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Excess IL-1 Signaling Enhances the Development of Th17 Cells by Downregulating TGF-β–Induced Foxp3 Expression

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IL-1R antagonist–deficient (*Il1rn*$^{-/-}$) mice develop autoimmune arthritis in which IL-17A plays a crucial role. Although many studies have shown that Th17 cell differentiation is dependent on TGF-β and IL-6, we found that Th17 cells developed normally in *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice in vivo. Then, we analyzed the mechanisms of Th17 cell differentiation in *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice. We found that IL-21 production was increased in the lymph nodes of *Il1rn*$^{-/-}$ mice, naive *Il6*$^{-/-}$ CD4$^+$ T cells differentiated into Th17 cells when cultured with TGF-β and IL-21, and the differentiation was greatly enhanced when IL-1 was added to the culture. Th17 cell differentiation was not induced by either TGF-β or IL-1 alone or in combination. IL-21 induced IL-1R expression in naive CD4$^+$ T cells, and IL-1 inhibited TGF-β–induced Foxp3 expression, resulting in the promotion of Th17 cell differentiation. Furthermore, IL-1 augmented the expression of Th17 cell–specific transcription factors such as *Nfkbiz* and *Batf*. These results indicate that excess IL-1 signaling can overcome the requirement of IL-6 in the differentiation of Th17 cells by suppressing Foxp3 expression and inducing Th17 cell–specific transcription factors. ***The Journal of Immunology*****, 2014, 192: 1449–1458.**

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory, autoimmune disorder exhibited most commonly in joints. Although genetic factors, environmental factors, and infectious agents have been suggested as causes of the disease (1), so far, the etiopathogenesis has not been completely elucidated. It is remarkable that the expression of proinflammatory cytokines, chemokines, and growth factors is augmented in affected joints, forming a complex cytokine network. IL-6 is one of those proinflammatory cytokines. Because IL-6–deficient mice resist the development of arthritis in many arthritis models, such as collagen-induced arthritis (CIA), human T cell leukemia virus 1 (HTLV-1) transgenic (Tg), and SKG mice, IL-6 is thought to be crucial in the development of arthritis (2, 3). In fact, an Ab against the IL-6R has been shown to be effective in the treatment of RA (4–6).

The importance of IL-1 in the development of RA is also suggested in animal models; IL-1R antagonist (IL-1Ra; an endogenous inhibitor of IL-1)–deficient mice (*Il1rn*$^{-/-}$ mice), in which excess IL-1 signaling is induced, spontaneously develop an autoimmune arthritis (7), and the deficiency of IL-1 suppresses the development of CIA and arthritis in HTLV-I Tg, SKG, and K/BxN mice (2). IL-1Ra is also effective in the treatment of RA, indicating the importance of IL-1 in humans (8, 9). However, the precise pathological roles of IL-1 and IL-6 in the development of arthritis still remain to be elucidated.

Th17 cells are a novel subset of CD4$^+$ Th cells that preferentially produce IL-17A (10, 11). They play important roles in several mouse autoimmune disease models, such as CIA, experimental autoimmune encephalomyelitis, experimental colitis, and imiquimod-induced psoriasis models and are supposed to be similarly important in human diseases as RA, multiple sclerosis, and psoriasis (2, 12). Abs against IL-17A are effective in the treatment of RA and psoriasis in clinical trials, suggesting that IL-17A also plays important roles in the development of autoimmune diseases in humans (13, 14).

Different from Th1 and Th2, in which differentiation is induced by IL-12 and IL-4, respectively, Th17 cell differentiation from naive CD4$^+$ cells is induced by TGF-β plus IL-6 or IL-21 (15). TGF-β induces Foxp3 and the retinoic acid–related orphan receptor γt (RORγt) (16, 17), which is the master regulator of Th17 cell differentiation (18), whereas IL-6, IL-21, and IL-23 signaling activates STAT3 (10). STAT3 directly activates *Il17a* expression by binding the promoter together with RORγt and also indirectly by activating *Rora* and *Irf4* expression (19). STAT3 also enhances the expression of other Th17 cell–specific transcription factors such as *Nfkbiz*, *Hif1a*, and *Ahr* (20–22). *Stat3*-deficient CD4$^+$ T cells, in which the induction of IL-21 and IL-23R is barely detected, fail to differentiate Th17 cells, indicating that STAT3 plays an essential role in Th17 cell differentiation (23, 24). It is now accepted that IL-23 is required for the growth, survival, and effector functions of Th17 cells and promotes IL-17A, IL-17F, and IL-22 production. IL-1 promotes IL-17A production through the activation of PI3K, NF-κB, and protein kinase Cθ (10, 25, 26) and also induces *Irf4* expression in combination with IL-6 (27). IL-1

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Abbreviations used in this article: Akt, protein kinase B; CIA, collagen-induced arthritis; Gata3, GATA-binding protein 3; HTLV-1, human T cell leukemia virus 1; IL-1Ra, IL-1R antagonist; LN, lymph node; MFI, mean fluorescence intensity; mTOR, mammalian target of rapamycin; RA, rheumatoid arthritis; rm, recombinant murine; RORγt, retinoic acid–related orphan receptor γt; Tg, transgenic; Treg, regulatory T; WT, wild-type.

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also antagonizes the inhibitory effects of IL-2 on Th17 cell differentiation (28). IL-6 induces IL-1R, whereas a single Ig IL-1R–related molecule is a negative regulator of IL-1R and suppresses Th17 cell proliferation via the inhibition of the mammalian target of rapamycin (mTOR) kinase (29). However, the mechanisms of how these cytokines promote Th17 cell differentiation have not been elucidated completely.

Foxp3 is the master regulator for regulatory T (Treg) cell differentiation and involved in the transcription of signature genes of Treg cells such as IL-10, CTLA-4, ICOS, and glucocorticoid-induced TNFR-related protein via regulating *Irf4* and *Blimp1* transcription (30, 31). Induced Foxp3$^+$ Treg cells develop in the periphery under subimmunogenic Ag stimulation, chronic inflammation, and physiological conditions of the gut (30). TCR signaling, IL-2, and TGF-β are important for the expression of Foxp3 and Treg cell differentiation. Under Th17 cell–polarization conditions, TGF-β–induced Foxp3 binds RORγt and inhibits its function, causing the inhibition of Th17 cell differentiation. IL-6, IL-21, and IL-23 relieve Foxp3-mediated inhibition, thereby promoting Th17 cell differentiation (16, 17, 32). Thus, it is suggested that there are two reciprocal developmental pathways for the generation of Th17 and Treg cells. In line with this concept, conditional deletion of Foxp3 in adult mice causes the increase of RORγt expression, resulting in the enhancement of IL-17A expression (33).

Il1rn$^{-/-}$ mice spontaneously develop arthritis resembling RA in humans (7). The development of arthritis is T cell dependent, and *Il1rn*$^{-/-}$ T cell transfer into nude mice can induce arthritis in the recipients (34). The arthritis is also dependent on IL-17A; IL-17A deficiency can suppress the development of arthritis completely (35). In this study, to elucidate the pathogenic mechanisms of the arthritis in *Il1rn*$^{-/-}$ mice, we examined the effects of IL-6 deficiency. Interestingly, we found that IL-6 deficiency does not affect the development of arthritis in this model and that Th17 cells develop normally in *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice as in *Il1rn*$^{-/-}$ mice. Furthermore, we showed that excess IL-1 signaling could overcome the deficiency of IL-6 in the development of Th17 cells by downregulating Foxp3 expression in an IL-6–independent manner. These findings reveal a novel function of IL-1 in the development of Th17 cells.

