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Journal

Immunity, 39(5)

ISSN

1074-7613

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Publication Date

2013-11-01

DOI

10.1016/j.immuni.2013.10.016

Peer reviewed

Immunity
Article

Self-antigen-Driven Activation Induces Instability of Regulatory T Cells during an Inflammatory Autoimmune Response

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http://dx.doi.org/10.1016/j.immuni.2013.10.016

SUMMARY

Stable Foxp3 expression is crucial for regulatory T (Treg) cell function. We observed that antigen-driven activation and inflammation in the CNS promoted Foxp3 instability selectively in the autoreactive Treg cells that expressed high amounts of Foxp3 before experimental autoimmune encephalitis induction. Treg cells with a demethylated Treg-cell-specific demethylated region in the *Foxp3* locus downregulated *Foxp3* transcription in the inflamed CNS during the induction phase of the response. Stable Foxp3 expression returned at the population level with the resolution of inflammation or was rescued by IL-2-anti-IL-2 complex treatment during the antigen priming phase. Thus, a subset of fully committed self-antigen-specific Treg cells lost Foxp3 expression during an inflammatory autoimmune response and might be involved in inadequate control of autoimmunity. These results have important implications for Treg cell therapies and give insights into the dynamics of the Treg cell network during autoreactive CD4$^+$ T cell effector responses in vivo.

INTRODUCTION

CD4$^+$CD25$^+$Foxp3$^+$ regulatory T (Treg) cells are crucial for self-tolerance and for maintaining balanced immune responses. Reduced numbers or function of Treg cells have been associated with the onset of autoimmunity (Long and Buckner, 2011; Tang et al., 2008), whereas increasing the number of Treg cells has had therapeutic success in models of autoimmunity and graft-versus-host disease (GVHD). In fact, Treg-cell-based therapies are currently being tested in clinical trials (Brunstein et al., 2011; http://clinicaltrials.gov/ct2/show/NCT01210664?term=type+1+diabetes+and+tregcells&rank=1). Treg cells constitutively express interleukin-2 (IL-2) receptor (IL-2R) and depend on IL-2 for survival and homeostasis in the periphery (Burchill et al., 2008; Cheng et al., 2011). Treg cells have attributes of activated conventional T (Tconv) cells with constitutively activated T cell receptor (TCR) signaling pathways and uncommitted chromatin marks on many "T effector" gene loci, such as interferon-γ (IFN-γ) (Moran et al., 2011; Salomon et al., 2000; Wei et al., 2009). The Treg cell transcription factor Foxp3 activates genes required for suppressor function and is essential for maintaining the transcriptional program of the Treg cell lineage (Gavin et al., 2007). In fact, targeted deletion of Foxp3 in Treg cells turns them into IFN-γ- or IL-2-producing T effector cells (Williams and Rudensky, 2007). In this regard, Treg cells deficient in single transcription factors or key signaling proteins have identified pathways crucial for Foxp3 expression and stability (Kitoh et al., 2009; Rudra et al., 2009; Vanvalkenburgh et al., 2011; Wang et al., 2011; Yao et al., 2007; Zanin-Zhorov et al., 2010; Zheng et al., 2010). These studies support the general concept that altered signaling can lead to Treg cell instability and the possibility that at least a subset of Treg cells could be unstable and lose Foxp3 expression once the cell lineage is fully established.

We, as well as others, have shown that a substantial subset of unmanipulated CD4$^+$ T cells express low levels of Foxp3 in vivo, especially in lymphopenic and inflammatory settings (Zhou et al., 2009; Miyao et al., 2012). With the use of lineage reporter and tracer mice for characterizing loss of Foxp3 expression and concomitant functional activity (Rubtsov et al., 2010; Sharma et al., 2010; Zhou et al., 2009), it was observed that Treg cell instability results in loss of the regulatory network that maintains self-tolerance. Additionally, cells that downregulate Foxp3 expression ("exFoxp3" cells) produce proinflammatory cytokines and can act as effector cells causing tissue destruction if they are self-reactive (Zhou et al., 2009), a characteristic of thymically derived Treg (tTreg) cells (Hsieh et al., 2006; Wong et al., 2007). Thus, the emergence of effector-cell-like characteristics in this population could have serious repercussions for autoimmunity in terms of both loss of regulation and potential pathogenic activity.

More recent studies have argued that many, if not all, of these exFoxp3 cells derive from an early, "aborted" T cell differentiation process that occurs prior to full Treg cell commitment rather

than from the instability of bona fide tTreg cells (Rubtsov et al., 2010; Miyao et al., 2012). These studies were conducted under largely homeostatic conditions in the steady state, in vitro, or the setting of acute lymphopenia, thus raising the question of whether the Treg cell instability observed by us and others could be related to the inflammatory pathogenic setting in our studies. Indeed, a number of reports have demonstrated Treg cell reprogramming and acquisition of pathogenic potential in autoimmunity, GVHD, and vaccination settings (Dominguez-Villar et al., 2011; Laurence et al., 2012; McClymont et al., 2011; Sharma et al., 2010; Zhou et al., 2009), consistent with the suggestion that active immunity might have direct effects on Treg cell stability. Therefore, in this study, we set out to examine Foxp3 stability in bona fide Foxp3^{hi} Treg cells responding to self-antigen within a polyclonal T cell repertoire and in the context of an active CD4$^+$ T cell autoimmune response. Using an experimentally induced autoimmune encephalomyelitis (EAE) model, we observed that antigen-driven activation and inflammation promoted Foxp3 instability selectively in the autoreactive Treg cells that expressed high levels of Foxp3 before EAE induction. Transfer experiments demonstrated that bona fide Treg cells with a demethylated Treg-cell-specific demethylated region (TSDR) in the *Foxp3* locus downregulated *Foxp3* transcription during the induction phase of the response. Stimulation with cognate autoantigen induced IFN-γ production by the exFoxp3 cells in the CNS at the peak of the response. Stable Foxp3 expression returned with the resolution of inflammation or was rescued by enhancement of IL-2 receptor signaling with IL-2-anti-IL-2 complex treatment during the antigen priming phase. These findings suggest that a subset of antigen-specific Treg cells participating in the control of an immune response can be reprogrammed and might play a role as potentially pathogenic cells during autoimmunity.

RESULTS

Unstable Foxp3 Expression during EAE in C57BL/6 Mice

Treg cells were analyzed in EAE induced in the C57BL/6 (B6) genetic background. The previously described Foxp3-lineage reporter mice (Zhou et al., 2009) were backcrossed more than eight generations onto the B6 background. In these bacterial artificial chromosome (BAC) transgenic mice, Foxp3 promoter and regulatory elements drive the Cre-recombinase-GFP fusion protein. These mice were bred to two different independent mouse strains that express either a yellow fluorescent protein (YFP) or a red fluorescent protein (RFP) transgene engineered with a stop codon flanked by *loxP* sites and inserted into the *Rosa26* locus. In the dual-expressing (Foxp3.GFP-Cre and Rosa26.YFP or Rosa26.RFP) reporter mice, any cell expressing Foxp3 expresses RFP or YFP for its lifetime, whereas GFP is expressed only in cells that currently express Foxp3. The CD4$^+$ T cell compartment of 6- to 8-week-old B6 Foxp3-Cre BAC transgenic mice crossed to Rosa26.RFP mice contained 0.5%–1.5% CD4$^+$ T cells with reduced or lost Foxp3 expression (termed exFoxp3 cells; Figure 1A) in the steady state. These data were confirmed in another line of B6 mice generated with Cre recombinase expressed in the *Foxp3* 3′ UTR (Rubtsov et al., 2008) and crossed to Rosa26.RFP mice (Figure S1, available online). These results demonstrate that Foxp3 downregulation occurred within the polyclonal Treg cell population in a lymphoreplete, intact immune environment, albeit in a small percentage of the cells.

Next, we induced EAE by immunizing B6 mice with MOG_{35-55} peptide in complete Freund's adjuvant (CFA) (MOG_{35-55}-CFA). Lymphocytes were harvested from the draining lymph nodes (LNs) and spleen and CNS tissues of immunized mice and were examined for evidence of antigen-specific T cell expansion and differentiation with a major histocompatibility complex (MHC)-peptide tetramer, I-A^b-MOG_{38-49}, which bound to MOG_{35-55}-peptide-specific T cells as previously described (Korn et al., 2007). Using this probe, we analyzed MOG_{38-49}-specific CD4$^+$ T cells among the polyclonal CD4$^+$ T cell population during the asymptomatic and inflammatory phases of MOG_{35-55}-induced EAE. After an enrichment step, MOG_{38-49}-reactive cells accounted for 4% of CD4$^+$ T cells in the peripheral T cell compartment after EAE induction (Figure 1B). The tetramer staining was specific given that control I-A^b:hClip tetramer staining was negligible in this population (data not shown). For further studies, we focused on an analysis of antigen-specific T cells within polyclonal populations.

