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Identification of T helper type 1–like, Foxp3+ regulatory T cells in human autoimmune disease

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Abstract

$CD4^+CD25^{high}CD127^{low/-}$ forkhead box p3 $(Foxp3)^+$ regulatory T cells (T_{reg} cells) possess functional plasticity. Here we describe a higher frequency of T helper type 1 (T_H1)-like, interferon-γ (IFN-γ)-secreting $Foxp3^+$ T cells in untreated subjects with relapsing remitting multiple sclerosis (RRMS) as compared to healthy control individuals. In subjects treated with IFN-β, the frequency of IFN-γ^+Foxp3^+ T cells is similar to that in healthy control subjects. *In vitro*, human T_{reg} cells from healthy subjects acquire a T_H1-like phenotype when cultured in the presence of interleukin-12 (IL-12). T_H1-like T_{reg} cells show reduced suppressive activity *in vitro*, which can partially be reversed by IFN-γ–specific antibodies or by removal of IL-12.

Multiple sclerosis is a genetically mediated chronic inflammatory disease of the central nervous system (CNS). Activated $CD4^+$ inflammatory cells in the circulation of affected individuals infiltrate into the CNS and damage both myelin and axons[1,2]. A general loss of immune regulation is commonly seen in human autoimmune diseases[3–6].

In light of recent findings suggesting that $Foxp3^+$ T_{reg} cells show functional and phenotypic plasticity and are capable of secreting proinflammatory cytokines[7–9], we were interested in analyzing *ex vivo* secretion of cytokines by T_{reg} cells from individuals with relapsing/remitting multiple sclerosis (RRMS) that had not been receiving any immunomodulatory treatment as compared to age-matched healthy controls (Supplementary Methods and **Supplementary Table 1**). We stimulated FACS-sorted $CD4^+CD45RA^-CD25^{high}CD127^{low/-}$ T_{reg} cells (Supplementary Fig. 1a) for 4 h with phorbol 12-myristate 13-acetate (PMA) and ionomycin. The percentage of T_{reg} cells producing IFN-γ was significantly higher in untreated individuals with RRMS as compared to healthy control individuals (Fig. 1a,b). The frequency of T_{reg} cells producing IL-17 did not differ between individuals with RRMS and control subjects. Analysis of the $Foxp3^+$ T_{reg} cell–specific demethylated region (TSDR) in sorted IFN-γ^+Foxp3^+ and IFN-γ^-Foxp3^+ multiple sclerosis T_{reg} cells revealed that IFN-γ–producing $Foxp3^+$ T_{reg} cells possessed a

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AUTHOR CONTRIBUTIONS

M.D.-V. designed and performed the experiments, analyzed data and wrote the manuscript; C.M.B.-A. and D.A.H. supervised the study and wrote the manuscript.

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The authors declare no competing financial interests.

similar pattern of demethylation in the TSDR region to that of IFN-γ^-Foxp3$^+$ T_{reg} cells (Fig. 1c) and healthy control Foxp3$^+$ T_{reg} cells (data not shown).

After a 4-h stimulation with PMA and ionomycin, T_{reg} cells isolated *ex vivo* from untreated subjects with RRMS showed a T_H1-like phenotype, including secretion of IFN-γ, upregulation of mRNA expression of the T_H1-associated transcription factor TBET (encoded by *TBX21*) and downregulation of *RORC* (encoding RAR-related orphan receptor C) and *TGFB1* (encoding transforming growth factor β1) mRNA (Supplementary Fig. 2 and **Supplementary Table 1**). *CXCR3*, but not *CCR5* or *IL10*, was upregulated in T_{reg} cells from subjects with RRMS as compared to healthy controls (Supplementary Fig. 2). T_{reg} cells from subjects with RRMS downregulated *CTLA4* (encoding cytotoxic T lymphocyte–associated protein 4) (271.8 ± 86.9 (arbitary units) in controls compared to 43.91 ± 11.7 in RRMS T_{reg} cells).

To ascertain whether IFN-γ secretion by RRMS T_{reg} cells reduces their suppressive activity, we cultured T_{reg} cells and responder T cells *ex vivo* in the presence of an IFN-γ–specific antibody. The suppressive activity of multiple sclerosis T_{reg} cells was significantly increased upon IFN-γ blockade, whereas healthy control T_{reg} cells were not affected (Fig. 1d).

