Synergistic Effect of IL-6 and IL-4 in Driving Fate Revision of Natural Foxp3+ Regulatory T Cells

Lauren Kastner, David Dwyer and F. Xiao-Feng Qin

*J Immunol* 2010; 185:5778-5786; Prepublished online 6 October 2010; doi: 10.4049/jimmunol.0901948

http://www.jimmunol.org/content/185/10/5778
Synergistic Effect of IL-6 and IL-4 in Driving Fate Revision of Natural Foxp3+ Regulatory T Cells

Lauren Kastner,* David Dwyer,* and F. Xiao-Feng Qin*†

Expression of forkhead transcription factor Foxp3 defines a distinct lineage of naturally arising regulatory T cells (nTregs) that is segregated from effector CD4+ T cells during early development in the thymus. It remains elusive whether nTregs can convert into effector cells by turning off their Foxp3 expression and, if so, whether Th17 is a default alternative fate choice. In this report we provide compelling evidence showing that effector T cell-polarizing cytokines IL-6 and IL-4 can act synergistically to induce marked downregulation and inactivation of Foxp3 gene expression in mouse nTregs, and consequently the loss of suppressor phenotype and functions. However, the resulting Foxp3+ cells are not polarized and do not express IL-17 or other Th17-associated genes. Therefore, nTreg fate revision is not restricted to the Treg-Th17 axis and is likely to represent a rather broad phenomenon with divergent outcomes. The Journal of Immunology, 2010, 185: 5778–5786.

S ustained expression of Foxp3 is essential for the cellular phenotype, suppressive function, and homeostatic maintenance of Foxp3+ regulatory T cells (Tregs). Unlike inducible Tregs (iTregs), expression of Foxp3 in naturally arising regulatory T cell (nTreg) is imprinted during their development in the thymus and is thought to be highly stable (1). However, several recent studies revealed the existence of Foxp3+IL-17 and Foxp3/RORyt double-positive cells (2–7), and others reported the appearance of IL-17Foxp3+ cells from Foxp3+ nTregs upon stimulation with IL-6 (8, 9). Nevertheless, it remains uncertain whether nTregs indeed have an inherent potential to switch off Foxp3 expression, thereby converting to effector T cells, and whether such conversion is primarily driven by the competing Th17 differentiation program. In this study, we report that Foxp3+ nTregs could lose Foxp3 gene expression and their suppressor properties when they were stimulated in the presence of IL-6 and IL-4. Although IL-6 and IL-4 could individually downregulate Foxp3 expression at a low extent, together they rendered a much more potent and stable inactivation of Foxp3 gene expression. By direct intracellular staining for Foxp3 protein expression in CFSE-labeled nTregs, we confirmed that the formation of Foxp3+ cells was not due to outgrowth of non-Treg contaminants, but indeed resulted from genuine inactivation of Foxp3 expression in the stimulated nTregs, and that transitional cells with intermediate levels of Foxp3 expression were readily detected. Furthermore, by using a cell cycle inhibitor we showed that proliferation or cell division was not required for Foxp3 downregulation. Interestingly, although the reprogrammed cells had lost their Foxp3 expression and suppressor function, they were not converted to polarized effector cells. Even though they acquired the expression of IL-2, IFN-γ, IL-4, T-bet, and Gata-3, the levels were considerably lower compared with normal Th1 and Th2 effector cells. More importantly, Th17 attributes were largely absent in the Foxp3+ cells, as no upregulation of IL-17, RORyt, or IL-21 gene expression was detected. Therefore, Th17–Treg dichotomy does not constitute a default mechanism that drives nTreg fate revision, and, in fact, effector differentiation does not appear to be a priori for the revision process.

Materials and Methods

Mice

C57BL/6 mice were from the National Cancer Institute (Bethesda, MD) and Foxp3-GFP reporter mice (Foxp3-ires-GFP knock-in strain on C57BL/6 background; see Ref. 10) were provided by Dr. Mohamed Oukka (Harvard Medical School). Mouse colonies were maintained in a specific pathogen-free barrier facility, and experimental procedures conformed to the Institutional Animal Care and Use Committee protocols.