Initial studies showed that there was virtually no detectable I-A^b:MOG_{38-49} tetramer$^+$CD4$^+$ T cells (0.2% of CD4$^+$ T cells after an enrichment step in vitro for tetramer-bound cells) in the LNs prior to immunization (data not shown). Thus, after immunization, MOG_{38-49}-specific CD4$^+$ T cells expanded in draining LNs and spleen and expressed CD44, indicative of antigen-driven activation. During the clinical phases of EAE, all CD4$^+$ T cells in the CNS expressed high amounts of the activation marker CD44, and MOG_{38-49}-specific cells accumulated and represented >4% of CD4$^+$ T cells in the CNS (without any enrichment step in vitro, as required for seeing the cells in the draining LNs) (Figure 1B). We assayed the "quality" of the self-antigen-specific Treg cells during an autoimmune response by tracing the kinetics of Treg cells during EAE and the stability of Foxp3 expression with lineage-traced Treg cells. We used Foxp3.Cre.GFP × Rosa26.RFP (Figure 1C) and Foxp3.Cre.GFP × Rosa26.YFP mice and saw no differences in the percentage of Treg and exFoxp3 cells when the data were pooled from the different lineage-tracer mice (Figure 1D). Equivalent to RFP$^-$YFP$^-$ Tconv cells, exFoxp3 cells were identified as expressing low to negative amounts of GFP (Figures 1C and 1E). exFoxp3 cells made up a higher proportion of MOG_{38-49}-specific cells than polyclonal cells at the preclinical stage of EAE in the LNs and spleen 8 days after immunization and at the peak stage of the disease in the CNS (Figures 1C–1E). During EAE resolution, the proportion of exFoxp3 cells in the MOG_{38-49}-specific CD4$^+$ T cells was higher than that of polyclonal CD4$^+$ T cells in the LNs and spleen but was similar to the proportion of polyclonal CD4$^+$ T cells in the CNS (Figures 1C–1E). The MOG_{38-49}-specific cells had a larger fraction of Treg cells than polyclonal cells at all the stages of EAE 7 days after immunization in the LNs and spleen, as well as in the CNS, LNs, and spleen during the peak and resolution of EAE. The "enrichment" of antigen-specific Treg cells was in agreement with a previous study that demonstrated that MOG_{38-49}-specific Treg cell expanded from a Foxp3^{hi} Treg cell population that existed in B6 mice prior to MOG immunization (Korn et al., 2007). The kinetics of the exFoxp3 cells in the MOG_{38-49}-specific niche mirrored Treg cells at the preclinical and peak stages of EAE, whereas the percentage of

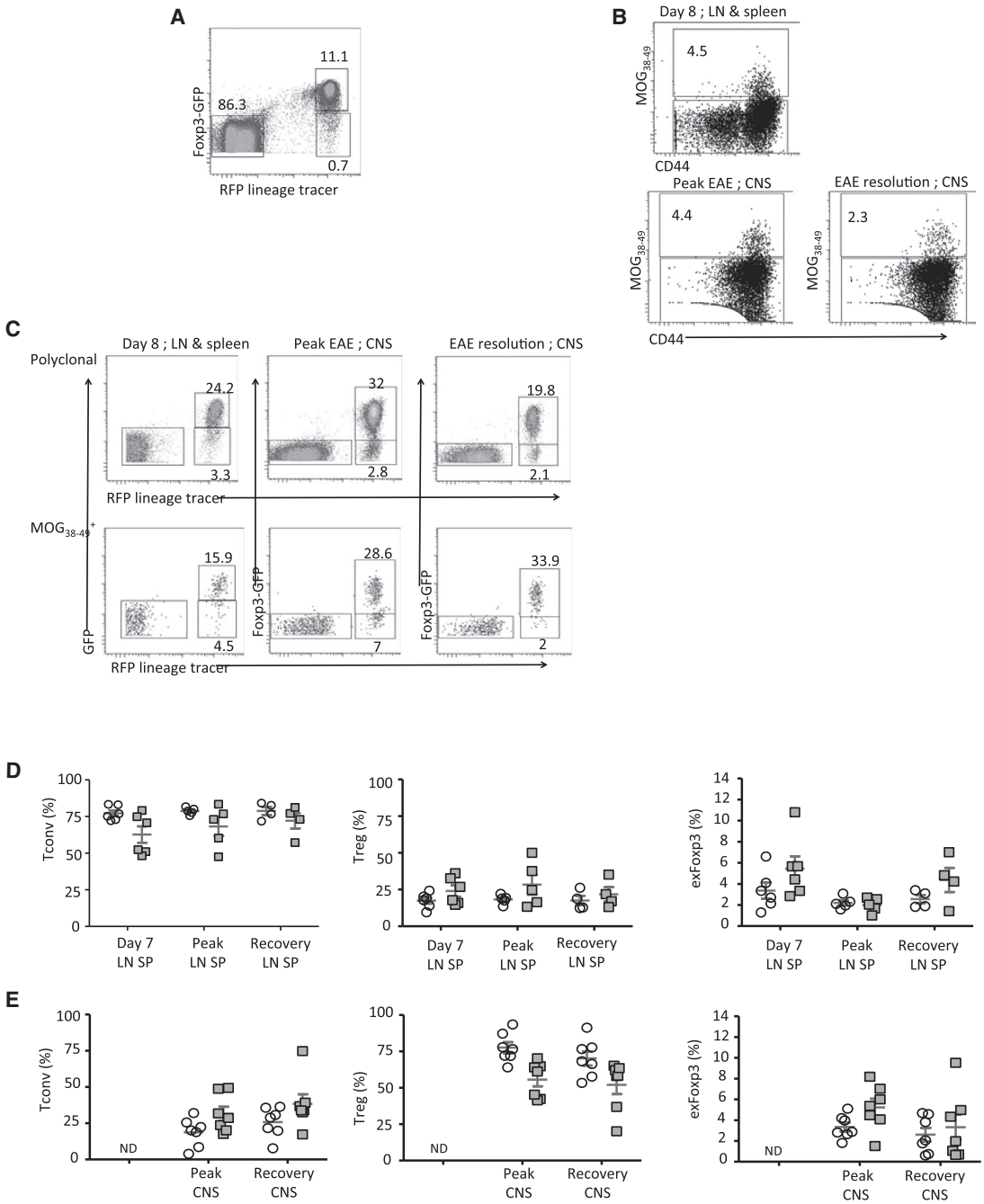

Figure 1. MOG_{38-49}-Specific Treg Cells Downregulate Foxp3 during EAE

(A) Expression of GFP and RFP in lymph node (LN) and spleen $CD4^+$ T cells of a 6-week-old B6 Foxp3.GFP.Cre.Rosa26.RFP mouse. Representative of 15 Foxp3.GFP.Cre.Rosa26.RFP or Foxp3.GFP.Cre.Rosa26.YFP mice. The percentages of Tconv ($RFP^-Foxp3.GFP^-$), Treg (RFP^+ $Foxp3.GFP^+$), and exFoxp3 ($RFP^+Foxp3.GFP^-$) cells are indicated.

(B) High-affinity MOG_{38-49}-specific T cells in the $CD4^+$ fraction were detected with $I\text{-}A^b$:tetramers at the indicated stages of EAE in LNs and spleen or the CNS (spinal cord and cerebellum). Dot plots are gated on $CD4^+$ T cells. Data are representative of three to six experiments.

(C) $CD4^+$ T cells were gated on $tetramer^-$ (polyclonal) (top) and MOG_{38-49}-specific (bottom) cells and analyzed for the frequency of Tconv, Treg, and exFoxp3 cells at the indicated stages of EAE in the LNs and spleen or the CNS. Data are representative of three to six experiments using Foxp3.GFP.Cre.Rosa26.RFP or Foxp3.GFP.Cre.Rosa26.YFP mice.

(D and E) The proportions of Tconv, Treg, and exFoxp3 cells in MOG_{38-49}-specific (filled square) and polyclonal (open circle) $CD4^+$ populations are shown in the LNs and spleen (D) and the CNS (E) at the indicated stages of EAE. Data points are individual Foxp3.GFP.Cre.Rosa26.RFP or Foxp3.GFP.Cre.Rosa26.YFP mice, and red lines show the mean ± SEM. The percentage of each population was not significantly different in polyclonal versus MOG_{38-49}-specific cells, as determined with a paired t test. ND, not done.

See also Figure S1.

