

# Development and characterization of IL-21-producing CD4<sup>+</sup> T cells

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**It has recently been shown that interleukin (IL)-21 is produced by Th17 cells, functions as an autocrine growth factor for Th17 cells, and plays critical roles in autoimmune diseases. In this study, we investigated the differentiation and characteristics of IL-21-producing CD4<sup>+</sup> T cells by intracellular staining. Unexpectedly, we found that under Th17-polarizing conditions, the majority of IL-21-producing CD4<sup>+</sup> T cells did not produce IL-17A and -17F. We also found that IL-6 and -21 potently induced the development of IL-21-producing CD4<sup>+</sup> T cells without the induction of IL-4, IFN- $\gamma$ , IL-17A, or IL-17F production. On the other hand, TGF- $\beta$  inhibited IL-6- and IL-21-induced development of IL-21-producing CD4<sup>+</sup> T cells. IL-2 enhanced the development of IL-21-producing CD4<sup>+</sup> T cells under Th17-polarizing conditions. Finally, IL-21-producing CD4<sup>+</sup> T cells exhibited a stable phenotype of IL-21 production in the presence of IL-6, but retained the potential to produce IL-4 under Th2-polarizing conditions and IL-17A under Th17-polarizing conditions. These results suggest that IL-21-producing CD4<sup>+</sup> T cells exhibit distinct characteristics from Th17 cells and develop preferentially in an IL-6-rich environment devoid of TGF- $\beta$ , and that IL-21 functions as an autocrine growth factor for IL-21-producing CD4<sup>+</sup> T cells.**

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Activated CD4<sup>+</sup> T cells differentiate into at least three distinct effector subsets as defined by their patterns of cytokine production (1–4). Th1 cells produce IFN- $\gamma$  and lymphotoxin and play a critical role in protective immunity against intracellular pathogens (1–4). Th2 cells produce IL-4, -5, and -13 and are essential for the expulsion of parasites (1–4). Newly identified Th17 cells produce IL-17A and -17F and play a pathogenic role in a variety of autoimmune diseases (1–5). Recently, it has been reported that IL-21 is another cytokine highly produced by Th17 cells and that it promotes the development of Th17 cells (6–9).

IL-21 is a four-helix-bundle type I cytokine with significant homology to IL-2, -15, and -4 (10–12). IL-21 has been demonstrated to be expressed in Th2 cells (13), follicular B helper

T cells (14), and NK T cells (15) and to exhibit pleiotropic effects on the proliferation, differentiation, and effector function of T, B, NK, and dendritic cells (10–12). In vivo, it has been shown that IL-21 is involved in several autoimmune disease models. For example, lymphopenia and compensatory IL-21-mediated homeostatic expansion of lymphocytes has been reported to be involved in the development of autoimmunity in NOD mice (16). The excessive production of IL-21 has been shown to be associated with the development of high titers of autoantibodies and a lupus-like pathology in *Sanroque* mice (17). Furthermore, a blockade of IL-21 signaling has been demonstrated to ameliorate mouse models of rheumatoid arthritis and lupus (18, 19). However, the characteristic of IL-21-producing CD4<sup>+</sup> T cells, especially in their relation to Th17 cells, is still unknown because a single-cell analysis of IL-21-producing cells has not been achieved yet.

A. Suto and D. Kashiwakuma contributed equally to this work.

In this study, by establishing the intracellular staining of IL-21, we found that although IL-21-producing CD4<sup>+</sup> T cells developed preferentially under Th17-polarizing conditions, a considerable number of IL-21-producing CD4<sup>+</sup> T cells were negative for intracellular IL-17A and -17F. We also found that IL-6 potently induced the development of IL-21-producing CD4<sup>+</sup> T cells without the induction of IL-4, IFN- $\gamma$ , IL-17A, or IL-17F production. On the other hand, TGF- $\beta$  inhibited the IL-6-induced development of IL-21-producing CD4<sup>+</sup> T cells. In addition, IL-2 significantly enhanced the development of IL-21-producing CD4<sup>+</sup> T cells under Th17-polarizing conditions. IL-21 itself also induced the development of IL-21-producing CD4<sup>+</sup> T cells. IL-21-producing CD4<sup>+</sup> T cells exhibited a stable phenotype of IL-21 production in the presence of IL-6, but still had a potential to produce IL-4 under Th2-polarizing conditions and IL-17A under Th17-polarizing conditions. Our results suggest that IL-21-producing CD4<sup>+</sup> T cells exhibit distinct characteristics from Th17 cells, that IL-21-producing CD4<sup>+</sup> T cells develop preferentially in an IL-6-rich environment devoid of TGF- $\beta$ , and that IL-21 functions as an autocrine growth factor for IL-21-producing CD4<sup>+</sup> T cells.

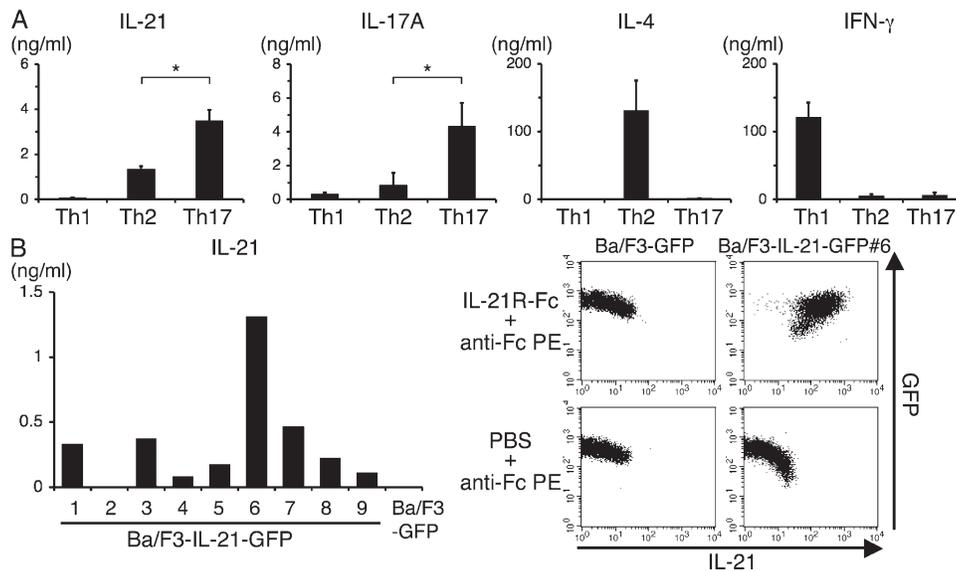
**RESULTS**

**IL-21-producing CD4<sup>+</sup> T cells develop preferentially under Th17-polarizing conditions, but the majority of them are negative for intracellular IL-17A and -17F**

Recently, it has been shown that Th17 cells produce a large amount of IL-21 compared with Th2 and Th1 cells, and that

IL-21 functions as an autocrine growth factor for Th17 cells (6–9). We also found that IL-21 was produced by activated CD4<sup>+</sup> T cells under Th17-polarizing conditions (in the presence of IL-6, TGF- $\beta$ , anti-IL-4 mAb, and anti-IFN- $\gamma$  mAb) much greater than under Th2-polarizing conditions or under Th1-polarizing conditions (Fig. 1 A). However, it was still unclear whether IL-21 is actually produced by Th17 cells and what is the developmental and phenotypic characteristic of IL-21-producing CD4<sup>+</sup> T cells because IL-21 production has not been determined at single-cell levels as yet. To address these issues, we established the intracellular cytokine staining of IL-21. We first generated several IL-21-producing Ba/F3 cell clones using a bicistronic retrovirus system and measured the levels of IL-21 in the culture supernatants by ELISA. Among these clones, Ba/F3-IL-21-GFP #6 cells produced a significant amount of IL-21 (Fig. 1 B, left), and we used this clone as a positive control for intracellular staining of IL-21. To detect intracellular IL-21, we used mouse IL-21R/human Fc-chimera (IL-21R-Fc) to capture IL-21 and anti-human Fc PE to visualize IL-21-IL-21R-Fc complexes. When cells were stained by this method, positive signals were detected in Ba/F3-IL-21-GFP #6 cells, but not in control Ba/F3-empty-GFP cells (Fig. 1 B, right). In the absence of IL-21R-Fc, no positive signal was detected (Fig. 1 B, right), confirming the correct detection of IL-21 by the intracellular staining.