T cell purification and cell sorting

Peripheral CD4+ T cells were isolated from pooled lymph nodes and spleen of Foxp3-GFP mice by CD4 MACS column (Miltenyi Biotec, Auburn, CA). MACS-enriched CD4+ cells were stained with PerCP Cy5.5-labeled anti-CD4 (L3T4) and sorted with a FACS aria cell sorter (BD Biosciences, San Jose, CA) to obtain CD4+GFP+ cells. To ensure unequivocal purity, the cells were subject to a second round of sorting, and the resulting CD4+ GFP+ nTregs were >99.9% pure with regard to GFP as well as Foxp3 protein expression. To obtain CD25+ subset of nTregs, the cells were also stained with allophycocyanin-labeled anti-CD25 (PC61), and CD25high CD4+GFP+ cells were sorted accordingly (two rounds of sorting). The thymic CD25+ Tregs were isolated from the thymus of Foxp3-GFP mice first by CD25 MACS column (Miltenyi Biotec). The enriched cells were further stained with PerCP Cy5.5-labeled anti-CD4 (L3T4), PE-labeled anti-CD8 (53-6.7), and allophycocyanin-labeled anti-CD25 (PC61) followed by two rounds of sorting to obtain CD25highCD4CD8+GFP+ cells. Spleen APCs were prepared from the spleen of C57BL/6 mice by depletion of T cells with anti-Thy1.2 Dynal beads (Invitrogen, Carlsbad, CA), and the resulting cells were gamma-irradiated (30 Gy) before culture.

Cell culture

T cell cultures were carried out in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 1 mM Na pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES [pH 7.04], 1×...
penicillin/streptomycin, 50 μM 2-ME: Invitrogen). T cells were stimulated with anti-CD3/anti-CD28–coated beads (mouse T cell expander; Dynal/Invitrogen) at a 2:1 beads/cell ratio. Next, 50 ng/mL rIL-6, 10 ng/mL rIL-4, 10 ng/mL rIL-12, 25 ng/mL rIFN-γ, 20 ng/mL rIL-21, 20 ng/mL rIL-1β, or 20 ng/mL rIL-23 was added either individually or in combinations as indicated from the beginning of culture. After the first 3 d, 200 U/mL rIL-2 was supplemented to all cultures (necessary to keep the cells alive). All recombinant cytokines are of murine origin, except IL-2, which is human. The cytokines were purchased from PeproTech (Rocky Hill, NJ) or R&D Systems (Minneapolis, MN). In some experiments, nTregs were labeled with CFSE dye (Invitrogen) according to the condition described before (11) for tracking cell division. To inhibit cell proliferation, 4 μM cell cycle inhibitor mycophenolic acid (MPA; Sigma-Aldrich, St. Louis, MO) was used.

FACS analysis
For Foxp3/GFP reporter expression analysis, the cells were harvested and stained with PerCP Cy5.5–labeled anti-CD4 and acquired with a FACSCalibur (BD Biosciences). To measure Foxp3 protein expression, the cells were subject to further intracellular staining with Alexa Fluor 647–labeled anti-CD3 Ab (FITC-16s; ebioscience, San Diego, CA) using the Fix/Perm buffers and conditions specified by the manufacturer (ebioscience). With the Fix/Perm buffer treatment, GFP reporter signal was largely destroyed, and hence there was no interference to CFSE fluorescence detection. For intracellular cytokine staining, the cells were stimulated for 4–5 h with 50 ng/mL PMA and 1000 ng/mL ionomycin (Sigma-Aldrich) in the presence of 2 μM monensin (GoGiStop; BD Biosciences). The stimulated cells were incubated first with Fc blocker and PerCP Cy5.5–labeled anti-CD4 for surface staining followed by intracellular staining using PE-labeled anti-mouse IL-2 (JES6-5H4), IFN-γ (XMGl.2), IL-4 (11B11), IL-10 (JES5-16E2), IL-17A (TC11-19H10), and isotype controls (BD Biosciences or ebioscience). For two-color cytokone staining, allophycocyanin-labeled anti-mouse IFN-γ (XMGl.2) was used together with other PE-labeled anti-mouse cytokine Ab described above. Flow cytometry acquisition was done with a FACSCalibur, and data analysis was performed with FlowJo software (Tree Star, Ashland, OR).

