,[1] Anton M. Jetten,[2] Qiang Tian,[3] and Chen Dong[1,*]
[1]Department of Immunology, M.D. Anderson Cancer Center, Houston, TX 77030, USA
[2]Cell Biology Section, Laboratory of Respiratory Biology, National Institute on Environmental Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA
[3]Institute for Systems Biology, Seattle, WA 98103, USA
*Correspondence: cdong@mdanderson.org
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SUMMARY

T helper (Th) 17 cells have been recently discovered in both mouse and human. Here we show that interleukin-1 (IL-1) signaling on T cells is critically required for the early programming of Th17 cell lineage and Th17 cell-mediated autoimmunity. IL-1 receptor1 expression in T cells, which was induced by IL-6, was necessary for the induction of experimental autoimmune encephalomyelitis and for early Th17 cell differentiation in vivo. Moreover, IL-1 signaling in T cells was required in dendritic cell-mediated Th17 cell differentiation from naive or regulatory precursors and IL-1 synergized with IL-6 and IL-23 to regulate Th17 cell differentiation and maintain cytokine expression in effector Th17 cells. Importantly, IL-1 regulated the expression of the transcription factors IRF4 and RORγt during Th17 cell differentiation; overexpression of these two factors resulted in IL-1-independent Th17 cell polarization. Our data thus indicate a critical role of IL-1 in Th17 cell differentiation and this pathway may serve as a unique target for Th17 cell-mediated immunopathology.

INTRODUCTION

Th17 cells are a unique lineage of T cells that have been shown to be important in autoimmunity and clearance of mucosal infection by producing proinflammatory cytokines IL-17, IL-17F, and IL-22 (Dong, 2008; Ouyang et al., 2008). Compared with other Th cell lineages, Th17 cells have unique genetic programs. The transcription factor STAT3 is essential for Th17 cell differentiation in mouse and human possibly via induction of two orphan nuclear receptors RORγt and RORα (Laurence et al., 2007; Ma et al., 2008; Milner et al., 2008; Yang et al., 2007, 2008b). RORγt was first identified as a Th17 cell-specific transcription factor that is sufficient and necessary for Th17 cell differentiation (Ivanov et al., 2006). RORα plays a synergistic and somewhat redundant function with RORγt during Th17 cell polarization (Yang et al., 2008c). Interferon regulatory factor 4 (IRF4) was originally reported to be important in Th2 cell differentiation (Lohoff et al., 2002; Rengarajan et al., 2002). However, a recent study revealed that IRF4-deficient T cells are completely impaired in Th17 cell polarization. Indeed, IRF4-deficient mice are resistant to induction of Th17 cell-mediated experimental autoimmune encephalomyelitis (EAE) (Brustle et al., 2007). An environmental toxin sensor, aryl hydrocarbon receptor (Ahr), was found to be highly expressed in Th17 and regulatory T (Treg) cells and to regulate Th17 cytokine production, especially IL-22 (Quintana et al., 2008; Veldhoen et al., 2008). In contrast, the regulatory T cell transcription factor Foxp3 inhibits Th17 cell polarization by binding to RORγt and RORα to inhibit their transcriptional activity (Yang et al., 2008b; Zhou et al., 2008).

IL-6 and transforming growth factor-β (TGF-β) have been reported as the minimal requirements for murine Th17 cell differentiation from naive $CD4^+$ T cells (Bettelli et al., 2006; Veldhoen et al., 2006). During Th17 cell differentiation initiated by TGF-β and IL-6, IL-1 appears to play an accessory role (Veldhoen et al., 2006). IL-21 induced by IL-6 acts in an autocrine fashion and drives Th17 cell generation together with TGF-β (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). However, human T cells were originally shown to require IL-1 plus IL-23 or IL-6 to differentiate into Th17 cells and addition of TGF-β inhibits this polarization (Acosta-Rodriguez et al., 2007; Wilson et al., 2007). More recent studies described that TGF-β is required for Th17 cell generation from naive $CD4^+$ T cells (Manel et al., 2008; Yang et al., 2008a). However, the different roles of IL-1 for Th17 cell commitment in mouse and human still remain elusive. Mice defective in IL-1R1 signaling were reported to be resistant to EAE and to exhibit a severe defect in the generation of IL-17-producing T cells (Sutton et al., 2006), suggesting that IL-1 is important for mouse Th17 cell regulation in vivo. However, it was not clear from this study whether IL-1 signaling in T cells was necessary for their differentiation into Th17 cells.

In the current study, we examined the role of IL-1 in murine Th17 cell regulation. Our results demonstrate that IL-1 signaling in T cells was required for early Th17 cell differentiation in vitro and in vivo. IL-1 regulated the expression of IRF4 and RORγt and overexpression of these two factors resulted in IL-1-independent Th17 cell differentiation. Therefore, similar to the human system, IL-1 plays a unique, nonredundant role during murine Th17 cell polarization.

RESULTS

IL-1R1 Expression Is Upregulated in Th17 Cells

In a search for genes upregulated in Th17 over Th1 cells, we found that IL-1 receptor 1 (IL-1R1) mRNA was expressed at

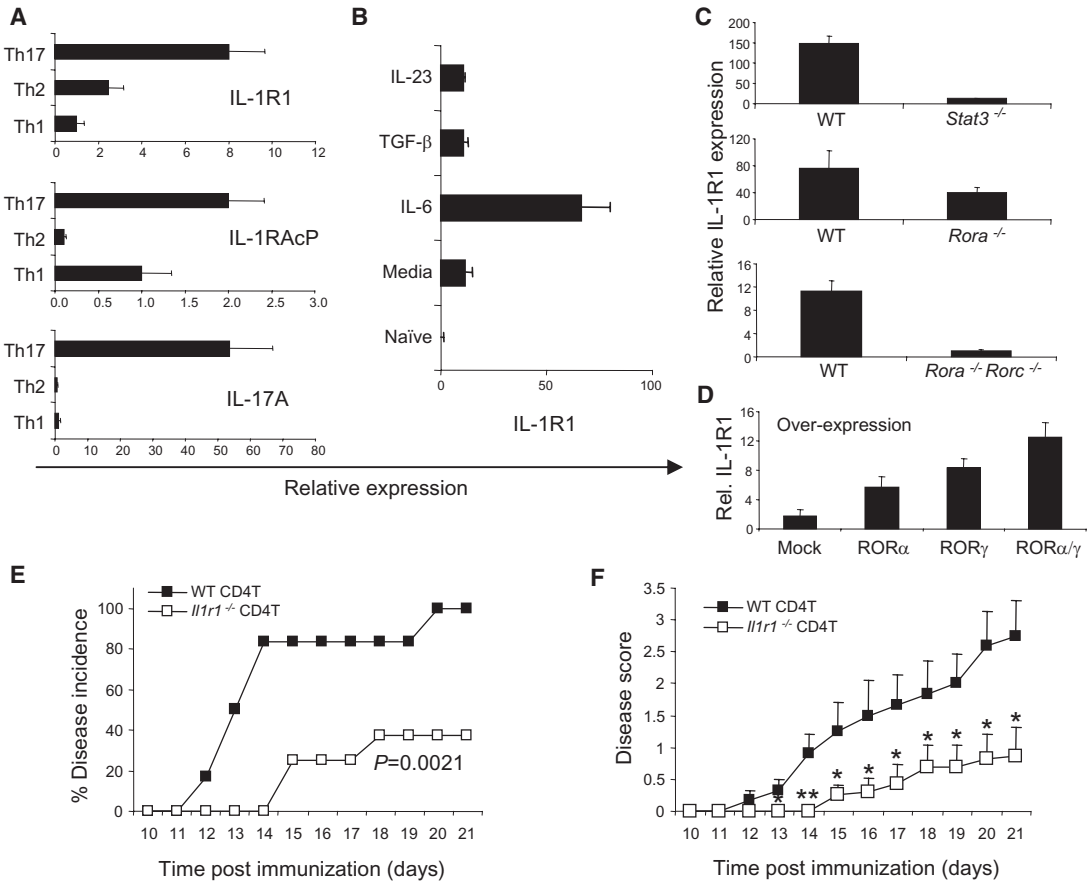

Figure 1. IL-1R1 Expression on Th17 Cells Is Critical for Induction of EAE

(A) $CD25^{-}CD44^{lo}CD62^{hi}CD4^{+}$ T cells (naive $CD4^{+}$ T) were FACS sorted and polarized under Th1, Th2, or Th17 cell condition for 4 days.

(B) Naive $CD4^{+}$ T cells were stimulated with anti-CD3 plus anti-CD28 in the presence of indicated cytokine for 24 hr before mRNA expression of IL-1R1 was analyzed.

(C) FACS-sorted naive $CD4^{+}$ T cells from WT or *Stat3*$^{-/-}$, *Rora*$^{-/-}$, or *Rora*$^{-/-}$*Rorc*$^{-/-}$ mice were cultured under Th17 cell condition for 4 days.

(D) Naive OT-II $CD4^{+}$ T cells were activated with Ova peptide-pulsed splenic APCs under the neutral (anti-IL-4 and anti-IFN-γ) and coinfected with two bicistronic retroviruses expressing RORα-GFP or GFP vector and RORγt-hCD2 or hCD2. $GFP^{+}hCD2^{+}$ cells were FACS sorted.