By using the intracellular staining of IL-21, we examined IL-21-producing CD4<sup>+</sup> T cells at single-cell levels. When

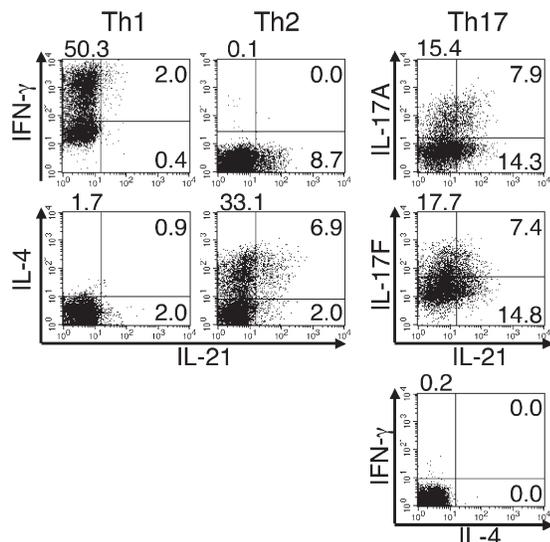


**Figure 1. Establishment of a single-cell analysis of IL-21-producing cells.** (A) IL-21 and -17A are produced by activated CD4<sup>+</sup> T cells under Th17-polarizing conditions. Purified CD4<sup>+</sup> T cells from lymph nodes of C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th1-, Th2-, and Th17-polarizing conditions. On day 5, 10<sup>6</sup> cells were restimulated with anti-CD3 mAb/anti-CD28 mAb for 24 h. The levels of cytokines in the culture supernatants were measured by ELISA. Data are the means  $\pm$  the SD from four independent experiments. \*, P < 0.01. (B) Establishment of a single-cell analysis of IL-21-producing cells. Ba/F3 cells were infected with pMX-IL-21-IRES-GFP retrovirus or control retrovirus (pMX-IRES-GFP). Nine clones of pMX-IL-21-IRES-GFP retrovirus-infected Ba/F3 cells (Ba/F3-IL-21-GFP cells) and one clone of control retrovirus-infected Ba/F3 cells (Ba/F3-GFP cells) were selected by limiting dilution. IL-21 in the culture supernatants was measured by ELISA (left). Ba/F3-GFP cells and Ba/F3-IL-21-GFP clone #6 cells were fixed, permeabilized, and incubated with IL-21R-Fc or PBS. After washing, cells were visualized with anti-Fc PE (right).

FACS-sorted naive CD4<sup>+</sup> T cells (CD44<sup>low</sup> CD25<sup>-</sup> CD4<sup>+</sup> T cells) were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th1-, Th2-, and Th17-polarizing conditions, IL-21-producing cells were detected under Th17-polarizing conditions with a high frequency and under Th2-polarizing conditions with a moderate frequency (Fig. 2), which is consistent with the levels of IL-21 in the supernatants of activated CD4<sup>+</sup> T cells under the corresponding culture conditions (Fig. 1 A). Under Th2-polarizing conditions, a significant number of IL-4-producing cells simultaneously produced IL-21 and >70% of IL-21-producing CD4<sup>+</sup> T cells were positive for intracellular IL-4 (Fig. 2). Under Th17-polarizing conditions, a significant number of IL-17A- and IL-17F-producing cells also simultaneously produced IL-21, but >60% of IL-21-producing CD4<sup>+</sup> T cells were negative for intracellular IL-17A and -17F (Fig. 2). IL-21-producing CD4<sup>+</sup> T cells under Th17-polarizing conditions were also negative for intracellular IL-4 and IFN- $\gamma$  (Fig. 2). These results suggest that although IL-21-producing CD4<sup>+</sup> T cells develop preferentially under Th17-polarizing conditions, the majority of IL-21-producing CD4<sup>+</sup> T cells do not produce IL-17A or -17F.

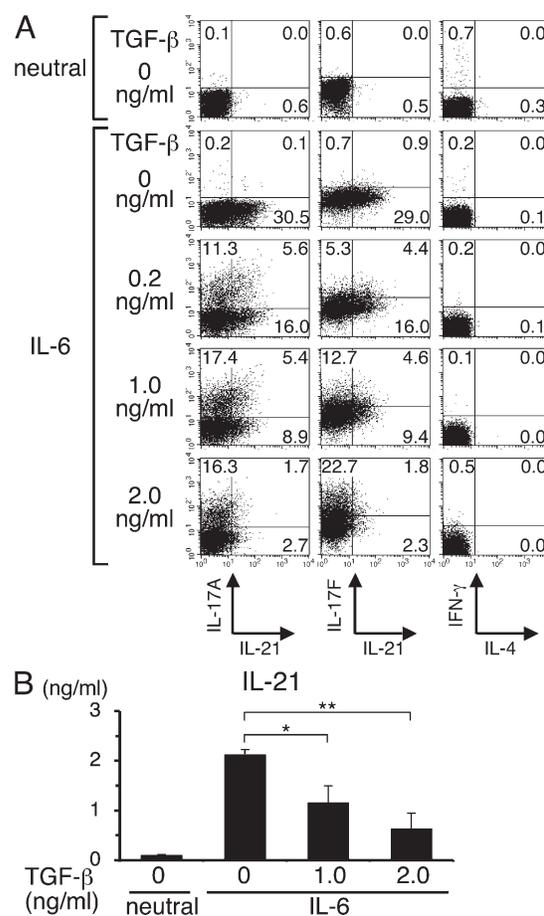
#### IL-6 induces, but TGF- $\beta$ inhibits, the development of IL-21-producing CD4<sup>+</sup> T cells

Because IL-21-producing CD4<sup>+</sup> T cells developed preferentially under Th17-polarizing conditions as compared with Th1- or Th2-polarizing conditions (Fig. 1 A and Fig. 2), we next examined the role of IL-6 and TGF- $\beta$  in the develop-



**Figure 2. CD4<sup>+</sup> T cells producing IL-21, but not IL-17A/IL-17F, are present under Th17-polarizing conditions.** Naive CD4<sup>+</sup> T cells from lymph nodes of C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th1-, Th2-, and Th17-polarizing conditions for 5 d. Cells were evaluated for the expression of the indicated cytokines by intracellular cytokine staining as described in the Materials and methods. Data are representative of three independent experiments.

