Cutting Edge

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Cutting Edge: An IL-17F-Cre^{EYFP} Reporter Mouse Allows Fate Mapping of Th17 Cells[1]

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The need for reporter lines able to faithfully track Th17 cells in vivo has become an issue of exceptional importance. To address this, we generated a mouse strain in which Cre recombinase is expressed from the IL-17F promoter. Crossing the IL-17F-Cre allele to a conditional enhanced yellow fluorescent protein (EYFP) reporter mouse yielded the IL-17F-Cre^{EYFP} strain, in which IL-17F expression is twinned with EYFP in live IL-17F-expressing cells. Although we demonstrate that IL-17F expression is restricted to CD4$^+$ T cells during experimental autoimmune encephalomyelitis, IL-17F-Cre^{EYFP} CD8 T cells robustly expressed IL-17F in response to TGF-β, IL-6, and IL-23. Fate mapping of IL-17F-expressing reporter T cells revealed a significant down-regulation of Th17 cytokines after homeostatic expansion in RAG1-deficient animals. Despite this loss of effector phenotype, committed Th17 cells were resistant to Foxp3 expression in vitro or in vivo. Thus, the IL-17F-Cre strain furthers our understanding of Th17 biology. ***The Journal of Immunology,*** **2009, 182: 1237–1241.**

An ever-increasing list of publications has resulted in the establishment of pathogenic Th17 cells, a T cell subset definable by expression of CD4, IL-17A, IL-17F, and to varying extents IL-6, IL-21, IL-22, TNF-α, and CCR6 (1, 2). The required signaling molecules for murine Th17 differentiation have already been characterized, with IL-6 or IL-21 in combination with TGF-β1 being the basic in vitro requirement (3). Further signaling through the IL-23 receptor is required to expand and render the new Th17 cells effective and pathogenic, such that experimental autoimmune encephalomyelitis (EAE)[3] can be passively transferred (4).

With the interest in Th17 cells escalating so vehemently, new questions have appeared concerning the location of in vivo generation, migration, expression profiles, proliferation, and the fate of these potentially pathogenic cells. Reporter mice for Th cell subsets including Th1 (5), Th2 (6) and regulatory T cells (Tregs) (7) have been generated. Until very recently, Th17 cells have lacked reporter strains able to account for their activity (8). In this study we introduce a new transgenic strain in which Cre recombinase is expressed exclusively from the IL-17F promoter. Crossing these mice to inducible ROSA26-enhanced yellow fluorescent protein (EYFP) reporter mice (9) allowed us to analyze the generation and location of Th17 cells. Cre-mediated highlighting of Th17 cells also induces a nonreversible fluorescence in IL-17F-expressing T cells, allowing us to map the fate of IL-17F-expressing cells in vivo. After ensuring that EYFP expression correlated with IL-17F expression, we use the IL-17F-Cre^{EYFP} system to describe the dynamics of Th17-related cytokine expression and lineage commitment.

Materials and Methods

Mice and induction of EAE

EAE was induced in 8-wk-old IL-17F-Cre^{EYFP} mice by a single s.c. injection at the tail base of myelin oligodendrocyte glycoprotein (MOG)$^{35–55}$ peptide (10) (50 μg/mouse) immersed in CFA and pertussis toxin in PBS (200 ng/mouse at days 0 and 2). Mice were sacrificed at day 14 after disease induction at varying clinical scores to obtain inflammatory CNS infiltrates, which were isolated using a Percoll (Invitrogen) gradient.

Generation of IL-17Cre^{EYFP} mice

The IL-17F-Cre allele was generated using recombineering on a bacterial artificial chromosome in *Escherichia coli*. Clones harboring the correct integration were confirmed using PCR analysis and Southern blotting. Bacterial artificial chromosome (BAC) DNA was isolated, linearized using PI-*Sce*I restriction digestion, and cleaned using Sepharose (GE Healthcare). Founder animals were obtained after pronuclear injection of the resulting BAC DNA at a final concentration of 2 ng/ml into pronuclei of a C57BL/6 DBA hybrid strain. Mice were backcrossed to the conditional ROSA26-EYFP reporter strain (9) before experimental analysis. Primers used for typing were 5′-ccccttcaggaagtggag tag-3′ for IL-17F-Cre forward and 5′-accgcgcgcctgaagatatag-3′ for IL-17F-Cre reverse.