IFN-β has been shown to affect the IL-12–IFN-γ axis in people with multiple sclerosis[10], among its other immunomodulatory effects. To examine the possible *in vivo* role of this axis in the generation of IFNγ^+ Foxp3$^+$ T cells in people with RRMS, we performed a cross-sectional investigation, isolating T_{reg} cells from patients with RRMS treated for at least 1 year with IFN-β and comparing them to T_{reg} cells from healthy control subjects. Intracellular staining revealed that the frequency of IFN-γ^+Foxp3$^+$ T cells in IFN-β–treated subjects with RRMS was similar to that in age- and sex-matched healthy control subjects (Fig. 1e). This was accompanied by an increase in the frequency of IL-17$^+$Foxp3$^+$ T cells in IFN-β–treated patients with RRMS compared to healthy controls (Fig. 1e). Although these alterations in cytokine release could be due to a direct effect of IFN-β on IL-17 secretion by T_{reg} cells, IFNβ–mediated decreases in the amount of IL-12 could also induce a change in the cytokine milieu, driving increased IL-17 production.

Individuals with autoimmune disease have elevated IL-12 expression, and T_{reg} cells isolated from such individuals show reduced suppressive activity[3,11,12]. To elucidate the mechanism by which T_{reg} cells produce IFN-γ, we hypothesized that IL-12, a cytokine associated with T_H1 responses, would induce a T_H1-like phenotype in T_{reg} cells, similar to the phenotype we observed *ex vivo* in T_{reg} cells from subjects with RRMS. We cultured T_{reg} cells from healthy control subjects in the presence or absence of IL-12. After 4 d, a significant (P = 0.009) percentage of IL-12–stimulated T_{reg} cells secreted IFN-γ (Fig. 2a and Supplementary Fig. 1b). This increase was even more pronounced when we stimulated T_{reg} cells from subjects with RRMS with IL-12 (Supplementary Fig. 1c), suggesting a higher intrinsic responsiveness to IL-12 in these individuals.

The capacity of T_{reg} cells from healthy subjects to secrete IFN-γ was not accompanied by loss of Foxp3 expression (Supplementary Figs. 1d and 3) and was dependent on the dose of IL-12 (Supplementary Fig. 4). IL-12 also induced a modest increase in IL-10, but not in IL-17 or IL-4, production (Fig. 2a and Supplementary Fig. 5). Other members of the IL-12 family of cytokines, including IL-23 and IL-27, did not induce either IFN-γ or IL-10 production (Supplementary Fig. 6). We confirmed at a single-cell level that IL-12 could induce IFN-γ and IL-10 production in T_{reg} cell clones (Supplementary Figs. 7 and 8).

As expected given the induction of IFN-γ secretion by IL-12, and similar to what we observed in *ex vivo* T_{reg} cells from subjects with RRMS IL-12–stimulated T_{reg} cells

expressed significantly more *TBX21* mRNA and protein and less *GATA3* mRNA as compared to T_{reg} cells not treated with IL-12 (Fig. 2b and Supplementary Fig. 9). There was no change in the expression of *FOXP3*, *RORC*, *IRF1* (encoding interferon regulatory factor 1) and *MAF*, a transcription factor related to IL-10 production (Fig. 2b and Supplementary Fig. 9). We obtained similar results with single-cell–derived T_{reg} cell clones stimulated for 10 d in the presence of IL-12 (Supplementary Fig. 10).

To examine whether IL-12–stimulated $Foxp3^+$T-bet^+ T_{reg} cells were functionally suppressive, we cocultured $CD4^+CD25^{low/-}$ responder T cells with T_{reg} cells that had been prestimulated for 4 d with IL-2 alone or with IL-2 and IL-12, along with antibody to CD3 and irradiated T cell–depleted peripheral blood mononuclear cells. After 3 d, T_{reg} cells cultured with IL-2 and IL-12 were significantly less effective at inhibiting responder T cell proliferation as compared to IL-2–treated T_{reg} cells (Fig. 2c and Supplementary Fig. 9c). Blocking IFN-γ increased the suppressive activity of T_{reg} cells cultured with IL-2 and IL-12 but not that of control cells treated with only IL-2. These data suggest that other mechanisms may also account for the defect in suppression. We obtained the same results in antigen-presenting cell–free coculture assays (Supplementary Fig. 11). We also observed diminished suppressive activity of IL-12–treated T_{reg} cell clones (Supplementary Fig. 12). Although our data point to a general defect in the suppressive activity of T_H1-like, $Foxp3^+$ T cells, it is unclear whether these cells are able to specifically suppress T_H1 responses, as has been shown in a mouse model of inflammation[13].

Next we examined the reversible nature of the T_H1-like T_{reg} cell phenotype. We collected T_{reg} cells cultured with IL-12 for 4 d and split them into two populations; one was washed to eliminate IL-12, whereas the other was cultured with IL-12 for 3 d longer. The frequency of IFN-γ^+Foxp3^+ T cells decreased when we cultured the cells without IL-12 for the last 72 h, and these T_{reg} cells reacquired suppressive activity (Supplementary Fig. 13). Viability staining of the populations excluded the possibility of selective apoptosis induced by the removal IL-12 (data not shown), suggesting that IL-12 did not induce permanent changes in the amount of IFN-γ expressed or the frequency of T_{reg} cells expressing IFN-γ.

