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Myelin-Reactive, TGF-β–Induced Regulatory T Cells Can Be Programmed To Develop Th1-Like Effector Function but Remain Less Proinflammatory Than Myelin-Reactive Th1 Effectors and Can Suppress Pathogenic T Cell Clonal Expansion In Vivo

Richard A. O'Connor,[*,†,‡] Melanie D. Leech,[*,†,‡] Janine Suffner,[§] Günter J. Hämmerling,[§] and Stephen M. Anderton[*,†,‡]

Interest in the use of regulatory T cells (Tregs) as cellular therapeutics has been tempered by reports of naturally occurring Tregs losing Foxp3 expression and producing IL-17, raising concerns over a switch to pathogenic function under inflammatory conditions in vivo. TGF-β–induced Tregs (inducible Tregs [iTregs]), generated in large numbers in response to disease-relevant Ags, represent the most amenable source of therapeutic Tregs. Using Foxp3-reporter T cells recognizing myelin basic protein (MBP), we investigated the capacity of iTregs to produce effector-associated cytokines under proinflammatory cytokine conditions in vitro and whether this translated into proinflammatory function in vivo. In contrast with naturally occurring Tregs, iTregs resisted conversion to an IL-17–producing phenotype but were able to express T-bet and to produce IFN-γ. iTregs initiated their T-bet expression during their in vitro induction, and this was dependent on exposure to IFN-γ. IL-12 reignited iTreg expression of T-bet and further promoted iTreg production of IFN-γ upon secondary stimulation. Despite losing Foxp3 expression and expressing both T-bet and IFN-γ, MBP-responsive IL-12–conditioned iTregs induced only mild CNS inflammation and only when given in high numbers. Furthermore, iTregs retained an ability to suppress naive T cell clonal expansion in vivo and protected against the development of experimental autoimmune encephalomyelitis. Therefore, despite bearing predictive hallmarks of pathogenic effector function, previously Foxp3$^+$ iTregs have much lower proinflammatory potential than that of MBP-responsive Th1 cells. Our results demonstrate that autoprotective versus autoaggressive functions in iTregs are not simply a binary relationship to be determined by their relative expression of Foxp3 versus T-bet and IFN-γ. ***The Journal of Immunology*, 2010, 185: 7235–7243.**

Mosmann and Coffman's (1) division of Th cells into the Th1 and Th2 subsets gave a mechanistic basis (differential cytokine production) for protective immune responses against intracellular pathogens and extracellular parasites, respectively. Lineage-specific transcription factors (T-bet for Th1 and GATA-3 for Th2) gave a molecular basis to this division. The impetus toward further subdivision of effector function has increased recently. Th17 cells express the transcription factors retinoic acid receptor related orphan receptor (ROR)γT and RORα (2, 3) and produce IL-17A, IL-17F, and IL-22 (reviewed in Ref. 4). Each of the above effector T cell "lineages" can have immunopathological properties if directed against self or otherwise innocuous non-self Ags (5–7). Counteracting these pathological consequences of CD4$^+$ Th cells, thymically derived naturally occurring CD4$^+$ regulatory T cells (nTregs) expressing the Foxp3 transcription factor limit the activation and expansion of autoreactive T cells, thereby providing dominant peripheral tolerance (8). Continual expression of Foxp3 is required within regulatory T cells (Tregs) for maintenance of their suppressive phenotype (9–11).

With a view to the therapeutic application of Tregs, the characterization of culture conditions (TGF-β plus IL-2 plus TCR stimulation) that drive the de novo expression of Foxp3 in so-called inducible Tregs (iTregs) was a major advance, providing the opportunity to expand freshly produced Tregs from non-Treg precursors (12). Proof of principle for Treg-mediated therapy was provided by prevention and cure of autoimmune pathology by instillation of autoantigen-reactive Tregs in models of diabetes (13) and gastritis (14) and in experimental autoimmune encephalomyelitis (EAE) (15). However, concerns have been raised recently over the potential of Treg-based therapy because of

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The online version of this article contains supplemental material.

Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; iTreg, inducible regulatory T cell; MBP, myelin basic protein; nTreg, naturally occurring CD4$^+$ regulatory T cell; pMOG, $MOG_{35–55}$; ROR, retinoic acid receptor related orphan receptor; Treg, regulatory T cell.

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convincing demonstrations that IL-6 (16) and other proinflammatory cytokines associated with the Th17 developmental pathway (IL-23, IL-1β, and TGF-β) can provoke nTregs to lose Foxp3 expression and produce IL-17 (16–18). This transition requires STAT3, RORγT, and RORα activity (18). Cells coexpressing Foxp3 and RORγT have been identified in humans (19), and stimulation of human $Foxp3^+$ cells in the presence of IL-1β, IL-23, and IL-21 has also been shown to promote their production of IL-17 (20, 21). In light of these results, it is important to question whether $Foxp3^+$ cells can develop pathogenic activity. A recent study has reported that "ex-$Foxp3^{+}$" cells were capable of effector cytokine production and displayed pathogenic potential upon transfer to naive lymphopenic mice (22). An extrapolation from that study is that in vitro-generated human iTregs may have similar plasticity in function. Moreover, activated autoantigen-reactive Tregs are the most effective agents for Treg-based therapy in the mouse (13–15, 23). A recent study by Zhang et al. (24) provided a precise dissection of the role of antigenic reactivity in the suppression of EAE and presented two important observations: Ag-reactive iTregs can expand in vivo upon encounter with their cognate Ag, and iTreg-mediated suppression in vivo is Ag-specific in nature. These findings strengthen the case for the therapeutic use of Ag-reactive iTregs and the attendant need to investigate the phenotypic stability of these cells. The production of IL-6 at sites of autoimmune inflammation has been reported to influence negatively the suppressive capacity of Tregs (25). Furthermore it is known that IL-6, IL-12, IL-23, and TGF-β are expressed in the inflamed CNS during EAE (26–28) and that these cytokines profoundly influence the function of effector T cells (28, 29). Thus it is possible that Tregs at inflammatory sites are exposed to cytokines that are not only capable of overcoming their suppressive function but may also favor their transition toward a proinflammatory phenotype. Therapeutic use of autoantigen-reactive human iTregs could have profound consequences if this potential for conversion to effector function were to be realized in a patient. This risk provides an imperative to understand the functional plasticity of iTregs in vitro and how this might translate into undesirable activity in autoimmune disease in vivo.