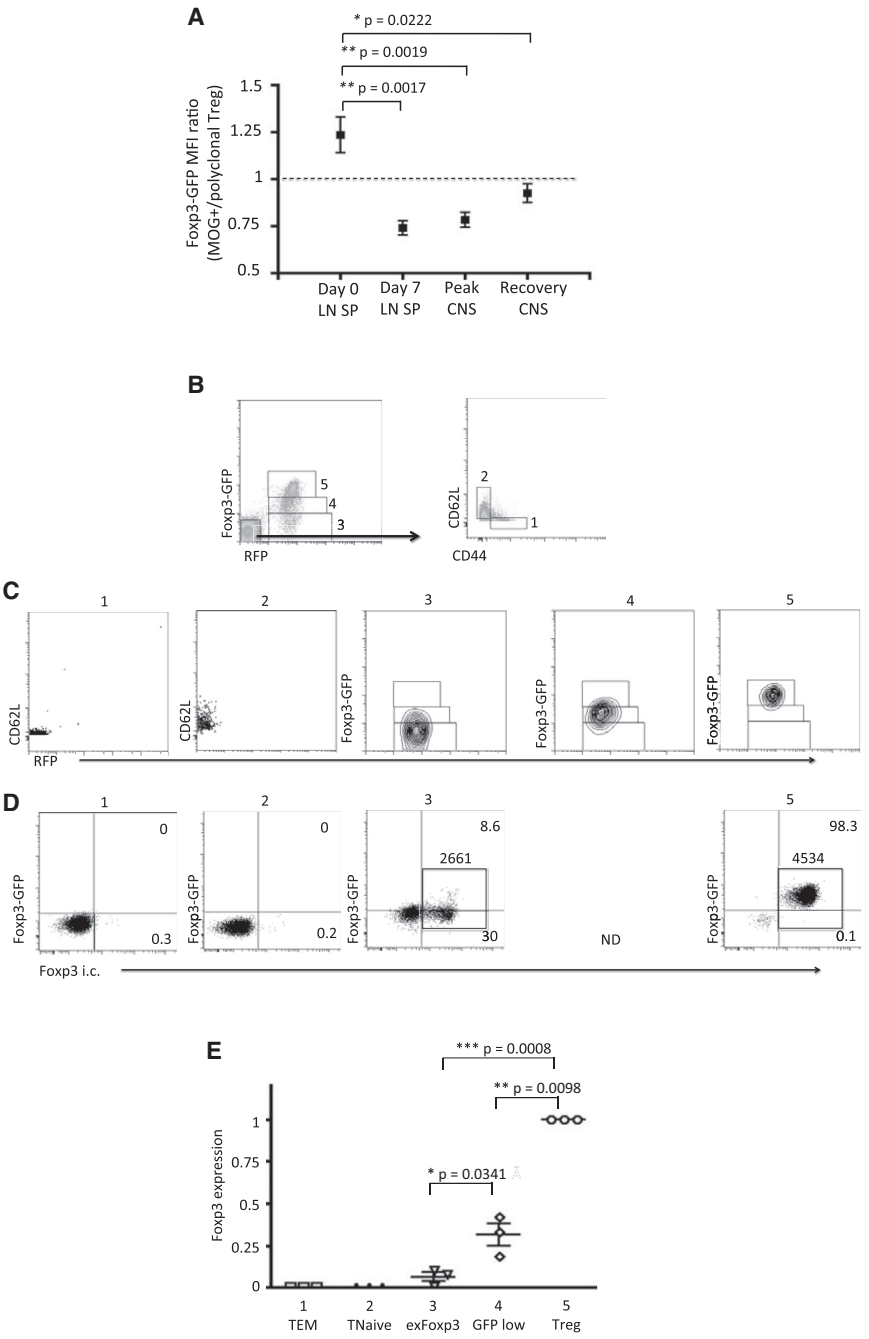

Figure 2. Reduced Foxp3 Expression in Antigen-Specific Treg Cells and Foxp3^{lo} Cells during EAE

(A) Ratio of GFP expression (MFI) in MOG_{38-49}-specific Treg cells versus polyclonal Treg cells. Mean ± SEM of four to six mice per time point is shown. p values are from an unpaired t test.

(B) Gating strategy for sort purification of LN and spleen CD4$^+$ T cell subsets 7 days after immunization with MOG_{35-55}-CFA. Data are representative of three experiments.

(C) Post-sort analysis of GFP and RFP expression in the sorted populations in (A).

(D) Post-sort analysis of intracellular (i.c.) Foxp3 and Foxp3-GFP levels in the sorted populations in (A). ND, not done.

(E) Foxp3 expression was measured by quantitative PCR and normalized to GAPDH. Each symbol represents one mouse. Error bars represent the mean ± SEM, and p values are from a two-tailed unpaired t test.

MOG_{38-49}-specific exFoxp3 cells was lower in the CNS during EAE resolution than during previous EAE stages, when the percentage of MOG_{38-49}-specific Treg cells continued to rise (Figures 1D and 1E). The data suggest that antigen triggering during inflammation induces antigen-specific Treg cells to accumulate and downregulate Foxp3 to compose a major population of exFoxp3 cells within the antigen-specific niche.

Treg Cells Downregulate *Foxp3* Transcription

To gain insight into the precursor-product relationship of MOG_{38-49}-specific exFoxp3 cells during EAE, we analyzed Foxp3 expression in Tconv, Treg, and exFoxp3 cells. According to studies using Foxp3.GFP-Cre lineage-tracer mice, Foxp3 expression is regulated at both transcriptional and protein levels, which allows for three readouts of Foxp3 regulation (Bailey-Bucktrout and Bluestone, 2011). First, GFP is a direct readout of *Foxp3* transcription given that its expression is controlled by the *Foxp3* locus promoter and enhancer activity. Second, the expression of YFP or RFP marks cells that currently express or historically expressed Foxp3. Third, the amounts of Foxp3 reflect translation and protein turnover at the cellular level. Together, these readouts allowed us to assess Foxp3 regulation in vivo. On the basis of median (data not shown) or mean fluorescence intensity (MFI), MOG_{38-49}-induced Treg cells had significantly lower levels of Foxp3-driven GFP than did the polyclonal Treg cells (Figure 2A), suggesting that the entire population of MOG_{38-49}-specific Treg cells downregulated *Foxp3* transcription after antigen exposure and inflammation.

We next analyzed the expression of *Foxp3* mRNA and Foxp3 in CD4$^+$ T cell populations in the LNs and spleen during preclinical EAE, when Foxp3-driven GFP was downregulated in Treg cells. *Foxp3* mRNA and Foxp3 were undetectable in sort-purified RFP$^-$CD62L^{hi}CD44$^-$ and CD62L^{lo}CD44$^+$ "naive" and "effector-memory" CD4$^+$ Tconv cells, respectively (Figures 2B–2E). The bulk population of RFP$^+$ cells was subdivided into three subsets on the basis of GFP expression. GFP^{hi} Treg cells expressed the highest levels of *Foxp3* mRNA and Foxp3, whereas RFP$^+$ cells with lower levels of GFP expression (GFP^{lo} cells) had significantly lower amounts of *Foxp3* mRNA than did GFP^{hi} Treg cells

(Figure 2E). Of note, RFP$^+$GFP$^-$ exFoxp3 cells contained a subpopulation of 30%–40% cells that still expressed Foxp3, although at lower levels than in GFP^{hi} Treg cells (2,661 versus 4,534 MFI), but compared with GFP^{hi} and GFP^{lo} cells, they had significantly downregulated *Foxp3* mRNA (Figure 2E). The data demonstrate that Foxp3-driven GFP faithfully reflects *Foxp3* transcription during EAE and further suggest a hypothesis wherein antigen-driven activation of Treg cells in an inflammatory environment results in the downregulation of *Foxp3* transcription in bona fide Treg cells prior to loss of longer-lived Foxp3. Once the residual Foxp3 is lost, Treg cells become functionally unstable. Finally, the Foxp3$^+$GFP$^-$RFP$^+$ cells produce IFN-γ amounts similar to those of the Foxp3$^-$ population (data not shown). Therefore, Foxp3 expression does not differentiate this phenotype per se. This result is consistent with a previous study (Ohkura et al., 2012).

Treg Cells Lose Foxp3

Recent studies have suggested that Treg cells are highly stable under homeostatic conditions with high expression of IL-2Rα and demethylated marks on the CpG motifs in the TSDR of the *Foxp3* locus (Miyao et al., 2012; Ohkura et al., 2012). To address the hypothesis that instability is a function of exposure to an inflammatory response, we examined Foxp3 expression in antigen-specific Treg cells during the active autoimmune response in EAE. Highly purified (>96%) Treg cells from naive mice were transferred into mice during the onset of EAE and were followed for changes in Foxp3. The Foxp3$^+$ Treg cells expressed high amounts of CD25 and a fully demethylated TSDR (Figures 3A and 3B) at the time of transfer. At the peak stage of EAE (Figure 3C), we used a congenic mark to identify the transferred Treg cells in the CNS infiltrate and analyze Foxp3 expression in the MOG$_{38-49}$-specific and polyclonal Treg cells (Figure 3D). The majority of the transferred polyclonal Treg cells in the CNS were stable for Foxp3 expression, given that 93.5% retained Foxp3 (Figure 3E). In contrast, Foxp3 expression was significantly lower in the adoptively transferred MOG-specific Treg cells. In fact, Foxp3 was undetectable in 39% of MOG$_{38-49}$-specific Treg cells at the peak of CNS inflammation (versus in <10% of the polyclonal Treg cells) (Figure 3D). It is important to note that even the small loss of Foxp3 expression within the polyclonal population could reflect antigen-driven effects given that I-A^b:MOG$_{38-49}$ tetramer staining does not detect all the MOG-specific or other neural antigen-specific Treg cells. These data formally demonstrate that a significant percentage of Treg cells, defined by high levels of Foxp3 and CD25 expression and a fully demethylated TSDR, lost Foxp3 after antigen exposure during an inflammatory response in vivo.

We demonstrated that Treg cells enriched with self-reactivity are biased for loss of Foxp3 expression during an autoimmune response, but the exFoxp3 cells that accumulate during EAE (Figure 1) could also have developed in a subset of antigen-specific cells with de novo Foxp3 expression as a result of antigen priming. It has been suggested in epigenetic-tracing studies that exFoxp3 cells arise from a Foxp3$^-$ T cell that transiently expresses Foxp3 (Miyao et al., 2012). Peripherally induced and in-vitro-induced CD4$^+$ Treg cells, termed pTreg and iTreg cells, respectively, that express Foxp3 during antigen recognition in extrathymic compartments have been described as being unstable for Foxp3 expression and suppressor activity (Chen et al., 2011; Josefowicz et al., 2012; Yadav et al., 2012). Therefore, we addressed the possibility that exFoxp3 cells generated during EAE arose from a Tconv cell population expressing Foxp3 for a transient but sufficient period of time to express Cre-recombinase and thus RFP. Congenically marked CD4$^+$GFP$^-$RFP$^-$ cells were transferred into mice with active EAE and were followed over time for determining whether CD4$^+$GFP$^-$RFP$^+$ cells could develop within the antigen-specific Tconv cell population. In four experiments, none of the MOG$_{38-49}^+$ tetramer cells expressed RFP (Figure S2). Thus, only the transfer of GFP$^+$RFP$^+$ Treg cells into the inflamed EAE setting led to loss of GFP (i.e., GFP$^-$RFP$^+$), whereas similar transfer of GFP$^-$RFP$^-$ Tconv cells did not result in a "transient" GFP$^-$RFP$^+$ Tconv cell population among the MOG-antigen-specific T cells. In contrast, MOG$_{38-49}^+$ exFoxp3 cells in the inflamed CNS at the peak of EAE arose from Treg cells. Thus, although exFoxp3 cells can be composed of populations of both uncommitted and previously committed cells, in the context of an autoimmune inflammatory response, such as EAE, the antigen-specific Foxp3$^{lo/-}$ population is derived overwhelmingly from previously committed, bona fide, Treg cells. These results are consistent with the fact that MOG$_{38-49}$-specific Foxp3$^+$ cells expanded from an antigen-specific Treg cell population that existed prior to autoimmunity, as well as with the fact that MOG$_{38-49}$-specific exFoxp3 cells were derived from an established Treg cell population and not transient Foxp3-expressing cells.

