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*J Immunol* 2009; 183:3583-3590; Prepublished online 26 August 2009;
doi: 10.4049/jimmunol.0900146
http://www.jimmunol.org/content/183/6/3583

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2009/08/26/jimmunol.0900146.DC1

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Immune Suppressive Activity and Lack of T Helper Differentiation Are Differentially Regulated in Natural Regulatory T Cells

Wei-ping Zeng, Chawshang Chang, and Jiann-jyh Lai

The mechanism for controlling Th cytokine expression in natural regulatory T (nTreg) cells is unclear. Here, it was found that under polarizing conditions Foxp3 did not affect Th1 cell, partially inhibited Th17 cell, but greatly inhibited Th2 cell differentiation of conventional CD4 T cells. Under the polarizing conditions, nTreg cells failed to differentiate into Th2 and Th17 cells, but differentiated into IFN-γ-producing cells. Such Foxp3-transduced CD4 T cells and nTreg cells expressed T-bet, GATA-3, or retinoic acid-related orphan receptor (ROR)γt, and retroviral GATA-3 and RORγt could not induce Th2 and Th17 differentiation from nTreg cells. However, regardless of their cytokine profiles, the Foxp3-transduced CD4 T cells and nTreg cells remained immune suppressive. These results suggested that it is possible to convert pathogenic Th cells to Treg-like cells for therapeutic application. In conclusion, our studies show that Foxp3 is sufficient for immune suppression, whereas the inhibition of cytokine expression requires additional mechanisms. The Journal of Immunology, 2009, 183: 3583–3590.

Lymphopoiesis in the thymus produces two separate lineages of CD4 T cells, the conventional naive CD4 T cells and the natural regulatory T (nTreg) cells (1). In the peripheral lymphoid tissues, upon Ag stimulation conventional naive CD4 T cells differentiate into functional Th subsets, the most prominent of which are the Th1, Th2, and Th17 cells (2). The differentiation of Th1, Th2, and Th17 cells is driven by the subset-specific transcription factors T-bet, GATA-3, and retinoic acid-related orphan receptor (ROR)γt/RORα, respectively, whose expression is either induced or up-regulated by external polarizing signals (3–6). Most of the functions of the Th subsets are mediated by the nonoverlapping sets of cytokines they produced. Th1 cells mainly produce IFN-γ, and Th2 cells characteristically produce IL-4, IL-5, and IL-13, whereas Th17 cells produce IL-17 (7–9).

The Th subsets are the cellular basis for the heterogeneity of immune responses that provide protective immunity against different pathogens, or cause the immunopathology in autoimmunity, infectious, and allergic diseases. Specifically, Th1 immune responses are required for the clearance of intracellular bacteria, Th2 immune responses are required for the clearance of helminth infection, whereas Th17 cells are important for mucosal defense against extracellular bacteria (10, 11). However, Th responses could also be detrimental to the hosts. Th1 and Th17 responses to self-Ags lead to organ-specific autoimmune diseases, and unbalanced Th2 responses to allergens and parasites cause allergic and parasitic diseases (2, 11).

To avoid autoimmunity, activation of the autoreactive T cells that have escaped central tolerance must be prevented by mechanisms of peripheral tolerance. nTreg cells are a major mechanism of peripheral tolerance. They can inhibit the proliferative responses of conventional CD4 and CD8 T cells in vitro, and they suppress autoimmune (12–14) and allergic (15–17) diseases in vivo. The transcription factor Foxp3 is both necessary and sufficient for the immune suppressive activity of the nTreg cells (18–20). Although nTreg cells are anergic in vitro, they proliferate readily in vivo in response to antigenic challenge or during homeostatic expansion (21–23). In fact, nTreg cells exhibit higher rates of homeostatic division than do conventional CD4 T cells (21, 22), and their TCR chains are highly phosphorylated (24), indicating that the TCRs of nTreg cells have higher affinities to self-Ags. Indeed, the most frequently used TCR in nTreg cells were found to be biased toward self-reactivity even though the overall TCR diversity was similar between nTreg cells and the conventional CD4 T cells (25, 26). The high affinity of TCR to self-Ags is important for the thymic development of nTreg cells (27), and presumably allows nTreg cells to be efficiently activated by self-Ags to suppress autoreactive T cells (28).

However, the self-reactivity of nTreg cells makes it necessary that they do not differentiate into Th effector cells after activation by self-Ags. Otherwise, nTreg cells themselves could cause autoimmunity. In this regard, earlier studies found that nTreg cells and Foxp3-transduced CD4 T cells produced little Th effector cytokines (19, 23, 29). However, these studies may have underestimated the potential of nTreg cells to produce Th effector cytokines, because the T cells were not differentiated under Th subset polarizing conditions in some cases, and in others the effect of cell number reduction, due to poor cell division of Treg cells in vitro, on the amounts of cytokine produced was not taken into consideration. Nonetheless, these studies have led to a popular view that Foxp3 inhibits the expression of T-bet, GATA-3, and RORγt and therefore attenuates Th subset differentiation (30), but this remains a hypothesis that has not been substantiated by direct evidence. On
the other hand, Foxp3-deficient Treg lineage cells in scurfy mice and immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients could become autoreactive effector cells and contribute to the autoimmune diseases. Based on these considerations, we have conducted studies to reexamine the potential of nTreg cells to produce Th effector cytokines after differentiation under Th subset polarizing conditions, and investigated the role of Foxp3 in controlling Th subset differentiation. The results showed that while Foxp3 is sufficient for immune suppression, it plays a limited role in suppressing Th subset differentiation.