In (A), (C), and (D), cells were restimulated with plate-bound anti-CD3 for 4 hr before mRNA expression was analyzed by real-time PCR. Data were normalized with expression amounts of *Actb* (mean ± SD).

(E and F) $CD4^{+}$ T cells were sorted from WT or *Il1r1*$^{-/-}$ mice and i.v. transferred into *Rag1*$^{-/-}$ mice. The recipient mice were induced EAE and disease incidence (E) and score (F) were measured daily and means ± SEM of all mice in each group were shown (F).

Data represent two independent experiments with consistent results. *$p < 0.05$; **$p < 0.01$ in comparison with WT recipients.

higher amounts in Th17 cells when compared with Th1 cells (data not shown). We then examined the expression of IL-1 receptor components in in vitro polarized Th1, Th2, and Th17 cells. We observed that IL-1R1 mRNA was highly expressed in Th17 cells compared with Th1 and Th2 cells (Figure 1A). Expression of IL-1R-accessory protein (IL-1RAcP) was also moderately increased. To understand the regulation of IL-1R1 expression, we activated naive $CD4^{+}$ T cells in the presence of anti-CD3 and anti-CD28 with or without IL-6, IL-23, or TGF-β. TCR and costimulation signaling elevated IL-1R1 mRNA expression (Figure 1B). Among the cytokines tested, IL-6 appeared to be a critical factor for IL-1R1 upregulation whereas IL-23 or TGF-β alone minimally affected its expression (Figure 1B).

Th17 cell differentiation requires several transcription factors such as STAT3, RORα, and RORγ (Ivanov et al., 2006; Yang et al., 2007, 2008c). To determine whether these transcription factors are required for IL-1R1 expression in $CD4^{+}$ T cells, we isolated naive $CD4^{+}$ T cells from mice defective in these transcription factors and cultured them in a Th17 cell-polarizing condition (Yang et al., 2007, 2008c). As shown in Figure 1C, IL-1R1 expression was severely impaired in *Stat3*$^{-/-}$ T cells. *Rora*$^{-/-}$ T cells showed a moderate decrease in IL-1R1 expression and *Rora*$^{-/-}$*Rorc*$^{-/-}$ T cells exhibited a more severe defect in IL-1R1 expression. Consistently, when we overexpressed RORα, RORγ, or both in T cells cultured under neutral conditions (Yang et al., 2008c), both RORα and RORγ induced the upregulation of IL-1R1 mRNA in T cells (Figure 1D).

IL-1 Signaling in T Cells Is Necessary for Th17 Responses In Vivo

IL-1R1 is necessary for the induction of EAE disease in mice (Brustle et al., 2007), which could be a result of defective IL-1 signaling in

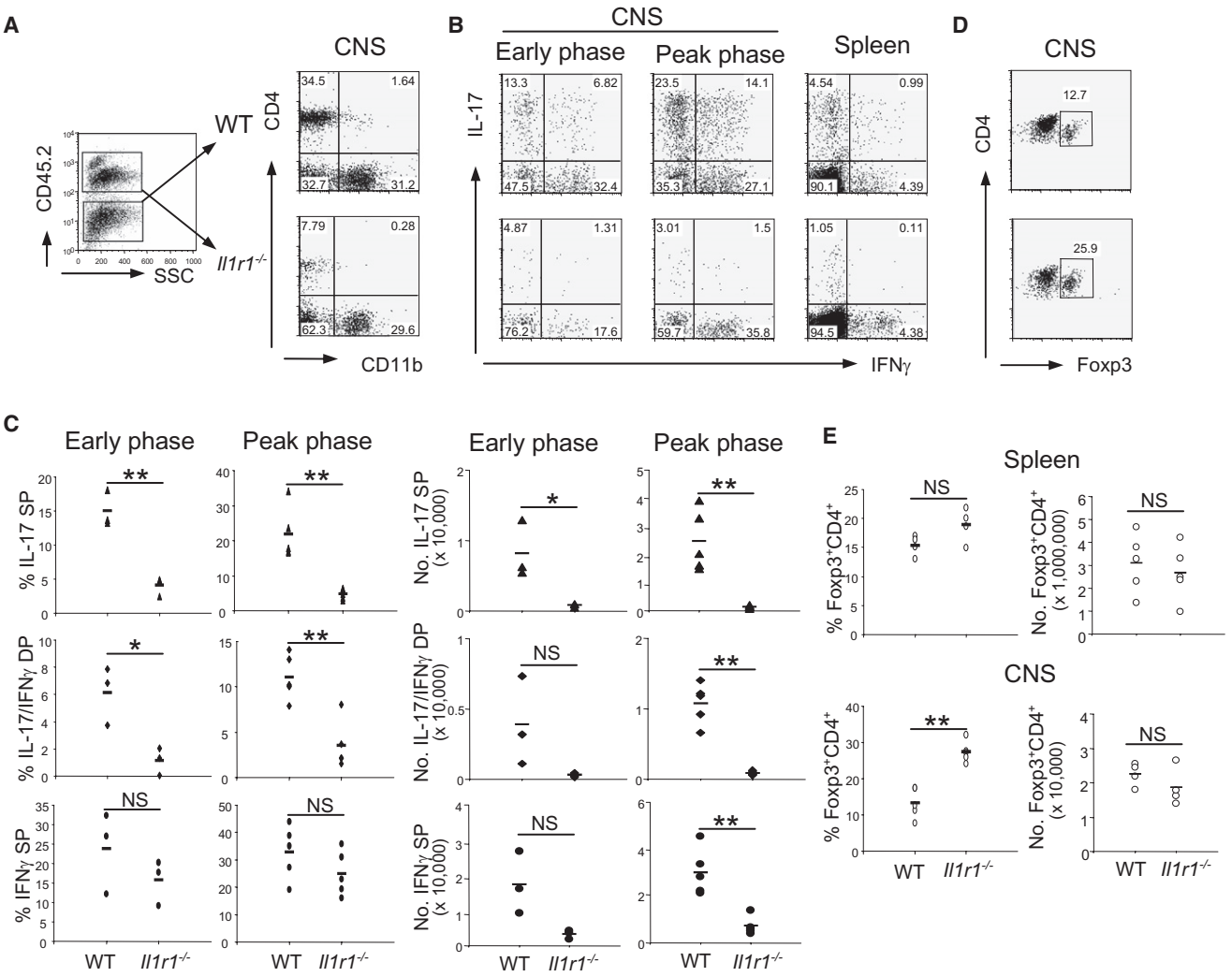

Figure 2. IL-1 Signaling in T Cells Is Required for Th17 Cell Development in EAE Model

Mixed bone marrow chimeric mice were generated and induced EAE. Mononuclear cells in central nervous system (CNS) and spleen were stained with anti-CD45.2 to distinguish WT and *Il1r1*$^{-/-}$ compartments and analyzed for CD4$^+$ and CD11b$^+$ cell presence (A), cytokine production profiles in CD4$^+$ T cells after PMA plus ionomycin (B and C) or MOG stimulation (B, only for spleen), or Foxp3$^+$ cell in CD4$^+$ T cells (D and E). The mice were divided into two groups based on the disease severity (score, 0.5–2 for early phase; 2.5–4.0 for peak phase). *$p < 0.05$; **$p < 0.01$ in comparison with WT compartments. Mean values are shown as horizontal bars (C and E). Data shown represent two independent experiments with consistent results.

the innate immune system and/or in T cells. Because Th17 cells highly express IL-1R1 and IL-1RAcP, we next sought to determine the role of IL-1R1 in CD4$^+$ T cells by adoptive transfer of CD4$^+$ T cells from wild-type (WT) or *Il1r1*$^{-/-}$ mice into *Rag1*$^{-/-}$ mice. Upon immunization with MOG, all recipient mice containing WT T cells developed EAE whereas only 3 out of 8 (37.5%) mice with *Il1r1*$^{-/-}$ T cells developed EAE, with significantly delayed onset (Figure 1E). Moreover, the severity of EAE was lower in mice with *Il1r1*$^{-/-}$ T cells than in those with WT cells (Figure 1F).