Antibodies, flow cytometry, and cytokine measurement

Lymph node cells, splenocytes and CNS-isolated cells were surface stained with anti-CD4, CD8, CD90.2, B220, F480, CD11c, CD19 (BD Biosciences), CD62L (Immunotools), and anti-CD11b (homemade). Intracellular staining

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; BAC, bacterial artificial chromosome; EYFP, enhanced yellow fluorescent protein; MOG, myelin oligodendrocyte glycoprotein; Tc, cytotoxic T cell; Treg, T regulatory cell; iTreg induced Treg.

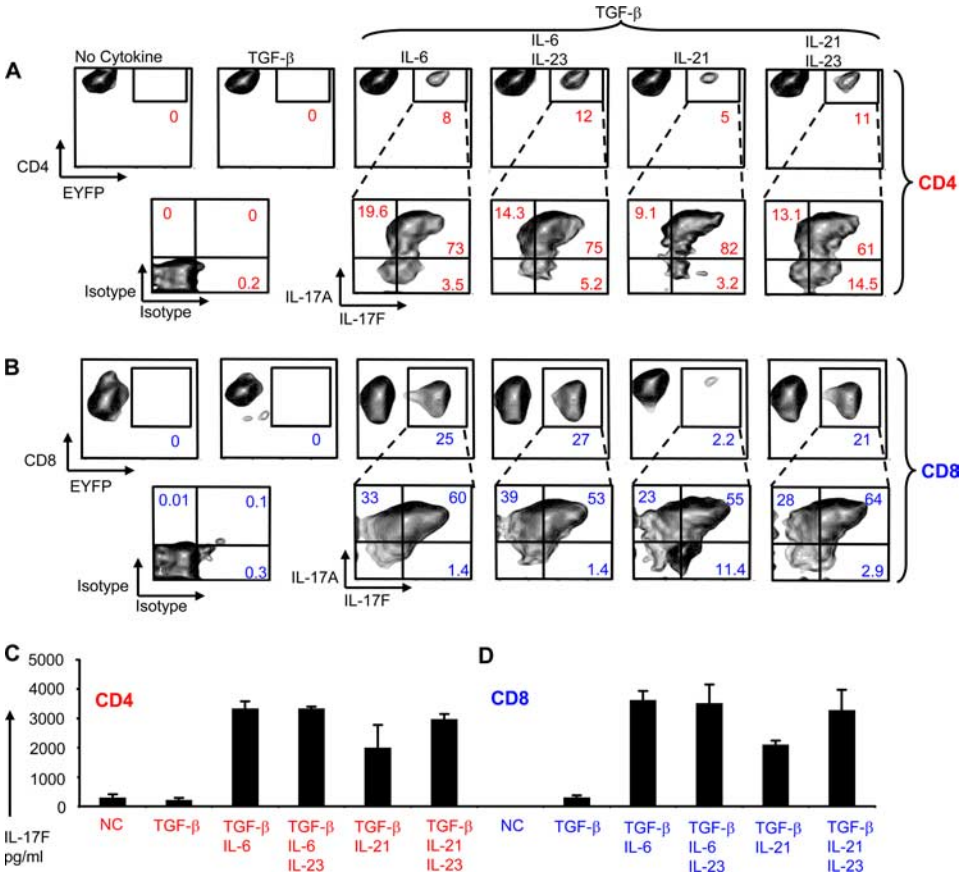

FIGURE 1. IL-17F-Cre^{EYFP} T cells express EYFP in response to Th17-polarizing cytokines. *A* and *B*, Purified naive CD4 (*A*) or CD8 (*B*) T cells were activated in the presence of the indicated cytokine combinations. EYFP induction is shown in the gates. The same cells were stained for expression of IL-17A and IL-17F. *C*, Supernatants from these cultures were assayed for IL-17F secretion.