[^3H]Thymidine uptake-based suppression assay
The [^3H]thymidine uptake-based suppression assay was described before (11). Specifically, CD4[^+^]CD25[^+^] G FP[^+] target cells were prepared from Foxp3–GFP mice by cell sorting. The target cells (5 × 10[^5^]) were cultured either alone or with different numbers of Treg effector cells in the presence of irradiated spleen APCs (5 × 10[^4^]) and 0.5 μg/mL anti-CD3 (145-2C11; BD Biosciences) in 96-well plates with complete RPMI 1640 medium. The cells were cultured for 72 h, and [^3H]thymidine (1 μCi/well; GE Healthcare, Piscataway, NJ) was added in the last 16 h of 72 h of culture. Results are expressed as the mean of triplicate wells ± SEM.

Quantitative PCR
Quantitative PCR (qPCR) was carried out by SYBR Green gene expression assays using the Universal PCR Master mix (Applied Biosystems, Foster City, CA) and the ABI Prism 7500 sequence detector system (PerkinElmer, Wellesley, MA) as previously described (11). The primer sequences are available upon request.

Statistical analysis
GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used for data analysis. The p values were derived from an unpaired two-tailed t test with a 95% CI.

Results

**Loss of Foxp3 expression in nTregs upon anti-CD3 stimulation in the presence of effector-polarizing cytokines**

Our previous study has shown that Foxp3 induction and formation of iTregs can be potently antagonized by Th1, Th2, as well as Th17 effector-polarizing cytokines (11). Therefore, we investigated whether Foxp3 expression and the fate of nTregs could be altered by exposure to those cytokines. To ensure unequivocal purity of nTregs, we isolated the peripheral Foxp3[^+^] nTregs from Foxp3–GFP reporter mice (10) by two rounds of FACS sorting for CD4[^+^]GFP[^+] cells. Postsort analysis confirmed that the purity of our nTreg preparations was >99.9% (Supplemental Fig. 1). The purified nTregs were then stimulated with anti-CD3/anti-CD28–coated beads (mouse T cell expander; Dynal/Invitrogen) plus Th17-polarizing cytokine IL-6, Th1-polarizing cytokines IFN-γ or IL-12, or Th2-polarizing cytokine IL-4. After 4–5 d of culture, we found that although Foxp3 expression was not affected in nTregs stimulated without polarizing cytokine, in the presence of IL-6 or IL-4 there was a moderate but reproducible loss of Foxp3/GFP reporter expression (10–16%; Fig. 1A, 1C). In contrast, downregulation of GFP reporter in cultures containing IFN-γ or IL-12 was minimal (Fig. 1A, 1C). In addition to measuring Foxp3 expression by GFP reporter, we also performed intracellular staining with anti-Foxp3 Ab and the same results were obtained (Fig. 2 and data not shown). Therefore, Th17- and Th2-, but not Th1- polarizing cytokines can selectively induce the downregulation of Foxp3 expression in nTregs. Indeed, qPCR analysis further confirmed that the loss of Foxp3 expression occurred at the mRNA level (see Fig. 3).

Synergistic effect of IL-6 and IL-4, and the requirement of Ag receptor engagement for turning off Foxp3 expression

To test whether IL-6 and IL-4 together can induce stronger downregulation of Foxp3, we added both cytokines to the culture, and the result showed a much higher level of Foxp3 downregulation than that of the individual ones (40–50%; Fig. 1B, 1D). Thus, IL-6 and IL-4 could act synergistically in turning off Foxp3 expression in the stimulated nTregs. To further investigate the essential signals required for the inactivation of Foxp3 expression in nTregs, we stimulated the cells either with anti-CD3/CD28 beads alone (anti-CD3/CD28 only) or with IL-6 and IL-4 alone (IL-6/IL-4 only). We found that there was no downregulation of Foxp3 expression that resulted from the beads or cytokines-only treatment (Fig. 1B, 1D). Therefore, both Ag receptor and effector-polarizing cytokine stimulation synergisms are essential for triggering the downregulation of Foxp3 gene expression.