To illustrate more precisely the functional defects of *Il1r1*$^{-/-}$ T cells in EAE, we generated mixed bone marrow (BM) chimeras by transferring a mixture of CD45.1$^+$ WT and CD45.2$^+$ *Il1r1*$^{-/-}$ BM cells into sublethally irradiated *Rag1*$^{-/-}$ mice (Figure S1 available online). Eight weeks later, we induced EAE in the recipient mice. To analyze the T cell phenotypes during disease progression, we divided the EAE mice into two groups—early and peak phases—based on the disease scores (score, 0.5–2 for early phase; 2.5–4.0 for peak phase). In CNS, the numbers of CD4$^+$ T cells were higher in WT population than in *Il1r1*$^{-/-}$ population ($9.1 \times 10^4 \pm 3.4 \times 10^4$ versus $3.3 \times 10^4 \pm 1.0 \times 10^4$ cells, $p = 0.016$), whereas the numbers of CD11b$^+$ macrophages were comparable between the two populations ($9.4 \times 10^4 \pm 1.3 \times 10^4$ versus $10.2 \times 10^4 \pm 1.6 \times 10^4$ cells, $p = 0.105$). Moreover, we observed that WT CD4$^+$ T cells contained a higher percentage of IL-17 producers compared to *Il1r1*$^{-/-}$ CD4$^+$ T cells (Figures 2B and 2C). The percentage of IFN-γ^+IL-17$^+$ cells was also remarkably higher in WT compared with the *Il1r1*$^{-/-}$ CD4$^+$ T cell population whereas the percentages of IFN-γ^+IL-17$^-$ cells were not different. Moreover, the absolute numbers of IL-17-producing WT CD4$^+$ T cells were profoundly higher than those found in

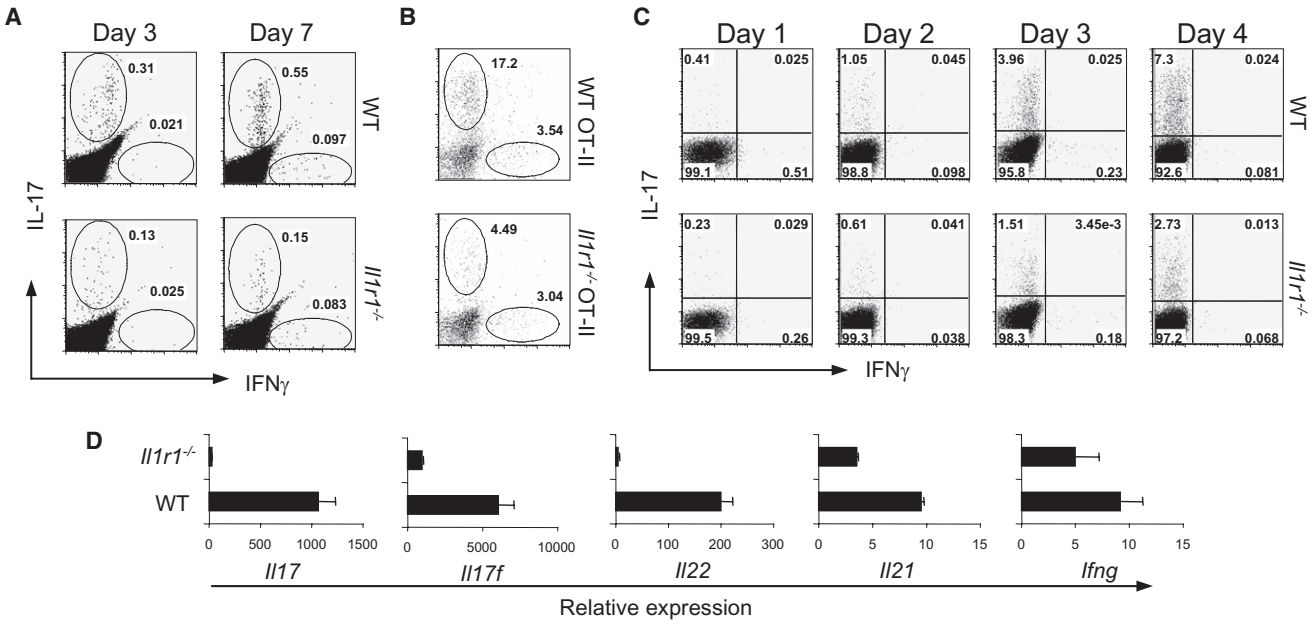

Figure 3. IL-1 Signal Is Required for Early Differentiation of Th17 Cells

(A) WT and *Il1r1*$^{-/-}$ mice were subcutaneously immunized with KLH in CFA. Three or seven days later, lymphoid cells from draining lymph nodes were restimulated with KLH overnight and IL-17- or IFN-γ-expressing cells were measured by intracellular staining.

(B) FACS-sorted naive OT-II T cells from WT or *Il1r1*$^{-/-}$ OT-II mice were intravenously transferred into congenic (CD45.1) mice. The recipient mice were immunized with $OVA_{323-339}$ peptide in CFA. Three days later, lymphoid cells from draining lymph nodes were restimulated with PMA plus ionomycin for 5 hr and IL-17- or IFN-γ-expressing cells were measured by intracellular staining. Data shown are on gated CD45.1$^+$ CD4$^+$ T cells.

(C and D) FACS-sorted naive CD4$^+$ T cells from WT or *Il1r1*$^{-/-}$ mice were cocultured with BM-derived DCs (WT) in the presence of soluble anti-CD3 Ab (0.2 μg/ml) and LPS (100 ng/ml) plus TGF-β (1 ng/ml). IL-17- or IFN-γ-expressing cells were measured by intracellular staining (C). On day 4, mRNA expression was assessed by real-time RT-PCR after restimulation of T cells by anti-CD3 for 4 hr (D).

Data shown represent two independent experiments and normalized with expression amounts of *Actb* (mean ± SD).

Il1r1$^{-/-}$ CD4$^+$ T cell population (Figure 2C). Because Treg cells can also migrate into the inflamed CNS in a CCR6-dependent manner (Yamazaki et al., 2008), we also analyzed the Treg cell population by staining Foxp3. Of note, we observed a higher percentage of Foxp3$^+$ cells in *Il1r1*$^{-/-}$ relative to WT population and this pattern was more obvious in the inflamed CNS (Figures 2D and 2E). However, the absolute number of Foxp3$^+$ T cells in WT and *Il1r1*$^{-/-}$ population was comparable because of the lower percentage of total *Il1r1*$^{-/-}$ CD4$^+$ T cell in the CNS (Figures 2A and 2E). Therefore, *Il1r1*$^{-/-}$ CD4$^+$ T cells migrated into the inflamed tissue much less efficiently than did WT CD4$^+$ T cells and were defective in IL-17 production. Further examination of MOG-reactive T cells in spleen revealed that IL-17- but not IFN-γ-expressing CD4$^+$ T cells were also reduced in the *Il1r1*$^{-/-}$ population (Figure 2B), suggesting that IL-1 signaling in CD4$^+$ T cells is necessary for proper Th17 cell differentiation in vivo.

IL-1 Signaling in T Cells Functions to Promote Early Th17 Differentiation

Il1r1$^{-/-}$ CD4$^+$ T cells were defective in generating Th17 cells in the EAE model. IL-1 signaling may mediate early Th17 cell differentiation or the maintenance and expansion of polarized Th17 cells. To test these two possibilities, we immunized WT and *Il1r1*$^{-/-}$ mice with KLH in CFA and analyzed IL-17 and IFN-γ-producing cells upon ex vivo KLH restimulation. As depicted in Figure 3A, as early as day 3, the *Il1r1*$^{-/-}$ CD4$^+$ T cell population contained a significantly lower frequency of IL-17$^+$ cells compared to WT. On day 7, increased percentages of IL-17$^+$ cells were found in WT but not in *Il1r1*$^{-/-}$ mice whereas the percentage of IFN-γ$^+$ cells was comparable between WT and *Il1r1*$^{-/-}$ mice. The Th17-specific defect of *Il1r1*$^{-/-}$ T cells was confirmed with supernatants from KLH-restimulated splenocytes (Figure S2). To directly ask whether the observed defect in the early Th17 polarization is T cell intrinsic, we transferred naive CD4$^+$ T cells from either WT or *Il1r1*$^{-/-}$ mice carrying the chicken ovalbumin (OVA)-specific TCR transgene (OT-II) into congenic mice and immunized the recipients with $OVA_{323-339}$ peptide in CFA. As shown in Figure 3B, the frequency of IL-17-producing WT OT-II T cells was significantly higher compared with *Il1r1*$^{-/-}$ OT-II cells within 3 days after immunization (15.07 ± 4.06 versus 4.19 ± 2.35, $p = 0.045$). These data overall indicate that the requirement of IL-1R1 signaling for early differentiation of Th17 cells in vivo is CD4$^+$ T cell intrinsic.

The above results suggest that IL-1 signaling is required in the early phase of Th17 cell differentiation in vivo. This is surprising considering that in vitro, IL-1 only moderately enhances Th17 cell differentiation in purified CD4$^+$ T cells (Veldhoen et al., 2006). We thus turned to a different Th17 cell differentiation system in which T cells were cocultured with dendritic cells (DCs) in the presence of soluble anti-CD3, LPS, and TGF-β (Veldhoen et al., 2006). Naive WT or *Il1r1*$^{-/-}$ CD4$^+$ T cells were activated with either WT or *Il1r1*$^{-/-}$ bone marrow-derived DCs.