MOG$_{38-49}$-Specific and Polyclonal exFoxp3 Cells in the CNS Are Differentially Demethylated at the TSDR

Next, we examined the methylation status of CpG motifs in the TSDR of the *Foxp3* locus (Huehn et al., 2009). Previous studies have shown that Treg cells exhibit demethylated CpG sites in the TSDR, whereas the CpG sites in this intron are fully methylated in the overwhelming majority of Tconv cells and pTreg cells with unstable or transient Foxp3 expression (Floess et al., 2007; Haribhai et al., 2011; Ohkura et al., 2012).

Using an assay developed for low cell numbers (Figure S3), we determined the TSDR methylation status in polyclonal and MOG$_{38-49}$-specific Tconv, Treg, and exFoxp3 cells in the CNS at the peak of EAE. As expected, both polyclonal and MOG$_{38-49}$-specific RFP$^-$Foxp3.GFP$^-$ Tconv cells were fully methylated at the TSDR, and both polyclonal and MOG$_{38-49}$-specific RFP$^+$Foxp3.GFP^{hi} Treg cells were fully demethylated at the TSDR (Figure 4). The MOG$_{38-49}$-specific RFP$^+$Foxp3.GFP$^-$ exFoxp3 cells in the CNS at the peak of EAE were more demethylated at the TSDR than the polyclonal exFoxp3 cells at the same location (Figure 4). In addition, the TSDR was predominantly demethylated in MOG$_{38-49}$-specific exFoxp3 cells from the CNS in two out of three mice. Thus, these data support the hypothesis that Treg cells with a fully demethylated TSDR can lose Foxp3 expression and become exFoxp3 cells.

Autoreactive exFoxp3 Cells Produce Potentially Pathogenic Cytokine

T effector cells in the CNS of mice with EAE produce cytokines, including IFN-γ and IL-17A (Ivanov et al., 2006), that induce a cascade of inflammatory cell recruitment, glial cell activation, and death of myelin-producing oligodendrocytes and their

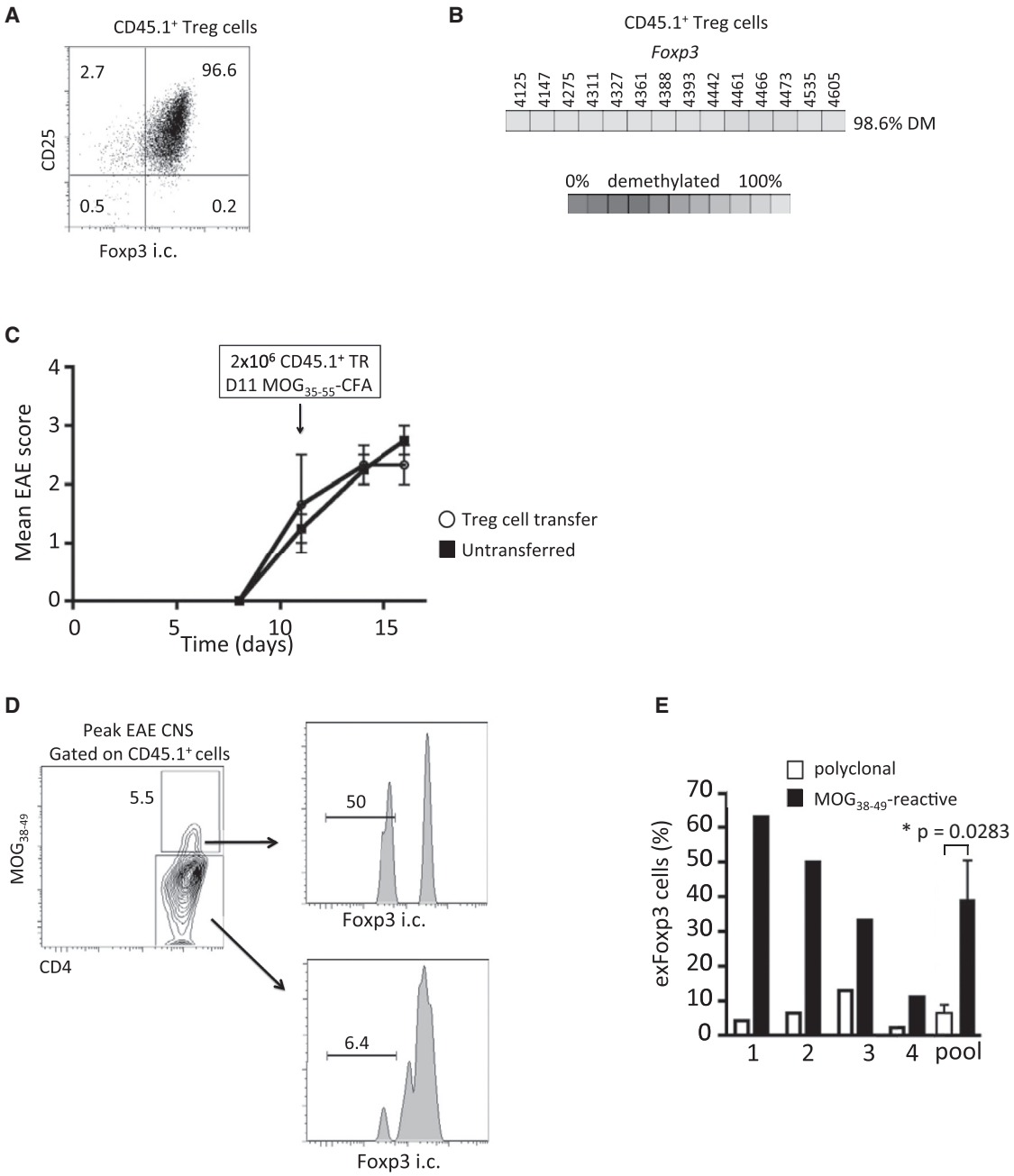

Figure 3. MOG-Specific exFoxp3 Cells Are Generated from Foxp3^{hi} Treg Cells

(A) Foxp3 and CD25 staining of sort-purified CD45.1$^+$CD4$^+$CD25^{hi}GITR^{hi} Treg cells.

(B) CpG demethylation status of the TSDR in the CD45.1$^+$ Treg cells depicted in (A). The location of each CpG in the *Foxp3* locus is depicted.

(C) EAE scores of mice that received two million Treg cells depicted in (A) and (B) (circle) and untransferred mice (square). Data represent the mean ± SEM of four mice per group. The EAE score between the groups at each time point was not significantly different in a paired t test. The difference between the groups was not significant.

(D) At day 16 of EAE, as depicted in (C), CNS cells were isolated and Foxp3 intracellular (i.c.) staining shown in CD45.1$^+$CD4$^+$ gated cells. Data are representative of four mice.

(E) Percentage of Foxp3 loss in polyclonal (unfilled bar) and MOG$_{38-49}$-specific (filled bar) CD45.1$^+$ transferred Treg cells in the CNS at the peak of EAE. Individual mice and pooled data (mean ± SEM) are shown. p values are from an unpaired t test.

See also Figure S2.

precursors. To address whether at least some of the cytokine-producing, and thus potentially pathogenic, T cells might be derived from the MOG-specific exFoxp3 population, we examined the production of IFN-γ by Tconv and lineage-tracer-positive cells isolated from the CNS after MOG$_{35-55}$ antigen recall in vitro. During the peak of EAE, 3.4% of the exFoxp3 cells

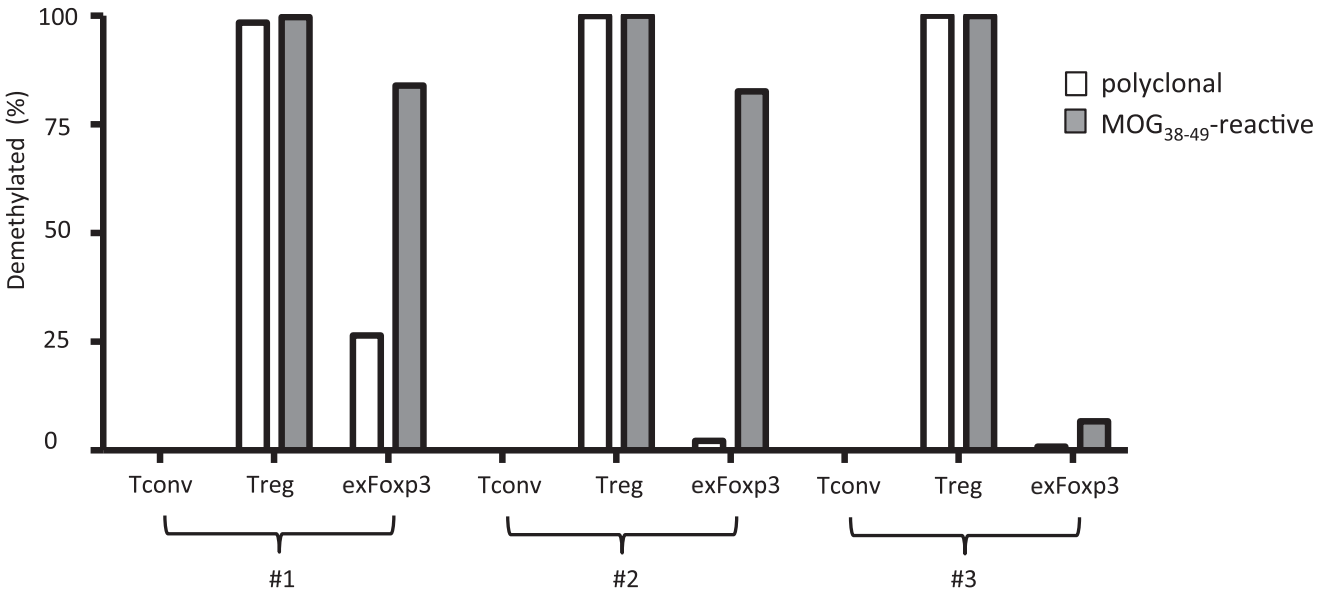

Figure 4. Distinct TSDR CpG Demethylation Marks in Autoreactive and Polyclonal exFoxp3 Cells in the CNS during EAE