was conducted on in vitro differentiated or ex vivo CNS-derived T cells using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's recommendations. Cells were restimulated at 1×10^6 cells/ml using a combination of PMA (50 ng/ml) and ionomycin (750 ng/ml). Brefeldin A was added at 1 μg/ml. Th17 intracellular stainings were performed using allophycocyanin-conjugated (eBioscience) or PE-conjugated anti-IL-17A or IL-17F Abs (Becton Dickinson). Intracellular FoxP3 staining was performed using a FoxP3 staining kit (eBioscience). Measurement of IL-17F was performed by an ELISA kit obtained from R&D Systems.

Cytokines and cell culture

Abs against CD3 (1 μg/ml) and CD28 (6 ng/ml) used for in vitro T cell activation were generated in our laboratory. Recombinant murine IL-6 (20 ng/ml), recombinant murine IL-23 (20 ng/ml), recombinant murine IL-2 (10 ng/ml), and recombinant human TGF-β1 (5 ng/ml) were all purchased from R&D Systems. Neutralizing anti-IFN-γ Ab was a gift from B. Becher (University of Zurich, Zurich, Switzerland) and was used at 10 μg/ml.

Real-time expression analysis and measurement

Total RNA from FACS-sorted cells (CD4$^+$EYFP$^+$ or EYFP$^-$) was isolated using TRIzol (Invitrogen). mRNA coding for IL-17A, IL-17F, IL-23R, IRF4, and Foxp3 were analyzed with primers from Qiagen using the QuantiTect SYBR Green RT-PCR kit. Changes in gene expression were calculated relative to that of GAPDH.

Results and Discussion

Generation and characterization of IL-17F-Cre^{EYFP} reporter mice

To track IL-17F-expressing cells in vivo, we opted for a Cre-loxP-mediated approach in which Cre recombinase would be expressed from the IL-17F promoter (supplemental Fig. 1*A*).[4] Upon crossing to a conditional EYFP reporter strain (9), IL-17F expression would result in EYFP expression from the ubiquitous ROSA26 promoter following Cre-mediated excision of a transcriptional stop cassette in IL-17F-expressing cell types (supplemental Fig. 1*B*), yielding the IL-17F-Cre^{EYFP} reporter strain. We did not observe EYFP$^+$ cells in lymph node (data not shown) or splenic B-lymphocytes, macrophages, dendritic cells, or CD8 T cells in naive 12-wk-old IL-17F-Cre^{EYFP} mice (supplemental Fig. 1*C*). No EYFP expression was ever detectable in ROSA26-EYFP$^+$IL-17F-Cre$^-$ littermate controls (data not shown).

IL-17F is coexpressed by a subpopulation of IL-17A-expressing Th17 cells in a number of Th17-polarizing culture conditions and in the context of in vivo inflammation models (11, 12). Our interest also extended to CD8 T cells, which are also known to express IL-17A in response to inflammatory stimuli and play a role in autoimmune inflammation (13). To investigate whether CD4 and CD8 T cells isolated from IL-17F-Cre^{EYFP} mice were able to express IL-17F, splenocytes from these mice were cultured under in vitro Th17-polarizing conditions for 5 days. To follow the induction of IL-17F expression in Th17-differentiated, spleen-derived T cell populations, cell culture wells were sampled from day 3 to day 5. A time-dependent increase in EYFP expression was observed in IL-17F-Cre^{EYFP} CD4 T cells and to a greater extent in CD8 T cells (supplemental Fig. 1*D*). This supports data describing IL-17 production from CD8 T cells in response to TGF-β and IL-6-signaling (14). Greater than 95% of the EYFP$^+$ CD4 T cells were positive for IL-17A and/or IL-17F following intracellular staining (supplemental Fig. 1*E*), confirming fidelity of the reporter construct. Given that expression of Cre recombinase from the IL-17F-Cre transgene occurs in parallel with IL-17F,

[4] The online version of this article contains supplemental material.