In this study, we compared nTregs and iTregs in their responses to stimulation in the presence of proinflammatory cytokines. iTregs proved to be resistant to the induction of IL-17 production in vitro but readily deviated from Foxp3 expression toward a Th1-like phenotype, particularly in the presence of IL-12. We tested the consequence of this in the setting of EAE and found that reactivation in the presence of IL-12 provided $Foxp3^+$ iTregs with the capacity to home to the CNS and to initiate low-grade clinical pathology. However, these "IL-12–conditioned iTregs" also retained some suppressive capacity, the ability to suppress the proliferation of naive T cells. Collectively, these data show that although iTregs can gain effector function, their pathogenic potential in vivo is limited compared with that of primary Th1 effectors of the same specificity.

Materials and Methods

Mice, Ags, and tissue culture medium

C57BL/6 (either CD45.2 or CD45.1), Foxp3-GFP reporter mice (30), Tg4 mice (31) (either CD45.2, CD45.1, or CD90.1), B10.PL×C56BL/6 mice, and IFN-$\gamma^{-/-}$ mice were used. Foxp3.LuciDTR-4 mice (32) were crossed with CD45.1 Tg4 mice to obtain $CD45.1^+$ Tg4×Foxp3.LuciDTR-4 mice. All mice were bred under specific pathogen-free conditions at the University of Edinburgh (Edinburgh, U.K.). All experiments had local ethical approval and were performed in accordance with U.K. legislation. The myelin basic protein (MBP) Ac1-9 and $MOG_{35–55}$ (pMOG) peptides were obtained from Cambridge Research Biochemicals (Cleveland, U.K.). Tissue culture medium (RPMI 1640 medium) was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol (all from Invitrogen Life Technologies, Paisley, U.K.) and 10% FCS (Sigma, Poole, U.K.).

Active induction of EAE

B10.PL×C57BL6 mice received 1×10^6 Tg4 $CD4^+$ T cells with or without 1×10^6 Tg4 iTregs treated as indicated in the text. One day later, mice received 100 μg Ac1-9 peptide emulsified in CFA containing 50 μg heat-killed *Mycobacterium tuberculosis* H37Ra (Sigma-Aldrich, Poole, U.K.) at a final volume of 100 μl injected s.c. into the hind legs. On the day of immunization and 48 h later, each mouse also received 200 ng pertussis toxin (Health Protection Agency, Dorset, U.K.) in 0.5 ml PBS i.p. C57BL/6 mice were immunized as above, using 100 μg pMOG per mouse. Clinical signs of EAE were assessed daily with the following scoring system: 0, no signs; 1, flaccid tail; 2, impaired righting reflex and/or gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, hind limb paralysis with partial front limb paralysis; and 6, moribund or dead.

Cell culture and passive induction of EAE

For Th1 polarization, Tg4 splenocytes were cultured at 4×10^6 cells ml with 10 μg/ml MBP(Ac1-9), 25 ng/ml rIL-12, 0.5 ng/ml rIL-2 (both from R&D Systems, Minneapolis, MN), and 25 ng/ml rIL-18 (MBL, Nagoya, Japan) as described previously (33). Cells were harvested after 72-h culture and 3×10^6 blasts transferred i.v. Some mice also received IL-12–conditioned iTregs as indicated in the text. On the day of cell transfer, each mouse also received 200 ng pertussis toxin (Health Protection Agency, Dorset, U.K.) in 0.5 ml PBS i.p. Clinical signs of EAE were assessed as described earlier.

iTreg generation

$CD4^+$ T cells were purified using magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) prior to surface staining and sorting by FACS. GFP^- $CD4^+$ T cells were cultured for 5 d on anti-CD3 (clone 145.2C11; eBioscience, Hatfield, U.K.) plus anti-CD28 (clone 37.51; e-Bioscience) coated plates in the presence of 5 ng/ml TGF-β (R&D Systems) and 100 U/ml IL-2 at a density of 0.25×10^6 to 0.5×10^6/well in 24-well plates. After 5 d of culture, GFP-expressing cells were purified by FACS sorting. In some experiments, GFP^+ iTregs received secondary stimulation with anti-CD3 in the presence of IL-12 (25 ng/ml), IL-6 (30 ng/ml), IL-23 (30 ng/ml), IL-1β (10 ng/ml), TGF-β (2.5 ng/ml) (all R&D Systems) individually or in combination as described in the text. In some cases (as indicated), cultures were supplemented with anti–IFN-γ (clone XMG1.2; Bio X Cell, West Lebanon, NH) at 10 μg/ml. The level of cytokine production during culture was assessed by ELISA.

Preparation of CNS-infiltrating mononuclear cells

Mice were sacrificed by CO_2 asphyxiation, perfused with cold PBS, and mononuclear cells were prepared from brain and spinal cord as described previously (34).

Abs and FACS analysis

Cells were stained using the following Abs and isotype controls (all from eBioscience, except where stated): anti-CD4–allophycocyanin, anti-CD4–PE, anti-CD4–PerCP, anti-CD4–AF700 (BD Pharmingen, Oxford, U.K.) anti-CD45.1–(FITC/PE/PerCPCy5.5/allophycocyanin), anti-CD25–(FITC/PE) (clone 7D4; Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD25–(PE/allophycocyanin) (clone PC61; BD Pharmingen), anti-CD90.1–(PE/allophycocyanin), anti-CD62L–FITC, anti–IFN-γ–FITC, anti–IL-17–PE, anti–IL-17–allophycocyanin (Biolegend, San Diego, CA), anti-GITR–allophycocyanin, anti-CTLA4–PE, anti–T-bet–PerCPCy5.5 (clone ebio410), anti–Mac-1–efluor 450, anti-Foxp3–efluor 450, rat IgG1–(FITC/PE/allophycocyanin/PerCPCy5.5), and rat IgG2b–PE. FACS data were collected on FACSCalibur, FACSCanto, LSR-II, or Fortessa flow cytometers (BD Biosciences, Franklin Lakes, NJ). FACS sorting was performed using Aria/Aria II cell sorters (BD Biosciences), and all data were analyzed using FlowJo software (Tree Star, Ashland, OR).