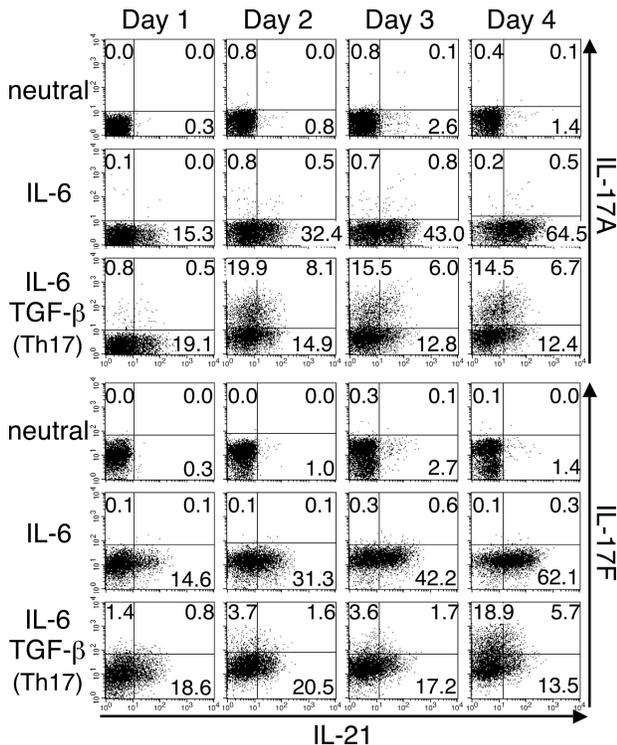
ment of IL-21-producing CD4<sup>+</sup> T cells. Naive CD4<sup>+</sup> T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti-IL-4 mAb, and anti-IFN- $\gamma$  mAb with or without TGF- $\beta$ . We found that IL-6 together with the blocking antibodies to IL-4 and IFN- $\gamma$  strongly induced the development of IL-21-producing CD4<sup>+</sup> T cells without the induction of IL-4, IFN- $\gamma$ , IL-17A, or IL-17F production (Fig. 3 A). Furthermore, unexpectedly, TGF- $\beta$  inhibited the development of IL-21-producing CD4<sup>+</sup> T cells in a dose-dependent manner (Fig. 3 A). Measurement of IL-21 levels in the supernatants confirmed the TGF- $\beta$ -mediated inhibition of IL-21 production (Fig. 3 B). TGF- $\beta$  also inhibited the development of IL-21-producing CD4<sup>+</sup> T cells under



**Figure 3. IL-6 induces, but TGF- $\beta$  inhibits, the development of IL-21-producing CD4<sup>+</sup> T cells.** (A and B) Naive CD4<sup>+</sup> T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of 10  $\mu$ g/ml anti-IL-4 mAb and 10  $\mu$ g/ml anti-IFN- $\gamma$  mAb (neutral condition) with or without 100 ng/ml IL-6. Where indicated, 0.2–2 ng/ml TGF- $\beta$  was added. (A) 4 d later, cells were stimulated with PMA/ionomycin, and intracellular staining for the indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments. (B) 4 d later, cells were washed and stimulated with PMA/ionomycin for 8 h at  $2 \times 10^6$  cells/ml. The levels of IL-21 in the culture supernatants were measured by ELISA. Data are the mean  $\pm$  the SD ( $n = 3$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

Th2-polarizing conditions (unpublished data). In contrast, consistent with previous reports (20–22), TGF- $\beta$  together with IL-6 induced the development of IL-17A- and IL-17F-producing Th17 cells (Fig. 3 A). These results indicate that IL-6 induces the development of IL-21-producing CD4<sup>+</sup> T cells, but on the contrary, TGF- $\beta$  inhibits IL-6-induced development of IL-21-producing CD4<sup>+</sup> T cells.

We next examined the kinetics of IL-21, -17A, and -17F production from naive CD4<sup>+</sup> T cells upon stimulation with anti-CD3 mAb/anti-CD28 mAb. In the presence of IL-6, anti-IL-4 mAb, and anti-IFN- $\gamma$  mAb, IL-21-producing CD4<sup>+</sup> T cells were detected at a low frequency at day 1 after stimulation, and then the frequency of IL-21-producing CD4<sup>+</sup> T cells was increased until day 4 in a time-dependent manner without the production of IL-17A and -17F (Fig. 4). Under Th17-polarizing conditions, IL-21-producing CD4<sup>+</sup> T cells were also detected at day 1, but the frequency of IL-21-producing CD4<sup>+</sup> T cells was not increased thereafter (Fig. 4). On the other hand, IL-17A-producing CD4<sup>+</sup> T cells were detected at day 2 under Th17-polarizing conditions (Fig. 4). IL-17F-producing CD4<sup>+</sup> T cells were also detected at day 2 under Th17-polarizing conditions and were increased at day 4 (Fig. 4).



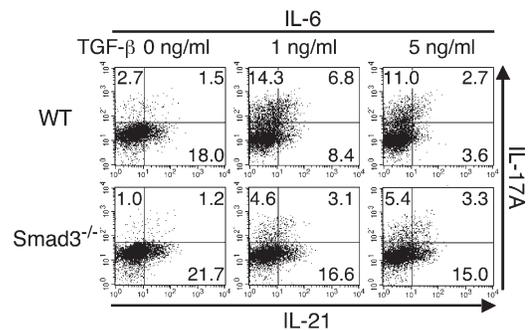
**Figure 4. Kinetics of IL-21 production from CD4<sup>+</sup> T cells.** Naive CD4<sup>+</sup> T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb and anti-IFN- $\gamma$  mAb (neutral condition) with 100 ng/ml IL-6 or IL-6 plus 1 ng/ml TGF- $\beta$ . At indicated times after stimulation, cells were stimulated with PMA/ionomycin, and intracellular staining for the indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments.

**Smad3 is involved in TGF- $\beta$ -mediated inhibition of IL-21-producing CD4<sup>+</sup> T cells**

Smad2 and Smad3 are phosphorylated by TGF- $\beta$  receptor and heterodimerize with Smad4 (23, 24). The activated Smad complex then translocates into the nucleus and regulates the transcription of target genes (23, 24). We then used the mice lacking Smad3 (Smad3<sup>-/-</sup> mice) to determine whether Smad3 is required for the TGF- $\beta$ -mediated suppression of IL-21-producing CD4<sup>+</sup> T cells. Purified CD4<sup>+</sup> T cells from WT mice or asymptomatic Smad3<sup>-/-</sup> mice, in the latter of which lymph node CD4<sup>+</sup> T cells exhibit a normal phenotype regarding the activation markers such as CD69 (unpublished data) (25), were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti-IL-4 mAb, anti-IFN- $\gamma$  mAb, and anti-IL-2 mAb with or without TGF- $\beta$  and the development of IL-21-producing CD4<sup>+</sup> T cells and Th17 cells was evaluated by intracellular staining. As shown in Fig. 5, not only TGF- $\beta$ -mediated induction of Th17 cell development but also TGF- $\beta$ -mediated suppression of IL-21-producing CD4<sup>+</sup> T cells were reduced in Smad3<sup>-/-</sup> CD4<sup>+</sup> T cells compared with those in littermate WT mice. These results indicate that TGF- $\beta$  signaling induces Th17 cell development, but inhibits the development of IL-21-producing CD4<sup>+</sup> T cells, in part via Smad3-dependent pathways.