IL-12–stimulated T_{reg} cells showed a T_H1 chemokine receptor profile[14,15] characterized by expression of both *CXCR3* and *CCR5* and decreased *TGFB1* mRNA and protein expression (Fig. 2d and Supplementary Fig. 14). In contrast to T_{reg} cells from subjects with RRMS, T_{reg} cells upregulated *CTLA4* mRNA and protein expression in response to IL-12 (Supplementary Fig. 15).

These data provide a general mechanism by which proinflammatory cytokines such as IL-12 can rapidly alter the phenotype and function of T_{reg} cells, decreasing their suppressive activity. Our results suggest one possible mechanism to account for the diminished suppressive activity of T_{reg} cells from individuals with multiple sclerosis[3–6], although prospective studies on the frequency of IFN-γ^+Foxp3^+ T cells in populations at risk for developing multiple sclerosis and in patients before and after treatment with IFN-β are needed to confirm whether these T_H1-like T_{reg} cells are associated with disease pathogenesis. Taken together, our results underscore the plasticity of T_{reg} cells in a proinflammatory environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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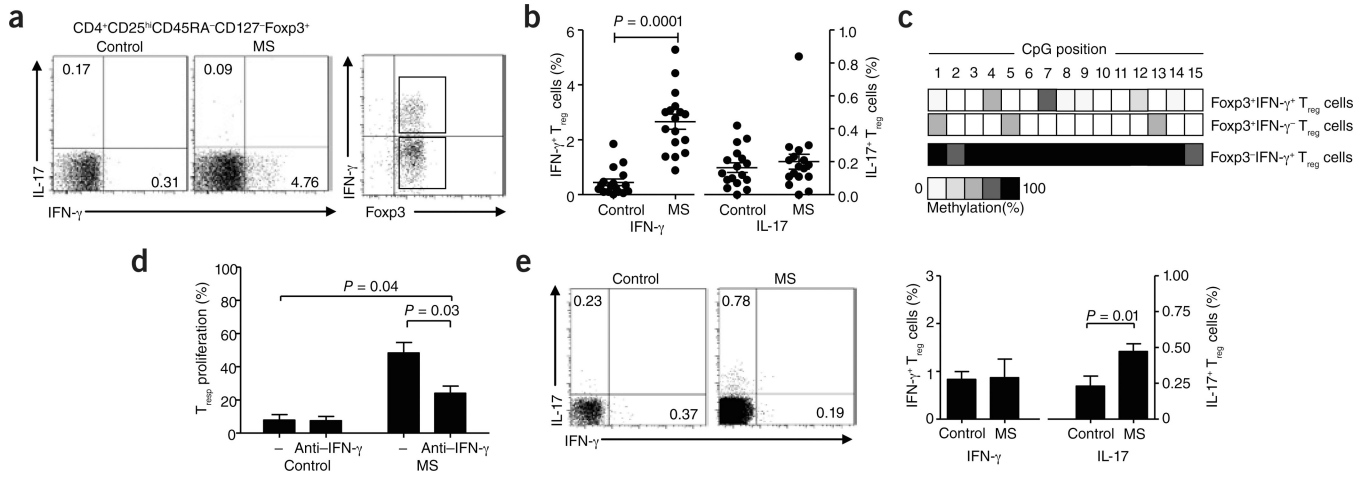

Figure 1.

T_{reg} cells from individuals with RRMS secrete IFN-γ *ex vivo*. (**a**) The frequency of FACS-sorted IFN-γ^+ and IL-17^+ T_{reg} cells in healthy control individuals (left) and untreated individuals with RRMS (middle, $n = 17$) gated on $Foxp3^+$ T_{reg} cells. Right, purity analysis of the sorted IFN-γ^+Foxp3^+ and IFN-γ $Foxp3^+$ populations from subjects with RRMS used for methylation analysis in **c**. (**b**) Percentage of IFN-γ^+Foxp3^+ and IL-17^+Foxp3^+ T_{reg} cells ($n = 17$) as a proportion of total $Foxp3^+$ T_{reg} cells. (**c**) Representative example of methylation analysis of the TSDR region of the *FOXP3* locus in sorted IFN-γ^+Foxp3^+ and IFN-γ^- $Foxp3^+$ T_{reg} cells from subjects with RRMS. An analysis of IFN-γ^+Foxp3^- memory T cells from subjects with RRMS is shown as a control. (**d**) Proliferation of responder T (T_{resp}) cells cultured with *ex vivo* FACS-sorted T_{reg} cells from healthy control subjects and untreated subjects with multiple sclerosis (MS; T_{reg} cell:T_{resp} cell ratio of 1:2) in the presence or absence of an IFN-γ–specific antibody ($n = 4$). (**e**) The frequency of IFN-γ^+ and IL-17^+ T_{reg} cells in healthy control subjects (left) or IFN-β–treated patients with RRMS (right) as assessed by intracellular cytokine staining and FACS analysis. The bar diagram (right) shows the percentage of IFN-γ^+Foxp3^+ and IL-17^+Foxp3^+ cells as a proportion of total $Foxp3^+$ T_{reg} cells in healthy controls or IFN-β–treated patients with RRMS ($n = 12$). Approval for studies was obtained from the Brigham and Women's Hospital Institutional Review Board, and informed consent was obtained from all donors.