T cells will be IL-17F and Cre positive but remain EYFP$^-$ until Cre mediates recombination of the STOP cassette. The latter may explain why a small population of IL-17F-expressing T cells remain EYFP$^-$ (supplemental Fig. 1*E*).

To analyze the expression signatures of Th17 and cytotoxic T (Tc)17 cells from IL-17F-Cre^{EYFP} mice, real-time PCR analysis was performed on total RNA isolated from sorted CD4$^+$ EYFP$^+$ and CD8$^+$EYFP$^+$ T cells after 5 days in Th17-polarizing culture conditions. Significant up-regulations in the expression of *il17a*, *il17f*, and *il23r* were abundantly clear in CD4$^+$EYFP$^+$ and CD8$^+$EYFP$^+$ T cells when compared with control-stimulated CD4$^+$EYFP$^-$ T cells (supplemental Fig. 1*F*). This coincided with a down-regulation of the regulatory T cell marker *foxP3* and an increase in the *irf4*, a transcription factor shown to be essential for Th17 differentiation (15).

Th17 conditions induce IL-17F expression in both CD4 and CD8 IL-17F-Cre^{EYFP} T cells

Most published Th17 differentiations show expression of IL-17A. We wanted to observe the expression of IL-17F under conditions shown to promote Th17 differentiation and to examine how this expression relates to that of its relative, IL-17A. In addition to TGF-β, both IL-21 and IL-6 have been shown to promote Th17 differentiation (3), with IL-23 being thought to drive expansion of newly formed Th17 cells (4). To this end, we purified CD4$^+$CD25$^-$ or naive CD8 T cells and activated them in the presence of Th17-promoting cytokine cocktails for 5 days. We observed IL-17F expression by both CD4 and CD8 T cells from IL-17F-Cre^{EYFP} mice under all Th17-polarizing conditions. The majority of IL-17F-expressing Th17 cells also expressed IL-17A (Fig. 1*A*). CD8 T cells also robustly expressed IL-17F in response to the same cytokine mixtures. As observed in Th17 cells, the majority of Tc17 cells coexpress IL-17A and IL-17F. However, a greater proportion of Tc17 cells down-regulate IL-17F and express IL-17A alone (Fig. 1*B*). In addition to the intracellular staining for IL-17F, we measured cytokine secretion to confirm that EYFP expression correlated with IL-17F protein secreted from IL-17F-Cre^{EYFP} T cells. A strong correlation was observed between EYFP$^+$ cells and IL-17F secretion (Fig. 1*C*). Thus, EYFP$^+$ T cells generated from IL-17F-Cre^{EYFP} mice are bona fide Th17 cells.

IL-17F expression is restricted to CD4$^+$ T cells during MOG-induced EAE

IL-17A is thought to be a major contributor to a number of inflammatory disease models (16, 17). Following clarification of the IL-17F expression fidelity in the IL-17F-Cre^{EYFP} strain and the finding that CD8 T cells robustly express IL-17F and IL-17A, we investigated the localization of IL-17F-expressing T cells during MOG-induced EAE. A completely CD4 T cell-restricted (Fig. 2*A*) and, more specifically, CD90.2$^+$CD4$^+$ CD62L$^-$ T cell-restricted IL-17F expression was observed in spleen and blood (Fig. 2*B*). Thus, peripheral CD8 T cells readily express IL-17F in vitro in response to culture conditions designed to induce Th17 cells, but not during EAE, a predominantly Th1- and Th17-mediated disease.