Cytokines IL-2, IL-12, IL-21, IL-1β, and IL-23 have been implicated in modulating Foxp3[^+^] Treg maintenance and adaption in several previous reports (2–7). To examine potential effects of these cytokines on downregulating Foxp3 expression in nTregs, we carried out further experiments by adding IL-2, IL-12, IL-21, IL-1β, and IL-23 alone, or together with IL-6 and IL-4, to stimulate nTregs. Results showed that only IL-21 by its own could induce a moderate downregulation of Foxp3 expression, whereas others had no significant effect (Fig. 1E, upper panel). When added together with IL-6 and IL-4, IL-21 resulted in further increase of the percentage of Foxp3[^−^] cells (Fig. 1E, lower panel). However, the effect appeared to be largely additive rather than synergistic. Interestingly, although IL-1β and IL-23 had been reported to play a role in promoting Th17 differentiation, in our experiments when added in combination with IL-6 and IL-4, these two cytokines actually showed a moderate but reproducible effect on preventing Foxp3 downregulation. Thus, the outcome of combinatorial stimulation by multiple cytokines can be complex; certain combinations could result in synergistic downregulation of Foxp3 expression, whereas others would only generate an additive effect, and yet some other combinations might act in a mutually antagonistic manner. Despite the complexity, the effect of IL-6 and IL-4 stimulation appeared to be dominant over other cytokines tested thus far in our studies.

Loss of Foxp3 expression occurs along with cell proliferation, but proliferation and cell division are not required for the downregulation process

As stimulation with anti-CD3/CD28 beads leads to proliferative expansion of nTregs, we therefore wanted to investigate whether

The Journal of Immunology 5779
Foxp3 inactivation is associated with cellular proliferation. To this end, we labeled the purified nTregs with CFSE so that proliferation and cell division could be directly tracked along time. To directly measure Foxp3 protein levels, we conducted intracellular staining with anti-Foxp3 Ab labeled with Alexa Fluor 647. This staining condition is compatible with CFSE detection for nTregs isolated.

FIGURE 1. Loss of Foxp3 expression in peripheral nTregs upon in vitro stimulation in the presence of IL-6 and IL-4. Peripheral Foxp3+ nTregs were isolated from the spleen and lymph nodes of Foxp3–GFP reporter mice by FACS sorting. The cells were stimulated in vitro with anti-CD3/CD28–coated beads in the presence of various effector-polarizing cytokines as indicated. The stimulated cells were harvested on day 4, stained with PerCP Cy5.5-labeled anti-CD4 Ab, and analyzed for GFP expression by FACS. A and E, Cells were stimulated with anti-CD3/CD28–coated beads plus indicated cytokines or no cytokine as a control. B, The cells were stimulated only by the beads (anti-CD3/CD28 only) or IL-6 and IL-4 (IL-6/IL-4 only), or by beads plus the two cytokines (*IL-6/IL-4*). Foxp3 expression was determined by GFP reporter signal represented in CD4/GFP density plots and GFP histograms. C, Statistical results of A, showing the average percentage of Foxp3+ cells (mean, SEM) induced by different stimulation conditions. Significance of the difference between different treatment conditions is indicated by p value. Data were pooled from four different experiments. D, Statistical results of B. Data were derived from three independent experiments.
from the GFP reporter mice, as GFP fluorescence is largely eliminated by the fixation and permeabilization treatment (see Materials and Methods). With this method, we found that downregulation of Foxp3 expression could be detected in +IL-6+IL-4–stimulated nTreg culture starting at day 3 (Fig. 2). Time course experiments further showed that the Foxp3\(^+\) population increased progressively over time accompanied with cellular proliferation. Although both Foxp3\(^{+}\) and Foxp3\(^{+}\) cells divided extensively after 6 d of stimulation in the presence of the cytokines, especially with +IL-6 and +IL-6+IL-4, the Foxp3\(^{+}\) population appeared to divide faster (Fig. 2). This phenomenon might be due to the fact that once Foxp3 expression is downregulated, the anergic state is reversed and the cells also become nonsuppressive (see below). Interestingly, although most Foxp3\(^{+}\) cells had gone through several rounds of cell division at day 6, a small fraction of yet undivided cells (CFSE\(^{\text{high}}\)) were found to have downregulated Foxp3 expression as well (Fig. 2). Furthermore, transitional cells with intermediate levels of Foxp3 expression were detected, which were particularly evident at the day 6 time point (Fig. 2). To determine whether proliferation and cell division are necessary for Foxp3 downregulation, we performed a further experiment using cell cycle inhibitor MPA, which inhibits G\(_1\)–S phase transition and DNA synthesis of activated T cells (12). CFSE labeling confirmed that cell division was completely blocked in the presence of MPA (Fig. 4). Nonetheless, a substantial fraction of nTregs stimulated with the cytokines downregulated Foxp3 expression. More significantly, contour plots of anti-Foxp3 staining revealed a clear spectrum of transitional cells with various intermediate levels of Foxp3 expression. Therefore, this experiment unequivocally demonstrated that downregulation of Foxp3 expression in nTregs stimulated by Ag receptor plus polarizing cytokine treatment was independent of cell proliferation. Moreover, it also ruled out any remaining possibilities that Foxp3\(^{+}\) cells were derived from outgrowth of non-Treg contaminants.