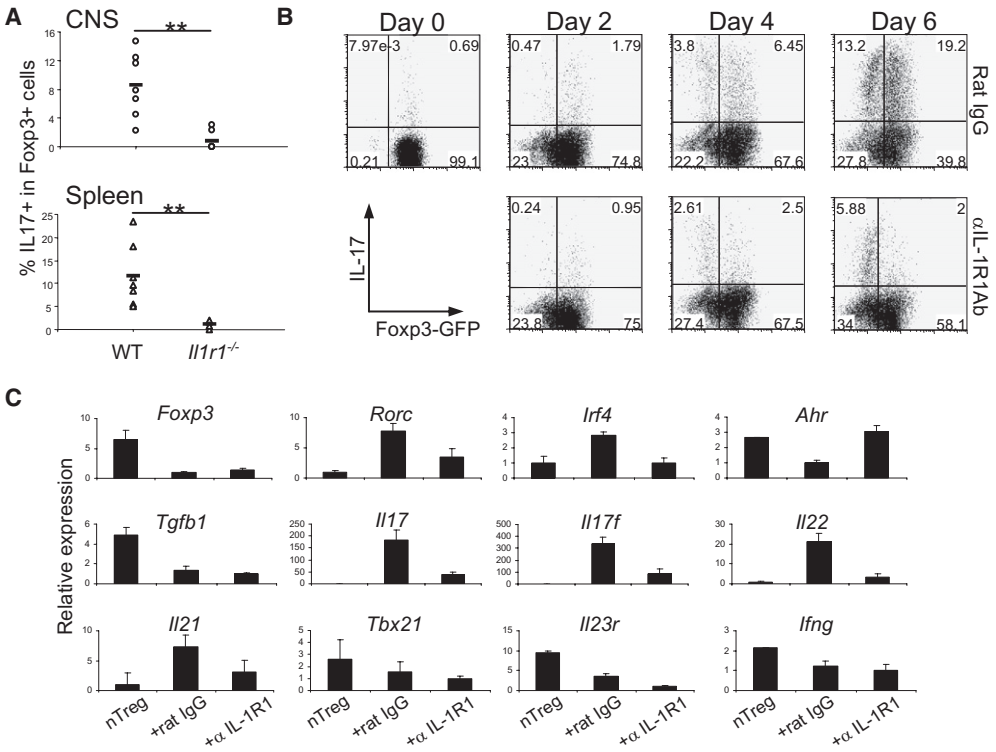

Figure 4. Conversion of Foxp3⁺ T Cells into Th17 Cells Requires IL-1 Signal

(A) Mononuclear cells in EAE-induced mixed bone marrow chimeric mice as described in Figure 2 were analyzed for IL-17 and Foxp3 expression. Data shown are on gated $CD4^+$ $Foxp3^+$ cells. Mean values are shown as horizontal bars. **$p < 0.01$ in comparison with WT compartments. Data shown represent two independent experiments with consistent results.

(B and C) FACS-sorted $CD4^+GFP^+$ cells from naive Foxp3-GFP reporter mice were cultured with DCs and in the presence of soluble anti-CD3, LPS, and TGF-β plus anti-IL-1R1 or rat IgG as a control. IL-17- or GFP-expressing cells were measured by intracellular staining (B). On day 4, mRNA expression was assessed by real-time RT-PCR after restimulation of T cells by anti-CD3 for 4 hr (C). mRNA expression in fresh FACS-sorted $CD4^+GFP^+$ cells, stimulated with anti-CD3 for 4 hr, was used as a control.

Data shown represent two independent experiments and normalized with expression amounts of *Actb* (mean ± SD).

Deficiency of IL-1R1 on T cells but not on DCs resulted in a great reduction in all Th17 cytokines including IL-17, IL-17F, IL-22, and IL-21 (Figure S3). Therefore, the IL-1 signal in $CD4^+$ T cells rather than DCs is required for Th17 cell generation.

To further address the role of IL-1 during early Th17 cell commitment, we utilized the same DC-T cell coculture system as described above and examined the kinetics of Th17 cell generation. IL-17-producing T cells started to appear 2 days after culture with evident difference in WT and *Il1r1*$^{-/-}$ T cells and thereafter (Figure 3C). Moreover, the staining intensity of anti-IL-17 in WT cells was higher than that of *Il1r1*$^{-/-}$ (mean fluorescence intensity on day 4, WT; 402 versus *Il1r1*$^{-/-}$; 211), indicating that WT Th17 cells are more potent IL-17 producers. Real-time PCR analysis after anti-CD3 restimulation showed an efficient induction of all Th17 cytokines (IL-17, IL-17F, IL-22, and IL-21) in WT but not in *Il1r1*$^{-/-}$ T cells (Figure 3D). These in vivo and in vitro experiments together demonstrate that IL-1 signaling is critical for the early differentiation stages of the Th17 cell lineage.

Conversion of Treg Cells into IL-17-Producing Cells Requires IL-1 Signaling

In our EAE study with mixed bone marrow chimeras, we observed higher percentages of $Foxp3^+$ T cells in the *Il1r1*$^{-/-}$ population, compared with WT, particularly in the inflamed CNS (Figures 2D and 2E). This observation led us to hypothesize that IL-1 signaling might be involved in a reciprocal regulation between Treg and Th17 cells. IL-1 does not seem to be involved in the generation of naturally occurring $Foxp3^+$ Treg cells because naive *Il1r1*$^{-/-}$ mice have normal numbers of $Foxp3^+$ T cells in secondary lymphoid organs (data not shown). Recent studies showed that $Foxp3^+$ T cells can be reprogrammed into IL-17-producing cells (Yang et al., 2008b). To examine whether IL-1 signaling is necessary for the conversion of $Foxp3^+$ cells into IL-17-producing T cells in vivo, we analyzed $IL\text{-}17^+$ cells that coexpressed Foxp3 in the $CD4^+$ T cells from our mixed BM-chimeric mice after EAE induction. We reasoned that $IL\text{-}17^+Foxp3^+$ T cells were likely to be Treg cells on their way to become Th17 cells because very few $Foxp3^+$ T cells in naive mice produce IL-17. As shown in Figure 4A, ~10% of splenic and CNS $Foxp3^+$ WT T cells produced IL-17 after EAE induction; however, this population was almost completely absent within *Il1r1*$^{-/-}$ $Foxp3^+$ T cells in the same animals.

To directly determine the role of IL-1 in the induction of IL-17 in $Foxp3^+$ T cells, we utilized Foxp3-GFP reporter mice (Fontenot et al., 2005). $CD4^+GFP^+$ T cells from naive Foxp3-GFP reporter mice were cultured with DCs in the presence of soluble anti-CD3,

LPS, and TGF-β. In some samples, we added anti-IL-1R1 in the culture to block IL-1 signaling. IL-17$^+$ cells started to appear on day 2 after activation of Treg cells (Figure 4B). Blocking IL-1R1 greatly reduced the IL-17$^+$ population, especially within the GFP$^+$ (Foxp3$^+$) population. This pattern became more apparent on day 6 with more than 50% reduction of IL-17$^+$ cells in GFP$^-$ population and nearly 90% reduction in GFP$^+$ population (Figure 4B). Of note, little difference in GFP downregulation was observed between cells treated with control Ab and IL-1R1 blocking Ab, indicating no role of IL-1 signaling in Foxp3 downregulation. In support of these data, real-time RT-PCR analysis revealed that Foxp3$^+$ Treg cells after activation remarkably downregulated Foxp3 and TGF-β1 expression with no difference between control Ab and IL-1R1Ab treatment (Figure 4C). In contrast, those cultured in the presence of control Ab expressed increased levels of RORγ and IRF4 transcripts whereas cells treated with IL-1R1 blocking Ab did not. Moreover, the expression of IL-17, IL-17F, IL-22, and IL-21 was greatly increased in T cells in the absence of IL-1R1 blockade (Figure 4C). The induction of Th17 cell transcription factors and cytokines appeared to be specific as indicated by the fact that T-bet and IFN-γ expression remained largely unchanged.

Collectively, these data demonstrate that similar to naive T cells, IL-1 signaling is critical for inducing the phenotypic conversion of natural Treg into Th17 cells, which is independent of Foxp3 downregulation.

IL-1 Signaling Maintains Th17 Cells in the Absence of TCR Stimuli

The regulation of Th17 cell maintenance after initial differentiation has not been well understood. IL-23 has been suggested as a key factor required for Th17 cell maintenance rather than differentiation (Veldhoen et al., 2006). On the other hand, IL-1R1 expression, similar to that of IL-23R, is also upregulated by IL-6 (Figure 1B), raising the possibility that IL-1 signaling may have an additional function in polarized Th17 cells. To address this hypothesis, naive CD4$^+$ T cells from IL-17F-RFP reporter mice (Yang et al., 2008b) were stimulated under Th17 cell conditions and RFP$^+$ cells were sorted to enrich Th17 cells. More than 50% of the sorted cells expressed Th17 cytokines (IL-17, IL-17F) with less than 1% of Foxp3$^+$ cells and virtually no IFN-γ$^+$ cells (Figure 5A). These cells were labeled with CFSE and cultured with IL-23 or IL-1 alone, IL-23 plus IL-1, or IL-6 plus TGF-β in the absence of TCR stimulation. Of interest, Th17 cells cultured with IL-1 but not IL-23 proliferated in the absence of TCR stimulation, which was enhanced by IL-23 (Figure 5B, top). Compared with the starting cells, Th17 cells cultured with medium alone produced reduced amounts of IL-17 and IL-17F (Figures 5A and 5B). In Th17 cells cultured with IL-23 alone or IL-6 plus TGF-β, there was no improvement over the medium-only condition. However, addition of IL-1 to the culture effectively maintained the cytokine production in Th17 cells (Figure 5B). Moreover, Th17 cells cultured with IL-1 plus IL-23 exhibited enhanced maintenance of Th17 cytokine profiles. None of the culture condition showed any increased expression of IL-4, IFN-γ (Figure 5B, bottom), or Foxp3 (data not shown).