For intracellular staining in response to peptide, cells were resuspended at 1×10^7/ml in the presence or absence of 20 μg/ml Ac1-9 or pMOG. After overnight culture, 1 μl/ml brefeldin A (eBioscience; 1000× stock) was added for the last 4 h of culture. For staining at the end of polarizing culture, 50 ng/ml PMA, 50 ng/ml ionomycin, and 1 μl/ml brefeldin A was added for the last 4 h of culture. Cells were washed once in FACS buffer (PBS, 2% FCS, 0.01% NaN_3) and surface stained prior to processing for Foxp3 staining using proprietary buffers according to the manufacturer's instructions (eBioscience). After overnight incubation in Foxp3 fix/perm buffers, cells were stained for intracellular Ags. Due to loss of GFP activity as a result of fixation, cells from Foxp3.LuciDTR-4 mice were stained with anti-Foxp3.

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In vitro proliferation and suppression assays

Freshly isolated CD4$^+$CD25$^-$ cells or various Treg populations were cultured at 2×10^4 per well for 96 h with 1×10^5 irradiated (30 Gy) splenic APCs and 1 μg/ml anti-CD3 (clone 145.2C11; eBioscience). [^{3}H]Thymidine deoxyribose (0.5 μCi; Amersham Biosciences, Amersham, U.K.) was added for the final 18 h of culture, and incorporation was measured using a liquid scintillation β-counter (Wallac, Turku, Finland). Mean cpm of triplicate cultures are shown in Fig. 5*A*.

For suppression assays, 2×10^5 CFSE-labeled CD45.1$^+$CD4$^+$ responder cells were cocultured at a 1:1 ratio with CD45.2 IL-12–conditioned iTregs or with control iTregs that had received an identical secondary stimulation but without inclusion of IL-12, 1 μg/ml anti-CD3, and irradiated APCs. Cultures were plated in triplicate in 48-well plates, and CFSE dilution in the CD45.1$^+$ responder cells was measured after 72-h culture.

Statistical analysis

Statistical analysis of results was performed using the Mann–Whitney *U* test, the two-tailed Student *t* test, and one-way ANOVA with Bonferroni's multiple comparison test.

Results

nTregs produce IL-17, whereas iTregs produce IFN-γ upon secondary stimulation

There are conflicting reports concerning the capacity of iTregs to produce IL-17 (16–18). To provide populations of Ag-reactive Tregs, Tg4 mice (31) that express a transgenic TCR recognizing the Ac1-9 peptide of MBP in association with A^u were crossed with Foxp3.LuciDTR-4 mice (BAC transgenics expressing eGFP under the Foxp3 promoter) (32), thereby producing Tg4×Foxp3. LuciDTR-4 mice. To compare directly the ability of nTregs and iTregs to produce IL-17, we generated highly purified populations of Foxp3$^+$ iTregs and freshly isolated nTregs and stimulated them in the presence of a cytokine mixture reported to give optimal induction of IL-17 in nTregs (18) (see schematic Fig. 1*A*). As anticipated, these conditions provoked approximately one in three nTregs (sorted CD4$^+$CD62L^{hi}GFP$^+$ cells from Tg4×Foxp3. LuciDTR-4 mice) to produce IL-17. Of these IL-17 producers, around two thirds retained Foxp3 expression (Fig. 1*B*, *left panels*). To our surprise, although exposure to Th17-inducing conditions led to loss of Foxp3 expression in around 75% of iTregs, this did not allow them to produce IL-17. Instead, there was a clear population producing IFN-γ, of which approximately two thirds were Foxp3$^-$ (Fig. 1*B*, *right panels*). Thus, there was a striking difference in how nTregs and iTregs responded to pro-Th17 conditions; nTregs produced IL-17, but not IFN-γ, whereas iTregs produced IFN-γ, but not IL-17.

The frequency of GFP$^+$ cells after the initial iTreg sort was routinely >97% and in some experiments >99% (see Supplemental Fig. 1). Furthermore, "spiking" experiments, in which known frequencies of traceable GFP$^-$ cells (sorted from parallel cultures under iTreg conditions) were added to sorted GFP$^+$ cells, confirmed that the ultimate GFP$^-$ population was derived chiefly from conversion of GFP$^+$ cells, rather than an outgrowth contaminating GFP$^-$ cells remaining postsort (Supplemental Fig. 1).

Having uncovered the ability of iTregs to produce IFN-γ under Th17 conditions, we next tested whether this ability could be magnified under alternative in vitro cytokine conditions. iTregs (sorted for Foxp3 expression after the initial iTreg culture) produced IFN-γ upon secondary TCR stimulation, even in the absence of exogenous cytokines. The cytokines composing the Th17 mixture did not markedly increase the proportion of IFN-γ–producing cells when used singly or in combination (Fig. 1*C*). However, IL-12 induced a pronounced increase in the frequency of IFN-γ–producing cells (Fig. 1*C*). IL-17 production among

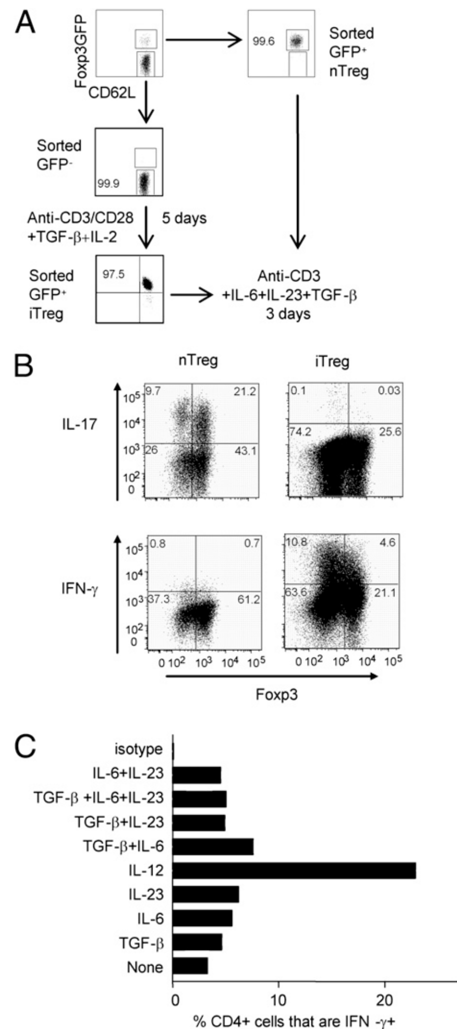

FIGURE 1. nTregs and iTregs respond differently to stimulation in the presence of proinflammatory cytokines. *A*, Schematic outlining the preparation of Foxp3$^+$ nTreg and iTreg populations. *B*, Expression of Foxp3, IL-17, and IFN-γ by nTregs (*left*) and iTregs (*right*) after stimulation with plate bound anti-CD3/anti-CD28 in the presence of IL-6, IL-23, and TGF-β. *C*, The percentage of iTregs producing IFN-γ after 72-h stimulation in the presence of the indicated cytokines. The proportion of cells producing IFN-γ in the presence of IL-6, IL-23, and TGF-β varied between experiments from 5.5 to 25% giving a mean of 15.4 ± 6.6%. Data are from one of three experiments giving consistent results.

iTregs was not seen in any of the conditions tested (data not shown). The majority of cells lost expression of Foxp3 under all conditions tested, even when stimulated in the presence of TGF-β alone (data not shown). Given that it is present in many tissues undergoing autoimmune inflammation, the ability of IL-12 to trigger production of IFN-γ in iTregs is highly pertinent to their role in modulating inflammation in vivo.