**Inactivation of Foxp3 expression by +IL-6+IL-4 stimulation leads to the loss of suppressor phenotype and function but without gaining effector cytokine expression**

The hallmark of Foxp3\(^{+}\) nTregs is suppression, anergy, and lack of effector cytokine expression (1). To investigate whether inactivation of Foxp3 expression in nTregs leads to their fate revision, we performed functional analyses to examine those essential properties. Using an in vitro CD4\(^{+}\) T cell suppression assay, we found that nTregs stimulated by the +IL-6+IL-4 condition became largely
nonsuppressive, while +IL-6– or +IL-4–stimulated cells only had modest reduction of suppressive activity compared with the control (Fig. 5A). In parallel with the loss of suppressive function, the anergic state of +IL-6–IL-4–stimulated cells was also reversed (Fig. 5B). In contrast, +IL-6– or +IL-4–stimulated cells remained anergic (Fig. 5B). Thus, these results demonstrated the synergy of IL-6 and IL-4 in driving nTreg reprogramming at the functional level. Consistently, cell surface expression of a panel of Treg-associated markers, including CD25, GITR, CD39, FR4, and CTLA-4, was all downregulated on +IL-6+IL-4–stimulated nTregs, and the down-regulation appeared to correlate with the reduction of Foxp3 expression (Fig. 5C). To further examine the nature of Treg fate revision at the gene expression level, we performed qPCR analysis on an extensive panel of effector cytokine genes as well as the key T cell lineage-specific transcriptional factors. We found that +IL-6+IL-4–treated nTregs acquired the expression of a mixture of cytokine genes belonging to Th1 and Th2 effector cells (Fig. 3). However, expression levels of those cytokine genes were rather low compared with normally polarized Th1 and Th2 cells generated from naive CD4+ T cells. Furthermore, consistent with the cytokine expression profile, a low-level expression of Th1 and Th2 transcription factors T-bet and Gata-3 was detected (Fig. 3). Surprisingly, however, very little expression of Th17-associated genes, including IL-17, IL-21, and RORγt, was induced (Fig. 3). Thus, the gene expression pattern revealed by qPCR analysis suggested a paucity of effector polarization of Foxp32 revertant cells. However, low mRNA levels of T helper transcription factor and cytokine genes detected by qPCR could be due to the fact that only a small fraction of the reprogrammed Foxp32 cells were able to express effector T cell genes at high levels, or might indeed reflect low expression of the genes in most of the reprogrammed cells. To distinguish these two possibilities, we carried out intracellular cytokine staining experiments on IL-6– and IL-4–stimulated Tregs. Single cytokine staining analysis revealed that with +IL-6+IL-4 stimulation, only a small percentage of the cells acquired the ability to express IL-2, IFN-γ, IL-4, and IL-10 (Fig. 6A). Furthermore, in agreement with the qPCR results, no significant IL-17-positive cells were detected (Fig. 6A). Similarly, even lower numbers of cells

![FIGURE 3. mRNA expression profiles of cytokine and transcription factor genes in nTreg revertants. qPCR was performed to measure gene expression levels. The data are expressed as relative expression levels normalized to the housekeeping gene ubiquitin. nTreg treatment was as described in Fig. 1. Additionally, freshly isolated nTregs, naive CD4+ T cells, and effector T cells differentiated from naive CD4+ T cells were included as controls. Each RNA sample was pooled from at least two independently treated cell preparations. qPCR was carried out by SYBR Green gene expression assays using the Universal PCR Master mix (Applied Biosystems).](http://www.jimmunol.org/)