Materials and Methods

Mice

The generation of *Il1rn*$^{-/-}$ (*Il1rn*^{tm1Yiw}) and *Il1αβ*$^{-/-}$ (*Il1a*^{tm1Yiw}/*Il1b*^{tm1Yiw}) mice was described previously (7, 36). *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice were generated in our laboratory by crossing *Il1rn*$^{-/-}$ mice with *Il6*$^{-/-}$ (*Il6*^{tm1Kopf}) mice, which were kindly provided by M. Kopf (37). These mice were backcrossed for more than eight generations to BALB/cA mice (CLEA Japan, Tokyo, Japan). *Myd88*$^{-/-}$ (*Myd88*^{tm1Aki}) mice were kindly provided by S. Akira and also backcrossed for more than eight generations to C57BL/6J mice (38). Mice, matched by age, sex, and genetic background, were used for experiments, and wild-type (WT) C57BL/6J mice (Nihon, Shizuoka, Japan) or BALB/cA mice (CLEA Japan) were used as controls. All mice were kept in specific pathogen-free conditions at the Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo. Experiments were carried out according to the institutional ethical guidelines for animal experiments and rDNA guidelines and were approved by the institutional committees.

T cell differentiation assays

WT or *Il6*$^{-/-}$ CD4$^+$ T cells were purified from pooled lymph nodes (LNs) with mouse CD4 microbeads, according to the manufacturer's directions (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4$^+$ T cells were stimulated with plate-coated 4 μg/ml anti-CD3 (clone 145-2C11) or soluble 1 μg/ml anti-CD28 (clone 37.51; BioLegend, San Jose, CA) and supplemented with 10 ng/ml recombinant murine (rm)IL-1α (PeproTech, Rocky Hill, NJ), 10 ng/ml rmIL-1β (PeproTech), 10 μg/ml anti–IFN-γ (clone R4-6A2), and 10 μg/ml anti–IL-4 (clone 11B11) for 5 d in X-VIVO 20 (Lonza, Basel, Switzerland).

Naive CD4$^+$ T cells were purified with a combination of cell-sorting techniques. Briefly, a single-cell suspension was prepared from the LNs and spleen of BALB/cA background WT, *Il6*$^{-/-}$, and *Il1αβ*$^{-/-}$ mice and C57BL/6J background *Myd88*$^{+/-}$ and *Myd88*$^{-/-}$ mice, and CD4$^+$ T cells were purified with biotin-conjugated anti-mouse B220, anti-mouse CD8α, anti-mouse CD11b, anti-mouse DX5, and anti-mouse Ter^{119} Abs (BD Biosciences, San Jose, CA) using an AutoMACS (Miltenyi Biotec). Then, these CD4$^+$ T cells were further fractionated with PE-Cy7–conjugated anti-mouse CD4 (BioLegend), Pacific Blue–conjugated anti-mouse CD62L (BioLegend), and PE-conjugated anti-mouse CD25 Abs (eBioscience, San Diego, CA) by an FACSAria (BD Biosciences), and the CD4^{hi}CD25^{lo} CD62L^{hi} fraction was defined as naive CD4$^+$ T cells. Unless otherwise indicated, T cells were cultured in the X-VIVO 20 (Lonza) medium. Th17 cell differentiation was induced in naive CD4$^+$ cells under Th17 cell–polarizing conditions by thorough culturing in 4 μg/ml anti-CD3–coated (clone 145-2C11) plates with 1 μg/ml soluble anti-CD28 (clone 37.51; BioLegend), 10 μg/ml anti–IFN-γ (clone R4-6A2), 10 μg/ml anti–IL-4 (clone 11B11), 3 ng/ml recombinant human TGF-β1 (PeproTech), and 100 ng/ml rmIL-21 (PeproTech). In some experiments, the following reagents were added as indicated in the figure legends: 40 ng/ml rmIL-6 (PeproTech), 10 ng/ml rmIL-1α (PeproTech), 10 ng/ml rmIL-1β (PeproTech), 10 ng/ml rmIL-23 (R&D Systems, Minneapolis, MN), rmIL-21R subunit/Fc chimera (R&D Systems), and/or 50 ng/ml rapamycin (LC Laboratories, Woburn, MA). In restimulation experiments, viable T cells were recovered on days 4 to 5 of the first or second culture and subcultured with a fresh medium containing cytokines and Ab mixtures as indicated.

LN cell culture

LN cells were purified from *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice. LNs cells were cultured with 5 ng/ml rmIL-1α (PeproTech), 5 ng/ml rmIL-1β (PeproTech), 5 ng/ml rmIL-23 (R&D Systems), or medium alone for 2 d in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS.

Flow cytometric analyses

Cells were collected and, where indicated, stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 5 h in the presence of 2 μM monensin (Sigma-Aldrich). These cells were blocked with anti-FcγRII/III receptor mAb (clone 2.4G2) and stained with cell lineage–specific Abs against cell-surface molecules. We used 2 μg/ml 7-aminoactinomycin D (Sigma-Aldrich) to exclude dead cells in flow cytometric analyses. After being washed with FACS solution, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, resuspended in the permeabilization buffer (0.1% saponin-containing FACS solution), and stained with anti-cytokine Abs for 40 min at 4°C. Abs used for the cell lineage–specific staining were as follows: allophycocyanin-conjugated anti-CD4 (BD Biosciences), PE-Cy7–conjugated anti-CD4 (BioLegend), FITC-conjugated anti-TCRβ (BioLegend), FITC-conjugated anti-CD8a (eBioscience), allophycocyanin-Cy7–conjugated anti-B220 (BioLegend), PE-conjugated anti-mouse CD11c (BioLegend), FITC–anti-mouse γδ TCR (eBioscience), and PE-conjugated anti–IL-1R1 (BioLegend). Those used for intracellular staining were as follows: FITC- or PE-conjugated anti–IFN-γ (BioLegend), FITC- or Pacific Blue–conjugated anti–IL-17A (BioLegend), allophycocyanin- or PE-conjugated anti-Foxp3 (BD Biosciences), and Alexa Fluor 647–conjugated anti–IL-21 (BioLegend). In Foxp3 expression analysis, we used the Foxp3 Staining Kit according to the manufacturer's instructions (eBioscience). Flow cytometry analysis was performed on an FACSCanto II (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR)

CFSE labeling

T cells (1×10^6–1×10^7 cells/ml) were incubated for 6 min at 37°C with 5 μM CFSE (Invitrogen, Carlsbad, CA) in PBS. Then, after the addition of an equal volume of FBS, cells were collected by centrifugation. After being washed, cells were resuspended in the culture medium at a density of 8×10^5 cells/ml and cultured with combinations of cytokines. Cell proliferation was monitored by fluorescence intensity using an FACSCanto II (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

Real-time RT-PCR

Total RNA was extracted with a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. RNA was reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time RT-PCRs were performed with SYBR Premix Ex Taq (TaKaRa, Shiga, Japan) and an iCycler System (Bio-Rad, Hercules, CA). The primer sequences were as follows: *Gapdh*,

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5′-TTCACCACCATGGAGAAGGC-3′ and 5′-GGCATGGACTGTGGTCATGA-3′; *Foxp3*, 5′-AGAAGCTGGGAGCTATGCAG-3′ and 5′-TACTGGTGGCTACGATGCAG-3′; *Il17a*, 5′-CTCCAGAAGGCCCTCAGACTAC-3′ and 5′-GGGTCTTCATTGCGGTGG-3′; *Rorc*, 5′-AGCAGTGTAATGTGGCCTAC-3′ and 5′-GCACTTCTGCATGTAGACTG-3′; *Il1r1*, 5′-ACCTTCCCACAGCGGCTCCACATT-3′ and 5′-TTGTCAAGAAGCAGAGGTTTACAG-3′; GATA-binding protein 3 (*Gata3*), 5′-CTTATCAAGCCCAAGCGAAG-3′ and 5′-CATTAGCGTTCCTCCTCCAG-3′; *Tbx21*, 5′-GGTGTCTGGGAAGCTGAGAG-3′ and 5′-CCACATCCACAAACATCCTG-3′; *Il21*, 5′-GCCAGATCGCCTCCTGATTA-3′ and 5′-CATGCTCACAGTGCCCCTTT-3′; *Il22*, 5′-TGACGACCAGAACATCCAGA-3′ and 5′-AGCTTCTTCTCGCTCAGACG-3′; *Batf*, 5′-CCAGAAGAGCCGACAGAGAC-3′ and 5′-GAGCTGCGTTCTGTTTCTCC-3′; and *Nfkbiz*, 5′-CCTCCGATTTCTCCTCCACT-3′ and 5′-GTTCTTCACGCGAACACCTT-3′.