Unexpectedly, in the above experiment, we detected increased levels of IL-17, IL-17F, and IL-22 in the supernatants from cells treated with IL-1 (Figure 5C). Therefore, polarized Th17 cells produced Th17 cytokines upon IL-1 signal in the absence of TCR stimulation. Consistent with intracellular staining profiles, IL-23 alone did not induce these Th17 cytokines but greatly synergized with IL-1, especially in inducing IL-17 and IL-22 secretion (Figure 5C). After anti-CD3 restimulation, cells cultured with IL-1 alone or together with IL-23 produced Th17 cytokines at least comparable to original Th17 cells whereas the other conditions failed to do so (Figure 5D). These observations indicate that IL-1 signaling not only maintains Th17 cell phenotypes after polarization but also triggers Th17 cytokine production even in the absence of TCR stimulation.

IL-1 Regulates Th17 Cell Differentiation in the Absence of Exogenous TGF-β

Our in vivo and in vitro data thus far have revealed an essential role of IL-1 signaling for early Th17 cell differentiation. However, it was also shown that purified T cells differentiated into Th17 cells in the presence of TGF-β and IL-6. To better understand the IL-1 effect on T cell polarization, we treated naive CD4$^+$ T cells with anti-CD3 and anti-CD28 in the presence of defined cytokine combination. In this experimental setting, IL-6 plus TGF-β induced comparable IL-17$^+$ cells between WT and *Il1r1*$^{-/-}$ T cells, indicating no autocrine effect of IL-1 in this condition. However, addition of IL-1 in WT but not *Il1r1*$^{-/-}$ culture greatly increased the IL-17$^+$ population and induced higher amounts of IL-17 as measured by mean fluorescence intensity (Figure 6A). Because TGF-β is essential for Th17 cell commitment, downregulation of Foxp3 by IL-21 or IL-6 is one of the crucial steps during Th17 cell differentiation. Foxp3 induction by TGF-β was not hampered by addition of IL-1 (Figure 6B), suggesting that IL-1 contributes to Th17 cell polarization via a mechanism distinct from IL-6 and IL-21.

In the absence of exogenous TGF-β, IL-6 and IL-23 induced only low levels of IL-17 production (Figure 6C), whereas IL-1 and IL-23 barely induced IL-17 (data not shown). However, the combination of IL-6, IL-23, and IL-1 greatly induced IL-17$^+$ cells (Figure 6C). This was dependent on TGF-β as indicated by the fact that neutralizing anti-TGF-β completely abolished IL-17 induction. Th17 cells generated by combination of IL-6, IL-23, and IL-1 produced all Th17 cytokines (IL-17, IL-17F, IL-22) whereas Th17 cells generated by IL-6+TGF-β produced little amount of IL-22 (Figure 6D). Therefore, IL-1 synergizes with IL-6 and IL-23 to induce Th17 cell differentiation at a low concentration of TGF-β.

IL-1 Signaling Induces IRF4 and RORγt Expression during Early Th17 Polarization

To understand the molecular mechanism by which IL-1 signaling induces Th17 cell polarization, we stimulated naive CD4$^+$ T cells in the presence of anti-CD3 plus anti-CD28 with or without IL-1, IL-6, and/or IL-23. Cells stimulated with IL-6 moderately upregulated RORγt but not IRF4 expression; however, the combination of IL-6 and IL-1 significantly increased the expression of these transcription factors, particularly IRF4 (Figure 7A). The combination of IL-6, IL-1, and IL-23 further enhanced the expression of RORα and Ahr.

The above data suggest that IL-1 may synergize with IL-6 to induce IRF4 and RORγt expression during Th17 cell polarization. To further test this idea, we compared gene expression of WT and *Il1r1*$^{-/-}$ CD4$^+$ T cells during Th17 cell differentiation activated by DCs in the presence of LPS and TGF-β. Real-time

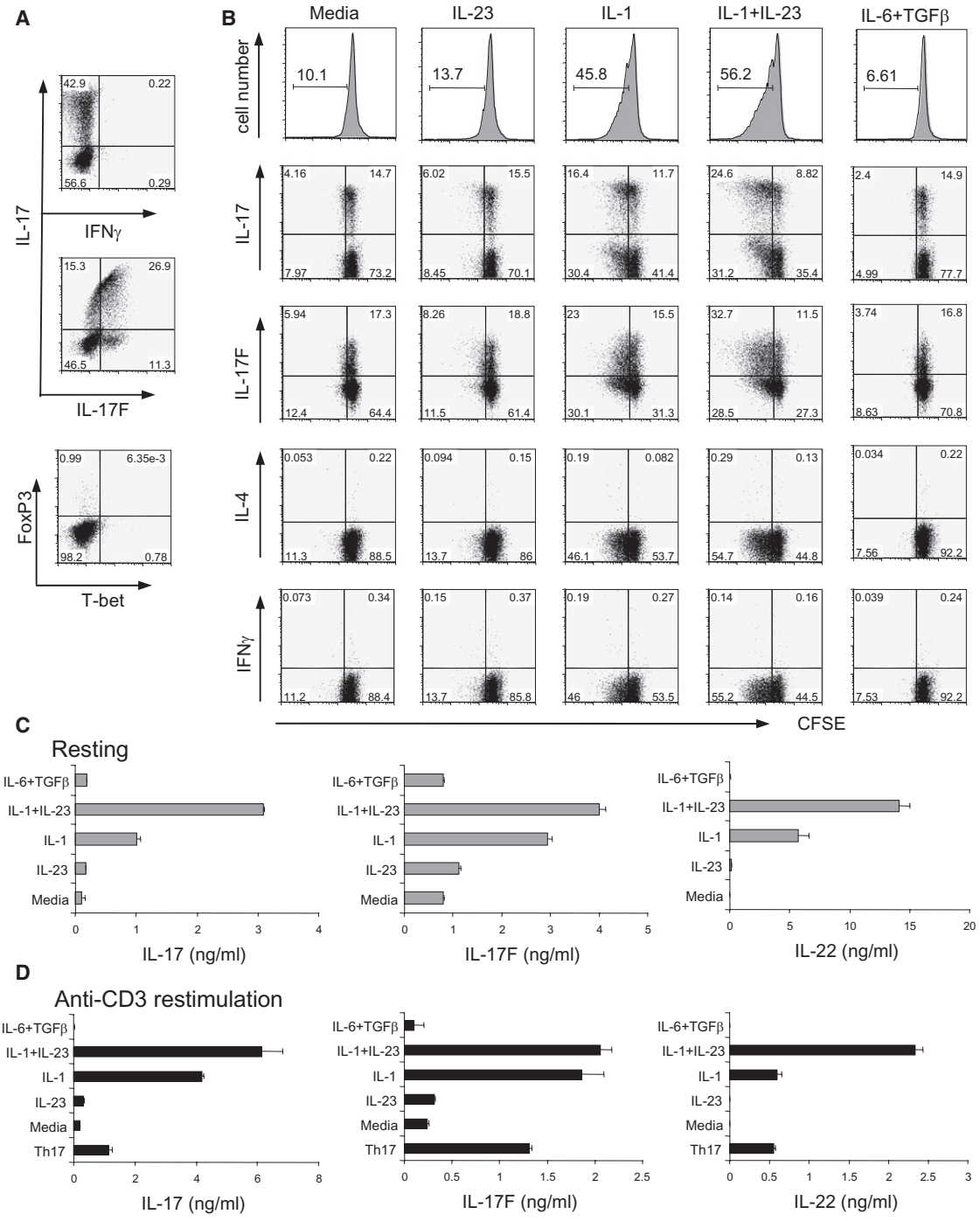

Figure 5. IL-1 Signal Expands and Maintains Th17 Cell Program in the Absence of TCR Stimulation

FACS-sorted naive $CD4^+$ T cells from IL-17F-RFP reporter mice were stimulated under Th17 cell conditions (anti-IL-4, anti-IFN-γ, IL-6, TGF-β, IL-23).

(A) On day 4, RFP^+ cells were sorted and the expression of IL-17, IL-17F, IFN-γ, Foxp3, or T-bet was analyzed by intracellular staining.

(B–D) The sorted RFP^+ cells were labeled with CFSE and cultured with medium alone, IL-23, IL-1 alone, IL-23 plus IL-1, or IL-6 plus TGF-β for additional 3 days.

(B) IL-17-, IL-17F-, IL-4-, IFN-γ-expressing cells were analyzed by intracellular staining after PMA plus ionomycin stimulation for 4 hr.

(C) Cytokines in the supernatant of 3 day additional culture were measured by ELISA.

(D) Cells were further restimulated with plate bound anti-CD3 overnight and cytokines in the supernatant were measured by ELISA. "Th17" designates the supernatant of the sorted RFP^+ cells after anti-CD3 overnight stimulation.

Values are the mean ± SEM (C and D). Data shown represent four independent experiments.