At day 14 after EAE induction, IL-17F-Cre^{EYFP} mice were sacrificed at a mean clinical score of 3.5 (hind limb paralysis) and brain and spinal cord were analyzed. CNS infiltrates were surface-stained for CD45.2, CD11b, CD4, and intracellular IL-17A. IL-17A and IL-17F expression was restricted to

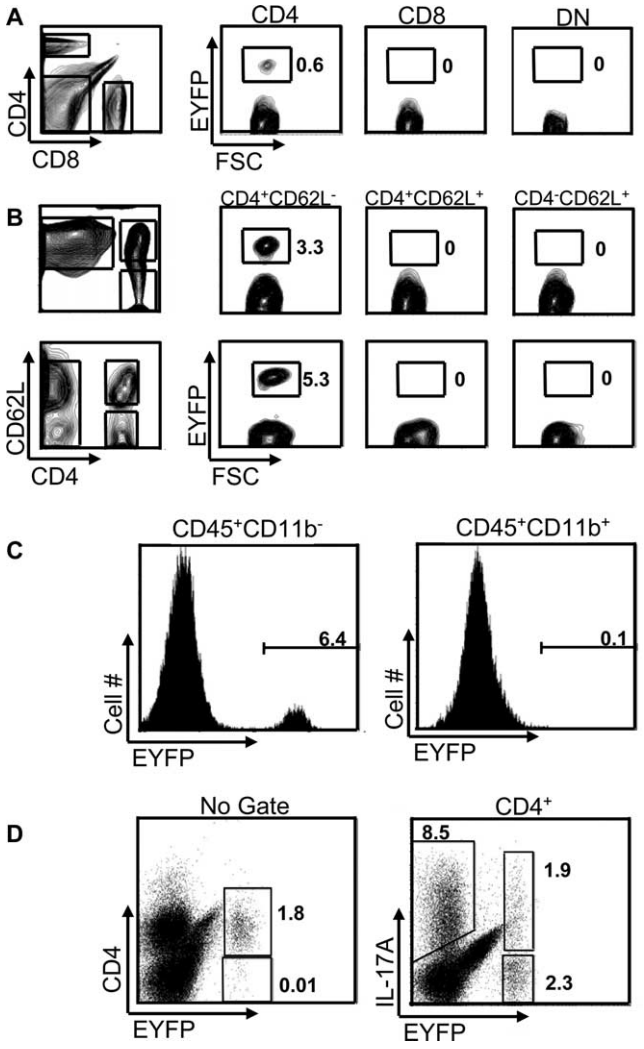

FIGURE 2. IL-17F expression is restricted to CD4 T cells during EAE. *A*, Splenocytes from EAE-sick mice (day 14) were stained for CD4 and CD8. Percentages of gated cells expressing EYFP are given in the gates. *B*, Splenocytes (*upper row*) and PBMC (*lower row*) were stained for CD62L and CD4. The percentages of gated cells are shown in the regions. *C*, CNS-infiltrating cells were isolated and stained for CD45.2 and CD11b. EYFP expression in the indicated populations is shown in the histograms. *D*, Total CNS-isolated cells were stained for CD4 and IL-17A. EYFP expression is shown in the indicated cell types.

CD45.2$^+$CD11b$^-$ lymphocytes (Fig. 2*C*). Of these, the vast majority were CD4 positive (Fig. 2*D*). This confirmed a peripherally derived and CD4 T lymphocyte-restricted expression of IL-17F in the inflamed CNS during EAE. Infiltrating cells were additionally stained for intracellular IL-17A. Of CNS-isolated T cells from EAE-afflicted mice, the majority of Th17 cells present, as defined by expression of IL-17A, IL-17F, or both, expressed only IL-17A. A proportion of CD4 T cells were coexpressers of both IL-17A and IL-17F, whereas another population expressed IL-17F exclusively (Fig. 2*D*).