Clinical assessment of arthritis

Arthritis development in each paw was graded by macroscopic evaluation as follows: 0, no change; 1, mild swelling; 2, obvious joint swelling; and 3, severe joint swelling and ankylotic changes. The arthritic severity score is the sum of four paw scores.

Statistical analysis

Unless otherwise specified, all results are shown as a mean and the SEM. Unpaired Student *t* tests or χ^2 tests were used for the statistical analysis of the results. Differences were considered significant at $p < 0.05$.

Results

IL-6 deficiency does not influence the development of arthritis and Th17 cell differentiation in Il1rn$^{-/-}$ mice

We previously reported that the expression of proinflammatory cytokines including IL-1β, IL-6, TNF, and IL-17A was augmented in the arthritic joints of *Il1rn*$^{-/-}$ mice, and arthritis development was suppressed in TNF- or IL-17A–deficient mice (34, 35). To elucidate the role of IL-6 in the development of arthritis in *Il1rn*$^{-/-}$ mice, we generated IL-1Ra and IL-6 double-deficient mice by crossing *Il1rn*$^{-/-}$ mice with *Il6*$^{-/-}$ mice. We found that *Il1rn*$^{-/-}$ *Il6*$^{-/-}$ mice fully developed arthritis (Fig. 1A), indicating that IL-6 is dispensable in the development of arthritis in *Il1rn*$^{-/-}$ mice.

The total number of lymphocytes in the peripheral LNs as well as the numbers of B220$^+$ cells, CD4$^+$ cells, and CD8$^+$ cells of *Il1rn*$^{-/-}$ and *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice were similarly increased compared with WT mice (Supplemental Fig. 1A). To examine Th17 cell development in *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice, we next investigated the cytokine profile of CD4$^+$ cells in the draining LNs in these mice by FACS analysis. The proportion as well as the number of CD4$^+$ IL-17A$^+$ cells (Th17 cells) were similar in *Il1rn*$^{-/-}$ and *Il1rn*$^{-/-}$ *Il6*$^{-/-}$ mice, although the content was not so high compared with CD4$^-$IL-17A$^+$ T cells (Fig. 1B, 1C, Supplemental Fig. 1B). These results indicate that IL-6 is not necessarily required for the development of Th17 cells in *Il1rn*$^{-/-}$ mice.

Excess IL-1 signaling induces Th17 cell differentiation in an IL-6–independent manner

Because Th17 cell expansion was observed in *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice, we assumed that excess IL-1 signaling might enhance Th17 cell differentiation in an IL-6–independent manner. To examine this possibility, CD4$^+$ T cells were purified from WT and *Il6*$^{-/-}$ mouse LNs, and the effects of IL-1 on Th17 cell differentiation were examined in vitro. Exogenous IL-1 induced CD4$^+$IL-17A$^+$ T cell differentiation from *Il6*$^{-/-}$ T cells, consistent with a previous report (28) (Fig. 2A).

We found that IL-21 mRNA expression was significantly increased in LN cells from *Il1rn*$^{-/-}$ as well as *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice compared with those from WT mice, indicating that excess IL-1 signaling induces IL-21 expression (Fig. 2B). Because IL-23 expression is elevated in *Il1rn*$^{-/-}$ mice due to excess IL-1 signaling (39), we examined the effect of IL-1 and IL-23 on the IL-21 expression in γδ T cells, CD4$^+$ T cells, and CD11c$^+$ cells (Fig. 2C). We found that the expression of IL-21 was enhanced in γδ T cells by the treatment with IL-1 and IL-23, suggesting that IL-1–induced IL-23 is involved in the induction of IL-21 in *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mouse LNs.

Because IL-21 can substitute for the function of IL-6, and IL-21 plus TGF-β can induce Th17 cell differentiation (40), we examined the involvement of IL-21 in IL-6–independent Th17 cell differentiation in vitro. Neither IL-1 or TGF-β alone nor IL-1 plus TGF-β could induce Th17 cell differentiation from naive T cells, in contrast to LN CD4$^+$ T cells (Fig. 2D, Supplemental Fig. 2). In contrast, TGF-β plus IL-6 or TGF-β plus IL-21 significantly induced Th17 cell differentiation from naive T cells, and IL-1 enhanced Th17 cell differentiation induced by TGF-β plus IL-6 or TGF-β plus IL-21 (Fig. 2D).

Because IL-1 could not induce differentiation of naive CD4$^+$ T cells, we examined the expression of IL-1R1 on naive T cells after treatment with various cytokines. We found that IL-1R1 was not expressed in naive T cells, and its mRNA expression, and the expression on the cell surface was induced by IL-6 or IL-21 in a dose-dependent manner (Fig. 2E, 2F, Supplemental Fig. 3A). These results suggest that IL-6 or IL-21 induces IL-1R expression on naive T cells, and IL-1 enhances Th17 cell differentiation by acting on these Th17-committed cells.

IL-1 downregulates Foxp3 expression in vitro

We next investigated the effects of IL-1 on the expression of transcriptional factors under Th17 cell differentiation conditions. We analyzed the expression of mRNAs of *Tbx21*, *Gata3*, *Rorc*, and *Foxp3*, the characteristic transcription factors for Th1, Th2, Th17, and Treg cells, respectively, using real-time PCR after the induction of IL-1R1 by incubating naive T cells with TGF-β plus IL-21 for 5 d (first culture) followed by treatment with various cytokines for 2 d (second culture). *Il17a* expression was induced by TGF-β plus IL-1, but only in low levels by TGF-β plus IL-21 or TGF-β alone, in the second culture (Fig. 3A). The expressions of *Tbx21*, *Gata3*, and *Rorc* were not affected by IL-1. Interestingly, Foxp3 was strongly downregulated by IL-1, but not by IL-21. Decreased Foxp3 expression due to IL-1 was also confirmed by FACS analysis (Fig. 3B). The mean fluorescence intensity (MFI) of the PE-labeled anti-Foxp3$^+$ cells was significantly lower in TGF-β/IL-1/IL-21– than TGF-β or TGF-β/IL-21–stimulated T cells (Fig. 3C).

Because IL-1 can promote T cell growth (41), it is possible that IL-1 only promotes Foxp3$^-$ cell growth, resulting in the expansion of Foxp3$^-$ cells. Therefore, we analyzed the proliferation dependency of Foxp3 downregulation. Naive T cells were labeled with CFSE and after being cultured for 96 h under induced Foxp3$^+$ Treg or Th17 cell–polarizing conditions, Foxp3 expression was analyzed after cell divisions. The proportion of Foxp3$^+$ cells decreased greatly in the cultures of TGF-β/IL-21, TGF-β/IL-21/IL-1, and TGF-β/IL-6–treated cells compared with TGF-β–treated cells (Fig. 3D). The Foxp3$^+$ population rather increased with cell divisions 1–3 in TGF-β/IL-1/IL-21, excluding the possibility that IL-1 selectively promotes Foxp3$^-$ cell proliferation.