**IL-2 does not inhibit the development of IL-21-producing CD4<sup>+</sup> T cells**

It has recently been reported that IL-2 signaling via STAT5 inhibits the development of Th17 cells (26). We next examined the role of IL-2 in the development of IL-21-producing CD4<sup>+</sup> T cells. When naive CD4<sup>+</sup> T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti-IL-4 mAb, and anti-IFN- $\gamma$  mAb, neither IL-2 nor anti-IL-2 mAb significantly affected the development of IL-21-producing CD4<sup>+</sup> T cells (Fig. 6). On the other hand,



**Figure 5. Smad3 is involved in TGF- $\beta$ -mediated suppression of IL-21 production from CD4<sup>+</sup> T cells.** Purified CD4<sup>+</sup> T cells from Smad3<sup>-/-</sup> mice or littermate WT mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb, anti-IFN- $\gamma$  mAb, and anti-IL-2 mAb with 100 ng/ml IL-6 or IL-6 plus 1 or 5 ng/ml TGF- $\beta$  for 3 d. Cells were then stimulated with PMA/ionomycin and intracellular staining for IL-21 versus IL-17A was performed. Shown are representative FACS profiles from three independent experiments.

under Th17-polarizing conditions, IL-2 significantly enhanced the development of IL-21-producing CD4<sup>+</sup> T cells (Fig. 6). In contrast, IL-17A- and IL-17F-producing CD4<sup>+</sup> T cells were decreased by IL-2 and were increased by anti-IL-2 mAb under Th17-polarizing conditions (Fig. 6), which is consistent with the previous study showing that the genetic deletion or antibody blockade of IL-2 promotes the differentiation of IL-17A-producing CD4<sup>+</sup> T cells (26). These results suggest that, in contrast to the development of Th17 cells, IL-2 instead exerts an enhancing effect on the development of IL-21-producing CD4<sup>+</sup> T cells under Th17-polarizing conditions.

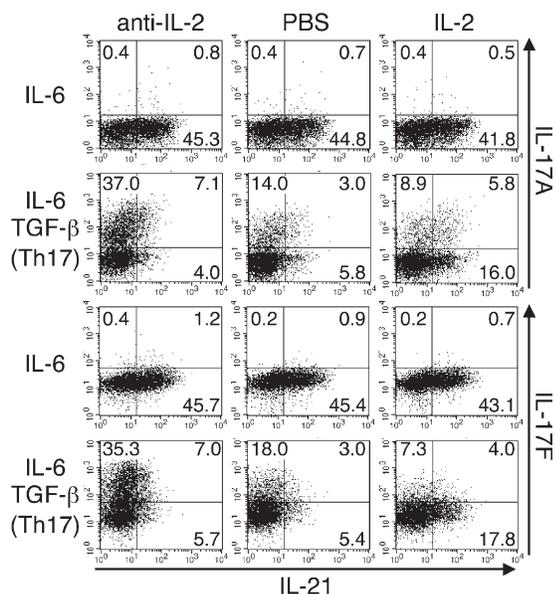
### IL-21 functions as an autocrine growth factor for IL-21-producing CD4<sup>+</sup> T cells

It was recently reported that IL-21 functions as an autocrine growth factor for the development of Th17 cells (6–9). We determined the role of IL-21 on the development of IL-21-producing CD4<sup>+</sup> T cells. IL-21, in the presence of the blocking antibodies to IL-4 and IFN- $\gamma$ , induced the development of IL-21-producing CD4<sup>+</sup> T cells from WT CD4<sup>+</sup> T cells, but not from IL-21R-deficient (IL-21R<sup>-/-</sup>) CD4<sup>+</sup> T cells (Fig. 7 A). In addition, IL-21-induced development of IL-21-producing CD4<sup>+</sup> T cells was inhibited by TGF- $\beta$  (Fig. 7 A), like IL-6-induced development was (Fig. 3). Soluble IL-21R-Fc, which neutralizes IL-21 (10), also inhibited the development of IL-21-producing CD4<sup>+</sup> T cells both in the

presence of IL-6, anti-IL-4 mAb, and anti-IFN- $\gamma$  mAb and under Th17-polarizing conditions by 20–30% (Fig. 7 B). These results suggest that IL-21 and -6 both induce the development of IL-21-producing CD4<sup>+</sup> T cells, the effect of the latter of which is mediated in part by IL-21 production.

In contrast, the blockade of IL-21 signaling by soluble IL-21R-Fc resulted in the decreased development of Th17 cells (Fig. 7 B), which is consistent with previous reports (6–9). Together with our finding that a considerable number of IL-21-producing CD4<sup>+</sup> T cells are negative for intracellular IL-17A and -17F under Th17-polarizing conditions (Fig. 2), these results suggest that IL-21 functions not only as an autocrine growth factor but also as a paracrine growth factor for the development of Th17 cells.

We also examined the effect of anti-IL-6 antibody on the differentiation of IL-21-producing CD4<sup>+</sup> T cells from naive CD4<sup>+</sup> T cells cultured under Th2-polarizing conditions. As shown in Fig. 7 C, a neutralizing antibody against IL-6 decreased the number of IL-21-producing CD4<sup>+</sup> T cells by 50% without any significant change in the number of IL-4 single-positive cells. Moreover, although soluble IL-21R-Fc itself did not significantly decrease the number of IL-21-producing CD4<sup>+</sup> T cells under Th2-polarizing conditions (unpublished data), IL-21R-Fc together with anti-IL-6 antibody significantly suppressed the differentiation of IL-21-producing CD4<sup>+</sup> T cells under Th2-polarizing conditions to a greater extent than anti-IL-6 antibody alone (Fig. 7 C). These results suggest that during Th2 cell differentiation, IL-6 produced by Th2 cells promotes the differentiation of IL-21-producing CD4<sup>+</sup> T cells from Th2 cells and non-Th2 CD4<sup>+</sup> T cells.



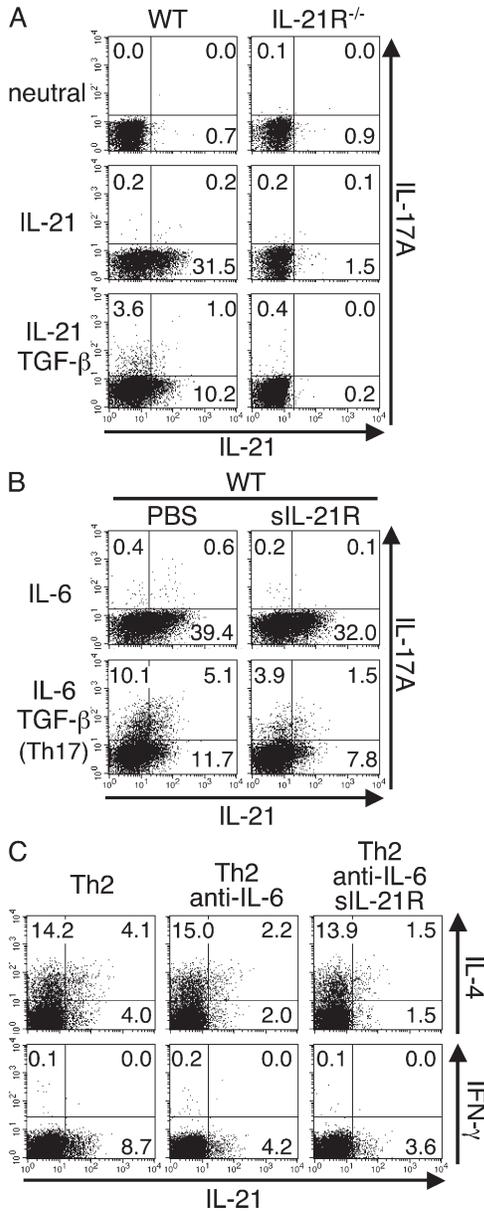
**Figure 6. IL-2 does not inhibit the development of IL-21-producing CD4<sup>+</sup> T cells.** Naive CD4<sup>+</sup> T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb and anti-IFN- $\gamma$  mAb with 100 ng/ml IL-6 or IL-6 plus 1 ng/ml TGF- $\beta$ . Where indicated, either 10 ng/ml IL-2 or 10  $\mu$ g/ml anti-IL-2 antibody was added to the culture. 4 d later, cells were stimulated with PMA/ionomycin and intracellular staining for the indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments.