Materials and Methods

Mice

BALB/c mice were purchased from the National Cancer Institute. C.B-17 Scid mice were purchased from the National Cancer Institute and bred in the University of Rochester animal facility. BALB/c.Thy1.1 congenic mice and BALB/c.Ca ^−/− mice were gifts from Dr. D. Fowell.

In vitro T cell culture

Polarizing conditions for Th1, Th2, and Th17 cell differentiation were described previously (31–33). CD25^−/− and/or CD25^+ CD4 T cells with a "naive" phenotype (CD45RB+brightCD44low) from BALB/c or BALB/c.Thy1.1 mice were isolated by FACS. The T cells were activated by anti-CD3 Abs (0.5 μg/ml) and irradiated spleen cells from BALB/c.TCR Ca ^−/− mice plus IL-2 (100 U/ml). Cultures containing CD25^− nTreg cells were replenished with fresh IL-2 (100 U/ml) daily after 2–3 days of differentiation. After 5 days, the cells were rested overnight in fresh medium containing IL-2 (50 U/ml) before intracellular cytokine staining or restimulation with anti-CD3 Ab and APC. Retroviral infection of T cells was conducted as previously described (34). Typically, we infected CD4 T cells 16–22 h after T cell activation and obtained the same results. MigR1 Foxp3 was a gift from Dr. S. Sakaguchi. The Foxp3 cDNA was excised from MigR1 Foxp3 and cloned into retroviral vector Mit with Thy1.1 as the marker gene (gift from Dr. M. Boothby). Retroviral vector T-body and MigR1 ROR-γt were CD45RB+brightCD44low naive CD4 T cells under Th polarizing conditions and infected with Foxp3-expressing retrovirus. In contrast, Foxp3-transduced CD4 T cells with a naive phenotype (CD45RB+brightCD44low) from BALB/c or BALB/c.Thy1.1 mice were isolated by FACS and restimulated by anti-CD3 Ab and APC to collect Th subset polarizing conditions for Th1, Th2, and Th17 cell differentiation. The results showed that while Foxp3 is sufficient for immune suppression, it plays a limited role in suppressing Th subset differentiation.

Analysis of cytokine expression

Detection of IFN-γ, IL-4, and IL-17 in cytokine supernatants was conducted by ELISA as previously described (34). Intracellular cytokine staining without Foxp3 staining was performed using the Cytofix/Cytoperm kit (BD Biosciences). All Abs against cytokines were purchased from BD Biosciences. Anti-Foxp3 Abs and staining reagents were purchased from eBioscience, and Foxp3 and cytokine staining was conducted following the manufacturer’s instructions.

Detection of transcription factor expression

Messenger RNA levels of T-bet, GATA-3, and ROR-γt were measured by SYBR Green-based real-time PCR. Transcripts relative to β-tubulin V (Tubb5) were calculated using the 2 −ΔΔCT method. Proteins of the transcription factors were detected by Western blot analyses using Abs against T-bet, GATA-3 (Santa Cruz Biotechnology), or ROR-γt (eBioscience).

Adaptive cell transfer

Retrovirus-infected CD4 T cells were isolated by FACS and allowed to rest overnight in fresh medium containing IL-2 (100 U/ml). FACS-sorted CD25 CD45RB+brightCD44 low T cells (4 × 10^5/mouse), either alone or with the sorted retrovirus-infected CD4 T cells (1 × 10^5/mouse), were transferred into C.B-17 Scid mice by tail vein injection. Colonoscopy of the recipient mice were fixed in formalin, and paraffin sections were analyzed by H&E staining. The microscopic sections were graded as described (35) to determine the severity of colitis.

Proliferation assays

[3H]Thymidine incorporation assay was performed as described (14). For CFSE dilution assay, labeled responder CD4 T cells (5 × 10^6/ml) from BALB/c.Thy1.1 mice were activated by anti-CD3 Ab and APC in the presence of FACS-sorted Foxp3-expressing CD4 T cells (Foxp3 GFP−CD45RB+brightCD44low) from the BALB/c.Thy1.1 mouse. After 5 days, the cells were harvested by flow cytometry. In vivo proliferation of CD4 T cells in the spleen was measured by BrdU staining, using the BrdU flow kit (BD Biosciences) following the manufacturer’s instructions.