To confirm that this downregulation of Foxp3 expression was caused by IL-1, we next investigated the effects of the deficiency of MyD88, the adaptor molecule involved in IL-1 signal transduction. FACS-sorted naive CD4$^+$ T cells from *Myd88*$^{+/-}$ and *Myd88*$^{-/-}$ mice were cultured under Th17 cell–polarizing conditions (TGF-β plus IL-21) for 4 d, followed by restimulation with anti-CD3, anti-CD28, TGF-β, and IL-1 for 2 d, and Foxp3 expression was analyzed by a flow cytometer. As shown in Fig. 3E, Foxp3 downregulation was completely abolished in MyD88-deficient

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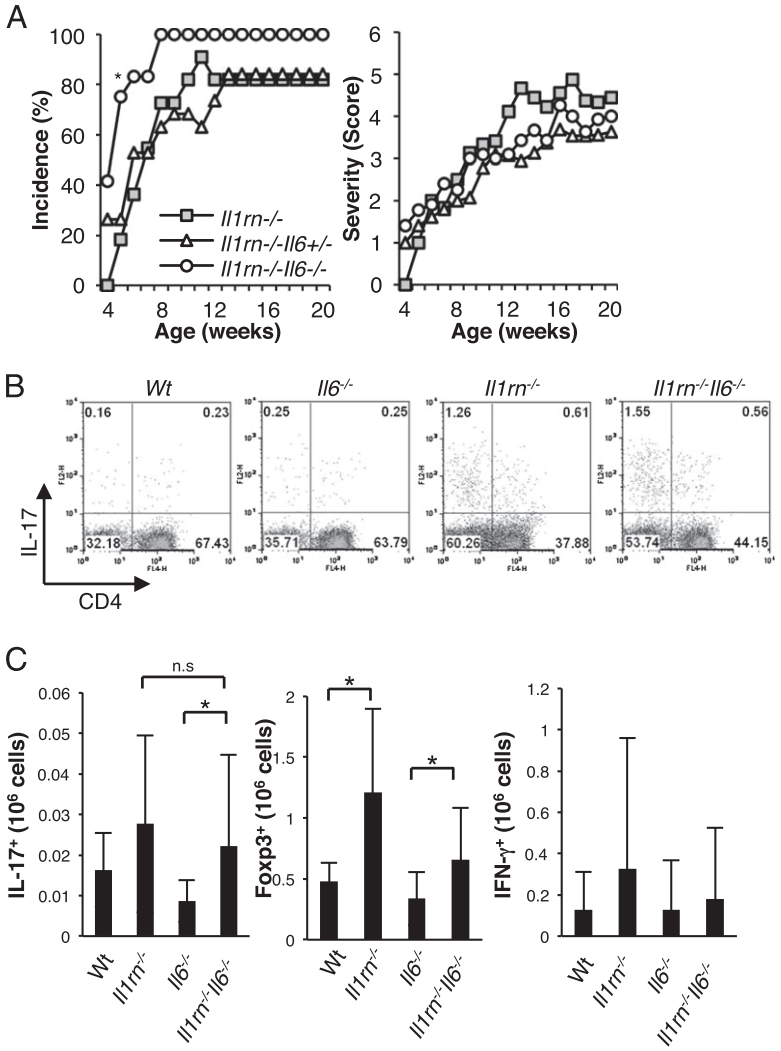

FIGURE 1. Arthritis development and Th17 cell differentiation were normally observed in *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice. (**A**) *Left panel*, Incidence of arthritis. *Right panel*, Arthritic severity score of the affected mice. $n = 15$–22/group. $*p < 0.05$: *Il1rn*$^{-/-}$ versus *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice, χ^2 tests. The data are representative of two independent experiments. (**B**) Intracellular IL-17A expression in LN cells from WT, *Il6*$^{-/-}$, arthritic *Il1rn*$^{-/-}$, and arthritic *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice at the age of 7–14 wk were examined by FACS after PMA/ionomycin stimulation in vitro. Numbers indicate percentage of cells in each area. (**C**) CD4$^+$TCR-β$^+$ T cell population was purified from LN cells of WT, arthritic *Il1rn*$^{-/-}$, *Il6*$^{-/-}$, and arthritic *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice at the age of 7–14 wk, and intracellular IFN-γ, IL-17A, and Foxp3 were determined by flow cytometry after PMA/ionomycin stimulation in vitro. $n = 14$–17/group. $*p < 0.05$, Student *t* tests.

mice, indicating that IL-1 downregulated Foxp3 expression through the MyD88-mediated pathway. As expected, IL-1R1 was expressed in both IL-17A$^+$ cells and Foxp3$^+$ cells after being cultured under Th17 cell–differentiation conditions (Supplemental Fig. 3B). These results suggest IL-1 promotes Th17 cell differentiation by suppressing Foxp3 expression.

IL-1 downregulates Foxp3 independently from IL-6 and IL-21

Because T cells produce IL-21, IL-6, IL-1, and IL-1Ra (34, 42, 43), and IL-6 and IL-21 activate STAT3, which downregulates Foxp3 expression (44, 45), we examined the autocrine effects of these cytokines on Foxp3 downregulation. First, to discriminate the function of IL-1 from that of IL-21, the dose-dependent effects of IL-21 and IL-6 on the induction of IL-17A and the suppression of Foxp3 were examined (Fig. 4A, *top panel*). IL-6 in collaboration with TGF-β efficiently induced IL-17A and almost completely suppressed Foxp3 expression in a dose-dependent manner. These effects were also observed in *Il1ab*$^{-/-}$ T cells, indicating that these activities of IL-6 are independent of endogenously produced IL-1 (Fig. 4A, *bottom panel*). In contrast, the IL-17A–inducing activity and Foxp3-suppressing activity of IL-21 were weak and plateaued at the concentration of 100 ng/ml. The effects of IL-21 were also independent of endogenous IL-1α/β.

Next, we examined the effects of IL-1 on the expression of IL-17A and Foxp3. To exclude the effects of endogenous IL-6 that are induced by IL-1, we used *Il6*$^{-/-}$ naive T cells. We found that IL-1 promoted IL-17A expression and suppressed Foxp3 expression even in the presence of the saturation concentration of IL-21 (400 ng/ml), indicating that the Th17 cell–inducing activity of IL-1 is not mediated by IL-21 or IL-6 (Fig. 4B). Furthermore, the inhibition of IL-21 activity by IL-21R–Fc did not completely inhibit the IL-1–induced Foxp3 suppression in *Il6*$^{-/-}$ cells that had been induced to differentiate to Th17 cells with TGF-β plus IL-21, suggesting that IL-1 itself can suppress Foxp3 expression (Fig. 4C). Consistent with this observation, IL-1 inhibited Foxp3 expression in the presence of an excess concentration of IL-21R–Fc (20 μg/ml) in a dose-dependent manner (Fig. 4D). Because IL-21R–Fc could relieve Foxp3 suppression, IL-21 itself was also suggested to be partly involved in the suppression (Fig. 4C). These results indicate that IL-1 suppresses Foxp3 expression independently from the actions of IL-6 and IL-21.

IL-1 enhances the expression of Th17 cell–specific genes

We next examined the effect of IL-1 on the expression of Th17 cell–specific cytokines and transcription factors in naive CD4$^+$ T cells after incubation with TGF-β plus IL-21 for 4 d. To avoid the possible effects of IL-1–induced IL-6 and to assess the IL-6–independent Th17 cell differentiation, we used IL-6–deficient T cells. IL-1 induced the expression of IL-17A and IL-22 mRNA, but not IL-21 mRNA (Fig. 5A). IL-1 also enhanced the expression of *Nfkbiz* and *Batf*, specific transcription factors for Th17 cells (20, 46), but not of *Rorc* nor *Foxp3* (Fig. 5B).