Percent demethylated TSDR CpG motifs in sort-purified MOG_{38-49}-specific (filled bar) and polyclonal (open bar) $CD4^+RFP^-$ Tconv, $CD4^+$ RFP^+ Foxp3.GFP^+ Treg, and $CD4^+RFP^+$ Foxp3.GFP^- exFoxp3 cells infiltrating the CNS at the peak of EAE. The results of three analyzed mice are shown.
See also Figure S3.

produced IFN-γ without antigen restimulation in vitro. In response to MOG_{35-55} stimulation, this percentage increased to 9.6% of the exFoxp3 cells, only slightly lower than the 11.6% of Tconv cells that produced IFN-γ in the same conditions (Figures 5A and 5B). IFN-γ production was never detected in antigen-stimulated $Foxp3^+$ Treg cells during EAE. Interestingly, IL-17A was undetectable in exFoxp3 T cells in this assay and was detected only after PMA and ionomycin stimulation (data not shown). Therefore, Foxp3 loss bestows MOG-specific Treg cells with IFN-γ production levels comparable to those of pathogenic T effector cells, which has also been observed in the diabetes setting (Zhou et al., 2009). These results further argue against the hypothesis that exFoxp3 cells are derived from a transient Foxp3-expressing population given that others have shown that these cells are marked by coexpression of Foxp3 and RORγt, the lineage-specific transcription factor for the IL-17-producing T helper 17 (Th17) cell subset (Zhou et al., 2008a).

exFoxp3 Cells Are Pathogenic, Causing EAE

In EAE, pathogenic T cells induce an inflammatory cell infiltrate in the spinal cord and cerebellum, resulting in tail and hind-limb dysfunction (Bailey et al., 2006). MOG_{35-55}-reactive $CD4^+$ Tconv and exFoxp3 cells produced equivalent levels of IFN-γ in the CNS at peak (Figure 5), suggesting that they might be pathogenic. The implication that Treg cell instability leads to pathogenicity in autoimmunity is of major significance because Treg cells are enriched with self-reactivity (Hsieh et al., 2006; Wong et al., 2007) and can express homing receptors to allow preferential migration to inflamed tissues (Dominguez-Villar et al., 2011; Josefowicz et al., 2012). To test the pathogenicity of exFoxp3 cells in EAE, we compared isolated exFoxp3 cells with Tconv and Treg cells to determine their ability to induce EAE after adoptive cell transfer. Congenically marked $CD4^+RFP^-$ Foxp3.GFP^- Tconv, $CD4^+$ RFP^+ Foxp3.GFP^+ Treg, and $CD4^+RFP^+$ Foxp3.GFP^- exFoxp3 cells were purified from the LNs and spleen of MOG_{35-55}-CFA-immunized mice by fluorescence-activated cell sorting and expanded in vitro with MOG_{35-55} for enriching antigen-reactive cells, then with anti-CD3 plus anti-CD28 polyclonal stimuli for generating enough cells for the experiment. Expansion was performed with IL-2 for Tconv and Treg cells and with IL-2 and IL-7 for exFoxp3 cells. IL-7 was needed for expanding exFoxp3 cells ex vivo, most likely because they express low levels of CD25 (IL-2Rα) and express CD127 (IL-7Rα) at higher levels (Zhou et al., 2009). No Th1- or Th17-cell-driving cytokines that might influence the effector function of the T cells were added. The individual cell populations remained stable for Foxp3.GFP expression during the expansion and were resorted to purify from feeder cells during the antigen-driven expansion (Figure S4). Individual cell populations were transferred into lymphodeficient recipients, which were immunized with MOG_{35-55}-CFA for inducing EAE. Immunization was necessary for inducing EAE because the cells were expanded in "neutral" conditions, and no population induced EAE without immunization. As expected, Treg cells did not induce EAE, whereas MOG-specific exFoxp3 and Tconv cells induced EAE with similar incidence and severity (Figures 6A and 6B). The onset of disease was slightly delayed in exFoxp3 cell recipients compared to Tconv cell recipients in the experiment depicted, but the difference was not significant when the experiments were pooled (data not shown). Infiltrates were isolated from the spinal cord and cerebellum (CNS) of recipients at the peak of EAE (Figure 6A), and the abundance and composition of the inflammatory cells were compared. The transferred exFoxp3 cells had a stable RFP^+GFP^- phenotype after the induction of EAE, and the majority of Tconv cells remained RFP^-, although 4.7% transiently expressed Foxp3 (Figure S4). This transient

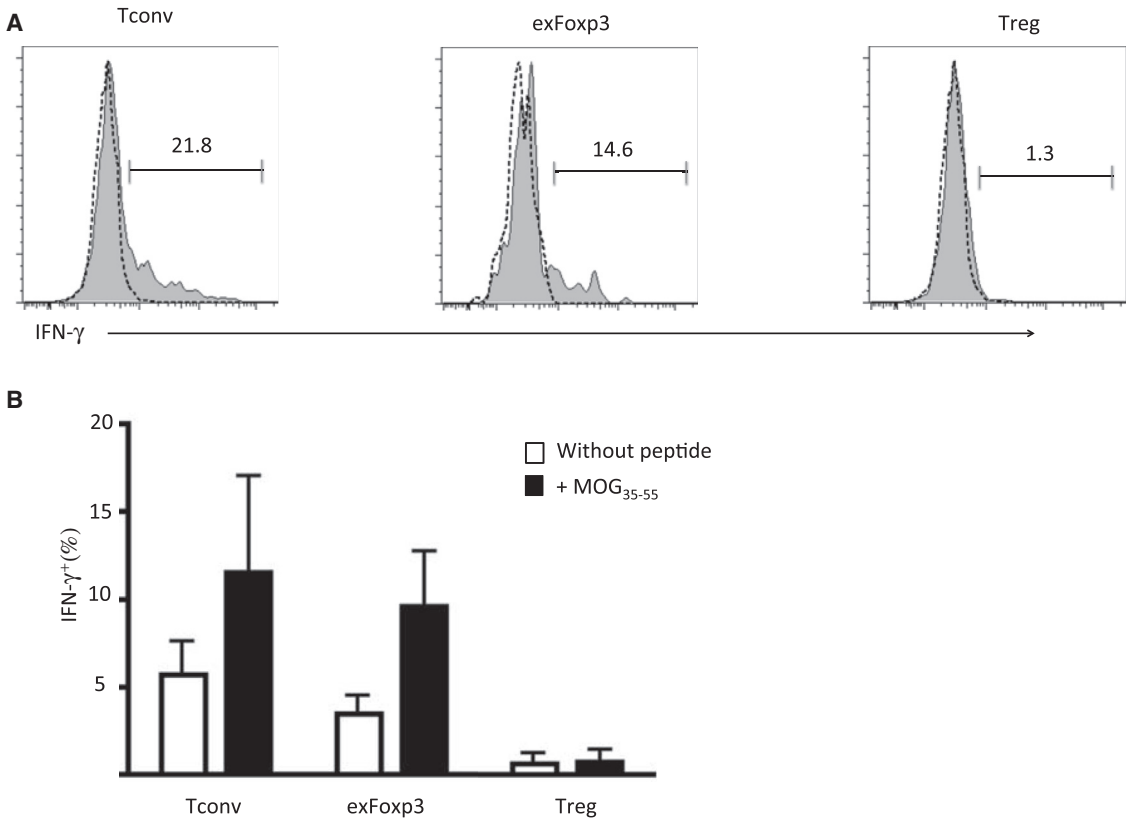

Figure 5. CNS exFoxp3 Cells Produce IFN-γ Similarly to Tconv Cells

(A) CNS cells were isolated at the peak of EAE and incubated for 16 hr with MOG_{35-55}, and Tconv, Treg, and exFoxp3 cell subsets were identified on the basis of staining for CD4, YFP or GFP, and Foxp3. IFN-γ (tinted histogram) and isotype control (dashed line) staining in the live $CD4^+$ population is shown. Data are representative of three experiments.

(B) Percentage of CNS $CD4^+$ T cells expressing IFN-γ as in (A) with (filled bar) or without (open bar) MOG_{35-55} stimulation in vitro. Data represent the mean ± SEM of three experiments with two to three mice pooled in each.

Foxp3 expression by a minor population of Tconv cells in a lymphodeficient environment is in agreement with previous studies (Miyao et al., 2012). The numbers of macrophages ($CD45^{hi}$, $CD11b^+$, $CD11c^-$), myeloid dendritic cells (mDCs; $CD45^{hi}$, $CD11b^+$, $CD11c^+$), plasmacytoid dendritic cells (pDCs; $CD45^{hi}$, $CD11b^-$, $CD11c^+$, $CD45R^+$), microglia ($CD45^{med}$, $CD11b^+$), and T cells in the CNS after EAE induction with Tconv or exFoxp3 cells were similar (Figure 6C), demonstrating that Tconv and exFoxp3 cells orchestrate similar cellular immune responses during autoimmunity. The majority of Tconv and exFoxp3 cells in the inflamed CNS produced IFN-γ (Figure 6D), which could have initiated the inflammatory cascade that caused EAE in the recipients. These results demonstrate that exFoxp3 cells can function as pathogenic effector cells producing IFN-γ in the CNS and that MOG-specific exFoxp3 cells induce EAE similarly to Tconv cells.