Endogenous production of IFN-γ during in vitro induction of iTregs

The ability to produce IFN-γ in response to IL-12 is governed by signals from both the TCR and the IFN-γ receptor, which initiate/stabilize T-bet expression. Our iTreg conditions provided a TCR stimulus (anti-CD3) but no additional IFN-γ. To test whether there was endogenous IFN-γ in iTreg cultures, we sorted Foxp3(GFP)$^-$ cells from naive mice, exposed them to iTreg conditions, and performed kinetic analyses for the expression of Foxp3, IFN-γ, and T-bet (Figs. 2, 3). A significant Foxp3$^+$ fraction could be seen

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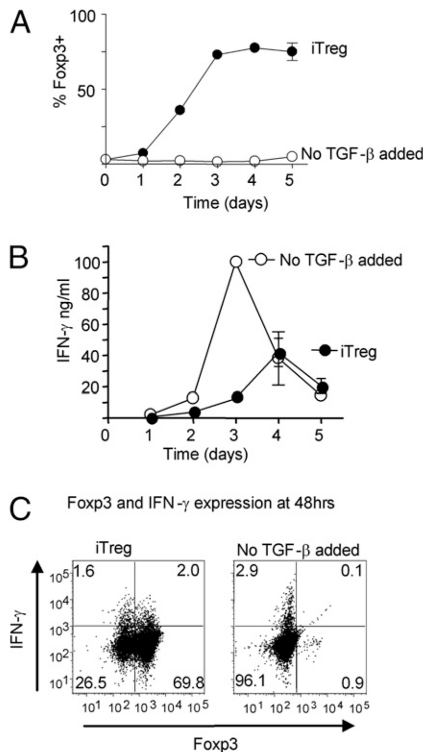

FIGURE 2. IFN-γ production is induced in Foxp3⁻ and Foxp3⁺ cells during the generation of iTregs. Sorted $CD4^{+}GFP^{-}$ cells were cultured with anti-CD3/anti-CD28 under iTreg conditions or without addition of TGF-β (No TGF-β added). *A*, Expression of Foxp3 over time. *B*, Production of IFN-γ over time as determined by ELISA. *C*, Intracellular staining for Foxp3 and production of IFN-γ in cells sampled after 48 h of culture. Data are from one of two experiments giving consistent results.

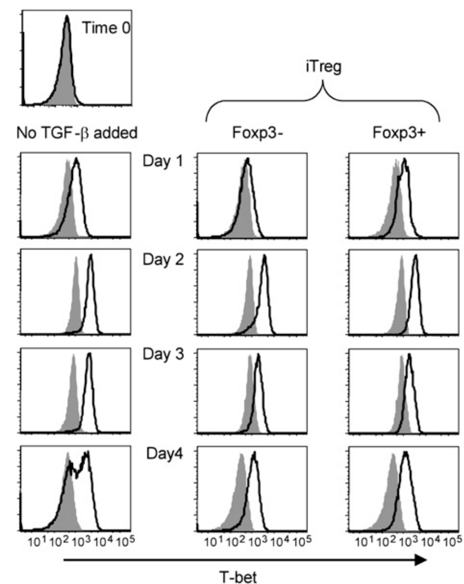

FIGURE 3. T-bet expression is induced in $Foxp3^{-}$ and $Foxp3^{+}$ cells during the generation of iTregs. Cultures were established as described for Fig. 2. Expression of T-bet (open histogram) over isotype control (gray histogram) prior to in vitro stimulation (*top left panel*) and over time in non-iTreg conditions (*left panels*) or under iTreg conditions gating on $Foxp3^{-}$ cells (*middle panels*) and $Foxp3^{+}$ cells (*right panels*). Data are representative of two experiments giving consistent results.

after 48 h, and this leveled at around 75% from 72 h onwards (Fig. 2*A*). Detectable IFN-γ appeared in iTreg supernatants from 72 h (Fig. 2*B*). Intracellular staining for Foxp3 and IFN-γ revealed that by 48 h, IFN-γ–producing cells were apparent under iTreg conditions, and, of these, about half were $Foxp3^{+}$ (Fig. 2*C*). Notably, when cells were stimulated under neutral conditions, IFN-γ was produced by $Foxp3^{-}$ cells only (Fig. 2*C*). Thus, there was a clear source of IFN-γ in the iTreg cultures and at least some of this was produced by cells that had already initiated Foxp3 expression.

T-bet expression in $Foxp3^{-}$ and $Foxp3^{+}$ cells during the generation of iTregs

Expression of the Th1-associated transcription factor T-bet has been identified in a minor population of nTregs (35), and epigenetic modifications of *Tbx21* have identified it as a gene poised for expression in nTregs (36). Having found $Foxp3^{+}$ iTregs to be responsive to IL-12 and capable of IFN-γ production, we questioned whether iTregs also expressed T-bet. We sorted $Foxp3^{-}(GFP^{-})$ $CD4^{+}$ T cells from naive mice and monitored T-bet expression over time during stimulation under iTreg-inducing conditions. Prior to stimulation, there was no evidence of T-bet expression in the starting population (Fig. 3, *top left panel*). Expression of T-bet was increased after 24 h in the absence of TGF-β (Fig. 3, *left panels*) and more markedly by 48 h. There was also an increase in T-bet expression under iTreg conditions that, although less marked at 24 h, was clearly upregulated by 48 h and maintained at 72 h (Fig. 3). It was notable that the increase in T-bet expression occurred to a similar extent in both $Foxp3^{-}$ and $Foxp3^{+}$ cells.