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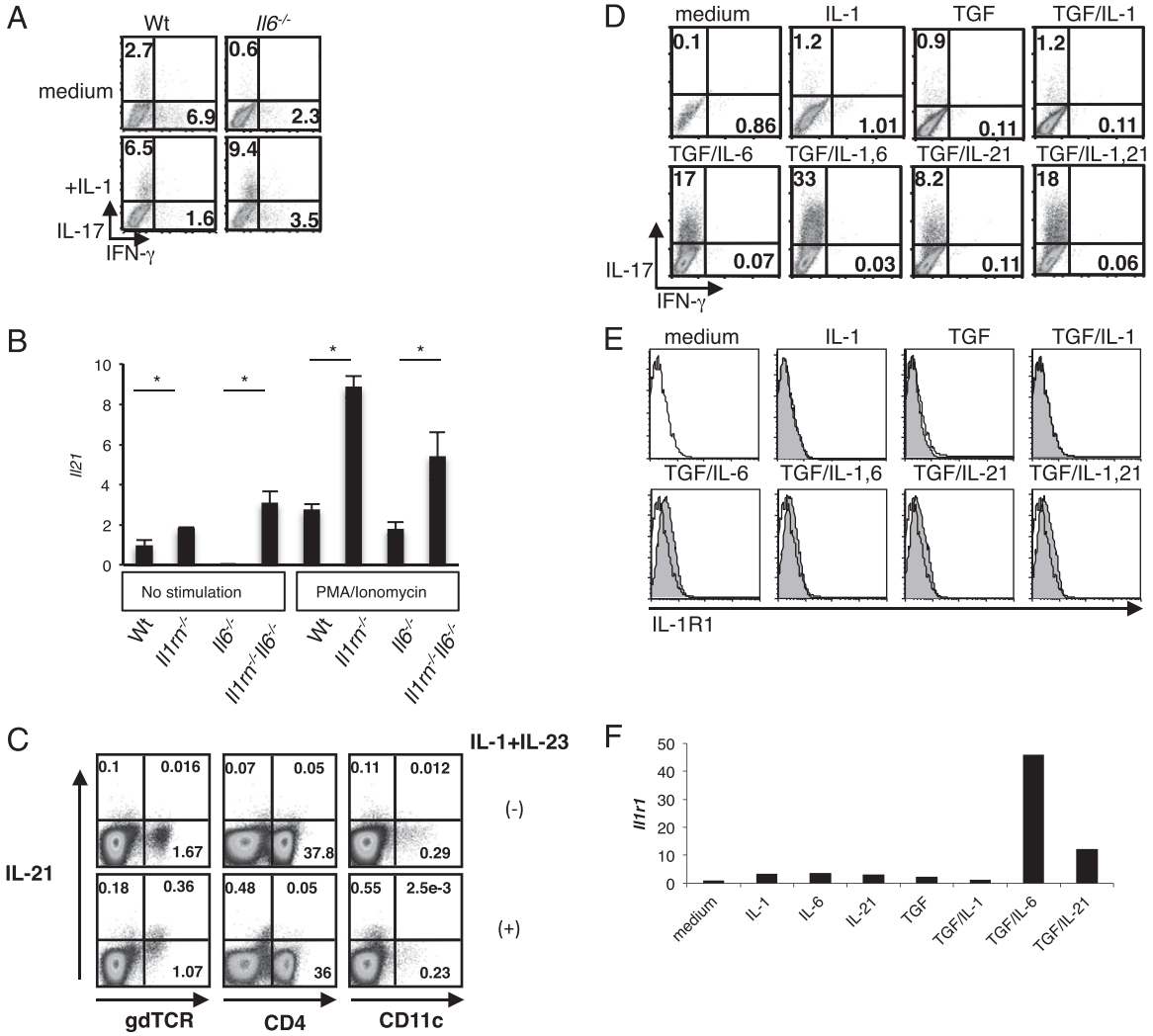

FIGURE 2. IL-1–induced Th17 cell differentiation in an IL-6–independent manner. (**A**) $CD4^+$ T cells were purified from WT or $Il6^{-/-}$ mouse LNs and cultured in the presence of anti-CD3, anti-CD28, anti–IL-4, and anti–IFN-γ for 5 d with or without IL-1α plus IL-1β. Numbers in quadrants indicate percentage of cells in each area. One of two representative experiments is shown. (**B**) mRNA was prepared from LNs of WT, $Il1rn^{-/-}$, $Il6^{-/-}$, and $Il1rn^{-/-}$ $Il6^{-/-}$ mice, and *Il21* expression was determined by real-time RT-PCR. All of the data were normalized to *Gapdh* mRNA. $n = 3$. The data are representative of two independent experiments. (**C**) $Il1rn^{-/-}Il6^{-/-}$ LNs cells were cultured for 2 d with or without IL-1α plus IL-1β + IL-23. Numbers in quadrants indicate percentage of cells in each area. One of two representative experiments is shown. (**D–F**) In vitro Th17 cell differentiation assay. FACS-sorted naive $CD4^+$ T cells from WT mice were cultured with anti-CD3, anti-CD28, anti–IL-4, and anti–IFN-γ for 5 d in the presence of indicated reagents. Intracellular IL-17A and IFN-γ (D) and cell-surface IL-1R1 (E) were stained after PMA/ionomycin activation and analyzed by an FACS. (D) Numbers in quadrants indicate percentage of cells in each area. The data are representative of two independent experiments. (E) Anti–IL-1R1: filled histogram; control (medium cultured cells): solid line. (F) The expression of *Il1r1* mRNA after treatment with indicated cytokine(s) was determined by real-time RT-PCR. All of the data were normalized to GAPDH RNA. The data are representative of two independent experiments. $*p < 0.05$.

IL-1 enhances Th17 cell differentiation and maintains long-term survival

Over the last few years, it has been shown that Th17 cells are plastic and change their cellular characteristics when they are cultured in vitro or transferred into other mice. Under certain conditions, they even acquire the ability to secrete Th1 cytokines (e.g., IFN-γ) (47). Therefore, we next examined the effects of IL-1 on the maintenance of Th17 cell lineage. Naive T cells were cultured under Th17 cell–differentiation conditions (TGF-β plus IL-21) for 5 d; then, cells were subcultured under defined cytokine conditions for an additional 5 d (second round) or 10 d (third round) (Fig. 6A). As reported (47), TGF-β was required for the maintenance of Th17 cells, and cells that restimulated with IL-1 in the absence of TGF-β did not maintain IL-17A expression (Fig. 6A). However, in collaboration with TGF-β, IL-1 enhanced IL-17A production and significantly prolonged IL-17A expression. These enhancements of IL-17A expression and Th17 cell survival were independent of IL-21 action, because IL-21 was not required in the second- and third-round cultures. These results suggest IL-1 activates IL-17A expression and maintains Th17 cell lineage survival in collaboration with TGF-β.

It was reported that IL-1 activates the protein kinase B (Akt)–mTOR pathway that suppresses Treg cell differentiation (48). So, we analyzed the effects of rapamycin, an inhibitor of mTOR, on the Foxp3 suppression by IL-1. As shown in Fig. 6B, rapamycin suppressed the Foxp3-inhibitory activity of IL-1, suggesting that the Akt-mTOR pathway is involved in the IL-1–induced Foxp3 suppression.

Discussion

The development of arthritis in RA models such as HTLV-I Tg mice, SKG mice, and CIA models are dependent on IL-6, although