IL-17-expressing T cells down-regulate their effector phenotype during homeostatic proliferation

A major advantage of using a Cre-loxP-mediated reporter system is the ability to follow the fate of Cre-expressing Th17 cells. In addition to this, the effector function of Th17 cells is a major consideration in models relying on adoptive transfer of Th17

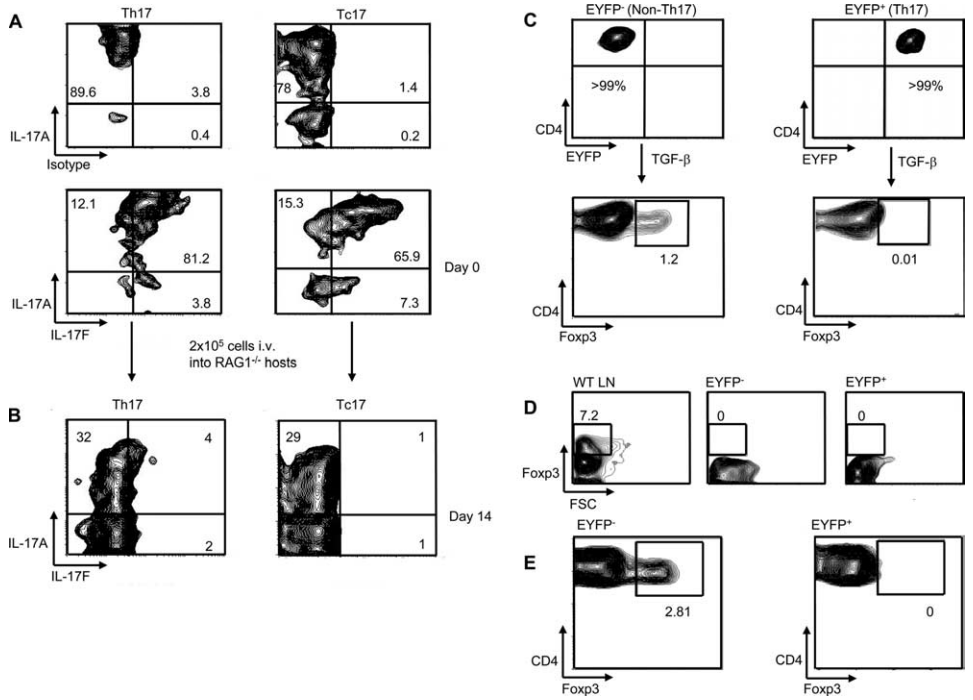

FIGURE 3. Th17 cells are resistant to Foxp3 up-regulation. Th17 and Tc17 cells were raised in a culture from pooled splenocytes isolated from MOG-CFA-immunized IL-17F-Cre^{EYFP} mice. *A*, Whole splenocytes were restimulated using MOG peptide (50 μg/ml) TGF-β, IL-6, and anti-IFN-γ for 4 days then for a further 3 days with IL-23 and IL-2. After this culture period, extracted cell samples were stained intracellularly for IL-17A, IL-17F, and a fluorochrome-matched isotype control Ab after restimulation with PMA, ionomycin, and brefeldin A. Contour plots are gated on Th17 ($CD4^+EYFP^+$) or Tc17 ($CD8^+EYFP^+$) cells. *B*, $CD90.2^+EYFP^+$ cells were cell sorted from these cultures (>99%), after which 2×10^5 cells were injected into the tail vein of $RAG1^{-/-}$ mice. Contour plots represent IL-17F and IL-17A expression of the transferred Th17 or Tc17 cells after a 14-day period of expansion. Percentages of $EYFP^+$ gated cells are shown in the representative quadrants. *C*, Magnetically enriched IL-17F-Cre^{EYFP} $CD4^+$ cells from spleen and lymph nodes were cultured for 5 days with anti-CD3, anti-CD28, TGF-β, IL-6, and neutralizing anti-IFN-γ or TCR-stimulated in the absence of Th17 cytokines. After 5 days, cells were sorted from the cultures according to $CD4^+EYFP^+$ or $CD4^+EYFP^-$. The sorted cells were switched into 2 ng/ml TGF-β-containing medium. After a further 3 days, Th17 and control cells were analyzed for up-regulation of Foxp3. Numbers in the quadrants represent percentages of $CD4^+$ gated cells. *D*, $CD90.2^+EYFP^-CD25^-$ or $CD90.2^+EYFP^+$ cells (1×10^5) from Th17 cultures were FACS sorted (99%) and stained for Foxp3. Percentages of $Foxp3^+$ T cells are given in the gates. *E*, Cells sorted as in *D* (1×10^5) were injected i.v. into RAG-deficient hosts. After 14 days, recovered cells were stained for Foxp3. Quadrant numbers represent the percentage of Foxp3-expressing CD4 T cells.