iTreg expression of T-bet is IFN-γ dependent

Having identified an endogenous source of IFN-γ in iTreg cultures, we tested whether this was required for T-bet expression by iTregs and/or their subsequent ability to produce IFN-γ upon restimulation. During iTreg generation, cells from $IFN\text{-}\gamma^{-/-}$ mice did not express T-bet (Fig. 4*A*). Moreover, whereas wild-type iTregs showed a marked ability to upregulate T-bet upon secondary stimulation in the presence of IL-12 (i.e., IL-12–conditioned iTregs), this was disabled in $IFN\text{-}\gamma^{-/-}$ iTregs (Fig. 4*B*). Ab-mediated neutralization of IFN-γ during initial iTreg cultures led to reduced T-bet expression in wild-type cells (Fig. 4*C*). Notably, anti–IFN-γ also increased the frequency of $Foxp3^{+}$ cells achieved in the initial iTreg cultures (Fig. 4*D*), an enhancement also seen when using $IFN\text{-}\gamma^{-/-}$ cells (data not shown), and increased production of IL-17 (Fig. 4*E*). Finally, when $CD25^{-}CD4^{+}$ T cells from CD45.2 $IFN\text{-}\gamma^{-/-}$ mice were cultured together with CD45.1 IFN-γ–sufficient cells under iTreg conditions, both populations were able to express T-bet (Fig. 4*F*). We therefore can conclude that exposure to IFN-γ during iTreg culture is a prerequisite for T-bet expression and subsequent sensitivity to IL-12–mediated conversion to an IFN-γ–producing phenotype.

IL-12–conditioned iTregs lose their anergic phenotype but still suppress naive T cell expansion in vitro and in vivo

A large proportion of $Foxp3^{+}$ iTregs lose expression of Foxp3 on secondary stimulation in vitro (Fig. 1*B*). The continued expression of functional Foxp3 is necessary to maintain suppressive function and to prevent adoption of effector cytokine production in nTregs (11). With this in mind, we sorted $Foxp3^{+}$ cells from primary iTreg cultures, exposed them to secondary TCR stimulation in the presence of IL-12 (IL-12–conditioned iTregs), and assessed their suppressive capacity. IL-12–conditioned iTregs differed from nTregs or iTregs in that they lost their anergic phenotype when given a tertiary stimulation with anti-CD3 (Fig. 5*A*). However, they behaved similarly to iTregs that had been maintained in

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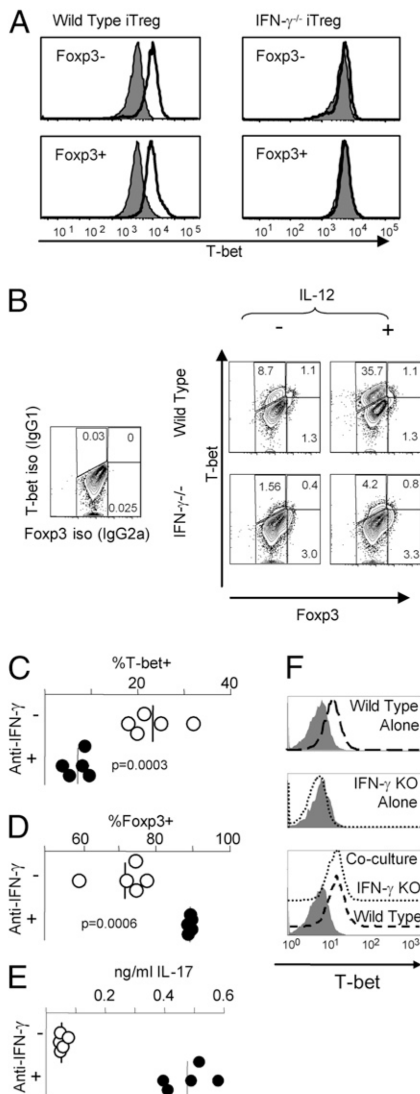

FIGURE 4. IFN-γ induces T-bet expression and responsiveness to IL-12 in iTregs. *A*, Expression of T-bet (open histogram) over isotype control (gray histogram) among Foxp3$^-$ and Foxp3$^+$ cells from wild-type (*left*) and IFN-$\gamma^{-/-}$ (*right*) mice cultured under initial iTreg conditions (sampled after 5 d). *B*, iTregs from wild-type and IFN-$\gamma^{-/-}$ mice then received secondary stimulation with anti-CD3 in the presence or absence of IL-12. Expression of T-bet in wild-type (*upper panels*) and IFN-$\gamma^{-/-}$ (*lower panels*) iTregs 72 h after secondary stimulation is shown. The level of background staining using appropriate isotype controls for T-bet and Foxp3 is shown in the *left-hand panel*. *C–E*, iTregs were generated from wild-type mice by initial culture under iTreg conditions in the presence or absence of anti–IFN-γ (clone XMG1.2). Frequency of cells expressing T-bet (*C*) and Foxp3 (*D*) and the presence of IL-17 in culture supernatants (*E*) was assessed after 72 h. *F*, iTregs were generated by initial culture under iTreg conditions of CD25$^-$CD4$^+$ T cells from wild-type (CD45.1) and IFN-$\gamma^{-/-}$ (CD45.2) mice either alone (*upper panels*) or in coculture at a 1:1 ratio (*bottom panel*). T-bet expression was assessed after 72 h. Data are from one of three experiments giving consistent results.

medium without IL-12 in their ability to inhibit the proliferation of naive CFSE-loaded T cells upon in vitro coculture (Fig. 5*B*). IL-12–conditioned iTregs maintained expression of CTLA4 and GITR, although at lower levels than those of iTregs (data not shown).

We next assessed the capacity of IL-12–conditioned iTregs to limit the Ag-driven expansion of CD4$^+$ T cells in vivo. First, we established that primary iTregs showed suppressive activity