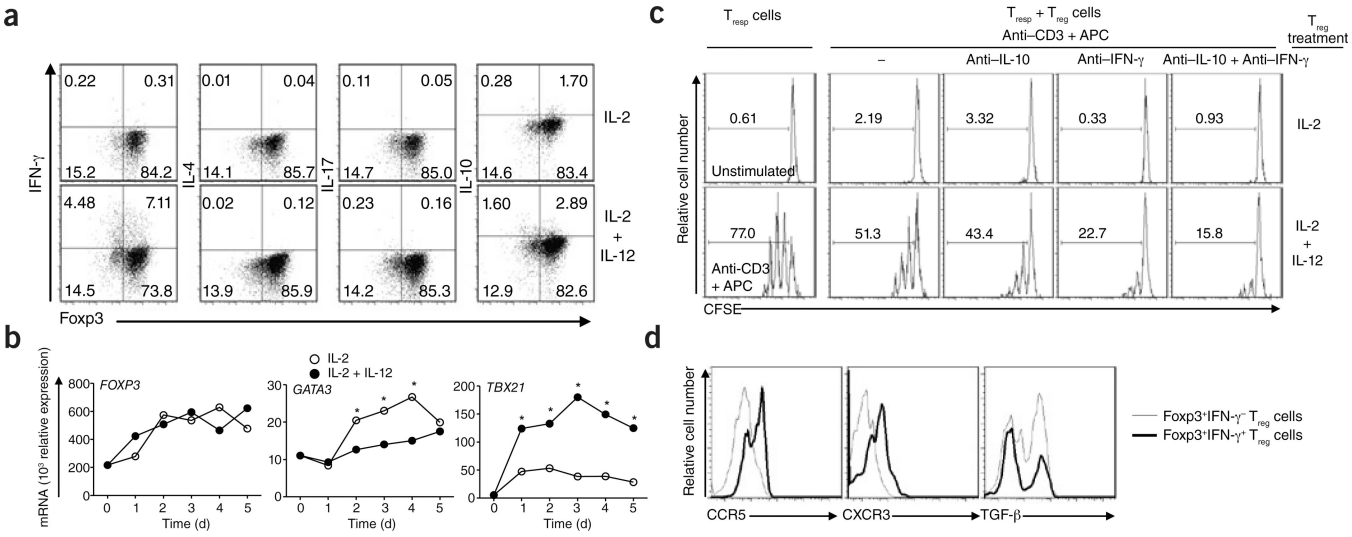

Figure 2.

Characterization of IL-12–driven, IFN-γ^+Foxp3$^+$ T_{reg} cells *in vitro* from healthy controls. (**a**) Intracellular staining for IFN-γ, IL-4, IL-17 and IL-10 of untreated (upper row) and IL-12-stimulated (bottom row) human T_{reg} cells from healthy controls at day 4. (**b**) mRNA expression of *FOXP3* (left), *GATA3* (middle) and *TBX21* (right) in T_{reg} cells stimulated in the presence or absence of IL-12 for 5 d (data are a representative example of three experiments performed with similar results; $*P < 0.05$). (**c**) Proliferation of T_{resp} cells cocultured for 3 d with T_{reg} cells (T_{reg} cell:T_{resp} cell ratio of 1:2) previously treated with IL-2 (top) or IL-2 + IL-12 (bottom), as assessed by carboxyfluorescein succinimidyl ester (CFSE) dilution. Histograms depict unstimulated T_{resp} cells alone (top left), T_{resp} cells stimulated with antibody to CD3 (anti-CD3) and antigen-presenting cells without T_{reg} cells (bottom left) and T_{resp} cell and T_{reg} cell cocultures without blocking antibodies (second column), in the presence of an IL-10–specific blocking antibody (anti–IL-10; third column), IFN-γ–specific blocking antibody (anti–IFN-γ; fourth column) or both antibodies (fifth column). (**d**) Representative example of staining for T_H1-associated chemokines(CCR5 and CXCR3) and TGF-β on IFN-γ^+Foxp3$^+$ and IFN-γ^- Foxp3$^+$ T_{reg} cells (data are representative of three experiments).