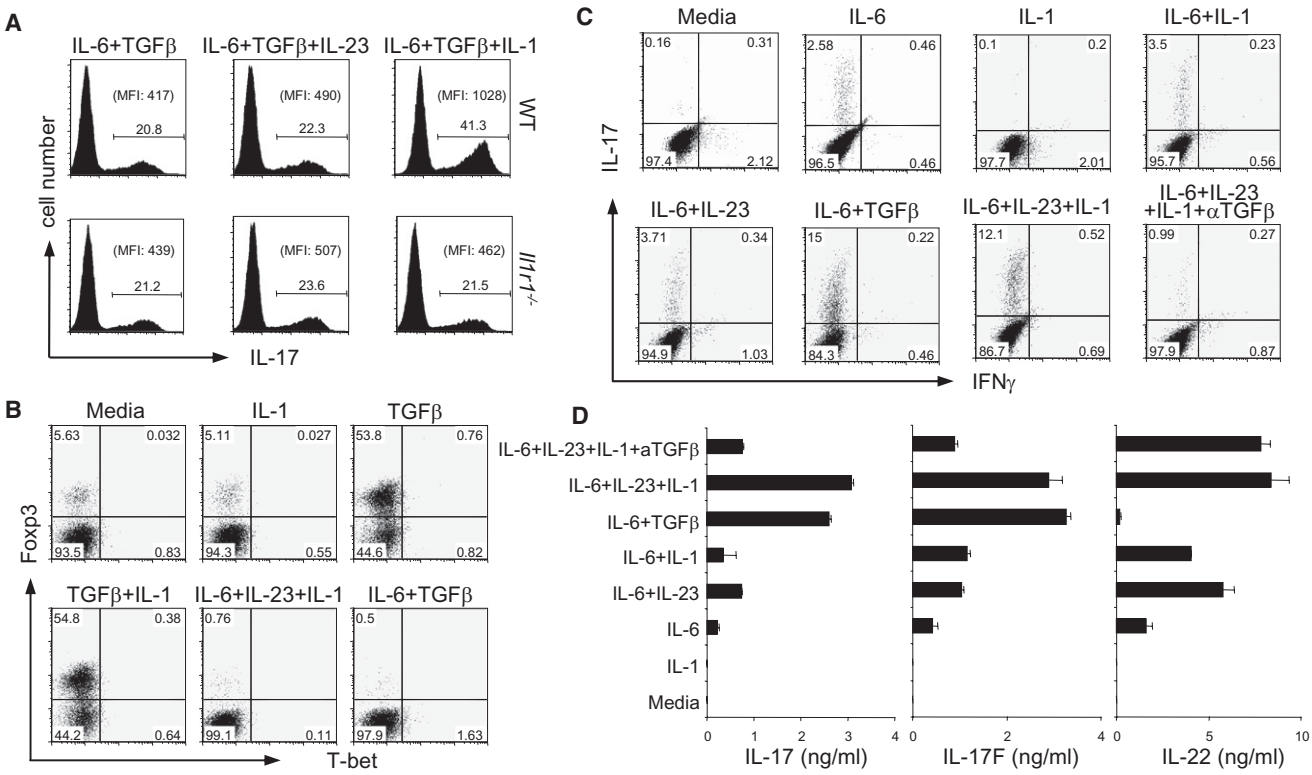

Figure 6. IL-1 Signal Regulates Th17 Cell Cytokine Production in the Absence of Exogenous TGF-β

(A) FACS-sorted naive CD4$^+$ T cells from WT or *Il1r1*$^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 plus anti-IL-4 and anti-IFN-γ in the presence of indicated cytokines for 4 days. IL-17-producing cells were analyzed by intracellular staining. Values are the percentage of IL-17$^+$ cells and mean fluorescence intensity (MFI) of the IL-17 staining of the gated cells.

(B) Foxp3- and T-bet-expressing cells were analyzed by intracellular staining.

(C) IL-17- and IFN-γ-expressing cells were analyzed by intracellular staining.

(D) On day 4, cells stimulated under the condition described in (C) were harvested and restimulated with plated bound anti-CD3 overnight and cytokines in the supernatant were measured by ELISA ([D], mean ± SEM). Data shown represent at least two independent experiments.

RT-PCR analysis demonstrated comparable induction of RORα in WT and *Il1r1*$^{-/-}$ T cells (Figure 7B). In contrast, *Il1r1*$^{-/-}$ T cells failed to express RORγt. Similarly, an almost complete lack of IRF4 expression was observed in *Il1r1*$^{-/-}$ T cells compared with WT T cell as early as day 2 (Figure 7B). Moderate increase in IL-23R expression was observed in WT but not in *Il1r1*$^{-/-}$ T cells (data not shown). In contrast, Foxp3 and Ahr expression was higher in *Il1r1*$^{-/-}$ T cells than those of WT T cells.

We next asked whether overexpression of IRF4 and/or RORγt in T cells would overcome IL-17 deficiency in *Il1r1*$^{-/-}$ cells. We utilized retroviral overexpression during the DC-T cell coculture. Analysis of cytokine expression in T cells infected with empty vector showed a great reduction of IL-17$^+$ population in *Il1r1*$^{-/-}$ T cells compared with WT T cells (Figure 7C). RORγt overexpression greatly improved the percentages of the IL-17-producing population in both WT and *Il1r1*$^{-/-}$ T cells. Interestingly, overexpression of IRF4 moderately increased the IL-17$^+$ population in *Il1r1*$^{-/-}$ T cells, with little effect in WT T cells (Figure 7C). Because we observed severe defects in both RORγt and IRF4 expression in *Il1r1*$^{-/-}$ T cells and overexpession of either one only partially restored IL-17 production in *Il1r1*$^{-/-}$ T cells, we further assessed whether combined expression of IRF4 and RORγt induces IL-17 production in *Il1r1*$^{-/-}$ T cells. After infection, dually infected cells were sorted and analyzed. Again, overexpression of IRF4 alone increased the IL-17$^+$ population only in *Il1r1*$^{-/-}$ but not in WT T cells (Figure 7D). In WT T cells, RORγt overexpression increased the IL-17$^+$ population with no further increase by coexpression of IRF4. In contrast, the IL-17$^+$ population in *Il1r1*$^{-/-}$ T cells was remarkably higher under conditions where RORγt and IRF4 were coexpressed compared to RORγt or IRF4 single overexpression condition. Therefore, RORγt and IRF4 can synergistically restore the Th17 cell polarization in the absence of IL-1R1 signal.

DISCUSSION

In the present study, we addressed the role of IL-1 in the Th17 cell lineage differentiation pathway. We found that Th17 cells expressed higher amounts of IL-1R1 mRNA, which was dependent on STAT3, RORα, and RORγt. *Il1r1*$^{-/-}$ CD4$^+$ T cells failed to induce EAE, which was associated with a selective defect in IL-17-producing T cells and an accumulation of Treg cells in the inflamed tissue. IL-1R1 signal was required for the early differentiation of Th17 cells and conversion of Foxp3$^+$ T cells into IL-17-producing cells. After polarization, IL-1 also allowed Th17 cells to maintain their cytokine secretion profile. The IL-1R1 signaling in T cells functioned by upregulating IRF4 and RORγt.