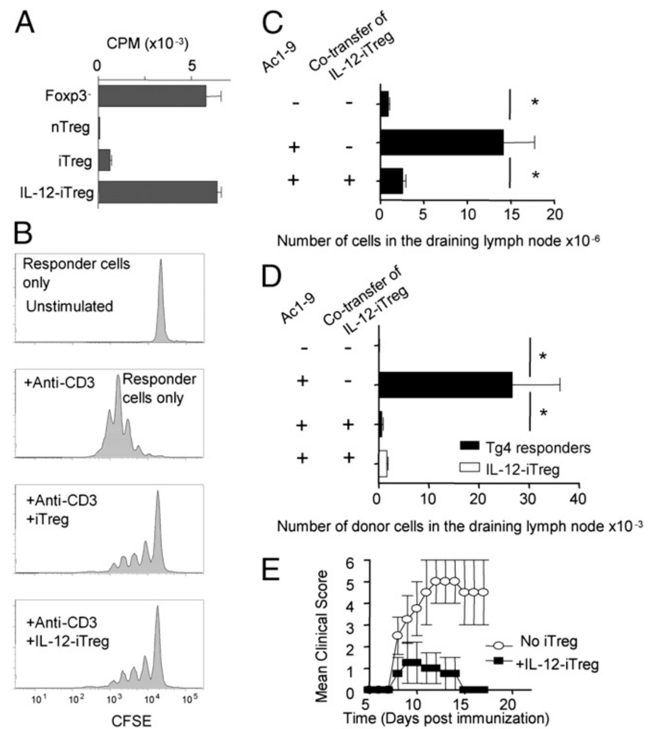

FIGURE 5. IL-12–conditioned iTregs suppress the proliferation of naive responder cells in vitro and in vivo. *A*, Proliferation of freshly isolated Foxp3$^-$ cells, freshly isolated nTregs, iTregs, and IL-12–conditioned iTregs (IL-12-iTreg) in response to anti-CD3. *B*, GFP$^+$ iTregs received secondary stimulation for 72 h with anti-CD3 in the absence (iTreg) or presence (IL-12-iTreg) of IL-12. These cells were then cultured for 72 h at a 1:1 ratio with freshly isolated, CFSE-labeled CD45.1$^+$CD4$^+$ CD25$^-$ responder cells and irradiated APCs, with or without anti-CD3. *C–E*, Naive CD90.1$^+$CD45.1$^-$ Tg4 T cells (1×10^6) were transferred to B10.PL×C57BL/6 hosts either alone or together with 1×10^6 CD90.1$^-$ CD45.1$^+$ Tg4 IL-12–conditioned iTregs 1 d prior to immunization with Ac1-9 for the induction of EAE. *C*, Cellularity of the draining lymph nodes 6 d postimmunization. *D*, Number of CD90.1$^+$Tg4 T cells (solid bars) and CD45.1$^+$Tg4 IL-12–conditioned iTregs (open bar) recovered from the draining lymph nodes 6 d after immunization. *E*, Clinical course of EAE ($n = 4$ per group). Data are from one of two experiments giving consistent results. $^*p \leq 0.05$ as determined by ANOVA.

in vivo. We transferred traceable (CD90.1$^+$) MBP-reactive Tg4 T cells (representing our "responder" population) into B10. PL×C57BL/6 hosts, either alone or in the presence of primary Tg4 iTregs (generated from Tg4×Foxp3.LuciDTR-4 mice) prior to immunization with the MBP Ac1-9 peptide in CFA. This is an informative model system because the host mice only develop EAE in response to immunization with the Ac1-9 peptide if they have first received a cohort of naive Tg4 T cells (37). This means that the Tg4 cells are necessary for the initiation of the CNS inflammation and that analysis of their fate is highly pertinent to disease outcome. The co-transfer of iTregs reduced the clinical severity of disease, and this correlated with the presence of fewer MBP-reactive CD90.1$^+$ responder cells in both the spleen and the CNS (Supplemental Fig. 2). These data are consistent with the reported ability of iTregs to limit the clonal expansion of naive T cells in other experimental models of pathology (14, 38).

Using the same model system, we found that co-transfer of CD45.1$^+$ IL-12–conditioned iTregs with CD90.1$^+$ "responder" Tg4 T cells significantly inhibited both the increase in cellularity of the draining lymph nodes (Fig. 5*C*) and the expansion of the responder Tg4 cells seen upon immunization (Fig. 5*D*). Only very low numbers of IL-12–conditioned iTregs could be recovered

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from the draining lymph node (Fig. 5D) suggesting competition for Ag does not account for the observed suppression. The ability to limit the expansion of MBP-reactive responder cells was reflected in the reduced incidence and severity of EAE seen in recipients of IL-12–conditioned iTregs (Fig. 5E). Thus, despite only 2% of their number being Foxp3$^+$ at the time of transfer (data not shown), IL-12–conditioned iTregs can suppress the in vivo expansion of autoreactive T cells and thereby limit the development of pathology.

IL-12–conditioned iTregs display limited pathogenic potential and do not prevent passive transfer of EAE

Having observed IL-12–conditioned iTreg expression of T-bet and capacity for IFN-γ production, we sought to determine whether IL-12–conditioned iTregs displayed any pathogenic potential themselves. We used a well-characterized model of passive EAE induction whereby disease is initiated by the transfer of activated Tg4 T cells (33). This allowed us to assess both the pathogenic potential of our MBP-reactive Tg4 IL-12–conditioned iTregs and their ability to influence the pathogenicity of preformed effector T cells.

Th1 polarized Tg4 cells readily induced disease when transferred in numbers as low as 1 million (Fig. 6A). In contrast, transfer of 3 million IL-12–conditioned iTregs did not result in any overt pathology (Fig. 6A). Transfer of more than 4 million Th1 polarized cells leads to a high mortality rate in this model, precluding titration to higher numbers of Th1 cells. When we transferred higher numbers of IL-12–conditioned iTregs (6 million), recipient mice developed clinical signs of EAE, but disease was slower in onset, milder in severity, and shorter in duration than that seen with lower numbers of bona fide Tg4 effectors (Fig. 6B). IL-12–conditioned iTregs were enriched within the CNS, lacked Foxp3 expression (Fig. 6C), and could produce IFN-γ in response to peptide restimulation ex vivo (Fig. 6D). Thus, despite having a similar capacity for IFN-γ production as primary Th1 cells at the time of their infusion (Supplemental Fig. 3), IL-12–conditioned iTregs have limited pathogenic potential in this model. This could not be accounted for by a protective effect of iTreg-derived IL-10 because we could not detect any IL-10 production by these cells as measured by ELISA (data not shown).

To determine whether IL-12–conditioned iTregs influenced the development of pathology initiated by Th1 effector cells, host mice were given Tg4 Th1 effectors either alone or together with an equal number of either IL-12–conditioned Tg4 iTregs or Tg4 iTregs that had not been conditioned further with IL-12. No significant differences between these groups were seen in incidence or severity of disease (Fig. 6E). The relative lack of pathogenic potential in IL-12–conditioned iTregs is further illustrated by the fact they did not exacerbate disease in co-transferred mice. Tg4 Th1 cells that received secondary in vitro stimulation in the presence of IL-12 maintained their encephalitogenic function in vivo (Supplemental Fig. 4). This confirmed that the limited pathology seen with IL-12–conditioned iTregs was not simply a function of their exhaustion imposed by repeated triggering of their TCR in vitro. Notably, co-transfer of Th1 cells significantly increased the frequency of IL-12–conditioned iTregs recovered from the CNS suggesting that they are actively recruited to sites of Th1 inflammation (Supplemental Fig. 5).