### Expression of transcription factors in CD4<sup>+</sup> T cells cultured in the presence of IL-6

We also examined the expression pattern of transcription factors in CD4<sup>+</sup> T cells that were cultured in the presence of IL-6, anti-IL-4 mAb, and anti-IFN- $\gamma$  mAb. Consistent with previous reports (5), CD4<sup>+</sup> T cells cultured under Th17-polarizing conditions expressed ROR $\gamma$ t, but not Foxp3, T-bet, or GATA3 (Fig. 8). In contrast, CD4<sup>+</sup> T cells cultured in the presence of IL-6, anti-IL-4 mAb, and anti-IFN- $\gamma$  mAb expressed a moderate level of ROR $\gamma$ t, but not Foxp3, T-bet, or GATA3 (Fig. 8). As expected, Th1 cells expressed T-bet, Th2 cells expressed GATA3, and regulatory T (T reg) cells expressed Foxp3 (Fig. 8).

### The frequency of IL-21-producing cells is increased with the rounds of cell cycle progression

It has been shown that the production of cytokines such as IL-4 is correlatively increased in CD4<sup>+</sup> T cells with cell divisions (27, 28). We next examined the correlation of IL-21 production and cell cycle progression of CD4<sup>+</sup> T cells using a CFSE-labeling method. As shown in Fig. 9, the frequency of cell division was indistinguishable in CD4<sup>+</sup> T cells cultured in the presence of IL-6, anti-IL-4 mAb, and anti-IFN- $\gamma$  mAb and



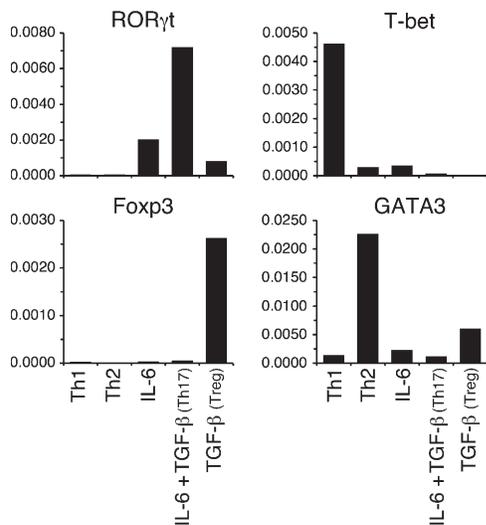
**Figure 7. IL-21 induces the development of IL-21-producing CD4<sup>+</sup> T cells.** (A) Naive CD4<sup>+</sup> T cells from IL-21R<sup>-/-</sup> mice or littermate WT mice on a BALB/c background were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb and anti-IFN-γ mAb (neutral condition) with 100 ng/ml IL-21 or 1 ng/ml IL-21 plus TGF-β. 4 d later, cells were stimulated with PMA/ionomycin and intracellular staining for IL-21 versus IL-17A was performed. Shown are representative FACS profiles from three independent experiments. (B) Naive CD4<sup>+</sup> T cells from BALB/c mice were stimulated with anti-CD3 mAb/anti-CD28 mAb for 4 d in the presence of anti-IL-4 mAb and anti-IFN-γ mAb with IL-6 or IL-6 plus TGF-β. Where indicated, soluble IL-21R-Fc (10 μg/ml) was added. Cells were stimulated with PMA/ionomycin and intracellular staining for IL-21 versus IL-17A was performed. Shown are representative FACS profiles from three independent experiments. (C) Naive CD4<sup>+</sup> T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th2-polarizing conditions for 5 d. Where indicated, a neutralizing antibody against 10 μg/ml IL-6 and/or soluble IL-21R-Fc were added. Cells were

under Th17-polarizing conditions. IL-21-producing CD4<sup>+</sup> T cells were observed in all rounds of cell cycle progression in the presence of IL-6, anti-IL-4 mAb, and anti-IFN-γ mAb, but the frequency of IL-21-producing cells was increased with the rounds of cell cycle progression (Fig. 9). Similarly, under Th17-polarizing conditions, IL-17A- and IL-17F-producing cells were observed in all rounds of cell cycle progression, but the frequency of them was also increased with the rounds of cell cycle progression (Fig. 9).

**IL-21-producing CD4<sup>+</sup> T cells do not lose the ability to produce IL-21 when they are restimulated under Th1- or Th2-polarizing conditions**

Fully differentiated Th1 cells and Th2 cells exhibit a stable phenotype regarding the pattern of cytokine production (29, 30). Finally, we examined whether IL-21-producing CD4<sup>+</sup> T cells exhibited a stable phenotype of IL-21 production. To address this issue, CD4<sup>+</sup> T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti-IL-4 mAb, and anti-IFN-γ mAb twice, at a 5-d interval, and IL-21 production was assessed by intracellular staining after restimulation with anti-CD3 mAb. As shown in Fig. 10 A (top), IL-21-producing CD4<sup>+</sup> T cells did not lose their ability to produce IL-21 at 10 d and did not produce a significant amount of IFN-γ, IL-4, or IL-17A. This stable phenotype of IL-21-producing CD4<sup>+</sup> T cells was observed at least for 20 d of culture (unpublished data). We also examined whether IL-21-producing CD4<sup>+</sup> T cells exhibited a stable phenotype against Th1, Th2, or Th17 polarization. After CD4<sup>+</sup> T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti-IL-4 mAb, and anti-IFN-γ mAb for 5 d, these cells were restimulated under Th1-, Th2-, or Th17-polarizing conditions for 5 d. As shown in Fig. 10 A (bottom), IL-21-producing CD4<sup>+</sup> T cells that were restimulated under Th1-polarizing conditions produced IL-21, but not IFN-γ, indicating that IL-21-producing CD4<sup>+</sup> T cells are stable against Th1 polarization. In contrast, when IL-21-producing CD4<sup>+</sup> T cells were restimulated under Th2-polarizing conditions, a considerable population of cells produced both IL-21 and -4 (Fig. 10 A), suggesting that IL-21-producing CD4<sup>+</sup> T cells may have a potential for IL-4 production. When IL-21-producing CD4<sup>+</sup> T cells were restimulated under Th17-polarizing conditions, IL-21-producing CD4<sup>+</sup> T cells were decreased and IL-17A-producing cells were increased (Fig. 10 A), suggesting that IL-21-producing CD4<sup>+</sup> T cells may also have a potential for the differentiation of Th17 cells. Collectively, these results indicate that IL-21-producing CD4<sup>+</sup> T cells exhibit a stable phenotype of IL-21 production in the presence of IL-6, but may still have a potential for IL-4 and -17A production.