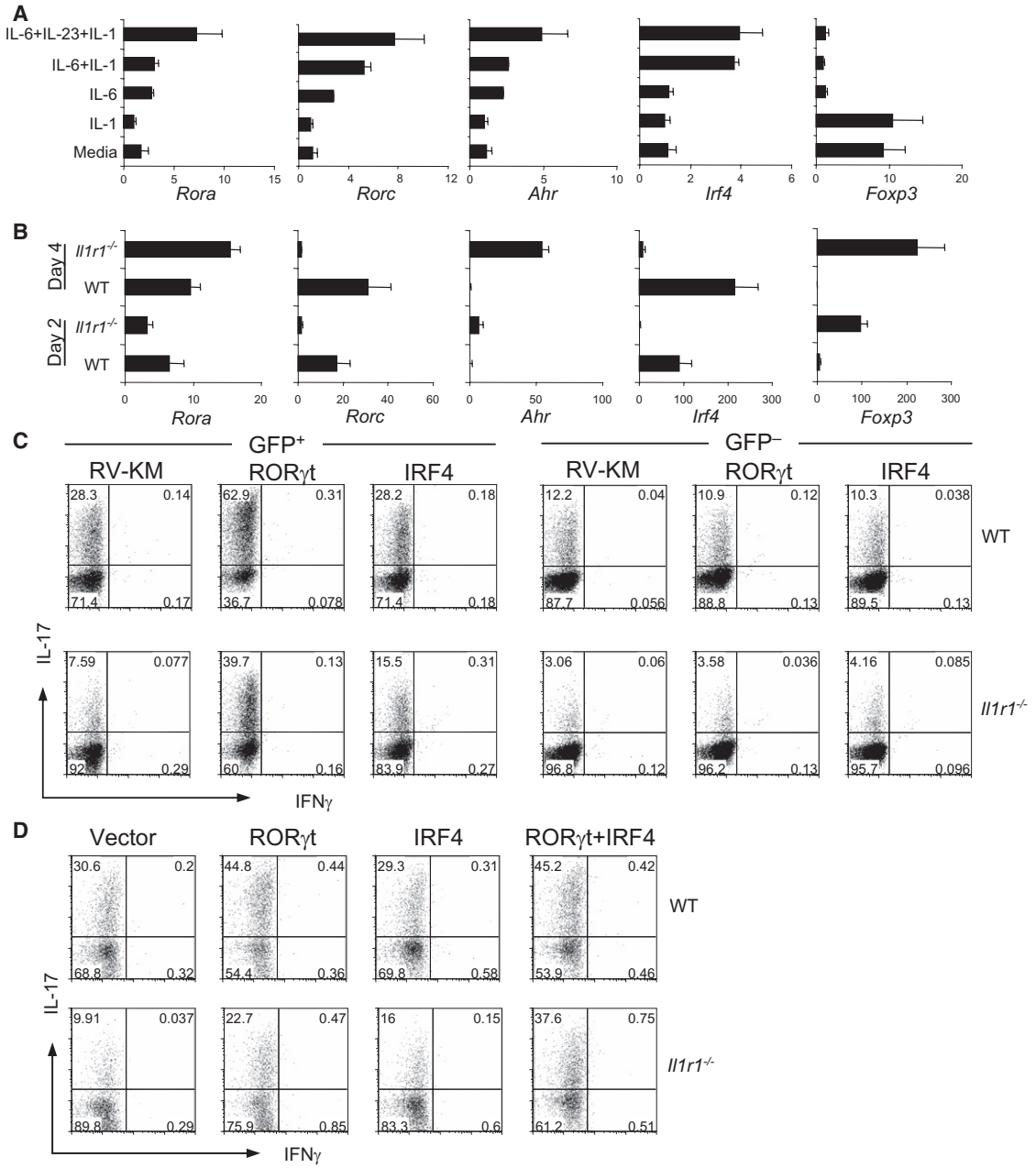

Figure 7. IL-1R1 Signal Regulates Upregulation of RORγt and IRF4 Expression

(A) FACS-sorted naive CD4$^+$ T cells from WT mice were stimulated with anti-CD3 and anti-CD28 plus anti-IL-4 and anti-IFN-γ in the presence of indicated cytokines for 4 days. mRNA expression was assessed by real-time RT-PCR.

(B) FACS-sorted naive CD4$^+$ T cells from WT or *Il1r1*$^{-/-}$ mice were cocultured with BM-derived DCs (WT) in the presence of soluble anti-CD3 and LPS plus TGF-β. On days 2 and 4, mRNA expression was assessed by real-time RT-PCR. Data shown represent two independent experiments and are normalized with expression amounts of *Actb* (mean ± SD).

(C and D) FACS-sorted naive CD4$^+$ T cells from WT or *Il1r1*$^{-/-}$ mice were cocultured with BM-derived DC (WT) in the presence of soluble anti-CD3 and LPS plus TGF-β and infected with bicistronic retrovirus expressing GFP vector (RV-KM), RORγt-GFP, or IRF4-GFP. Four days after infection, IL-17- or IFN-γ-expressing cells were assessed by intracellular staining. Data shown are gated on GFP$^+$ or GFP$^-$ cells. Cells were coinfected with two bicistronic retroviruses expressing IRF4-GFP or GFP vector and RORγt-hCD2 or hCD2 (D). GFP$^+$ and hCD2$^+$ cells were sorted and assessed by intracellular staining. Data represent two independent experiments with consistent results.

IL-1 is a pleiotropic cytokine with many target cells. Although IL-1 was shown previously to be important in Th17 cell generation in vivo, its exact action has not been understood. Higher IL-1R1 expression on Th17 cells has been observed in the SKG mouse strain that spontaneously develops arthritis (Hirota et al., 2007). On the other hand, IL-1R1 expression on DCs has

been reported to be critical for onset of autoimmune myocarditis (Eriksson et al., 2003), a Th17 cell-mediated autoimmune disorder (Rangachari et al., 2006). In our study, we clearly demonstrated that IL-1R1 expression in $CD4^+$ T cells but not on DCs is critically required for Th17 cell generation in vitro. Moreover, *Rag1*$^{-/-}$ mice receiving *Il1r1*$^{-/-}$ $CD4^+$ T cells displayed significantly attenuated EAE compared with recipients of WT T cells. Taken together, these observations indicate that IL-1 responsiveness in T cells is required for Th17 cell development and Th17 cell-mediated autoimmunity.

The regulation of IL-1R1 expression has not been well understood. In the current study, we show that although TCR plus costimulation activation resulted in elevated IL-1R1 mRNA expression, IL-6, but not IL-23 or TGF-β, serves a unique role to further enhance its expression. Consistent with this observation, IL-1 functioned only in the presence of IL-6 in upregulating Th17 cell-specific genes. IL-6 is an essential Th17 cell priming cytokine—it can not only initiate Th17 cell differentiation and downregulate Foxp3 expression, but it also has been shown to allow $CD4^+$ T cells to produce IL-21 (Nurieva et al., 2007; Zhou et al., 2007) and respond to IL-1 and IL-23 (Yang et al., 2007; Zhou et al., 2007). Interestingly, naive $CD4^+$ T cells stimulated with IL-6, IL-23, and IL-1 can be differentiated into Th17 cells in the absence of exogenous TGF-β. Although this type of Th17 cell differentiation is still dependent on TGF-β, most likely from T cell itself or contained in serum, it led to greater IL-22 expression than those polarized by only TGF-β and IL-6. This finding suggests flexibility of Th17 cell differentiation. As previously shown in the literature, Th17 cells can be generated in the presence of IL-6 and relatively high concentrations of TGF-β, which could be found at sites such as the intestine or tumor microenvironment. On the other hand, with low TGF-β concentrations but with strong innate responses, IL-1, along with the other proinflammatory cytokines, can also drive Th17 cell polarization. This idea is supported by the early defect of Th17 cell differentiation in vivo in the absence of IL-1 signaling. However, the function and regulation of Th17 cells generated by these two different conditions remain to be understood. Interestingly, IL-1-induced IL-22 expression in Th17 cells differentiated in the presence of exogenous TGF-β, suggesting the plasticity of IL-22 expression. Most importantly, our results also indicate that similar to human Th17 cells, mouse Th17 cells can be developed in the presence of proinflammatory cytokines IL-6, IL-1, and IL-23 in the absence of exogenous TGF-β.

IL-23 has been suggested to support Th17 cell expansion and maintenance (Veldhoen et al., 2006). In the present study, we observed that IL-1 regulates early Th17 cell differentiation and maintenance of polarized effector Th17 cells. For the latter, IL-1 may have an overlapping function with IL-23. However, IL-23 alone had little effect on Th17 cells in the absence of TCR stimulation whereas the combination of IL-1 and IL-23 greatly synergized to expand Th17 cells and maintain their cytokine profiles. As *Il1r1*$^{-/-}$ T cells expressed lower IL-23R, IL-1 may function to enhance the IL-23R expression on Th17 cells. Nevertheless, the expansion and cytokine production of Th17 cells by IL-1 in the absence of TCR stimulation suggests an important role of IL-1 in the homeostatic maintenance of Th17 cells. Because tissue inflammation induces abundant IL-1 and IL-23 expression, it would be interesting to surmise that Th17 cells that are not antigen specific in inflamed tissue might expand and produce Th17 cytokines in a TCR-independent manner, and thus aggravate tissue damage. Further in vivo studies will clarify the role of inflammatory cytokines on Th17 cells.

A recent study demonstrates that IRF4 is necessary for Th17 cell differentiation. *Irf4*$^{-/-}$ T cells fail to produce IL-17 in Th17 cell conditions but produces IFN-γ in Th1 cell conditions (Brustle et al., 2007). Moreover, overexpression of RORγt in *Irf4*$^{-/-}$ T cells only partially restores IL-17 production (Brustle et al., 2007). The physiological factor(s) regulating IRF4 induction during Th17 cell polarization has been unclear. In the present study, we utilized a DC-T cell coculture system and observed a severe defect in IRF4 expression in *Il1r1*$^{-/-}$ T cells compared to WT T cells. Overexpression of IRF4 or RORγt alone partially overcame Th17 cell polarization in *Il1r1*$^{-/-}$ T cells. Interestingly, coexpression of these two transcription factors enhanced Th17 cell polarization in *Il1r1*$^{-/-}$ but not in WT T cells. Therefore IL-1 from activated antigen-presenting cells delivers signals to T cells to upregulate IRF4 and RORγt during their early lineage programming and to sustain their differentiation. The present study unveiled IL-1 as a critical factor for inducing IRF4 in $CD4^+$ T cells during Th17 cell polarization. Lack of an effect after IRF4 overexpression in WT T cells is probably because IRF4 expression in WT T cells after stimulation by TCR and DCs was sufficient for Th17 cell polarization.