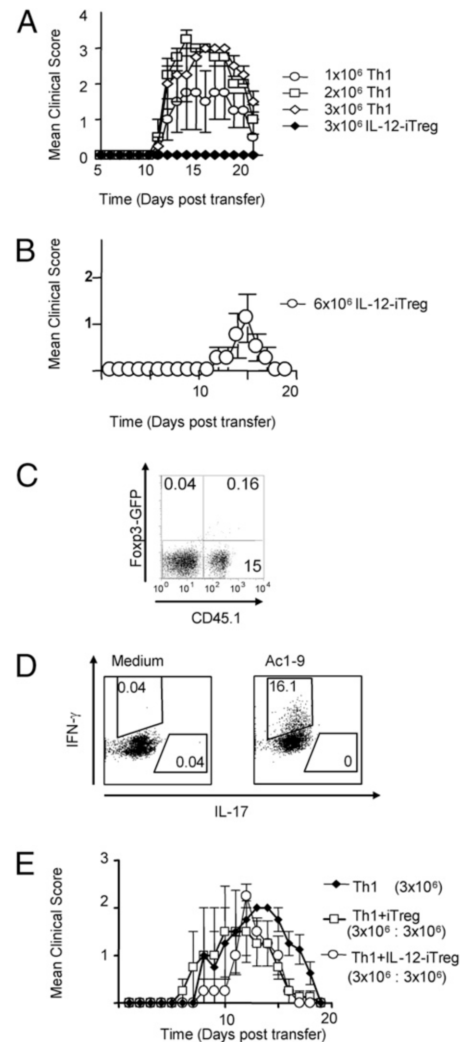

FIGURE 6. IL-12–conditioned iTregs display modest pathogenic potential and are recruited to the site of type 1 inflammation. *A*, Clinical course of EAE in mice receiving graded numbers of Th1 polarized Tg4 cells (as indicated [open symbols]) or IL-12–conditioned Tg4 iTregs (3×10^6 per mouse [solid symbols]). *B*, Clinical course of EAE in mice given 6×10^6 IL-12–conditioned Tg4 iTregs. *C*, Foxp3 (GFP) status of CD45.1$^+$ IL-12–conditioned Tg4 iTregs recovered from the CNS. *D*, Production of IFN-γ and IL-17 by IL-12–conditioned iTregs (gated on CD45.1$^+$ cells) recovered from the CNS in response to overnight stimulation in the presence (*right*) or absence (*left*) of Ac1-9. *E*, Clinical course of EAE in mice receiving Tg4 Th1 effectors alone (solid symbols) or in co-transfer with primary Tg4-iTreg (open squares) or IL-12–conditioned Tg4 iTregs (open circles), each at 3×10^6 per mouse. $n = 4$ to 6 mice per group. Data are from one of two experiments giving consistent results.

Discussion

We describe, to our knowledge, a novel functional division between nTregs and iTregs in their responses to proinflammatory cytokines. nTregs display a greater propensity for IL-17 production than that of iTregs, which are more predisposed to IFN-γ production in vitro. IFN-γ released during the in vitro generation of iTregs induces their coexpression of T-bet and Foxp3 and enhances their sensitivity to IL-12. IL-12 in turn heightens T-bet expression and induces high levels of IFN-γ production in previously Foxp3$^+$ cells. The gain of Th1-like effector function is accompanied by effector-like homing properties and sufficient proinflammatory activity to produce mild clinical CNS pathology. This previously unappreciated role for IFN-γ in conditioning the responsiveness of iTregs to cytokine stimulation highlights potential dangers in the therapeutic application of autoreactive iTregs.

The potential for Foxp3$^+$ nTregs to gain a capacity for IL-17 production after stimulation in the presence of inflammatory

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cytokines (principally IL-6, IL-23, and IL-1β) has been identified in murine (16–18) and human (20, 21) T cells. Although most studies of this transition have been carried out in vitro, cells with the potential to coexpress Foxp3 and IL-17 have been identified in mice in the lamina propria (39) and in human peripheral blood (19). The capacity of Foxp3$^+$ cells (key players in peripheral tolerance) to produce IL-17 presents an uncomfortable dichotomy in terms of functional classification. However, demonstrable effects of this change in terms of pathology have remained elusive.

The most successful experimental applications of Treg-based therapies have used Ag-reactive Tregs (13–15, 23, 24), and the most expedient means of obtaining such cells, in significant numbers, is via the TGF-β–mediated induction of Foxp3 expression in vitro (40). iTregs have shown their therapeutic potential in models of organ-specific autoimmune disease (14, 24, 41) and in preventing the development of pathology in scurfy mice (42). However, there is conflicting literature regarding the capacity of iTregs for IL-17 production. An initial report declared iTregs resistant to conversion (17), but a subsequent paper provided evidence that both nTregs and iTregs could be converted to “Th17” cells (18). We show here that iTregs are indeed resistant to conversion toward IL-17 production, with the previously unappreciated role of IFN-γ accounting for the above contradiction. Yang et al. (18) (unusually) included a neutralizing Ab to IFN-γ in their primary iTreg conditions, whereas Zheng et al. (17) used only TGF-β and IL-2. Without exposure to IFN-γ, iTregs fail to express T-bet during their in vitro generation and subsequently are able to produce IL-17 under pro-Th17 cytokine conditions, whereas initial exposure to IFN-γ promotes resistance to this transition. This unexpected conditioning of iTregs illustrates how differential responsiveness can be engendered in iTregs during their generation and offers a means of subtly programming responsiveness to future stimuli.

The pathogenic potential of Tregs is a key question, given that their continued expression of Foxp3 seems necessary to maintain full suppressive function (11, 43). Reduced or ablated Foxp3 expression can lead to Treg function shifting toward a Th2 or a Th1 profile, respectively (11, 43), rather than Th17. When Tregs with a deletion of Foxp3 were transferred to lymphopenic hosts, they proliferated and were found among tissue-infiltrating cells suggesting an involvement in pathology (11). Serial transfer experiments have shown that Foxp3$^+$ cells transferred to lymphopenic hosts lose Foxp3 expression and on secondary transfer (again into lymphopenic hosts) promote tissue infiltration and damage (44).