IL-2R Signaling Overrides Foxp3 Instability

IL-2 is critical for expression of Foxp3 in Treg cells and the homeostasis of Treg cells in the periphery. It has been previously shown that targeting IL-2 to Treg cells with low concentrations of IL-2-anti-IL-2 complexes prevents or reverses autoimmune diabetes and delays onset and severity of EAE by causing Treg cell expansion (Tang et al., 2008; Webster et al., 2009). We hypothesized that supplementing antigen-experienced Treg cells with exogenous IL-2 would stabilize Foxp3 expression in MOG-specific Treg cells and would thus prevent the de novo generation of exFoxp3 cells. Treatment with IL-2-anti-IL-2 complexes after immunization induced a unimodal increase in phosphorylated STAT5 levels in Treg cells but did not affect phosphorylated amounts of STAT5 in Tconv cells or the majority of exFoxp3 cells (Figure S5). IL-2-anti-IL-2 complex treatment resulted in a significant reduction in the proportion and number of MOG_{38-49}-specific Treg cells that lost Foxp3 expression and became exFoxp3 cells, but it did not affect the abundance of polyclonal exFoxp3 cells (Figures 7B–7D), which is consistent with the absence of phosphorylated STAT5 in these cells in response to treatment (Figure S5). As expected, there was a trend toward increased numbers of MOG_{38-49}-specific and polyclonal Treg cells (Tang et al., 2008; Webster et al., 2009); however, it was striking that the only significant difference was stabilized Foxp3 expression in MOG_{38-49}-specific Treg cells after treatment with IL-2-anti-IL-2 complexes (Figure 7). We conclude that Treg cell expansion does not result in Foxp3 instability per se because the increase in number of MOG_{38-49}-specific Treg cells did not result in increased numbers of exFoxp3 cells but rather a

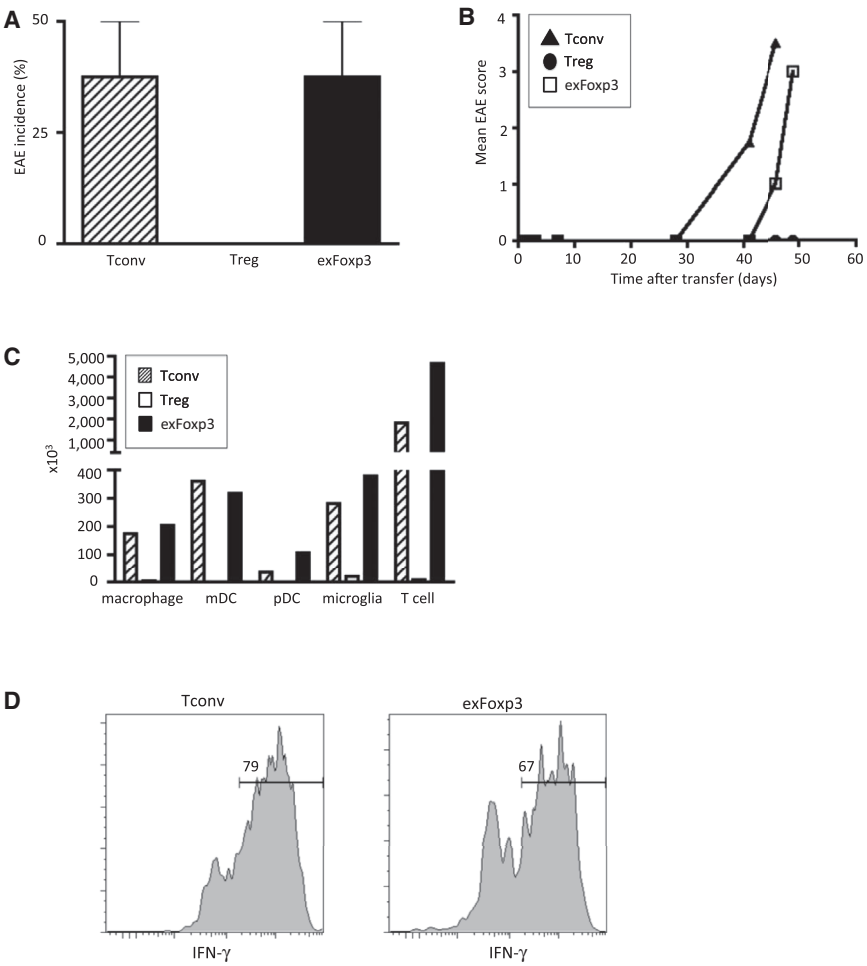

Figure 6. exFoxp3 Cells Cause EAE

(A) The proportion of mice that developed EAE after transfer of CD4$^+$RFP$^-$GFP$^-$ Tconv, RFP$^+$GFP$^+$ Treg, and RFP$^+$GFP$^-$ exFoxp3 cells. Data were pooled from two experiments with three mice per group and represent the mean ± SEM.

(B) EAE clinical score of mice that developed EAE as described in (A) in one of two experiments.

(C) The number of the indicated inflammatory cell populations in the spinal cords of mice shown in (B). Data are representative of two experiments.

(D) Staining and percentage of IFN-γ in CNS T cells from (B).

See also Figure S4.

sharp reduction in the frequency of these cells. We also conclude that Foxp3 instability in Treg cells responding to in vivo antigen is IL-2 sensitive. In a previous study, Webster et al. (2009) treated EAE prophylactically with IL-2 complexes to protect from disease; the conclusion was that the IL-2-expanded polyclonal Treg cells suppressed the development of the autoreactive effector T cell response during priming for EAE. The goal of our experiments was to target Treg cell instability, which, given the enrichment of MOG_{38-49}-reactive exFoxp3 cells compared with polyclonal cells, occurred within the initial 7 days after EAE induction (Figures 1C and 1D). Treg cells were targeted with IL-2-anti-IL-2 complexes in the 3 days after EAE induction, the MOG_{38-49}-reactive Treg cells retained high levels of Foxp3 expression, and the mice were protected from disease (Figure 7G), suggesting that the stability of antigen-specific Treg cells might be important for the course and control of autoimmunity.

DISCUSSION

Using lineage reporter and "fate-mapping" mice to characterize Foxp3 expression and downregulation within the same cell over-time, we previously showed that a subset of cells expressing Foxp3 could become Foxp3^{lo} (exFoxp3 cells), and previous studies by our group (Zhou et al., 2009) have led to the hypothesis that a subset of Treg cells could demonstrate highly reduced levels of Foxp3 and become functionally unstable in the context of autoimmunity and inflammation. However, this interpretation has been challenged by other studies, which concluded that "terminally differentiated" Treg cells are stable and exFoxp3 cells would instead derive from loosely committed Treg cells that have not fully acquired many features of bona fide Treg cells, such as high amounts of Foxp3 and CD25 and demethylation at the TSDR (Rubtsov et al., 2010; Miyao et al., 2012). Here, we address these apparently contradictory results in a model of autoimmunity by demonstrating that loss of Foxp3 and the resulting instability do occur in bona fide Treg cells but predominantly in a subset of self-antigen-specific Treg cells during responses initiated by immunization with this self-antigen. There is indeed evidence of a percentage of exFoxp3$^+$ cells derived from cells transiently expressing Foxp3, but these cells are not derived from the autoantigen-specific T cell population induced during EAE.

Rather, the Treg cells followed in this autoimmune setting expressed high amounts of CD25 and had a demethylated TSDR, markers that identify Treg cells that in the steady state maintained high levels of Foxp3 expression under lymphoreplete conditions in other studies (Miyao et al., 2012; Rubtsov et al., 2010). However, these studies were largely performed in the steady state and under a homeostatic setting where the immune system had not been perturbed by autoimmunity. Importantly, these studies did not focus on selective, antigen-specific Treg cells expanded in response to an inflammatory autoimmune reaction. We addressed whether committed Treg cells lose Foxp3 during a mouse model of autoimmunity, EAE, by interrogating antigen-specific Treg cells within the polyclonal repertoire. Using lineage tracing and cell transfers, we found that although the majority of polyclonal and antigen-specific Treg cells stably expressed Foxp3 during the autoimmune response in this mouse model of autoimmunity, a substantial fraction of antigen-specific Treg cells with "signature" features (Foxp3^{hi},