Results

Th subset differentiation from nTreg cells

To directly compare the potentials of nTreg cells and conventional CD4 T cells to differentiate into Th cells, CD25 + and CD25 − CD4 T cells with a naive phenotype (CD25CD44low) were mixed and stimulated under polarizing conditions for Th1, Th2, and Th17 differentiation (31–33). IL-2 was added to the cell cultures to maintain cell growth. After differentiation, nTreg cells and conventional CD4 T cells were distinguished by Foxp3 staining, and cytokine expression was analyzed by intracellular cytokine staining (Fig. 1a). Unexpectedly, similar percentages of IFN-γ + cells and intensities of IFN-γ staining were found between nTreg cells (Foxp3 CD4 + ) and conventional (Foxp3 CD4 + ) CD4 T cells after Th1 differentiation. In contrast, nTreg cells differentiated under Th2 and Th17 polarizing conditions did not express IL-4 and IL-17, respectively, whereas their Foxp3 counterparts expressed the cytokines. IFN-γ was also detected in nTreg cells in mice that have developed experimental autoimmune encephalomyelitis or type 1 diabetes, albeit at relatively low frequencies (supplemental Fig. 1). Note that although IL-2 has been shown to partially inhibit Th17 differentiation (36), substantial Th17 differentiation still occurred in the Foxp3 + cells in our coculture experiments.

We have also performed experiments to determine the cytokine expression of thymic nTreg cells. CD25 + mature CD4 single-positive thymocytes (HSAdCD4 + ) of BALB/c origin were differentiated together with BALB/c.Thy1.1 CD25 − naive CD4 T cells under Th polarizing conditions. The differentiated cells were analyzed for Foxp3 and cytokine expression. As shown in Fig. 1b (upper panels), Foxp3 expression remained in the Thy1.1 + CD4 T cell population with no significant conversion of the Thy1.1 + CD25 + CD4 T cells into Foxp3 + cells. Foxp3 expression within the Thy1.1 + CD4 T cell population was generally heterogeneous. After Th1 differentiation, significant numbers of both the Foxp3low−/− and Foxp3 highThy1.1 − CD4 T cells expressed IFN-γ. In contrast, significant expression of IL-4 and IL-17 was observed only in the Foxp3low−Thy1.1 − CD4 T cells after Th2 and Th17 differentiation, respectively (Fig 1b, lower panels). Therefore, nTreg cells directly isolated from the thymus behaved similarly to their peripheral counterparts.

Effects of Foxp3 on Th subset differentiation

To determine the role of Foxp3 in suppressing Th subset differentiation, CD25 − naive CD4 T cells were stimulated for Th1, Th2, and Th17 differentiation and infected with Foxp3-expressing (MigR1 Foxp3, GFP as marker) or control retrovirus (MigR1). After differentiation, the infected cells (CD45RB+brightCD44low naive CD4 T cells) were mixed and stimulated with anti-CD3 Ab and APC to collect cytokine supernatant for cytokine analysis. The cytokine concentrations were found among different cultures at the time of supernatant collection (data not shown). ELISA analyses showed that CD4 T cells differentiated under Th1 polarizing conditions and infected with the Foxp3-expressing or control retrovirus produced similar amounts of IFN-γ. Although modestly reduced, significant IL-17 production was also detected in CD4 T cells differentiated under Th17 polarizing conditions and infected with Foxp3-expressing retrovirus. In contrast, Foxp3-transduced CD4 T cells differentiated under Th2 polarizing conditions produced only minimum amounts of IL-4. The Foxp3-transduced cells expressed similar or somewhat higher levels of Foxp3 than did ex vivo CD25 −

4 The online version of this article contains supplemental material.
nTreg cells (supplemental Fig. 2). Therefore, the expression of IFN-γ and IL-17 in the Foxp3-transduced “Th” cells was not due to insufficient Foxp3 expression.

Intracellular cytokine staining was performed to further assess the expression of the Th cytokines at the single cell level in the retrovirus-infected CD4 T cells (Fig. 1d). Consistent with the ELISA results, after Th1 differentiation no clear reduction of IFN-γ staining was observed in cells infected with the Foxp3-expressing retrovirus (Mit Foxp3, Thy1.1 as marker) as compared with control retrovirus (Mit). After Th17 differentiation, similar percentages of IL-17+ CD4 T cells were detected between cells infected with the Foxp3-expressing and control retrovirus (14.39% vs 15.41% of the Thy1.1+ populations). However, the intensities of IL-17 staining were noticeably lower in cells infected with Foxp3-expressing retrovirus (mean fluorescence intensity (MFI) of 622.06 vs 879.50).