The question of whether pathology seen in the absence of “normal” Treg function is due to unhindered responsiveness of T cells that have never expressed Foxp3 or to the “would be” or “had been” Tregs themselves is not simple. An elegant investigation into this division used *scurfy* mice crossed with DEREG mice (which express GFP under the control of an additional Foxp3 promoter). These mice allowed the effector function and pathogenic potential of those cells that would normally have been Foxp3$^+$, but lacked functional Foxp3, to be assessed. Transfer of these “would be” Tregs to RAG1$^{-/-}$ mice did not transfer the scurfy phenotype, whereas transfer of conventional GFP$^-$ T cells did, indicating the pathogenic potential lies in the effector cells, not the defective Treg compartment (45). The strongest evidence that cells that have previously expressed Foxp3 can go on to gain some pathogenic activity comes from experiments using mice in which cells that have expressed Foxp3 at any time express YFP, and those cells currently expressing Foxp3 express GFP. Thus, previously Foxp3$^+$ and currently Foxp3$^-$ cells display a YFP$^+$ GFP$^-$ phenotype. When such ex-Foxp3$^+$ cells with specificity for a pancreatic islet autoantigen were expanded in vitro and transferred to NOD *Rag2*$^{-/-}$ mice, the recipients developed rapid and severe diabetes (22).

It is surely important to note that all of the above examples suggesting pathogenic activity among Treg populations have used transfer into lymphopenic hosts. Extrapolation from pathology observed in such unfettered lymphopenic environments to what might occur upon transfer to hosts with normal immune complements is difficult. To our knowledge, our study is the first to report pathogenic activity after transfer of former Foxp3$^+$ cells into lympho-replete hosts.

To date, there is no direct evidence for IL-17 production by Tregs having a detrimental function in vivo. Our approach assessed the function of iTregs of known provenance; iTregs that previously expressed Foxp3 gave rise to a population of cells expressing T-bet and IFN-γ, and these cells were able to provoke CNS inflammation. We found no evidence of IL-17 production by IL-12–conditioned iTregs prior to transfer or upon recovery from the CNS; however, this does not exclude the possibility that these cells may have produced IL-17 at some point posttransfer. Although endowed with Th1-like characteristics, our IL-12–conditioned iTregs were (on a per cell basis) less pathogenic than Th1 cells of the same specificity. The transfer of 6 million Th1 cells results in fatal disease (data not shown), whereas this number of IL-12–conditioned iTregs produced only mild pathology. However, though less pathogenic than bona fide effector cells, this remains, to our knowledge, the first evidence of previously Foxp3$^+$ cells inducing inflammation in an immunologically intact host.

Conditions favoring T-bet expression and IFN-γ production by Tregs can be generated by infection in vivo, and coexpression of Foxp3 and T-bet has also been associated with negative consequences during lethal infection with *Toxoplasma gondii* (46). However, there is a profound decrease in Treg numbers during this infection. In the face of an overwhelming Th1 response, it is therefore impossible to say whether IFN-γ production by the low numbers of remaining Tregs definitively contributes at all to pathology in that model (46). We found that MBP-reactive, previously Foxp3$^+$ cells (IL-12–conditioned iTregs) were capable of promoting inflammatory pathology in vivo in the absence of any additional “help” from T effectors. Given that IL-12–conditioned Tregs also retained suppressive activity (upon naive T cells in vivo) and had comparatively low virulence on transfer, we conclude that despite T-bet expression and IFN-γ production, they are not equivalent to primary Th1 effectors. It is notable that when co-transferred with pathogenic Th1 cells, IL-12–conditioned iTregs were recovered from the inflamed CNS in greater numbers than when they were transferred alone. In this setting, they neither attenuated nor exacerbated disease severity. Thus, unlike freshly isolated nTregs, iTregs have a capacity to target the CNS (41, 47), and we have shown that IL-12–conditioned iTregs retain this ability. The fact that CNS recruitment of IL-12–conditioned iTregs was enhanced by the co-transfer of pathogenic Th1 cells suggests that iTregs can show an inflammation-seeking phenotype, a desirable characteristic in cells aimed for therapeutic application.

Tbx21 expression shows a broad spectrum of epigenetic states suggesting complex regulation, and epigenetic modifications associated with gene activation and gene repression colocalize in the promoter region of *Tbx21* in Tregs (36). Such colocalization is thought to mark genes that are “poised” for expression, and nTregs can be induced to express T-bet and produce IFN-γ upon stimulation in the presence of IL-12. This state of readiness to express T-bet may be required to allow regulatory responses to be tuned appropriately to effector responses, and it is interesting to note that similar modifications were found in both *Rorc* and *Gata3* (36). Whether exposure to proinflammatory cytokines during an

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immune response influences the stability of Tregs via epigenetic modification is an important area of future study.

Increasingly, promiscuity in the expression of "subset specific" transcription factors in T cells is becoming appreciated (48). In this study, exposure to IFN-γ endowed iTregs with certain Th1-like characteristics (expression of T-bet and elevated responsiveness to IL-12). The idea that Tregs can "co-opt" elements of effector phenotype, perhaps via the empathetic expression of chemokine receptors, seems an efficient way to tune regulatory responses to the effector responses they aim to control. Of direct relevance to our study, coexpression of T-bet in a subset of $Foxp3^+$ cells is reported to promote efficient control of type 1 inflammation through their expression of CXCR3 (35). Thus, coexpression of Foxp3 and effector-associated transcription factors (perhaps in response to low levels of effector cytokines) can be beneficial in terms of regulation. As techniques allowing the expression history of genes of interest to be visualized become available, our understanding of the dynamics of coexpression should progress rapidly.

In conclusion, we provide, to our knowledge, the first evidence of previously $Foxp3^+$ cells eliciting inflammatory pathology in immunologically intact hosts. At face value, this strikes a cautionary note for the therapeutic development of iTregs. However, despite production of IFN-γ, previously $Foxp3^+$ cells were only weakly pathogenic compared with primary Th1 cells, they retained some suppressive function, and they showed an inflammation-seeking phenotype. Although our current markers of "regulatory" and "effector" cells are clearly showing their limitations, further clarity on the mechanistic basis for T cell subset plasticity will allow better prediction of in vivo outcome and new means to control the stability of Treg function therapeutically.

Disclosures

The authors have no financial conflicts of interest.

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