![FIGURE 4. Proliferation and cell division are not required for down-regulation of Foxp3 expression. nTregs were labeled and stimulated as in Fig. 2. Cell cycle inhibitor MPA (4 μM) was added to the cultures to inhibit cell proliferation. The cells were harvested on day 4 and analyzed by FACS as in Fig. 2 for cell division and Foxp3 expression. A, Cell division was completely blocked in the presence of MPA as shown by CFSE histograms, while downregulation and loss of Foxp3 expression in the undivided cells are revealed in the Foxp3 histograms and Foxp3/CFSE contour plots. B, Percentage numbers of Foxp3− cells averaged from two independent experiments. The p values are indicated.](http://www.jimmunol.org/)

![FIGURE 5.](http://www.jimmunol.org/)
positive for IL-2, IFN-γ, IL-4, IL-10, or IL-17 production were found to be induced by +IL-6 or +IL-4 stimulation (Fig. 6A). To determine whether some of the reprogrammed cells might express more than one cytokine, we costained +IL-6+IL-4–treated nTregs with anti–IFN-γ in combination with a second cytokine Ab. Indeed, small fractions of IFN-γ+IL-2+, IFN-γ+IL-4+, and IFN-γ+IL-10+ double-positive cells were found in addition to the single-positive populations (Fig. 6B). Thus, intracellular cytokine staining confirmed that indeed the reprogrammed nTregs were largely unpolarized, with only a small proportion of the cells showing a lower level and heterogeneous pattern of cytokine gene expression, which is reminiscent of the Th0 phenotype described for conventional CD4+ T cells activated under unpolarizing conditions (13).

**CD25+Foxp3+ nTregs are also able to undergo fate revision**

Foxp3+ nTregs consist of CD25+ and CD25− subpopulations. Although both CD25+Foxp3+ and CD25−Foxp3+ nTregs have been shown to be equally suppressive and interconvertible in vivo (1), expression of CD25 marker was originally used to define nTregs, and the CD25− subpopulation has been reported to have more stable Foxp3 expression (14). To determine whether CD25+ nTregs also have the potential to undergo fate revision by IL-6 and IL-4 stimulation, we isolated CD25highFoxp3+ nTregs either from spleen and lymph node tissues or the thymus of Foxp3−GFP reporter mice, and we performed the same experiment as described in Fig. 1. The results showed that both peripheral and thymic CD25highFoxp3+ nTregs behaved in a similar manner as did total Foxp3+ nTregs in which downregulation of Foxp3 could be induced by anti-CD3/CD28 plus cytokine stimulation (Fig. 7). Similar to what occurred to the total Foxp3+ Tregs, and while IL-6 or IL-4 could individually induce low levels of downregulation, more extensive inactivation of Foxp3 expression requires the synergistic action of both cytokines (Fig. 7). Therefore, the potential to switch off Foxp3 expression and undergo fate revision appears to be an inherent property for all nTregs, even though the actual propensity of fate revision might vary among different subpopulations in response to different stimulations.

**Discussion**

The initial indication that nTregs might be able to differentiate into effector cells came from the observations that Foxp3+IL-17+ and Foxp3+RORγt+ double-positive cells could be detected in mice (2–4) and humans (5–7). Complementing those findings, several groups reported that IL-17–producing Foxp3− cells were induced from Foxp3+ nTregs by IL-6 stimulation (8, 9). However, questions remain whether those double-positive cells indeed represent nTregs in the midst of fate revision, or whether they reflect a transitional state of naive precursor cells undergoing Th17 differentiation. Similarly, it has not been carefully ruled out that IL-17+Foxp3− cells generated in IL-6–treated nTregs cultures could have resulted from a minor contamination of non-Tregs, since two separate studies showed that nTregs could direct naive CD4+ T cells to become Th17 cells due to elevated TGF-β production by nTregs upon Ag receptor stimulation (8, 15). Nevertheless, there is a clear dichotomous relationship between Th17 and iTregs. Bettelli et al. (10) first showed that while TGF-β alone could stimulate iTreg formation from naive precursor cells, TGF-β plus IL-6 would induce Th17 differentiation. More importantly, subsequent studies have further revealed that the outcome of iTreg and Th17 differentiation is regulated by relative abundance of TGF-β and IL-6. On one hand, high TGF-β promotes iTregs while inhibiting Th17; on the other hand, strong IL-6 signaling stimulates Th17 differentiation but antagonizes the formation of iTregs (2, 16).