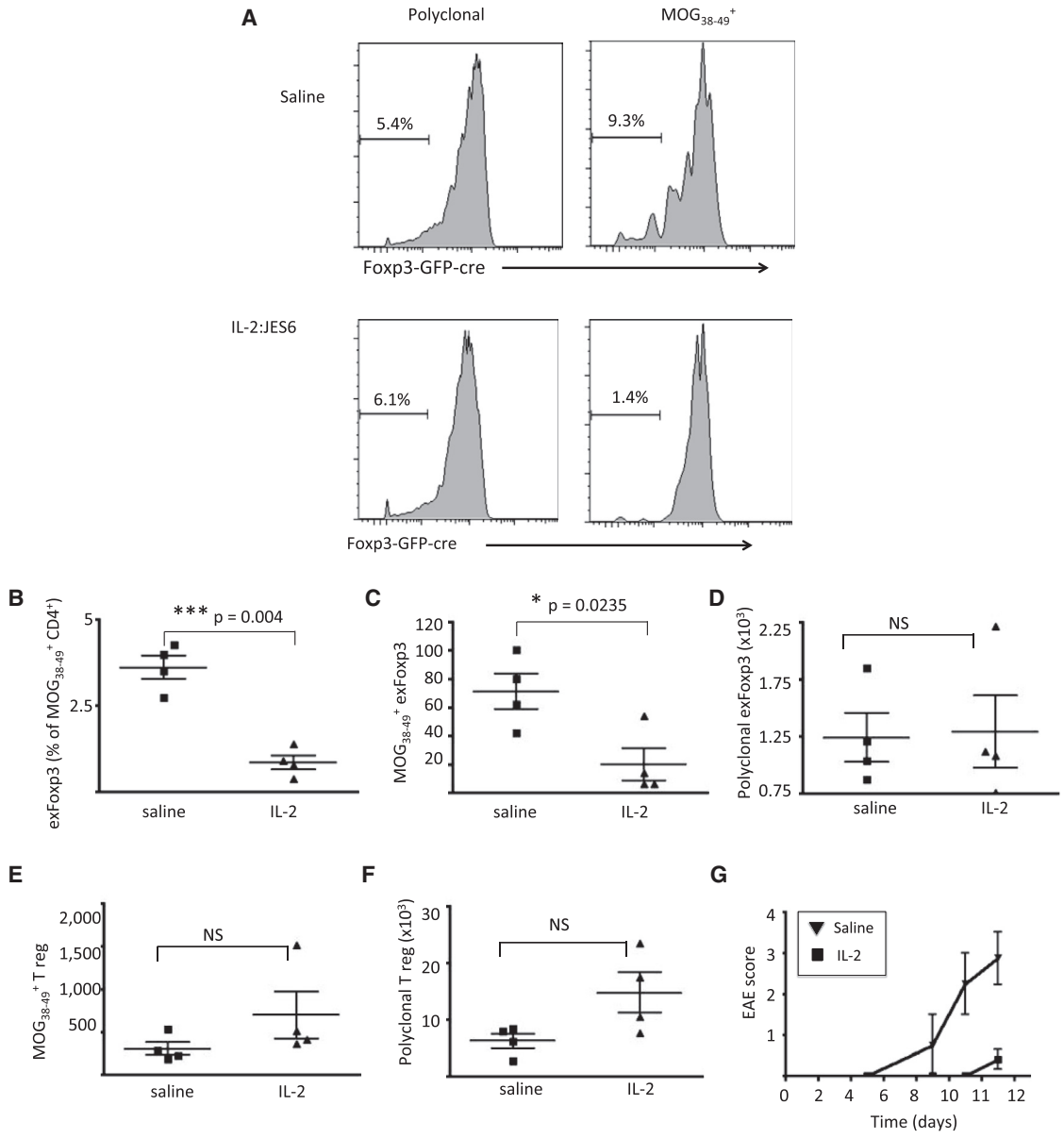

Figure 7. IL-2 Stabilizes Foxp3 during EAE

(A) Foxp3.GFP levels in polyclonal and MOG_{38-49}-specific $CD4^+RFP^+$ T cells from LNs and spleen 6 days after EAE induction and treatment with IL-2-anti-IL-2 complex or saline control on days 1, 3, and 5.

(B–F) Abundance of $CD4^+$ T cell populations in LNs and spleen from mice receiving saline or IL-2-anti-IL-2 complex treatment as in (A). (B) Percentage of $RFP^+GFP^-exFoxp3^+$ cells in MOG_{38-49}-reactive cells. (C) Frequency of MOG_{38-49}-reactive $RFP^+GFP^-exFoxp3^+$ cells. (D) Frequency of polyclonal $RFP^+GFP^-exFoxp3^+$ cells. (E) Frequency of MOG_{38-49}-reactive RFP^+GFP^+ Treg cells. (F) Frequency of polyclonal RFP^+GFP^+ Treg cells. Each point represents one sample, and lines represent the mean ± SEM of the pooled data. p values are from two-tailed t tests.

(G) EAE course with IL-2-anti-IL-2 complex treatment on days 1, 3, and 5. Triangles depict control (saline)-treated animals, and squares represent IL-2-treated animals. Data represent the mean ± SEM from two experiments with five animals in each.

See also Figure S5.

$CD25^{hi}$, demethylated TSDR) downregulated *Foxp3* transcription, lost Foxp3, and acquired effector T cell characteristics such as IFN-γ production and pathogenic potential in vivo. It should be highlighted that the exFoxp3 cells were only apparent in adoptive transfer experiments where bona fide $CD4^+CD25^{hi}$ Treg cells with a demethylated TSDR were transferred into mice with ongoing EAE. In some animals, up to one-third of the MOG $tetramer^+$ antigen-specific Treg cells lost Foxp3 expression. In contrast, transfer of highly purified $CD4^+GFP^-RFP^-$ Tconv cells did not result in any antigen-specific exTreg cells, suggesting that this population did not derive from a differentiating transient $Foxp3^+$ subset. These findings link Foxp3 instability in an antigen-specific subset of Treg cells to pathogenic responses during autoimmunity. In this regard, circulating

Treg cells that have altered characteristics including reduced suppressive ability, production of IFN-γ, and other features of Th1 cells are enriched in type 1 diabetes and multiple sclerosis in humans (Dominguez-Villar et al., 2011; McClymont et al., 2011). Interestingly, stable Foxp3 expression resumed during the remission phase of disease or after treatment with IL-2-anti IL-2 complexes that also ameliorate disease, suggesting potential therapeutic opportunities in these areas.

Even in the inflamed setting, a large number of MOG_{38-49}-specific Treg cells retained high amounts of Foxp3. This most likely reflects positive feedback of Foxp3 on its own expression (Zheng et al., 2010), and we hypothesize that once a low threshold of *Foxp3* transcription is reached, the Treg cell transcriptional landscape is lost and characteristics of exFoxp3 cells (e.g., loss of Foxp3 and acquisition of IFN-γ expression) become apparent (Zheng et al., 2007). Induced Treg cells or loosely committed $Foxp3^+$ cells are prone to Foxp3 instability and demonstrate a failure to imprint stable epigenetic marks at the *Foxp3* locus (Haribhai et al., 2011; Josefowicz et al., 2012, Ohkura et al., 2012). We thus investigated whether the Treg cells that downregulate *Foxp3* transcription during EAE might be predisposed because of a methylated TSDR. In two out of three mice, Foxp3 downregulation occurred predominantly in antigen-specific Treg cells with a demethylated TSDR, suggesting that lack of epigenetic imprinting in this region of the *Foxp3* locus is not a leading cause of loss of Foxp3 expression in Treg cells during EAE. In the third mouse, the antigen-specific exFoxp3 cells had a methylated TSDR, suggesting that the exFoxp3 cells derived from loosely committed Treg cells perhaps as a consequence of the local inflammatory environment, as previously reported (Miyao et al., 2012). Thus, exFoxp3 cells derive from bona fide Treg cells and loosely committed cells in individual mice, which could reflect differences in the local milieu that influence lineage differentiation and stability at the population level. The TSDR data further suggest that the majority of polyclonal exFoxp3 cells arise from transient or unstable Foxp3 expression (Miyao et al., 2012). The factors inducing Foxp3 instability in a subset of antigen-specific Treg cells remain unclear. Low CD25 expression and a loss of STAT5-driven Foxp3 expression have been implicated in Foxp3 instability during homeostasis (Miyao et al., 2012), but this does not appear to be the case in the generation of MOG-specific exFoxp3 cells during EAE. Treg cells responded to the IL-2 administered with unimodal phosphorylation of STAT5, suggesting that Treg cells are able to respond to IL-2 during EAE. Rescue of Foxp3 expression with IL-2-anti-IL-2 complexes suggests a deficiency in IL-2 available to Treg cells in vivo. Treg cells compete with T effector cells for local IL-2 during an immune response (O'Gorman et al., 2009), which is required for efficient Treg cell homeostasis (Barron et al., 2010; Zorn et al., 2006). The development of a strong effector cell response that consumes IL-2 might tip the balance of stability in Treg cells responding to antigen in a microenvironment with T effector cells. Consistent with this hypothesis, other studies have shown that Treg cell instability occurs during strong Th1-polarized responses (Oldenhove et al., 2009; Takahashi et al., 2011), and IL-2 deficiency has been implicated in one study (Oldenhove et al., 2009). IL-2-anti-IL-2 complex stabilization of Foxp3 in Treg cells correlated with reduced CNS inflammatory disease, further supporting that Treg cell instability is detrimental for tissue-specific tolerance. In this regard, autoimmune diabetes has also been associated with a local deficiency in Treg-cell-mediated immunoregulation and low Foxp3 expression in islet-infiltrating Treg cells (Tang et al., 2008; Zhou et al., 2009). Moreover, IL-2 therapy restored Foxp3 expression in pancreatic Treg cells and could prevent or cure new-onset diabetes in NOD mice (Grinberg-Bleyer et al., 2010; Tang et al., 2008), suggesting that local deficits in IL-2 signaling might be a general mechanism leading to downregulation of Foxp3 and Treg cell instability in autoimmune diseases.

Our data suggest that Foxp3 instability in Treg cells is induced and pronounced after antigen triggering and during inflammatory responses. Treg cell stability mirrors the resolution of autoimmune inflammatory disease. It is tempting to speculate that Treg cell stability drives the regulation of autoimmune responses. Recent studies of antigen-specific Treg cells have demonstrated a rapid expansion of Treg cells with a second wave of antigen availability (Rosenblum et al., 2011, Rowe et al., 2012). In line with a "priming and memory" model for Treg cells, we have shown that Treg cell instability occurs during the acute phases of inflammation, and this could remove inefficient, unstable Treg cells from the pool. The stable Treg cells that remain might be better equipped to control further flares of inflammation; indeed, Treg cell stability correlated with EAE resolution. We determined that the frequency of MOG-specific exFoxp3 cells declined during the peak and resolution phases and increased in the peripheral LNs during the same time frame. It is possible that the unstable Treg cells emigrate from the CNS during the decline of inflammation and take up residence in the secondary LN organs, as do effector cells during relapsing remitting EAE, although they have no further pathogenic consequence to the progression of the disease (Vanderlugt et al., 2000). Thus, instability at the individual cell level in a subset of Treg cells could be followed by improved stability and regulation at the population level in certain circumstances.