The balance between Treg and Th17 cells is important for the generation of immunity against extracellular bacteria and regulating autoimmunity. Recent studies by our own group and others showed a physical interaction between Foxp3 and RORγt or RORα (Yamazaki et al., 2008; Yang et al., 2008b; Zhou et al., 2008). Of note, $Foxp3^+$ cells can produce IL-17 in response to inflammatory stimuli. In the mixed bone marrow chimera study, we observed a higher percentage of $Foxp3^+$ T cells in the *Il1r1*$^{-/-}$ population compared with the WT population in the inflamed CNS. More importantly, about 10% of $Foxp3^+$ WT T cells in CNS and spleen expressed IL-17 whereas nearly no $Foxp3^+$ *Il1r1*$^{-/-}$ T cells expressed IL-17. It has been reported that $Foxp3^+$ IL-$17F^+$ cells are much less efficient in suppressing the activation of naive T cells (Yang et al., 2008b). Therefore, the IL-1R1 signal is critically required for $Foxp3^+$ T cells to produce IL-17. However, IL-1 did not affect the downregulation of Foxp3 or the TGF-β-induction of Foxp3, indicating that the mechanism of IL-1-mediated IL-17 expression is distinct from that of IL-6. IL-1R1 delivers a signal through the recruitment of MyD88 and TRAF6 leading to the activation of NF-κB and MAPK pathways, which is distinct from IL-6 and IL-23 signaling. This may explain the synergism of IL-1 with the other Th17 cell-inducing factors and their different effects on Foxp3, RORα, and RORγt. Ahr was recently discovered as a critical transcription factor for both Treg and Th17 cells. Of note, we observed higher Ahr expression in the *Il1r1*$^{-/-}$ T cells, suggesting an inhibitory role of IL-1 on Ahr. The precise regulation by IL-1 signaling on Ahr expression remains to be determined.

The present study unveiled the critical roles of IL-1 during early Th17 cell differentiation, expansion, and maintenance. Our results may explain the molecular mechanism of the spontaneous arthritis onset in IL-1Ra-deficient mice, which is a Th17-mediated autoimmune disorder (Nakae et al., 2003). Of interest, a recent study showed that administration of ATP, a well-known

inflammasome activator, drives Th17 cell differentiation in the gut (Atarashi et al., 2008), suggesting a role of IL-1 for the generation of Th17 cells in the gut. Our data corroborate these findings. The identification of IL-1 as a key regulator of Th17 cell biology may provide support for therapeutic targeting of IL-1 in autoimmune disorders.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6, OT-II, B6.SJL (CD45.1), *Il1r1*$^{-/-}$, and *Rag1*$^{-/-}$ mice were purchased from Jackson Laboratory. Foxp3-GFP reporter mice were generously provided by A. Rudensky (University of Washington) (Fontenot et al., 2005). IL-1R1-deficient and OT-II mice were bred to yield *Il1r1*$^{-/-}$ OT-II mice. Heterozygous *Staggerer* (*Rora*$^{sg/+}$) mice and *Rora*$^{sg/sg}$*Rorc*$^{-/-}$ mice were obtained as described (Yang et al., 2008c). *Stat3 fl* and *Tie2-Cre* mice were bred to yield *Stat3f/*Δ*Cre*$^{+}$ as described (Panopoulos et al., 2006; Yang et al., 2007). IL-17F-RFP reporter mice were generated as described (Yang et al., 2008b). The animal experiments were performed at the age of 6–10 weeks with protocols approved by Institutional Animal Care and Use Committee.

Adoptive Transfer and Mixed Bone Marrow Chimera Studies

CD4^{+} T cells from C57BL/6 or *Il1r1*$^{-/-}$ mice were sorted by AutoMacs and intravenously transferred into *Rag1*$^{-/-}$ mice (7 × 10^{6} cells/transfer). One day later, the recipient mice were induced EAE. To generate mixed bone marrow chimera, T cell-depleted bone marrow cells were obtained from C57BL/6 or *Il1r1*$^{-/-}$ mice and mixed 1:1 ratio before being transferred into irradiated *Rag1*$^{-/-}$ mice (10 × 10^{6} cells/transfer, 750 rad). Eight weeks later, the recipient mice were induced EAE.

In some experiments, we isolated naive CD4^{+} T cells from either wild-type OT-II or *Il1r1*$^{-/-}$ OT-II mice (CD45.2) and transferred into congenic mice (CD45.1). The recipients were immunized with $OVA_{323-339}$ peptide in CFA. Three days later, lymphoid cells from the draining lymph nodes of the recipients were stimulated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin in the presence of Golgi-stop for 4 hr, after which CD45.2^{+} CD4^{+} cells were analyzed for intracellular IL-17 and IFN-γ.

EAE Induction

Mixed bone marrow chimeras and CD4^{+} T cell reconstituted *Rag1*$^{-/-}$ mice were immunized subcutaneously at the dorsal flanks with 150 μg of MOG peptide emulsified in CFA at day 0 and day 7. Pertussis toxin was given intraperitoneally at day 1 and day 8 (500 ng/injection). Signs of EAE were assigned scores on a scale of 1–5 as follows: 0, none; 1, limp tail or waddling gait with tail tonicity; 2, wobbly gait; 3, hind-limb paralysis; 4, hind-limb and forelimb paralysis; 5, death. Disease incidence and scores were measured daily. For analysis of central nervous system infiltrates, both brain and spinal cord were collected from perfused mice, and mononuclear cells were prepared with 37% percoll gradient.

T Cell Differentiation

Dendritic cell and T cell coculture system was adopted from previous study (Veldhoen et al., 2006). In brief, naive CD4^{+}CD25^{-}CD62L^{hi}CD44^{lo} T cells from C57BL/6 and IL-1R1-deficient mice were FACS sorted and activated with 0.2 μg/ml anti-CD3 and bone marrow-derived DCs in the presence or absence of 1 ng/ml TGF-β (Peprotech), 100 ng/ml LPS. For anti-CD3/CD28-stimulated differentiation, naive CD4^{+} T cells were activated with plate-bound 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28 and in the presence of 5 ng/ml TGF-β (Peprotech), 40 ng/ml IL-6 (Peprotech), 50 ng/ml IL-23 (R&D systems), 5 μg/ml anti-IL-4 (11B11), 5 μg/ml anti-IFN-γ (XMG 1.2), 10 ng/ml IL-1β (Peprotech), 10 ng/ml IL-1α (Peprotech), or combination of these stimuli (in some experiments, 10 μg/ml of anti-IL-2 or anti-TGF-β was used). Four days after activation, cells were washed and restimulated with plate-bound anti-CD3 (2 μg/ml) overnight and cytokines in the supernatant were measured by ELISA. For intracellular staining, cells were stimulated with PMA and ionomycin in the presence of Golgi-stop for 4 hr, after which IL-17- and IFN-γ-producing cells were analyzed with intracellular staining. Intracellular staining for Foxp3 or T-bet was performed with a Foxp3 staining kit (eBioscience) and PE-conjugated anti-T-bet Ab (Santa Cruz).

Quantitative Real-Time RT-PCR

Total RNA was prepared from T cells with TriZol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized with Superscript reverse transcriptase and oligo(dT) primers (Invitrogen), and gene expression was examined with a Bio-Rad iCycler Optical System with iQ SYBR green real-time PCR kit (Bio-Rad Laboratories). The data were normalized to *Actb* reference. The following primer pairs were used: IL-1R1; forward, 5′-TGGAACAGAGCCAGTGTCAG-3′, reverse, 5′-CAGGAGAAGTCGCAGGAAGT-3′, IL-1RAcP; forward, 5′-TTGCCACCCCAGATCTATTC-3′, reverse, 5′- CCAGACCTCATTGTGGGAGT-3′, IRF4: forward, 5′-TCCTCTGGATGGCTCCAGATGG-3′, reverse, 5′-CACCAAAGCACAGAGTCACCTG-3′, TGF-β1; forward, 5′-GCAACATGTGGAACTCTACCAGA-3′, reverse, 5′-GACGTCAAAAGACAGCCACTCA-3′. The primers for *Ifng*, *Il17*, *Il17f*, *Il23r*, *Il22*, *Il21*, *Rora, Foxp3*, *Rorc*, *Tbx21*, and *Actb* were previously described (Nurieva et al., 2007; Yang et al., 2008c).

Retroviral Transduction

Genes encoding IRF4 (GenBank accession number NM013674) and RORγt (GenBank accession number AJ132394) were cloned into bicistronic retroviral vector pGFP-RV (Ouyang et al., 1998) or pMIG-hCD2 (Deftos et al., 1998) containing *IRES*-regulated *GFP* and human *CD2*, respectively. CD4^{+} CD25^{-}CD62L^{hi}CD44^{lo} T cells from C57BL/6 and *Il1r1*$^{-/-}$ mice were FACS sorted and activated with 0.2 μg/ml anti-CD3 and bone marrow-derived DCs in the presence or absence of 1 ng/ml TGF-β (Peprotech) and 100 ng/ml LPS (Veldhoen et al., 2006). Twenty-four hours after activation, the cells were infected by retroviruses expressing IRF4-GFP, RORγt-GFP, or control empty vector (containing only *IRES-GFP*). Four days after infection, the cells were restimulated with PMA and ionomycin in the presence of Golgi-stop for 4 hr, after which IL-17- and IFN-γ-producing cells were analyzed with intracellular staining on a GFP^{+} gate. Coinfection was performed with two bicistronic retroviruses expressing IRF4 or empty vector (both have *IRES-GFP*) and RORγt or control vector (both with *IRES-hCD2*). The analysis was performed on a GFP^{+}hCD2^{+} gate. RORα and RORγt single or double infection was performed as described (Yang et al., 2008c).

Statistics

The Kaplan-Meier method was used to determine the EAE incidence in CD4^{+} T cell transfer models, and the log-rank test was used for statistical analysis. The Student's t test was used to assess all other statistical values. p values were determined, and error bars represent standard error of the mean (SEM).

SUPPLEMENTAL DATA

Supplemental Data include three figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00142-3.

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