In this work, we found that IL-6 stimulation could indeed lead to a low degree of Foxp3 downregulation at both the protein and
mRNA levels in highly purified Foxp3+ nTregs, which is consistent with a more recent study described by Lal et al. (17). Similarly, another effector-polarizing cytokine, IL-4, which was shown to inhibit iTreg induction in previous studies (11, 18, 19), was also found to induce the loss of Foxp3 expression in nTregs. More significantly, together these two cytokines could act synergistically to drive a profound inactivation of Foxp3 gene expression.

With direct Ab staining for Foxp3 protein in CSFE-labeled nTregs, we identified a transitional state of the revision process in which nTregs progressively downregulated their Foxp3 protein expression along with cell proliferation. However, by using the cell cycle inhibitor MPA, our study demonstrated that proliferation or cell division was not essential for nTreg fate revision, at least the initial process of Foxp3 downregulation. Taken together, these results formally proved that the appearance of Foxp3− cells was not due to outgrowth of any potential contamination of non-nTregs. Interestingly, although the reprogrammed Foxp3− cells induced by "IL-6"IL-4 treatment had lost their suppressor function and phenotype, they did not appear to differentiate into polarized effector cells. Instead, they expressed a mixture of low levels of IL-2, IFN-γ, IL-4, and IL-10. More importantly, there was no conspicuous upregulation of IL-17 or other Th17-associated genes, including IL-21 and RORγt, in the Foxp3− revertant cells. Thus, nTreg fate revision is not necessary to be driven by the competing Th17 differentiation program. Clearly, Th17 is not the only alternative fate choice for nTregs.

The results from our study are consistent with earlier findings made from mouse genetic studies, which showed that genetic disruption or perturbation of Foxp3 expression in mature nTregs could lead to the generation of a mixed array of different effector cells, not only Th17, but also Th1- and Th2-like effector cells producing IL-2, IFN-γ, and IL-4 (20, 21). Additional support to the notion that nTregs might remain multipotential comes from more recent work showing that adoptive transfer of Foxp3− nTregs into lymphopenic hosts led to the appearance of Foxp3− cells of the donor origin exhibiting diverse effector phenotypes, including Th1, Th2, and Th17 in lymph node and spleen (14, 22–24). Interestingly, at certain anatomic locations, such as Peyer’s patches in small intestines, nTregs have also been found to differentiate into Foxp3− IL-21− follicular helper T cells rather than inflammatory Th17 cells (23). In addition to the lymphopenia setting, an elegant genetic marking study by Zhou et al. (25) recently showed that nTreg revision occurs constitutively in normal healthy animals and again the reprogrammed cells exhibit a mixed effector cytokine expression profile.

What physiological or pathophysiological conditions would promote nTreg fate revision? Our results suggest that IL-6 and IL-4
together might be one of the most potent cytokine milieus that drive Foxp3 downregulation and the loss of suppressor phenotype and function. This finding is in agreement with in vivo studies showing that the gastrointestinal tract is one of the favorable sites for nTreg revision. The gastrointestinal tract is known to have abundant IL-6 (26), and recent investigations further revealed that IL-6 as well as IL-4 levels might be modulated by commensal bacteria, and elevated levels of IL-6 and IL-4 appeared to be responsible for the inhibition of iTreg formation in gut mucosa (18, 26). Likewise, the lung and airway mucosa compartment also harbors high levels of IL-6 (26), and in the situation of severe airway inflammation in asthma, a vast increase of IL-6 and IL-4 can occur at the same time (27), thus constituting a conducive environment for driving nTreg revision. nTreg revision in this setting could lead to further exacerbation of disease development. Another notable circumstance for high levels of IL-6 and IL-4 is parasitic infection, which is often associated with strong Th2 skewing and chronic tissue damage. Modulation of nTreg function and maintenance under that condition is thought to serve as an important mechanism for balancing host immunity and tolerance and the control of immunopathology (28).