cells. To investigate the stability of IL-17A and IL-17F expression in vivo, Th17 and Tc17 cells were generated after restimulation of MOG-immunized IL-17F-Cre^{EYFP} splenocytes in the presence of Th17-polarizing cytokines and MOG peptide. Before injection, the Th17 phenotype of the $EYFP^+$ T cells was confirmed with respect to IL-17A and IL-17F expression (Fig. 3 *A*). Two weeks after transfer, we were able to recover both Th17 and Tc17 cells from spleen, mesenteric lymph nodes, and blood (supplemental Fig. 2). Most of the transferred Th17 and Tc17 cells significantly down-regulated expression of their hallmark cytokines after a period of homeostatic expansion in spleen (Fig. 3*B*) and mesenteric lymph nodes (data not shown). Interestingly, the down-regulation of IL-17F expression was more profound than that of IL-17A in MOG-specific Th17 and Tc17 T cells. Thus, a transient effector phenotype of Th17 and Tc17 T cells is a consideration when undertaking adoptive transfer experiments relying on pathogenicity of Th17 cells.

Th17 cells are resistant to expression of Foxp3 in vivo

The recently described relationship between the developmental pathways of both Th17- and Foxp3-expressing induced Tregs (iTregs) demonstrates TGF-β signaling as necessary for Foxp3 induction, but TGF-β in combination with IL-6 as a requirement for Th17 differentiation (18). It has also recently been demonstrated that naturally occurring Tregs can be driven to IL-17A expression in the presence of IL-6 (8). From IL-17F-Cre^{EYFP} mice, no EYFP expression was detectable in ex vivo isolated Tregs or in vivo differentiated iTregs (data not shown). We wanted to use the IL-17F-Cre^{EYFP} system to address whether or not fully differentiated Th17 cells could redifferentiate into Foxp3-expressing T cells while in culture or in vivo. IL-17F-Cre^{EYFP} splenocytes were incubated for 5 days in culture conditions favoring Th17 differentiation or were TCR-stimulated in the absence of the necessary cytokine milieu. After induction of IL-17F expression, $CD4^+EYFP^+$ or $CD4^+EYFP^-$ cells were sorted and recultured in the presence of TGF-β for a further 3 days. Th17 cells were unable to up-regulate Foxp3 under these circumstances despite an up-regulation in $CD4^+EYFP^-$ cells derived from non-Th17-stimulated cultures (Fig. 3*C*).

The IL-17F-Cre^{EYFP} model also provides the ability to observe a redifferentiation in vivo. We therefore sorted Th17 ($CD90.2^+EYFP^+$) or non-Th17 ($CD90.2^+CD25^-EYFP^-$) cells from Th17-polarized IL-17F-Cre^{EYFP} splenocyte cultures and injected them separately into RAG1-deficient mice. Before injection, samples of the sorted donor T cells were stained for Foxp3 to exclude the presence of naturally occurring Tregs within the transferred cells (Fig. 3*D*). Two weeks later, the T cells were reisolated from the hosts and stained for Foxp3. In line with our in vitro findings, none of the transferred Th17

cells were capable of expressing Foxp3 after expansion (Fig. 3*E*). Thus, Th17 cells are resistant to acquiring a regulatory T cell phenotype in vitro and in vivo. The recent data of iTregs and natural Tregs converting to Th17 cells (8) indicate that Tregs may represent a dynamic population capable of deciding whether to switch from suppression to inflammation. Our data indicate that this switch is unidirectional.

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Disclosures

The authors have no financial conflict of interest.

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