**Effects of Foxp3 on transcription factor-induced Th subset differentiation**

In the experiments described in Fig. 1, c and d, it was possible that the polarizing cytokines had already initiated the Th subset differentiation programs before retroviral expression of Foxp3. This could have rendered the retroviral Foxp3 inefficient to influence Th subset differentiation. To exclude this possibility, naïve CD4 T cells were stimulated and infected with retrovirus expressing T-bet, GATA-3, or RORγt (GFP as marker), and simultaneously the cells were infected with either control (Fig. 2a, upper panels) or Foxp3-expressing retrovirus (Thy1.1 as marker) (Fig. 2a, lower panels). In these experiments, Th subset differentiation was strictly induced by the retrovirally introduced Th subset-specific transcription factors (T-bet, GATA-3, or RORγt), because the culture conditions did not induce, but rather prevented, the expression of the endogenous genes of the transcription factors. Since the expression of Foxp3 and the Th subset-specific transcription factors was controlled by the same CMV promoter and other regulatory elements provided by the retroviral vectors, they had the same expression kinetics. Therefore, retroviral Foxp3 expression did not lag the potential initiation of the Th subset differentiation.

The results showed that retroviral coexpression of Foxp3 with T-bet did not cause obvious reduction of T-bet-induced IFN-γ expression. In contrast, GATA-3-induced IL-4 expression was inhibited by Foxp3. Similar to the findings in a recent report (37), Foxp3 reduced but did not abolish RORγt-induced Th17 differentiation, as both the percentage of IL-17+ cells (13.25% vs 31.5% of the Thy1.1+ populations) and the intensities of IL-17 staining (MFI of 836 vs 1449) were decreased, as compared with cells infected with the control retrovirus. In our experiments, we consistently noticed that the double-infected CD4 T cells (GFP+/Thy1.1+) tended to be better producers of IL-17. Thus, when the cells were double infected with RORγt-GFP and the control Mit-Thy1.1 retroviruses, the percentage of IL-17 producers in the Thy1.1+ population was much higher than that in the Thy1.1+ population. However, this percentage increase in the double-infected population was not seen in cells infected with the second retrovirus that expressed Foxp3-Thy1.1 (Fig. 2a, right panels). This demonstrated that Foxp3 decreased the percentage of IL-17 producers in RORγt-induced Th17 differentiation. We further analyzed the differences of RORγt-mediated Th17 differentiation between control (Mit) and Foxp3-transduced (Mit Foxp3) CD4 T cells in multiple experiments. Fig. 2c shows that the reduction of both the percentage and MFI of IL-17+ populations in the Foxp3-transduced cells was statistically significant.

Importantly, the inhibition of RORγt-induced Th17 differentiation by Foxp3 appeared to be greater than the inhibition by Foxp3
of Th17 differentiation induced by the external polarizing cytokines (TGF-β and IL-6) shown in Fig. 1d, in which only the levels of IL-17, but not the percentage of IL-17α cells, were decreased. Perhaps Th17 differentiation under the latter conditions was compensated by additional transcription factor(s) such as the other ROR isoforms (5). Examination of the expression of the retrovirally introduced T-bet, GATA-3, and RORγt showed their levels in the double-infected cells comparable to those in normal Th1, Th2, and Th17 cells (Fig. 2b). Therefore, the results were not caused by improper levels of expression of these factors.

Among the three Th subsets, only Th2 differentiation can be greatly inhibited by Foxp3. To further determine whether the inhibition is stable, the CD4 T cells double infected with GATA3-expressing and Foxp3-expressing (Mit Foxp3) or control (Mit) retrovirus under Th1 polarizing conditions were isolated by FACS. The isolated cells were restimulated and cultured for an additional 2 wk without polarizing cytokines or Abs, then analyzed for the expression of all three Th2 cytokines. While all three Th2 cytokines were expressed in GATA3-transduced CD4 T cells coinfected with the control retrovirus, their expression was completely inhibited in cells coinfected with the Foxp3-expressing retrovirus (supplemental Fig. 3).

Expression of T-bet, GATA-3, and RORγt in the Foxp3-transduced Th cells

The detection of IFN-γ and IL-17 in CD4 T cells infected with Foxp3-expressing retrovirus led to the question of whether Foxp3 can in fact suppress the expression of T-bet and RORγt. To answer this question, real-time RT-PCR was performed to determine the expression of T-bet and RORγt, as well as GATA-3, in the retrovirus-infected Th cells (Fig. 3a). Surprisingly, higher levels of T-bet and RORγt were detected in the Foxp3-transduced Th1 and Th17 cells, respectively, than in their control virus-infected counterparts. After Th2 differentiation, similar levels of GATA-3 were detected between the Foxp3-transduced Th2 cells and the control virus-infected Th2 cells. Western blot analyses also showed that the Foxp3-transduced Th cells expressed these three transcription factors (Fig. 3b).