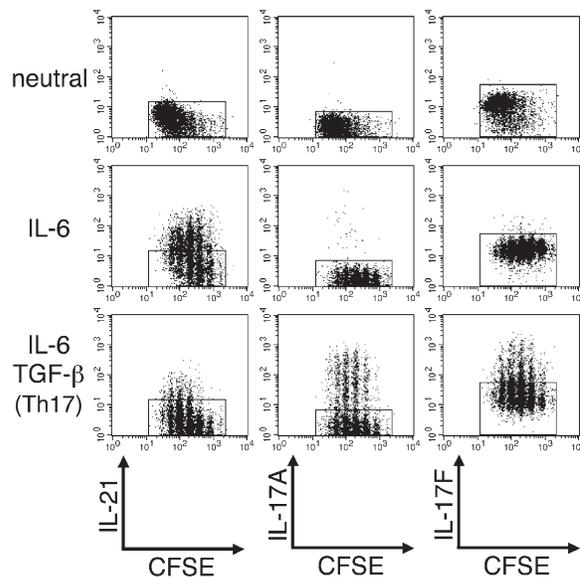
then stimulated with PMA/ionomycin and intracellular staining for the indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments.



**Figure 8. Expression of transcription factors in CD4<sup>+</sup> T cells cultured in the presence of IL-6.** Naive CD4<sup>+</sup> T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb and anti-IFN- $\gamma$  mAb with IL-6, IL-6 plus TGF- $\beta$ , or TGF- $\beta$  alone for 60 h. As controls, naive CD4<sup>+</sup> T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th1-polarizing conditions or Th2-polarizing conditions for 60 h. The expression of ROR $\gamma$ t, Foxp3, T-bet, and GATA3 was assessed by real-time PCR. Data are representative of three independent experiments.

## DISCUSSION

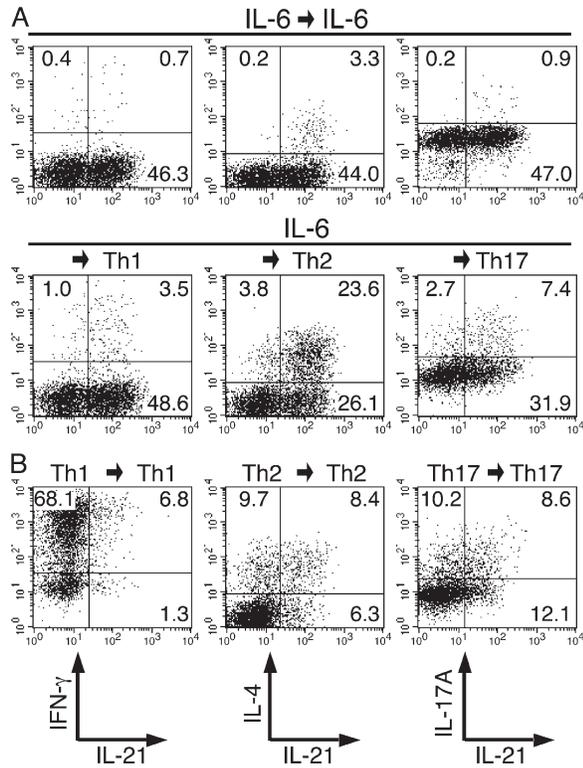
In this study, we investigated the differentiation and characteristics of IL-21-producing CD4<sup>+</sup> T cells. By the newly developed intracellular staining of IL-21, we found that although IL-21-producing CD4<sup>+</sup> T cells developed preferentially under Th17-polarizing conditions, a considerable number of IL-21-producing CD4<sup>+</sup> T cells were negative for intracellular IL-17A and -17F (Fig. 2). We also found that IL-6 strongly induced the development of IL-21-producing CD4<sup>+</sup> T cells without the induction of IL-4, IFN- $\gamma$ , IL-17A, and IL-17F production (Fig. 3). Furthermore, TGF- $\beta$  inhibited the IL-6-induced development of IL-21-producing CD4<sup>+</sup> T cells in a dose-dependent manner (Fig. 3). In contrast, consistent with previous reports (20–22), TGF- $\beta$  together with IL-6 induced the development of Th17 cells (Fig. 3). IL-21 itself also induced the development of IL-21-producing CD4<sup>+</sup> T cells from WT CD4<sup>+</sup> T cells, but not from IL-21R<sup>-/-</sup> CD4<sup>+</sup> T cells (Fig. 7). In addition, IL-21-induced development of IL-21-producing CD4<sup>+</sup> T cells was inhibited by TGF- $\beta$  (Fig. 7), like IL-6-induced development was (Fig. 3). Finally, CD4<sup>+</sup> T cells cultured in the presence of IL-6 expressed a moderate level of ROR $\gamma$ t, but not Foxp3, T-bet, or GATA3 (Fig. 8), and exhibited a stable phenotype of IL-21 production (Fig. 10). Collectively, these results suggest that IL-21-producing CD4<sup>+</sup> T cells exhibit distinct characteristics from Th17 cells, that IL-21-producing CD4<sup>+</sup> T cells develop preferentially in an IL-6-rich environment devoid of TGF- $\beta$ , and that IL-21 functions as an autocrine growth factor for IL-21-producing CD4<sup>+</sup> T cells.



**Figure 9. The frequency of IL-21-producing cells is increased with the rounds of cell cycle progression.** Naive CD4<sup>+</sup> T cells from C57BL/6 mice were labeled with CFSE, stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb and anti-IFN- $\gamma$  mAb (neutral condition) with IL-6 or IL-6 plus TGF- $\beta$  for 4 d. Cells were stimulated with PMA/ionomycin and intracellular staining for IL-21, -17A, and -17F was performed. Shown are representative FACS profiles from three independent experiments.

IL-21 was originally reported to be a product of activated CD4<sup>+</sup> T cells (10). Subsequently, it has been shown that among CD4<sup>+</sup> T cells, Th2 cells but not Th1 cells produce IL-21 (13). More recently, it has been demonstrated that Th17 cells produce higher levels of IL-21 than Th2 cells and that IL-21 functions as an autocrine growth factor for Th17 cells (6–9). However, all of these findings were based on the analysis on a mixed population of CD4<sup>+</sup> T cells because there has been no single-cell analysis for IL-21 production available as yet. In this study, we have established the intracellular cytokine staining of IL-21 and have shown that a considerable number of IL-21-producing CD4<sup>+</sup> T cells under Th17-polarizing conditions are negative for intracellular IL-17A and -17F (Figs. 2 and 3), providing a new insight into the cellular source of IL-21 in activated CD4<sup>+</sup> T cells.

We show that IL-6 and -21 both potently induce the development of IL-21-producing CD4<sup>+</sup> T cells (Figs. 3 and 7). In addition, IL-6-induced development of IL-21-producing CD4<sup>+</sup> T cells is mediated in part by IL-21 production because IL-6-induced development of IL-21-producing CD4<sup>+</sup> T cells was modestly, but reproducibly, decreased by the neutralization of IL-21 (Fig. 7). In a preliminary study, we also found that IL-21 enhanced the survival of IL-21-producing CD4<sup>+</sup> T cells (unpublished data). These results suggest that IL-21 may function as an autocrine growth factor for IL-21-producing CD4<sup>+</sup> T cells. In contrast, because IL-21 induces Th17 cell development (Fig. 7 A) (6–9), and because a considerable number of IL-21-producing CD4<sup>+</sup> T cells



**Figure 10.** IL-21-producing CD4<sup>+</sup> T cells do not lose their ability to produce IL-21 when they are restimulated under Th1- or Th2-polarizing conditions. (A) Purified CD4<sup>+</sup> T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb and anti-IFN- $\gamma$  mAb with IL-6 for 5 d. Cells were restimulated for another 5 d under the same condition (top) or Th1-, Th2-, or Th17-polarizing conditions (bottom). On day 10, cells were stimulated with anti-CD3 mAb and intracellular staining for indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments. (B) As positive controls, CD4<sup>+</sup> T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th1-, Th2-, or Th17-polarizing conditions for 5 d. Cells were restimulated under the same conditions for another 5 d. On day 10, cells were stimulated with anti-CD3 mAb and intracellular staining for indicated cytokines was performed.

under Th17-polarizing conditions are negative for IL-17A and -17F (Figs. 2 and 3), it is suggested that IL-21 functions on Th17 cells not only as an autocrine growth factor but also as a paracrine growth factor.