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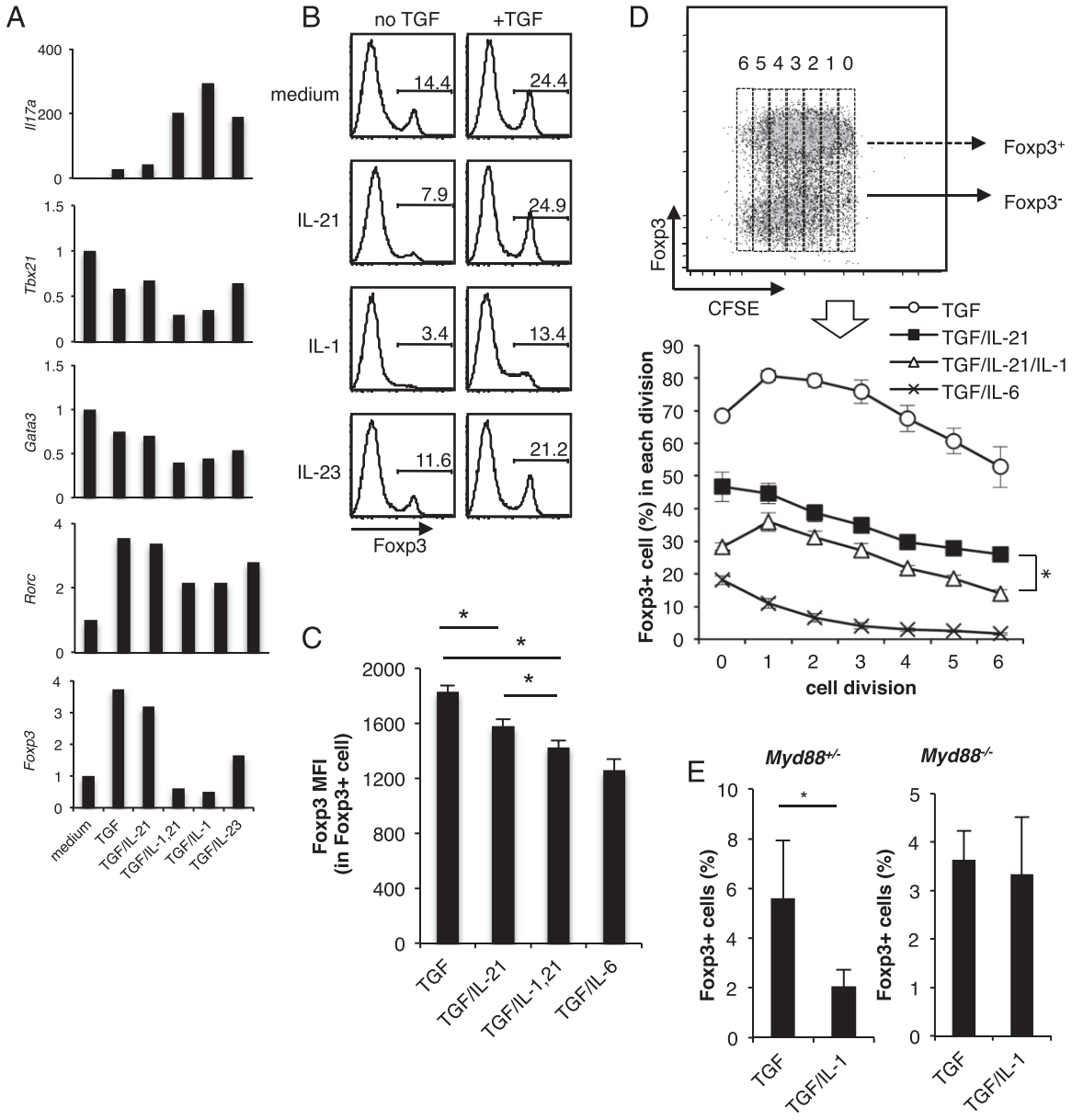

FIGURE 3. IL-1 suppressed Foxp3 expression. (**A** and **B**) FACS-sorted naive CD4$^+$ T cells from WT mice were cultured with anti-CD3 and anti-CD28 under Th17 cell–polarizing conditions (anti–IL-4, anti–IFN-γ, TGF-β, and IL-21) for 5 d. (A) Cells were restimulated with anti-CD3 and anti-CD28 in the presence of anti–IL-4, anti–IFN-γ, and indicated cytokines, and the expressions of *Il17a*, *Tbx21*, *Gata3*, *Rorc*, and *Foxp3* mRNAs were measured 48 h after restimulation. All data were normalized to *Gapdh* mRNA. (B) Cells were restimulated with anti-CD3 and anti-CD28 in the presence of anti–IL-4, anti–IFN-γ, and indicated cytokine(s) for an additional 2 d and stained for Foxp3 after PMA-ionomycin activation. *Left panel*, TGF-β was not added in the second-round stimulation. *Right panel*, TGF-β was added in the second-round stimulation. (**C** and **D**) FACS-sorted *Il6*$^{-/-}$ naive CD4$^+$ T cells labeled with CFSE were cultured with anti-CD3, anti-CD28, anti–IL-4, and anti–IFN-γ in the presence of indicated cytokines for 4 d, and the expression of Foxp3 was examined by intracellular staining. (C) MFI of the Foxp3 staining. (D) IL-1–induced Foxp3 downregulation was independent of cell proliferation. Cell division after CFSE labeling was calculated by the CFSE fluorescence intensity, and Foxp3$^+$ cell proportion was determined by flow cytometry in cells with different cell division. *$p < 0.05$: TGF-β + IL-21 versus TGF-β + IL-1α + IL-1β + IL-21 (Student *t* test). (**E**) FACS-sorted naive CD4$^+$ T cells from *Myd88*$^{+/-}$ and *Myd88*$^{-/-}$ mice were cultured with anti-CD3 and anti-CD28 under Th17 cell–polarizing conditions (anti–IL-4, anti–IFN-γ, TGF-β, and IL-21) for 4 d. Then, cells were restimulated with anti-CD3 and anti-CD28 in the presence of indicated cytokine(s) for 2 d and stained for Foxp3. These data are representative of two independent experiments. *$p < 0.05$ (Student *t* test).

IL-6 is not required in some T cell–independent RA models, such as human TNF-α Tg mice and collagen Ab–induced arthritis (2). Because the development of arthritis in these IL-6–dependent RA models also depends on IL-17A, it is suggested that IL-6 is required for the development of Th17 cells in vivo, as shown in vitro. Interestingly, we found that *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice develop arthritis of similar incidence and severity as *Il1rn*$^{-/-}$ mice. Because the development of arthritis in *Il1rn*$^{-/-}$ mice depends on IL-17A and T cells (34, 49), we examined the development of Th17 cells in these *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mice. We have demonstrated in this study that Th17 cells develop normally in *Il1rn*$^{-/-}$ mice even in the absence of IL-6. This is because excess IL-1 signaling induces Th17 cell differentiation synergistically with TGF-β by suppressing Foxp3 expression under Th17 cell–polarizing conditions. In addition, IL-1 can induce Th17 cell–specific transcription factors and support the maintenance of Th17 cell lineage synergistically with TGF-β. These results indicate that excess IL-1 signaling can overcome the deficiency of IL-6 in the development of Th17 cells.

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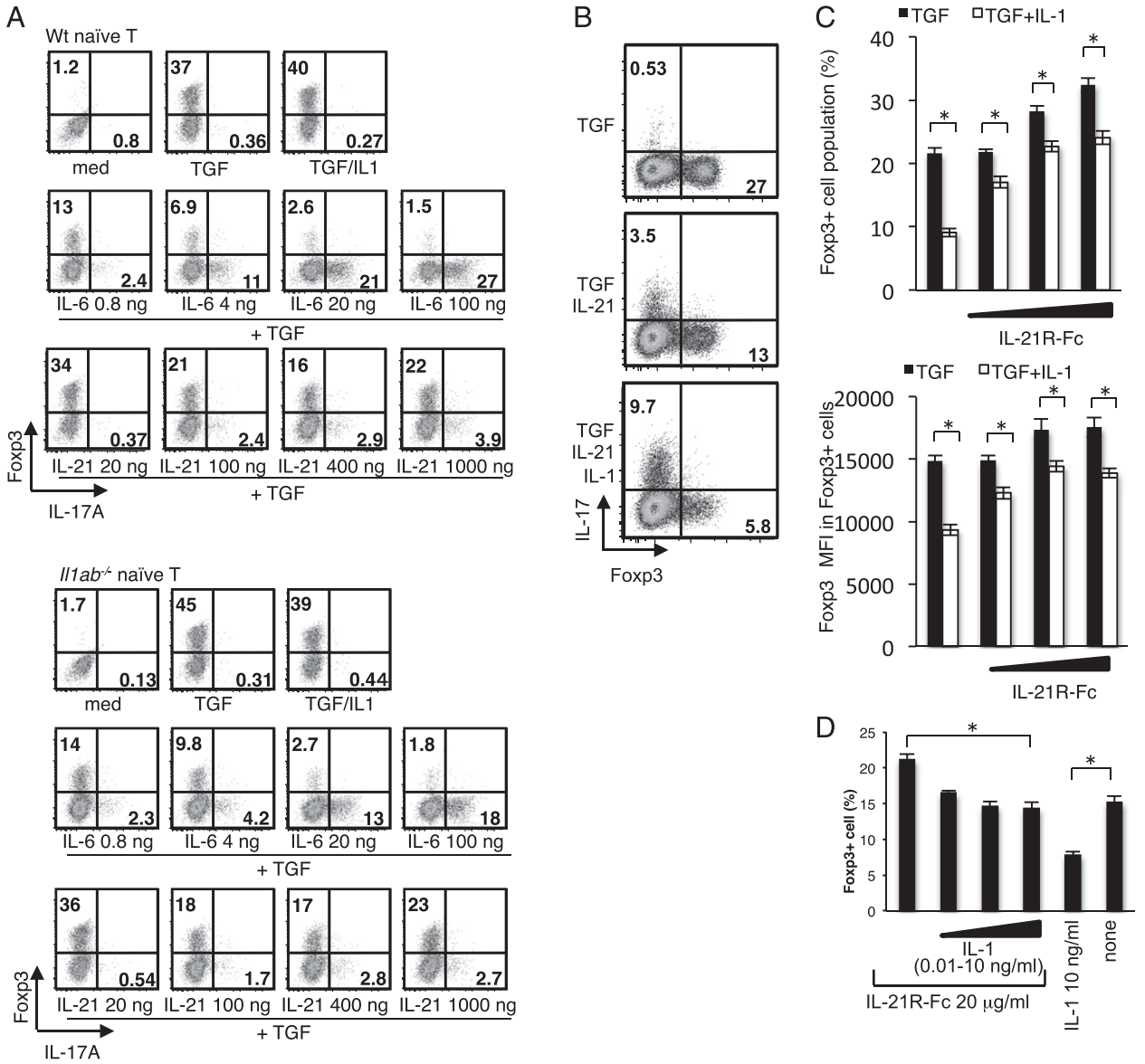