Expression and functions of GATA-3 and RORγt in nTreg cells

So far, the data suggested that the lack of Th2 and Th17 differentiation from nTreg cells (Fig. 1a) may not be caused by inhibiting the expression of GATA-3 and RORγt. To test this, experiments were performed to detect the expression of GATA-3 and RORγt in nTreg cells after Th2 and Th17 differentiation, respectively. For these experiments, conventional naive CD4 T cells (CD25−CD4+CD62LhighCD44low) and CD25high nTreg cells (CD25highCD4+CD62LhighCD44low) were isolated from naive BALB/c mice. Although some of the CD25low/nat CD4 T cells were Foxp3−, essentially all (>97%) CD25high CD4 T cells from naive mice were Foxp3+ (Fig. 4a) and therefore can be regarded as a homogeneous population of nTreg cells. The purified CD25high nTreg cells and the conventional naive CD4 T cells were stimulated separately under Th2 or Th17 polarizing conditions. After differentiation, the CD25high nTreg cells remained Foxp3+ and failed to express IL-4 and IL-17, respectively (Fig. 4b). The expression of GATA-3 and RORγt in these cells was analyzed by real-time RT-PCR. Compared with the CD25− CD4 T cells, nTreg cells after Th2 differentiation expressed ~2-fold higher levels of GATA-3, RORγt, and IL-17, but not the percentage of IL-17α-negative cells.
The cells were stained for CD4, Foxp3, and the indicated cytokines. dot plots of gated CD4 T cells are shown. Numbers in the plots indicate the percentages of the CD25\textsuperscript{high}Foxp3\textsuperscript{+} and Foxp3\textsuperscript{+} staining of gated CD4 T cells is shown. Numbers in the plot indicate the percentages of the CD25\textsuperscript{high}Foxp3\textsuperscript{+} and CD25\textsuperscript{high}Foxp3\textsuperscript{+} populations. b, FACS-sorted CD25\textsuperscript{−} conventional naive CD4 T cells and CD25\textsuperscript{high} naive nTreg cells were differentiated separately under Th2 or Th17 polarizing conditions. The differentiated cells were stained for CD4, Foxp3, and the indicated cytokines. dot plots of gated CD4 T cells are shown. Numbers in plots are the percentages of cells in the quadrants. c, Aliquots of the same cells as in b were analyzed for GATA-3 and ROR\gamma expression in the conventional CD4 T cells (open bars) and in the nTreg cells (hatched bars) by real-time RT-PCR. Mean values of transcripts relative to those of $\beta$-tubulin (Tubb5) are shown; error bars represent SDs. d, Naïve conventional CD4 T cells and nTreg cells were stimulated under Th1 (upper) and neutral (lower) conditions in mixed cultures and infected with retrovirus-expressing GATA-3 (MigR1 GATA-3) or ROR\gamma (MigR1 ROR\gamma). Th1 polarizing condition was used in the upper panel to ensure that Th2 differentiation was induced by retroviral GATA-3. After differentiation, the cells were stained for CD4, Foxp3, and the indicated cytokines. dot plots of the gated CD4\textsuperscript{+} GFP\textsuperscript{+} cells are shown. Numbers in the plots are the percentages of the cell populations. e, CD25\textsuperscript{+} T cells were isolated from BALB/c CD4 T cells by MACS and stimulated for Th1 and Th2 cell differentiation. After differentiation, the cells were stained for surface CD4 and intracellular Foxp3 and T-bet. dot plots of Foxp3 and T-bet staining on gated CD4 T cells are shown. Numbers in the plots indicate the percentages of the cell populations in the corresponding quadrants.

After Th17 differentiation, nTreg cells clearly expressed significant amounts of ROR\gamma, but the levels were lower than those of the conventional Th17 cells (Fig. 4c). Intracellular staining also detected T-bet expression in nTreg cells differentiated under Th1 conditions (Fig. 4e).

Experiments were also performed to retrovirally overexpress GATA-3 and ROR\gamma in nTreg cells in mixed cultures with CD25\textsuperscript{−} CD4 T cells. As expected, GATA-3 induced Th2 differentiation in the Foxp3\textsuperscript{−} CD4 T cells under Th1 polarizing conditions. However, it failed to do so in the Foxp3\textsuperscript{+} nTreg cells (Fig. 4d, upper panels). Similarly, ROR\gamma induced strong Th17 differentiation in the Foxp3\textsuperscript{−} CD4 T cells under neutral conditions, but it induced little, if any, IL-17 expression in the Foxp3\textsuperscript{+} nTreg cells (Fig. 4d, lower panels).

In vitro immune suppression by the Foxp3-transduced Th cells

It has been generally thought that immune suppressive activity and lack of Th cytokine expression are concurrent features of nTreg cells, and that Foxp3 is both necessary and sufficient for inhibiting the proliferation of conventional T cells (18–20). It was therefore interesting to learn whether the Foxp3-transduced Th cells described earlier remained immune suppressive. To address this issue, these cells were first analyzed for their own proliferative responses to stimulation by anti-CD3 Ab and APC. While the control retrovirus-infected Th cells were highly proliferative, the Foxp3-transduced Th cells were generally hypoproliferative. Nonetheless, the Foxp3-transduced Th17 cells did show a noticeable degree of proliferation. In multiple experiments, it appeared that cells differentiated under Th1 polarizing conditions proliferated poorly compared with cells differentiated under Th2 or Th17 polarizing conditions. On the other hand, the CD25\textsuperscript{+} nTreg cells differentiated under the Th subset polarizing conditions showed essentially no detectable proliferation (supplemental Fig. 4).