Treg-cell-derived MOG-specific exFoxp3 cells presented an effector cell phenotype with IFN-γ production and pathogenic potential after restimulation with their cognate antigen in vitro or in vivo. Although exFoxp3 cells are 10% of the frequency of Tconv cells that produce IFN-γ in the CNS during EAE, the local production of IFN-γ can provide amplifying feedback on local conventional Th1 cells (Takahashi et al., 2011). Indeed, exFoxp3 cells producing IFN-γ cause EAE with a severity similar to that of Tconv cells. Teleologically, it is possible that Foxp3 instability during normal immune responses could aid the generation of an antipathogen response, and an increase in protective cytokine production early in the response could provide a boost to protective immunity. However, as highlighted in this study, loss of Foxp3 correlates with, and might contribute to, the development of autoimmunity. Treg cell therapies are being tested in clinical trials in type 1 diabetes and GVHD, raising concerns that Treg cell instability could lead to unwarranted effects in patients and indicating that additional studies will be needed for determining the factors leading to instability. Moreover, Treg cell therapy might require the cover of a stabilizing factor such as IL-2, which has been used for directly modulating the frequency and proliferation of Treg cells in patients (Long et al., 2012; Koreth et al., 2011; Zorn et al., 2006).

EXPERIMENTAL PROCEDURES

Mice

Foxp3.GFP-Cre.ROSA26-YFP reporter mice have been described previously (Zhou et al., 2009) and were backcrossed greater than eight generations onto the B6 background. For the generation of new lineage reporter mice that had less spectral overlap than GFP versus YFP, allowing improved purification by flow cytometric sorting, B6 Foxp3.GFP-Cre mice (Zhou et al., 2009) and Foxp3.YFP.Cre mice (Rubtsov et al., 2008) were crossed with B6 ROSA26-RFP reporter mice (Luche et al., 2007). B6 TCR$\alpha^{-/-}$ mice were obtained from Jackson Laboratories. All mice were housed and bred under specific pathogen-free conditions at the University of California, San Francisco Animal Barrier Facility. The Institutional Animal Care and Use Committee of the University of California, San Francisco approved all animal experiments.

Antibodies

Labeled antibodies specific to CD4 (RM4-5), CD8 (Ly-2), CD25 (PC61), GITR (DTA-1), CD44 (pgp-1), FoxP3 (FJK-16 s), IL-17A (eBio17B7), IFN-γ (XMG1.2), phosphorylated STAT5 (pY694), and specific isotype controls were purchased from BD PharMingen or eBioscience. Intracellular GFP or YFP was stained with anti-GFP (rabbit polyclonal eBioscience catalog no. 14-6774-81) and Fab'2 anti-rabbit (goat polyclonal eBioscience catalog no. 11-4839-81).

Flow Cytometry

Stained single-cell suspensions were analyzed with a LSRII flow cytometer running FACSDiva (BD Biosciences), and FSC 2.0 files were analyzed and presented with FLOWJO Software (TreeStar). T cells were sorted with a MoFlo high-speed cell sorter (DakoCytomation) or FACSAria (BD Biosciences).

Phosphorylated STAT5 Flow

Spleen cells were harvested directly into fixative for ex vivo analysis of STAT5 phosphorylation, and staining was performed as described in O'Gorman et al. (2009).

Induction of EAE

B6 mice were immunized subcutaneously with 100 μl of emulsified CFA (BD Difco) supplemented with 4 mg/ml Mycobaterium tuberculosis H37Ra (BD Difco) and 200 μg MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK, Genemed Synthesis) and received intraperitoneal injections of 200 ng pertussis toxin from Bordetella pertussis (Sigma Aldrich) at the time of immunization and 48 hr later. Clinical disease was assessed by the scoring of ascending hind-limb paralysis as follows: no signs, score 0; flaccid tail, score 1; hind-limb weakness, score 2; partial hind-limb paralysis, score 3; complete hind-limb paralysis, score 4; and moribund mouse, score 5.

Transfer of CD4$^+$ Treg and Tconv Cells into EAE for Tracking Foxp3 Stability

Donor mice congenic for CD45.1 were immunized with MOG_{35-55} peptide emulsified in CFA similar to EAE induction; no pertussis toxin was administered. Five days after immunization, Treg cells were sort purified from axillary, inguinal, and brachial LNs and spleen and were gated as CD45.1$^+$, CD4$^+$, CD25^{hi}, or GITR^{hi}. For Foxp3-lineage tracing of Tconv cells, CD45.1$^+$ CD4$^+$GFP$^-$RFP$^-$ T cells were sort purified from axillary, inguinal, and brachial LNs and spleen 5 days after immunization of Foxp3.GFP.RFP reporter mice. A total of 2 × 10^6 CD45.1$^+$ Treg cells or CD4$^+$ Tconv cells were transferred intravenously into CD45.2 mice at the onset of EAE. At the peak of EAE, the CD45.1$^+$ transferred cells were analyzed for MOG-tetramer staining in the CNS as described below.

CD4$^+$ Tconv, Treg, and exFoxp3 Cell Expansion and Adoptive Transfer for EAE

Eight days after EAE induction in Foxp3-GFP.RFP reporter mice, CD4$^+$ T cell populations were sort purified from axillary, inguinal, and brachial LNs and spleen. Viable T cells were gated as CD4$^+$ cell, Treg cells were gated as GFP$^+$ RFP$^+$ cells, exFoxp3 cells were gated as GFP$^-$RFP$^+$ cells, and Tconv cells were gated as GFP$^-$RFP$^-$ cells. T cells were cultured at 2 × 10^5 cells/ml plus five times the number of lethally irradiated congenically marked splenocytes as feeder cells, 20 μg/ml MOG_{35-55}, and growth-factor cytokines: Tconv cells, 100 U/ml IL-2; Treg cells, 2,000 U/ml IL-2; and exFoxp3 cells, 2,000 U/ml IL-2 and 10 ng/ml IL-7. Growth-factor cytokines were refreshed every 2 days for 11 days. Cells were replated at 2 × 10^5 cells/ml and polyclonally expanded for 7 days with 1:1 bead:cell T-activator CD3/CD28 beads (Dynabeads, Life Technologies) and growth-factor cytokines as above. After 7 day, viable CD4$^+$ T cells were resorted for transfer as described above and shown in Figure S4. Sublethally irradiated (350 rads) B6 TCR$\alpha^{-/-}$ mice received 350,000–500,000 T cells intravenously. Recipients were immunized with MOG_{35-55}-CFA on the day of cell transfer and 7 days later. Mice were scored for EAE in a blind fashion every 2 days.

IL-2-Anti-IL-2 Complex Treatment

Mice immunized for EAE received an intraperitoneal injection of 1.5 μg recombinant mouse IL-2 (eBioscience) complexed with 5 μg anti-IL-2 (clone JES6-1A12, eBioscience) for 15 min at 37°C on days 1, 3 and 5.

MHC II Tetramer Staining

I-A^b:MOG_{38-49} and I-A^b:hClip tetramers were provided by the National Institutes of Health Tetramer Core Facility. CNS samples were stained with tetramer and analyzed directly, whereas tetramer-labeled LN and spleen samples were enriched for analysis as previously published (Moon et al., 2007).

TSDR Methylation Analysis

Methylation at the TSDR was evaluated by sequencing as described in Zhou et al., (2009) and by quantitative PCR (qPCR) as described in Yadav et al. (2012).

Foxp3 qPCR

To assay endogenous *Foxp3* transcription, we used a qPCR probe that recognizes a sequence in *Foxp3*'s tenth exon, which is not expressed by the BAC-Foxp3.GFP-Cre construct because it expresses a stop codon in exon 1 (Zhou et al., 2008b). RNA was isolated by TRIzol (Life Technologies), and cDNA was obtained with a first-strand cDNA synthesis kit (GE Healthcare Biosciences). qPCR was performed with TaqMan gene expression assays (Mm 00475165-m1, Life Technologies, Applied Biosystems) and analyzed with a 7500 Fast real-time PCR system.

Isolation of CNS Leukocytes

Mice were sacrificed with CO_2 and immediately perfused through the left ventricle with PBS until the effluent ran clear. Spinal cords were extruded by flushing of the vertebral canal with PBS, and cerebella were removed. Spinal cords and cerebella were diced, placed in Hank's balanced salt solution (HBSS) containing 25 mM HEPES, 300 Wunsch units/ml type-D clostridial collagenase (Life Technologies), and 50 μg/ml DNase I (Roche), and incubated for 30 min (37°C). The homogenate was resuspended in 30% isotonic Percoll (Pharmacia), underlaid with 70% Percoll, and centrifuged at 1,160 g at room temperature for 30 min. Mononuclear cells were collected from the Percoll interphase and washed twice in HBSS containing 2% fetal calf serum.

Cytokine Production

CNS mononuclear cells were incubated at 5 × 10^6 cells/ml with 1 μg/ml anti-CD28 (PV-1) with or without 10 μg/ml MOG_{35-55} for 16 hr at 37°C. Cells were activated for 2.5 hr with 50 ng/ml phorbol 12-myristate 13-acetate and 2 μM monensin before labeling with LIVE/DEAD fixable dead stain (Life Technologies), staining for CD11b (dump), CD4, Foxp3, IFN-γ, and GFP or YFP, and flow cytometric analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.10.016.

ACKNOWLEDGMENTS

We thank M. Lee, S. Jiang, and J. Paw for technical assistance; D. Fuentes for animal husbandry; Alexander Rudensky for the Foxp3-YFP-Cre mice; the

National Institutes of Health (NIH) Emory Tetramer Facility for providing I-Ab tetramers; and A. Abbas, Q. Tang, H. Bour-Jordan, M. Anderson, and members of the Bluestone laboratory for discussions. This work was supported by the NIH (R01 AI50834, RO1 AI46643, P01 AI35297, P30DK63720).

Received: March 2, 2012
Accepted: October 30, 2013
Published: November 14, 2013

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