Our results indicate that the developmental characteristic of IL-21-producing CD4<sup>+</sup> T cells is different from that of Th17 cells, although both T cells require IL-6 and/or IL-21 for their development (Figs. 3 and 7) (6–9). We demonstrate that TGF- $\beta$  inhibits IL-6- and IL-21-induced development of IL-21-producing CD4<sup>+</sup> T cells (Figs. 3, 4, and 7). In contrast, both IL-6 and TGF- $\beta$  are required for the development of Th17 cells (Fig. 3) (20–22). On the other hand, it has been shown that TGF- $\beta$  induces, but IL-6 inhibits, the development of Foxp3-expressing regulatory T cells (20, 31). In addition, IL-2 signaling inhibits the development of Th17 cells,

but not of IL-21-producing CD4<sup>+</sup> T cells (Fig. 6). These findings suggest that the development of IL-21-producing CD4<sup>+</sup> T cells, Th17 cells, and T reg cells is reciprocally regulated by the balance among IL-6 plus IL-21, TGF- $\beta$ , and IL-2 in an environment.

Molecular mechanisms underlying the development of IL-21-producing CD4<sup>+</sup> T cells remain to be elucidated. We show that IL-6 and -21, both of which use STAT3 as a signaling molecule (11, 12, 32), induce the development of IL-21-producing CD4<sup>+</sup> T cells (Figs. 3 and 7). It has also been reported that under Th17-polarizing conditions, IL-21 production is induced by a STAT3-dependent mechanism, but not a ROR $\gamma$ t-dependent mechanism (6, 7), suggesting that STAT3 is critical for the development of IL-21-producing CD4<sup>+</sup> T cells. Because IL-6 up-regulates NFAT transcriptional activity by increasing the levels of NFATc2 (33), and because NFATc2 activates IL-21 promoter (34), it is also plausible that IL-6-mediated activation of NFATc2 is involved in the induction of IL-21 gene expression. Because Foxp3, which is induced by TGF- $\beta$ -mediated signaling (31, 35), interacts with NFAT and down-regulates NFAT activity (36–38), TGF- $\beta$  may suppress the development of IL-21-producing CD4<sup>+</sup> T cells (Fig. 3) by producing Foxp3–NFATc2 complexes.

Recently, it has been shown that IL-22 is expressed in CD4<sup>+</sup> T cells under Th17-polarizing conditions (6, 39, 40) and that it mediates IL-23-induced dermal inflammation and hyperplasia of the epidermis in psoriasis (40). Interestingly, similar to IL-21, IL-22 is induced by IL-6 but is inhibited by TGF- $\beta$  (40). In contrast, IL-17A and -17F are expressed in the presence of IL-6 and TGF- $\beta$  (Fig. 3) (41, 42). However, at present, it is still unknown whether IL-21-producing CD4<sup>+</sup> T cells do produce IL-22 together. The detailed analysis of autoimmune disease models in IL-21-deficient mice, IL-17A/IL-17F double-deficient mice, and IL-17A/IL-17F/IL-21 triple-deficient mice may provide evidence for a non-redundant role of IL-21-producing CD4<sup>+</sup> T cells and IL-17A/IL-17F-producing CD4<sup>+</sup> T cells in the pathogenesis of autoimmune diseases.

Regarding the phenotypic stability of IL-21-producing CD4<sup>+</sup> T cells, we found that IL-21-producing CD4<sup>+</sup> T cells could produce IL-21 for at least 20 d of culture (Fig. 10 and not depicted). We also found that IL-21-producing CD4<sup>+</sup> T cells did not lose their ability to produce IL-21 even when they were restimulated under Th1- or Th2-polarizing conditions (Fig. 10). Interestingly, IL-21-producing CD4<sup>+</sup> T cells still had a potential to produce IL-4 when IL-21-producing CD4<sup>+</sup> T cells were restimulated under Th2-polarizing conditions, whereas IL-21-producing CD4<sup>+</sup> T cells did not produce IFN- $\gamma$  even when IL-21-producing CD4<sup>+</sup> T cells were restimulated under Th1-polarizing conditions (Fig. 10). These results indicate that IL-21-producing CD4<sup>+</sup> T cells exhibit a stable phenotype of IL-21 production and the silencing of IFN- $\gamma$  production. This is consistent with our previous finding that IL-21 inhibits IFN- $\gamma$  production in developing Th1 cells through the repression of Eomesodermin

expression (43). In contrast, when IL-21-producing CD4<sup>+</sup> T cells were restimulated under Th17-polarizing conditions, their ability to produce IL-21 was decreased and IL-17A-producing cells were increased (Fig. 10). Therefore, IL-21-producing CD4<sup>+</sup> T cells still have a potential for IL-4 and -17A production.

It has recently been shown that a subset of CD4<sup>+</sup> T cells, termed follicular B helper T cells (TFH cells), produce a large amount of IL-21 (14). TFH cells are distinguishable from other CD4<sup>+</sup> T cell populations by several criteria, including its location in B cell follicles and the expression of chemokine receptor CXCR5, and provide a helper function to B cells (14). In preliminary experiments, we found that CD4<sup>+</sup> T cells cultured in the presence of IL-6 were negative for CXCR5 expression (unpublished data). Therefore, although further studies are required, it is suggested that IL-21-producing CD4<sup>+</sup> T cells described in the present study seem to be different from TFH cells. The comparative analysis of their helper function to B cells, as well as the regulatory mechanism of IL-21 production, is required to further address the relationship between these populations.

We found that TGF- $\beta$ -mediated suppression of IL-21 production was reduced in Smad3<sup>-/-</sup> CD4<sup>+</sup> T cells compared with that in WT CD4<sup>+</sup> T cells (Fig. 5). We also found that TGF- $\beta$ -mediated induction of Th17 cells was reduced in Smad3<sup>-/-</sup> CD4<sup>+</sup> T cells (Fig. 5). These results indicate that TGF- $\beta$ -Smad3 signaling is involved not only in the induction of Th17 cell development but also in the suppression of IL-21-producing CD4<sup>+</sup> T cells. Interestingly, however, the inhibitory effects of TGF- $\beta$  were still observed in Smad3<sup>-/-</sup> CD4<sup>+</sup> T cells (Fig. 5). This is consistent with a previous study showing that TGF- $\beta$  exhibits partial effects on Smad3<sup>-/-</sup> CD4<sup>+</sup> T cells (44). Because the molecular functions of Smad3 are somewhat overlapping to Smad2 (23, 24), it is suggested that TGF- $\beta$  exhibits partial effects on Smad3<sup>-/-</sup> CD4<sup>+</sup> T cells by using Smad2 as a partner of Smad4.