Subsequently, the abilities of the Foxp3-transduced Th cells and the in vitro-differentiated nTreg cells to suppress the proliferation of conventional CD4 T cells in response to stimulation by anti-CD3 Ab plus APC were analyzed. Since the Foxp3-transduced Th17 cells showed noticeable proliferation, which could mask the results of inhibition in the $[^3]$H\textsuperscript{thymidine incorporation assay, CFSE dilution assay was performed. Responder CD4 T cells were derived from BALB/c.Thy1.1 congenic mice so that they can be distinguished from the inhibitors by Thy1.1 staining. Although the CFSE-labeled responder T cells (CD4\textsuperscript{+}Thy1.1\textsuperscript{+}) underwent one to two divisions initially, further divisions were stopped by the Foxp3-transduced Th cells. In contrast, the responder T cells had undergone extensive divisions and almost lost all CFSE labeling after ~3 days of culture in the absence of inhibitors. In control experiments, we used control retrovirus-infected Th cells as “inhibitors”. In such experiments, the division of the responder cells was delayed presumably due to competition for nutrients and growth factors by the control retrovirus infected Th cells (Fig. 5a). For the nTreg cells differentiated under polarizing conditions, because they themselves did not proliferate, $[^3]$H\textsuperscript{thymidine incorporation assay was used to assess their immune suppressive activities. Their presence completely inhibited $[^3]$H\textsuperscript{thymidine incorporation in the T cell cultures (Fig. 5b).

Prevention of lymphoproliferation and inflammatory bowel disease (IBD) by the Foxp3-transduced Th cells

We used the adoptive transfer model of IBD to investigate the immune suppressive activities of the Foxp3-transduced Th cells in vivo. In such experiments, CD25\textsuperscript{−}CD45RB\textsuperscript{high}CD4\textsuperscript{+} T cells of BALB/c.Thy1.1 origin were adoptively transferred into C.B-17 Scid mice. The recipient mice that received only these cells developed splenomegaly. However, cotransfer of the Foxp3-transduced Th cells prevented the splenomegaly (Fig. 6a). The Foxp3-transduced Th cells also inhibited BrdU uptake by the CD4 T cells in the recipient mice (Fig. 6b). As previously demonstrated (38), in such an adoptive transfer system, mice that received only the CD25\textsuperscript{−}CD45RB\textsuperscript{high} CD4 T cells developed IBD with massive...
inflammatory infiltration in the colon and destruction of the architecture of the intestinal walls (Fig. 6c). Splenomegaly and inflammatory infiltration of the intestines were also observed in mice cotransferred with Th cells infected with control retrovirus (data not shown). In contrast, as with splenomegaly, inflammatory infiltration was greatly reduced or absent, and the integrity of the intestinal walls was preserved in mice that received co-transfer of the Foxp3-transduced Th cells (Fig. 6c).

FIGURE 6. Prevention of lymphoproliferation and IBD by Foxp3-transduced Th cells. a. C.B-17 Scid mice received adoptive transfer of CD45RB<sup><b>hi</b></sup>/CD4<sup><b>+</b></sup> <b>T</b> cells (4 x 10<sup>5</sup>/mouse) either alone or together with Foxp3-transduced Th cells (1 x 10<sup>6</sup>/mouse). Two to 3 mo later, the spleens of the mice were harvested and their sizes were measured by gross examination. b. One hour before sacrifice, the recipient mice received 1 mg of BrdU per mouse i.p. Spleen cells were harvested and stained for CD4 and BrdU. Histograms of BrdU staining on gated CD4<sup><b>+</b></sup> T cells are shown. Numbers indicate the percentages of BrdU-positive cells and the MFI of BrdU staining. c. Representatives of histology (H&E staining) of the proximal colon (left panels) and the colitis scores (right panel) of the recipient mice are shown.
Discussion

The TCR of nTreg cells are known to be biased toward self-reactivity. Functionally, the high affinity to self-Ags allows efficient activation of the immune suppressive activity of nTreg cells against autoreactive conventional T cells, but it also necessitates that nTreg cells do not gain the destructive Th effector functions when activated by self-Ags. These two functional aspects are fundamental issues of the basic immunobiology of nTreg cells. Studies presented herein have examined the ability of nTreg cells to produce Th effector cytokines after differentiation under polarizing conditions. The results showed that nTreg cells did not differentiate into Th2 and Th17 cells, but unexpectedly differentiated into IFN-γ-producing cells. Contrary to a popular view that Foxp3 can inhibit the expression of T-bet, GATA-3, and ROR-γt (30), these transcription factors were highly expressed in the Foxp3-transduced Th cells. Despite the lack of Th2 and Th17 differentiation, nTreg cells expressed GATA-3 and ROR-γt after Th2 and Th17 differentiation, respectively. Furthermore, nTreg cells retrovirally over-expressing GATA-3 and ROR-γt did not differentiate into Th2 and Th17 cells. These results demonstrated that the lack of Th2 and Th17 differentiation from nTreg cells was not due to the inhibition of the expression of these two transcription factors.