FIGURE 4. IL-1–induced Foxp3 downregulation was independent of IL-6 and IL-21. (**A**) IL-6, but not IL-21, fully induced IL-17A and completely suppressed Foxp3 expression. FACS-sorted naive CD4$^+$ T cells from WT or IL-1α/β–deficient mice were cultured with anti-CD3, anti-CD28, anti–IFN-γ, and anti–IL-4 for 4 d in the presence of indicated reagents. Cells were stained for IL-17A and Foxp3 after PMA-ionomycin activation. *Top panel*, WT. *Middle and bottom panel*, *Il1ab*$^{-/-}$ T cells. The data are representative of two independent experiments. (**B**) FACS-sorted *Il6*$^{-/-}$ naive T cells were cultured for 4 d in the presence of anti–IFN-γ, anti–IL-4, plate-coated anti-CD3, and soluble anti-CD28 with indicated cytokine(s) (TGF-β, 3 ng/ml; IL-21, 400 ng/ml; IL-1α, 10 ng/ml; IL-1β, 10 ng/ml). Then, Foxp3 and IL-17A expression were analyzed by flow cytometry after restimulation with PMA and ionomycin. Data are representative of two independent experiments. (**C**) FACS-sorted *Il6*$^{-/-}$ naive CD4$^+$ T cells were cultured with anti-CD3 and anti-CD28 under Th17 cell–polarizing conditions (anti–IL-4, anti–IFN-γ, and TGF-β + IL-21) for 4 d. Cells were restimulated with anti-CD3 and anti-CD28 in the presence of TGF-β ± (IL-1α + IL-1β) and IL-21R–Fc (0, 1, 5, and 15 μg/ml) for 2 d. Dose-dependent effects of IL-21R–Fc on the expression of Foxp3 were determined by intracellular staining. *Top panel*, Percentage of Foxp3$^+$ cell population. *Bottom panel*, MFI of Foxp3 staining. *$p < 0.05$ (Student *t* test). (**D**) TGF-β + IL-21–treated *Il6*$^{-/-}$ cells as shown in (C) were restimulated with anti-CD3 and anti-CD28 in the presence of IL-1α and IL-1β (0, 0.01, 0.1, and 10 ng/ml each) and IL-21R–Fc (20 μg/ml), IL-1α plus IL-1β only (10 ng/ml each), or none for 2 d and stained for Foxp3. The data are representative of two independent experiments. *$p < 0.05$ (Student *t* test).

Importantly, IL-1 does not downregulate Foxp3 expression in naive T cells, because IL-1R1 is not expressed on the surface of these cells. We showed that TGF-β plus IL-21 or IL-6 can induce IL-1R1 expression on the surface of naive T cells, and IL-21 expression is augmented in γδ T cells from *Il1rn*$^{-/-}$*Il6*$^{-/-}$ mouse LNs upon treatment with IL-1 plus IL-23 (Fig. 2B, 2C). Consistent with our data (Fig. 2C), Sutton et al. (50) reported that γδ T cell–derived IL-21 augments IL-17 production by Th17 cells. Thus, in *Il1rn*$^{-/-}$ mice, IL-1Ra deficiency–induced excess IL-1 signaling activates dendritic cells and macrophages to produce IL-23, then IL-23 activates γδ T cells to produce IL-21, leading to the induction of IL-1R1 expression on naive T cells. In line with our notion, the involvement of IL-21 is implicated in various IL-17–dependent autoimmune diseases. Young et al. (51) showed that blockade of the IL-21/IL-21R pathway ameliorates CIA. IL-21 enhances experimental colitis by inducing Th17 cells and suppressing Foxp3 expression (52). IL-21–deficient mice fail to develop colitis due to the defect of Th17 cell differentiation, and IL-21 can substitute for IL-6 in driving IL-17 induction (53). In humans, Th17 cell differentiation from Foxp3$^+$ Treg cells is enhanced by IL-1β, IL-23,

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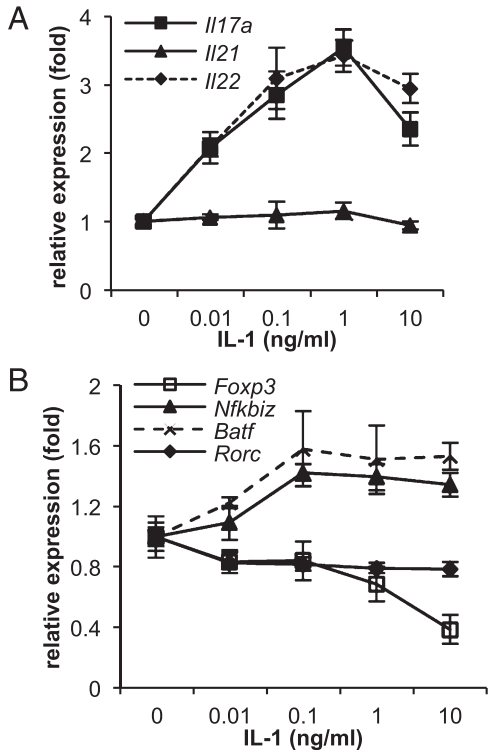

FIGURE 5. IL-1–induced the expression of Th17 cell–specific cytokines and transcription factors. (**A** and **B**) FACS-sorted naive CD4$^+$ T cells from *Il6*$^{-/-}$ mice were cultured with anti-CD3 and anti-CD28 under Th17 cell–polarizing conditions (anti–IL-4, anti–IFN-γ, TGF-β + IL-21) for 4 d. Cells were restimulated with anti-CD3 and anti-CD28 in the presence of anti–IL-4, anti–IFN-γ, and various concentrations of IL-1α and IL-1β (0–10 μg/ml each), and the mRNA of the indicated genes was measured at 24 h after stimulation. All of the data were normalized to *Gapdh* mRNA. Data are representative of two experiments.

and IL-21 (54). Anakinra, a human rIL-1Ra, significantly decreases serum levels of IL-17 and IL-21 and the percentages of Th17 cells, associated with clinical improvement in RA (55). Association of genetic variations of IL-21 region is reported in multiple autoimmune diseases such as systemic lupus erythematosus (56), RA, type 1 diabetes, inflammatory bowel diseases (57), and ulcerative colitis (58). These observations suggested that IL-21 is important for the differentiation of Th17 cells and development of autoimmune diseases.