In conclusion, we have shown that IL-6 and -21 preferentially induce the differentiation of IL-21-producing CD4<sup>+</sup> T cells and that the differentiation of IL-21-producing CD4<sup>+</sup> T cells and Th17 cells is differently regulated by TGF- $\beta$  and IL-2. Although further studies are required to address the physiological importance of IL-21-producing CD4<sup>+</sup> T cells in vivo, our results should add a new insight into the regulatory mechanism of helper T cell differentiation and the pathogenesis of autoimmune diseases.

## MATERIALS AND METHODS

**Mice.** BALB/c mice and C57BL/6 mice were purchased from Charles River Laboratories. IL-21R-deficient (IL-21R<sup>-/-</sup>) mice (45) on a BALB/c background and Smad3<sup>-/-</sup> mice (46) on a C57BL/6 background (provided by S. Honjo, M. Fujimoto, K. Kobayashi, and A. Sakamoto, Chiba University, Chiba, Japan) were described previously. All mice were housed in microisolator cages under specific pathogen-free conditions and animal procedures used in this study were approved by the Chiba University Animal Care and Use Committee.

**Reagents.** Antibodies to CD3 (145-2C11), CD25 (PC61), CD28 (37.51), CD44 (IM7), IL-4 (11B11), IL-6 (MP5-20F3), and IFN- $\gamma$  (XMG1.2) were

purchased from BD Biosciences. Murine IL-2, -4, -6, and -12 were purchased from PeproTech. Human TGF- $\beta$ , murine IL-21, murine IL-23, IL-21R-Fc chimera, and anti-IL-2 antibody (JES6-1A12) were purchased from R&D Systems.

**Cell isolation.** CD44<sup>low</sup> CD25<sup>-</sup> CD4<sup>+</sup> naive T cells were isolated from lymph nodes of mice, as previously described (40), with minor modifications. In brief, CD4<sup>+</sup> T cells were collected from lymph nodes of mice using CD4<sup>+</sup> T cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Purified CD4<sup>+</sup> T cells were stained with anti-CD44 mAb and anti-CD25 mAb, and CD44<sup>low</sup> CD25<sup>-</sup> cells were sorted on a FACSAria cell sorter (BD Biosciences). In the experiments shown in Fig. 1 A, Fig. 5, and Fig. 10, CD4<sup>+</sup> T cells were purified from lymph nodes of mice by EasySep mouse CD4<sup>+</sup> T cell enrichment kit (StemCell Technologies, Inc.) using an automated cell separator RoboSep (StemCell Technologies, Inc.). In both cases, the resultant cells were >98% pure CD44<sup>low</sup> CD25<sup>-</sup> CD4<sup>+</sup> T cells or CD4<sup>+</sup> T cells, respectively, by FACS analysis.

**Cell culture.** Naive CD4<sup>+</sup> T cells or purified CD4<sup>+</sup> T cells were stimulated with 1  $\mu$ g/ml plate-bound anti-CD3 mAb at 0.5–10<sup>6</sup> cells/ml in the presence of 1  $\mu$ g/ml anti-CD28 mAb in a 48-well plate. Where indicated, cells were cultured under either Th1-polarizing conditions (10 ng/ml IL-2, 1 ng/ml IL-12, and 10  $\mu$ g/ml anti-IL-4 mAb), Th2-polarizing conditions (10 ng/ml IL-2 and -4 and 10  $\mu$ g/ml anti-IFN- $\gamma$  mAb), or Th17-polarizing conditions (100 ng/ml IL-6, and 1 ng/ml TGF- $\beta$ , anti-IL-4 mAb, and anti-IFN- $\gamma$  mAb). When cells were cultured for >4 d, 20 ng/ml IL-23 was added under Th17-polarizing conditions. Where indicated, 100 ng/ml IL-6, 0.2–5 ng/ml TGF- $\beta$ , 10 ng/ml IL-2, 100 ng/ml IL-21, 10  $\mu$ g/ml anti-IL-2 mAb, 10  $\mu$ g/ml anti-IL-6 mAb, or 10  $\mu$ g/ml IL-21R-Fc was added to the culture.

**Retrovirus transduction.** Bicistronic retrovirus vector pMX-IRES-GFP was a gift from T. Kitamura (Tokyo University, Tokyo, Japan). The IL-21 cDNA was amplified by PCR and cloned into pMX-IRES-GFP (IL-21-IRES-GFP). The production of retroviruses and infection to Ba/F3 cells were performed as previously described (43).

**Intracellular cytokine analysis for IL-21.** Cultured cells were washed and restimulated with 20 ng/ml PMA (Calbiochem) plus 1  $\mu$ g/ml ionomycin (Calbiochem) or plate-bound anti-CD3 mAb at 37°C for 5 h in the presence of 2  $\mu$ M monensin (Sigma-Aldrich). Cells were fixed, permeabilized with Perm/Wash buffer (BD Biosciences), and incubated with IL-21R-Fc chimera for 30 min at 4°C. Cells were then washed with Perm/Wash buffer and stained with PE-conjugated affinity-purified F(ab')<sub>2</sub> fragment of goat anti-human Fc $\gamma$  antibody (anti-Fc PE; Jackson ImmunoResearch Laboratories) for 30 min at 4°C. Cells were washed twice and stained with anti-CD4 FITC and anti-IL-4 allophycocyanin (BD Biosciences), anti-IFN- $\gamma$  allophycocyanin (BD Biosciences), anti-IL-17A Alexa Fluor 647 (eBioscience), or anti-IL-17F Alexa Fluor 647 (eBioscience). Cytokine profile on CD4<sup>+</sup> cells was analyzed on a FACSCalibur using CellQuest Pro software (BD Biosciences). Intracellular cytokine staining for IL-4 versus IFN- $\gamma$  was performed using anti-IL-4 PE (BVD4-1D11; BD Biosciences) and anti-IFN- $\gamma$  allophycocyanin (XMG1.2, BD Biosciences) as previously described (43).

**Real-time PCR analysis.** Total cellular RNA was extracted with TRIzol solution (Invitrogen). Reverse transcription was performed using an iScript cDNA synthesis kit (BioRad Laboratories). Primers and TaqMan probes for T-bet, GATA3, ROR $\gamma$ t, Foxp3, and  $\beta$ -actin have been previously described (5, 6, 41, 43). Quantitative PCR was performed with an ABI PRISM 7300 sequence detection system (Applied Biosystems). The levels of T-bet, GATA3, ROR $\gamma$ t, or Foxp3 mRNA were normalized to the levels of  $\beta$ -actin mRNA.

**ELISA.** The amount of IL-4 and IFN- $\gamma$  in the culture supernatant was measured by ELISA kits from BD Bioscience. The amount of IL-17A and -21 in the culture supernatant was measured by ELISA kits from R&D Systems.

The assays were performed in duplicate according to the manufacturer's instruction. The minimum significant values of these assays were 8 pg/ml of IL-4, 30 pg/ml of IFN- $\gamma$ , 17 pg/ml of IL-17A, and 63 pg/ml of IL-21.

**CFSE-labeling of CD4<sup>+</sup> T cells.** Naive CD4<sup>+</sup> T cells were labeled with 1  $\mu$ M CFSE (Invitrogen) for 10 min at 37°C according to the manufacturer's instruction.

**Data analysis.** Data are summarized as the mean  $\pm$  the SD. The statistical analysis of the results was performed by the unpaired Student's *t* test. *P* values <0.05 were considered significant.

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