These experimental outcomes call for new theories to explain the lack of Th2 and Th17 differentiation from nTreg cells. For Th2 differentiation, it appears that Foxp3 can antagonize the functions of GATA-3 because coexpression of Foxp3 with GATA-3 completely blocked Th2 cell differentiation. The roles of GATA-3 in Th2 cell differentiation include the induction of transcription permissive chromatin structure at the Th2 cytokine gene locus and direct trans-activation of the Th2 cytokine gene promoters. It is possible that Foxp3 can somehow antagonize these functions of GATA-3 through either a direct effect on GATA-3 or an indirect effect on other factors, such as those important for chromatin remodeling and transcriptional gene activation.

Unlike Th2 differentiation, complete lack of Th17 differentiation was observed only in nTreg cells, whereas Foxp3-transduced conventional CD4 T cells could still undergo successful Th17 differentiation, albeit at lower efficiency. Therefore, the reciprocal relationship between Th17 and adaptive Treg differentiation from conventional CD4 T cells (31) may not be as stringent as previously thought. Nonetheless, several mechanisms may be responsible for the partial inhibition of Th17 differentiation from conventional CD4 T cells. First, a direct physical interaction between Foxp3 and ROR-γt may attenuate the function of ROR-γt (37, 39). Second, optional expression of IL-17 requires physical interaction and functional collaboration between ROR-γt and Runx1 (39). Foxp3 also interacts with Runx1 (40), which may further disrupt the function of ROR-γt. In nTreg cells other factors unique to this cell type may enhance these repressor activities of Foxp3 to achieve full suppression of Th17 differentiation. Conversely, nTreg cells may lack certain unknown factors necessary for IL-17 expression. It is also possible that Foxp3 may not be required for suppressing Th17 differentiation from nTreg cells, and the choice for a Th17 fate is already eliminated in the thymocytes upon their commitment to the nTreg lineage before Foxp3 expression. Such a scenario would be an interesting deviation from the current paradigm of Th subset differentiation, which maintains that cytokine genes in CD4 T cells remain plastic after thymic development, and the choice for them to become active or permanently silenced is made after Ag stimulation of the T cells in the periphery.

Unlike the lack of Th2 and Th17 differentiation, nTreg cells differentiated into IFN-γ-producing cells under Th1 polarizing conditions. Although it has been shown that adaptive Treg cells, as originated from the CD25+ conventional CD4 T cells, could produce IFN-γ (17, 41, 42), CD25+ nTreg cells, a completely separate lineage, have until now been considered unable to produce Th effector cytokines. In the present study, the expression of IFN-γ by nTreg cells was observed not only in the in vitro cultures, but, importantly, significant numbers of ex vivo nTreg cells from B6 and NOD mice that have developed experimental autoimmune encephalomyelitis and type 1 diabetes, respectively, also produced IFN-γ. The functional importance of the nTreg cell-derived IFN-γ in these autoimmune conditions remains to be discovered. However, the adaptive Treg cell-derived IFN-γ has been shown to be critical to the establishment of tolerance to allografts by inducing immune inhibitory activities of dendritic cells and macrophages (42, 43). IFN-γ has also been shown to convert CD25+ CD4 T cells to adaptive Treg cells (44). Therefore, by analogy one may speculate that under the autoimmune conditions, the nTreg cell-derived IFN-γ may contribute to ameliorating the severity of the autoimmune diseases by similar mechanisms of inducing immune inhibitory activities of dendritic cells and macrophages and converting neighboring CD25+ CD4 T cells into adaptive Treg cells. The role of IFN-γ in autoimmune diseases is complex (45). However, at least some studies have shown a protective role of IFN-γ against autoimmune diseases (46–49). Under these circumstances, there could be a potential link between Treg cells and the protective role of IFN-γ. Conversely, the expression of IFN-γ by Treg cells suggests that Treg cells may harbor intrinsic pro- and antiinflammatory potentials. The fact that IFN-γ was detected in Treg cells from mice with autoimmune diseases but not healthy naïve animals (supplemental Fig. 1) may be an indication of tipping the balance toward proinflammation under the autoimmune conditions. Following this logic, the progression to diabetes in aged NOD mice and the onset of experimental autoimmune encephalomyelitis despite normal numbers or accumulation of Treg cells (50, 51) could be at least partially attributable to the relative decline of the antiinflammatory activities and rise of proinflammatory activities of the Treg cells.