IL-6 induces the differentiation of Th17 cells in collaboration with TGF-β by activating STAT3 in the downstream. STAT3 activates Th17 cell–specific genes including *Il17a* and downregulates Foxp3, which inhibits Th17 cell differentiation by antagonizing the function of RORγt and RORα. IL-21 can also induce Th17 cell differentiation in collaboration with TGF-β and substitute for the function of IL-6 (40). We showed in this study that IL-1 also induced Th17 cell differentiation, but the function of IL-1 is different from that of IL-21 or IL-6. Although IL-1 can induce IL-21 and IL-6, the suppression of Foxp3 was not caused by IL-1–induced IL-21 or IL-6, because the suppression was also observed in IL-6–deficient T cells and IL-21R–Fc-treated T cells (Fig. 4). Furthermore, IL-6– or IL-21–induced Foxp3 downregulation occurred normally in IL-1α/β–deficient mice, indicating that the action of IL-6 or IL-21 is independent of IL-1 action (Fig. 4A). We also showed that this suppression was not a result of preferential Foxp3-negative T cell proliferation (Fig. 3D). These observations suggest that IL-1 itself has Foxp3-suppressing activity.

Regarding this, we showed that the Akt-mTOR pathway that suppresses Treg cell differentiation (48) is involved in IL-1–induced Foxp3 suppression (Fig. 6B). Furthermore, it was reported that IL-1 antagonizes the Th17 cell differentiation–inhibiting activity of IL-2, which induces Foxp3 expression by activating STAT5 (44).

We showed that IL-1 also activates Th17 cell–specific transcription factors, including *Nfkbiz* and *Batf*, but not *Rorc*, resulting in the upregulation of *Il17a* and *Il22* expression (Fig. 5A, 5B). This induction was not caused by IL-6 or IL-21, because we used IL-6–deficient T cells, and IL-1 from these Th17-polarized cells did not induce IL-21 expression. Recent studies have highlighted

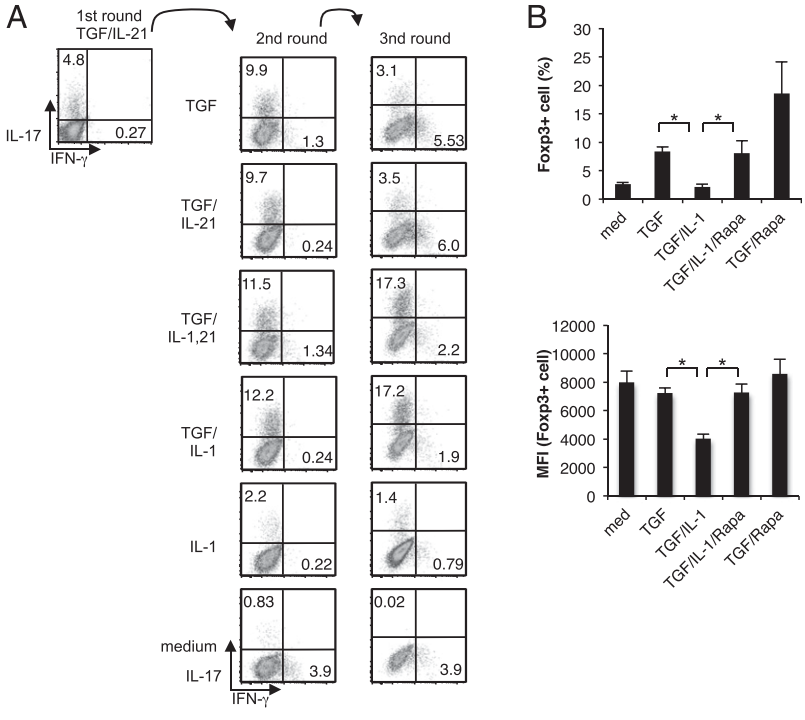

FIGURE 6. IL-1 maintained IL-17A expression synergistically with TGF-β. (**A**) FACS-sorted naive CD4$^+$ T cells from WT mice were cultured with anti-CD3 and anti-CD28 for 5 d under Th17 cell–polarizing conditions (TGF-β, IL-21, anti–IFN-γ, and anti–IL-4) (first-round culture). Cells were harvested and restimulated with anti-CD3 and anti-CD28 in the presence of indicated cytokine(s), anti–IFN-γ, and anti–IL-4 at the same concentration with or without IL-1α/β for an additional two rounds (5 d each) and stained for intracellular IL-17A and IFN-γ after PMA-ionomycin activation (post–third-round culture). The data are representative of three independent experiments. (**B**) IL-1 downregulated Foxp3 expression through mTOR. FACS-sorted naive CD4$^+$ T cells were cultured under Th17 cell–polarizing conditions (TGF-β, IL-21, anti–IFN-γ, and anti–IL-4) for 4 d. Cells were restimulated with anti-CD3 and anti-CD28 in the presence of indicated cytokine(s), anti–IFN-γ, anti–IL-4, and rapamycin (Rapa; 50 ng/ml) for an additional 5 d and stained intracellularly for Foxp3. *Top panel*, Percentage of Foxp3$^+$ cell population. *Bottom panel*, MFI of Foxp3 staining. The data are representative of two independent experiments. *$p < 0.05$ (Student *t* test). med, Medium.

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a critical role for IL-1 in the differentiation of Th17 cells (27, 59). We have also shown that IL-1 can maintain the survival of Th17 cells in collaboration with TGF-β. A much higher proportion of the IL-17A–producing T cell population was maintained in the presence of IL-1 than with TGF-β alone or IL-21 plus TGF-β (Fig. 6A). Consistent with our results, Striteski et al. (60) reported that IL-1β enhances IL-23–stimulated Th17 cell survival. These results suggest that IL-1 not only downregulates Foxp3 expression but also upregulates Th17 cell lineage–specific transcription factors, promoting differentiation and survival of Th17 cell lineage.

In this study, we showed the critical roles of IL-1 signaling during Th17 cell differentiation, providing a key link between IL-1 signaling and Foxp3 expression as well as the maintenance of Th17 cells. These findings should give more insight into the mechanisms of differentiation and maintenance of Th17 cells (Supplemental Fig. 4). Especially, the downregulation of Foxp3 expression by IL-1 may be important for the transition of Treg cells into Th17 cells. Zhou et al. (61) showed that Foxp3 expression in a substantial percentage of Treg cells is unstable under autoimmune conditions, and ex-Foxp3 cells generate pathogenic memory T cells and produce IFN-γ and IL-17. Furthermore, gut microenvironment or parasitic infection favors the reprogramming of $Foxp3^+$ Treg cells into effector Th17 and Th1 cells, and mTOR inhibition with rapamycin stabilizes Foxp3 expression in Treg cells and inhibits IL-17 expression (62). Because IL-1 expression is induced in autoimmune conditions and parasitic infection, we suggest that IL-1–mediated downregulation of Foxp3 expression is involved in the transition of Treg cells into Th17 cells in vivo.

However, the roles of these Th17 cells in the development of arthritis still remain to be elucidated, because IL-17A is also produced by γδ T cells in the affected joints of *Il1rn*$^{-/-}$ mice, and the IL-$17A^+CD4^+$ T cell content in LNs is low relative to IL-$17A^+$ $CD4^-$ T cells (Fig. 1). Furthermore, we recently found that both $CD4^+$ T cells and γδ T cells are required for the development of arthritis in *Il1rn*$^{-/-}$ mice (A. Akitsu and Y. Iwakura, unpublished observations). We are now analyzing the roles of IL-17A–producing γδ T cells in the development of arthritis in *Il1rn*$^{-/-}$ mice (A. Akitsu, H. Ishigame, S. Kakuta, S.H. Chung, S. Ikeda, K. Shimizu, S. Kubo, Y. Liu, H. Saito, M. Umemura, G. Matsuzaki, Y. Yoshikai, S. Saijo, and Y. Iwakura, unpublished observations).

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Disclosures

The authors have no financial conflicts of interest.

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