Several recent studies highlighted previously unrecognized heterogeneity and plasticity of Foxp3+ Tregs. It has been shown that nTregs can at least transiently acquire the expression of various effector T cell cytokines and transcription factors without losing suppressor activity or turning off Foxp3 expression (29–32). In fact, the expression or activation of effector transcription factors, including T-bet, IRF4, and Stat3, appears to be important for the regulatory function of nTregs to control corresponding effector type-specific immune responses (29–31). In contrast, nTregs can switch off Foxp3 expression and undergo fate revision under multiple different conditions. The choice of effector differentiation and consequence of such reprogramming are likely to be influenced by the host status, tissue microenvironment, antigenic stimulation, and cytokine milieus (14, 22–25, 33). Interestingly, although Foxp3 expression is tightly linked with the suppressor phenotype and function, effector differentiation is not a prerequisite for Foxp3 downregulation and nTreg fate revision. Our data obtained from intracellular cytokine staining analysis showed that most Foxp3+ revertants induced by +IL-6+IL-4 stimulation were negative for effector cytokine expression, although a sizable fraction of the cells did express low levels of Th1 and Th2 cytokines. These findings are consistent with in vivo study results reported by several different groups, nTreg revertants generated in normal healthy mice were not inflammatory and appeared to lack overt signs of polarization to Th1, Th2, or Th17 (14, 22–25). However, it is possible that some new types of T effector rather than unpolarized Th0 cells might be generated from nTregs by +IL-6+IL-4 treatment, as there is increasing evidence pointing to the unprecedented diversity and versatility of CD4+ helper T cells (34, 35). Global gene expression analysis in future work will help to address such issues. Nevertheless, despite this uncertainty, we found that +IL-6+IL-4–induced nTreg revertants were highly plastic, and they could be polarized to Th1, Th2, or Th17 effector cells during a second round of stimulation (data not shown). Thus, this raises a possibility that loss of Foxp3 expression/suppressor function and differentiation to effector cells can occur independently.

How IL-6 and IL-4 can act synergistically in downregulating Foxp3 expression is not known at present. Because no significant expression of RORγt or Gata-3 is induced in the Foxp3+ revertant cells by IL-6 and IL-4 treatment, it is unlikely that these two

**FIGURE 7.** Loss of Foxp3 expression in peripheral and thymic CD25+Foxp3+ Tregs by +IL6+IL-4 stimulation. A, Peripheral CD25+Foxp3+ Tregs were isolated from the spleen and lymph nodes of Foxp3–GFP reporter mice by sorting for the CD25+GFP+ subpopulation of Tregs. The cells were labeled with CFSE and stimulated in the same manner as in Fig. 1. At day 5 the stimulated cells were harvested and stained with anti-CD4 (PerCP Cy5.5) and anti-Foxp3 (Alexa Fluor 647) to measure Foxp3 expression and cell division by FACS. Foxp3/CFSE density plots and Foxp3 histograms are shown. B, Thymic CD25+Foxp3+ Tregs were isolated from the thymus of Foxp3–GFP reporter mice by sorting for the CD25+GFP+ fraction of Tregs. The cells were stimulated as in A but without CFSE labeling. At day 5 the stimulated cells were harvested, stained with anti-CD4 (PerCP Cy5.5), and analyzed by FACS for Foxp3–GFP reporter expression (GFP/CD4 density plots and GFP histograms). C and D, Average results of Foxp3– cell percentage of A and B derived from three and two independent experiments, respectively. The p values are indicated.
T helper lineage transcription factors may play any direct role in turning off Foxp3 gene expression. In contrast, Stat3 and Stat6 have been reported to be readily activated in nTregs upon exposure to IL-6 and IL-4, respectively (30, 31). Thus, one interesting possibility is that Foxp3 downregulation might be driven by strong activation of Stat3 and Stat6. In support to this view, previous studies have demonstrated that both Stat3 and Stat6 could potentially antagonize Foxp3 expression in naive CD4+ T cells induced by TGF-β stimulation (11, 16, 19, 36). Therefore, future studies are warranted to investigate how Stat3 and Stat6 might operate cooperatively to destabilize and eventually shut down the active transcription of Foxp3 gene in nTregs.

Acknowledgments

We thank David Z. He and Karen Ramirez for their assistance in cell sorting, Dr. Mohamed Oukka for providing Foxp3–GFP reporter mice, and Drs. Y.-J. Liu, Kai Voo, Eulogia Roman, and Shino Hanabuchi for discussions and critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References