Regardless of their Th cytokine profiles, Foxp3-transduced Th cells exhibited the typical antiproliferative and immune suppressive characteristics of Treg cells. They were hypoproliferative and suppressed the proliferation of conventional CD4 T cells in vitro. In adoptive transfer experiments, these cells prevented lymphoproliferation and colonic inflammatory infiltration caused by the cotransfer of the pathogenic conventional CD4 T cells. These findings showed that Foxp3 is sufficient for the immune suppressive activity even in CD4 T cells that have undergone effector differentiation. We think that this finding is of great clinical importance, as it is now widely appreciated that Treg cells represent a promising solution to allograft rejection and therapy for autoimmune and allergic diseases. However, most current studies seeking such clinical applications use polyclonal or nonspecific Treg cells. Such nonspecific Treg cells are often ineffective in suppressing Ag-specific immune responses (52). Furthermore, they could potentially cause generalized immune suppression, which increases the risk of opportunistic infection and cancer. Our present studies showed that it is possible to convert the patients’ own pathogenic CD4 effector T cells into adaptive Treg cells for therapy. Such converted adaptive Treg cells share the same antigenic reactivities as the pathogenic CD4 T cells, and therefore they can provide the most specific and effective immune suppression.

Acknowledgments

The authors thank Dr. S. Sakaguchi for providing the MigR1 Foxp3 plasmid, Dr. D. Littman for the MigR1 ROR-γt plasmid and anti-ROR-γt Abs, and Drs. L. Glimcher and S. Szabo for T-bet cDNA. The authors also thank Daniel Ryan for his support.
Disclosure

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. IFN-γ expression by nTreg cells from mice with autoimmune diseases. (a) Comparison of IFN-γ expression in nTreg cells from naïve B6 and B6 mice with experimental autoimmune encephalomyelitis (EAE). To induce EAE, B6 mice were immunized subcutaneously with MOG 35-55 peptide emulsified in IFA supplemented with *M. tuberculosis* (400 μg/ml). The mice also received i.p. injections of pertussis toxin (200 ng/mouse) on day 0 and day 2 after immunization. Draining lymph nodes (DLN) were harvested from mice that developed EAE with clinical scores greater than 2 (partial paralysis of hind legs). The DLN cells of EAE B6 mice and lymph node cells from naïve B6 mice were stimulated with PMA and ionomycin, then stained for CD4 and intracellular IFN-γ and Foxp3. Dot plots of gated CD4+ cells are shown, numbers show the percentages and mean fluorescence intensities of IFN-γ staining of cells in each quadrants. Notice that the IFN-γ+ cells in the Foxp3+ populations were 0.4% and 2.12% in naïve and EAE mice, respectively. (b) Spleenocytes of 21-week old female NOD mice were analyzed as in (a). The percentage of IFN-γ+ cells in the Foxp3+ population was 8.25%.
Supplementary Figure 2. Comparison of Foxp3 levels between Foxp3-transduced “Th” cells and ex vivo CD25+ nTreg cells. CD25− Balb/c naïve CD4 T cells were stimulated for Th1, Th2 or Th17 differentiation and infected with Foxp3-expressing retrovirus (MigR1 Foxp3). The infected (GFP+) CD4 T cells were isolated by FACS. The sorted cells (Th Foxp3) and fresh isolated CD25+ CD4 T cells (CD25+ nTreg) were stained for Foxp3. Histograms of the Foxp3 staining are shown.

Supplementary Figure 3. Stable inhibition of Th2 cytokine expression by Foxp3. Naïve CD4 T cells were stimulated under Th1 polarizing condition. Th2 differentiation was induced by retroviral expression of GATA-3. The cells were simultaneously infected with Foxp3-expressing or control retrovirus. CD4 T cells double-infected with GATA-3-expressing retrovirus (MigR1 GATA-3) and Foxp3-expressing (Mit Foxp3) or control (Mit) retrovirus were isolated by FACS. The sorted cells were re-stimulated and cultured for additional 2 weeks in the absence of polarizing cytokines and antibodies, then analyzed for the expression of IL-4, 5 and 13 by intracellular cytokine staining. Dot plots of Thy1.1 and cytokine staining are shown. Numbers show the percentages of the
cell populations in the corresponding quadrants. Notice that all three cytokines are expressed in cell infected with control virus (Mit), whereas their expression was inhibited in cells infected with Foxp3-expressing retrovirus (Mit Foxp3).

Supplementary Figure 4. In vitro hypoproliferation of Foxp3-tranduced CD4 T cells and nTreg cells differentiated under polarizing conditions. (a) CD25⁻ naïve CD4 T cells from Balb/c mice were stimulated for Th1, Th2 or Th17 differentiation and infected with either control (MigR1) or Foxp3-expressing retroviruses (MigR1 Foxp3). On day 5 of stimulation, the cells were washed and rested overnight in culture medium containing 50 u/ml IL-2. The infected cells (GFP⁺CD4⁺) were then sorted by FACS and stimulated with anti-CD3 antibody and APC for 72 hrs. ³H-thymidine was added to the cultures for the last 18 hrs. Values show means of ³H-thymidine incorporation of triplicate wells. Error bars represent standard deviations. We consistently observed that Th1-differentiated cells proliferated poorly compared with the other two subsets. (b) CD25⁺ nTreg cells (CD25⁺CD62L⇑ CD44↓CD4⁺) differentiated under the Th subset polarizing conditions, and fresh isolated CD4 T cells were stimulated, and proliferation was measured as in (a).