Cell

Control of the Inheritance of Regulatory T Cell Identity by a *cis* Element in the *Foxp3* Locus

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http://dx.doi.org/10.1016/j.cell.2014.07.031

SUMMARY

In multicellular organisms, specialized functions are delegated to distinct cell types whose identity and functional integrity are maintained upon challenge. However, little is known about the mechanisms enabling lineage inheritance and their biological implications. Regulatory T (Treg) cells, which express the transcription factor Foxp3, suppress fatal autoimmunity throughout the lifespan of animals. Here, we show that a dedicated *Foxp3* intronic element *CNS2* maintains Treg cell lineage identity by acting as a sensor of the essential Treg cell growth factor IL-2 and its downstream target STAT5. *CNS2* sustains Foxp3 expression during division of mature Treg cells when IL-2 is limiting and counteracts proinflammatory cytokine signaling that leads to the loss of Foxp3. *CNS2*-mediated stable inheritance of Foxp3 expression is critical for adequate suppression of diverse types of chronic inflammation by Treg cells and prevents their differentiation into inflammatory effector cells. The described mechanism may represent a general principle of the inheritance of differentiated cell states.

INTRODUCTION

Differentiated somatic cells exhibit distinct functions and behaviors that are specified by their developmental programs. In the past two decades, tremendous progress has been achieved in elucidating genetic and epigenetic mechanisms underlying differentiation of specialized cell lineages and organ development. However, little is known about how, and to what degree, the differentiated cells maintain their fate or lose their identity in response to changing environment or upon cell division, the two conditions that may disturb the inheritance of lineage-specifying factors (Sánchez Alvarado and Yamanaka, 2014). Consequently, factors that affect identity and function of a given cell type and molecular basis of their robustness upon environmental perturbations and its biological significance remain poorly understood.

The adaptive immune system with its somatic diversification of antigen receptors of essentially unlimited specificity affords vertebrates with an effective means of defense against previously encountered and new infectious agents. Potentially deleterious self-reactivity and "collateral" damage resulting in an impairment or loss of tissue function has been a tradeoff for the emergence of adaptive immunity. Central to limiting excessive immune responses and associated inflammation is their suppression mediated by regulatory T (Treg) cells, a subset of $CD4^+$ T cells expressing X-chromosome-encoded transcription factor Foxp3. Foxp3 is specifically expressed in Treg cells and plays a key role in their differentiation and function (Josefowicz et al., 2012). During the differentiation of Treg cells, Foxp3 is induced in response to TCR and IL-2 signaling (Josefowicz et al., 2012; Sekiya et al., 2013), and Foxp3 protein expression is required for Treg cell function (Gavin et al., 2007; Lin et al., 2007). In addition to conferring cellular identity and functional competence during differentiation of Treg cells, Foxp3 plays an essential role in their maintenance because deletion of a conditional *Foxp3* allele in differentiated Treg cells results in a loss of their function (Williams and Rudensky, 2007). Genetic fate mapping using inducible and constitutive Cre revealed heritable and stable Foxp3 expression in the Treg cell population in unchallenged mice as well as in the context of infection and autoimmune inflammation (Miyao et al., 2012; Rubtsov et al., 2010). In contrast, almost half of newly generated extrathymic Treg cells lose Foxp3 expression (Josefowicz et al., 2012). Thus, Treg cells represent a distinct cell lineage and Foxp3 is its late acting specification factor whose stable expression is a requisite for preserving Treg cell identity and functional integrity.

These findings also implied the existence of a distinct mechanism that ensures Treg cell lineage stability. A conserved intronic regulatory element *CNS2* is required for the maintenance of Foxp3 expression in the progeny of dividing Treg cells but does not affect Foxp3 induction and its amount on a per cell basis (Zheng et al., 2010). *CNS2* can be bound by numerous transcription factors, including STAT5, STAT3, and Foxp3, but how these factors regulate Foxp3 expression during cell division remains unknown (Samstein et al., 2012; Xu et al., 2010; Yao et al., 2007; Zheng et al., 2010).

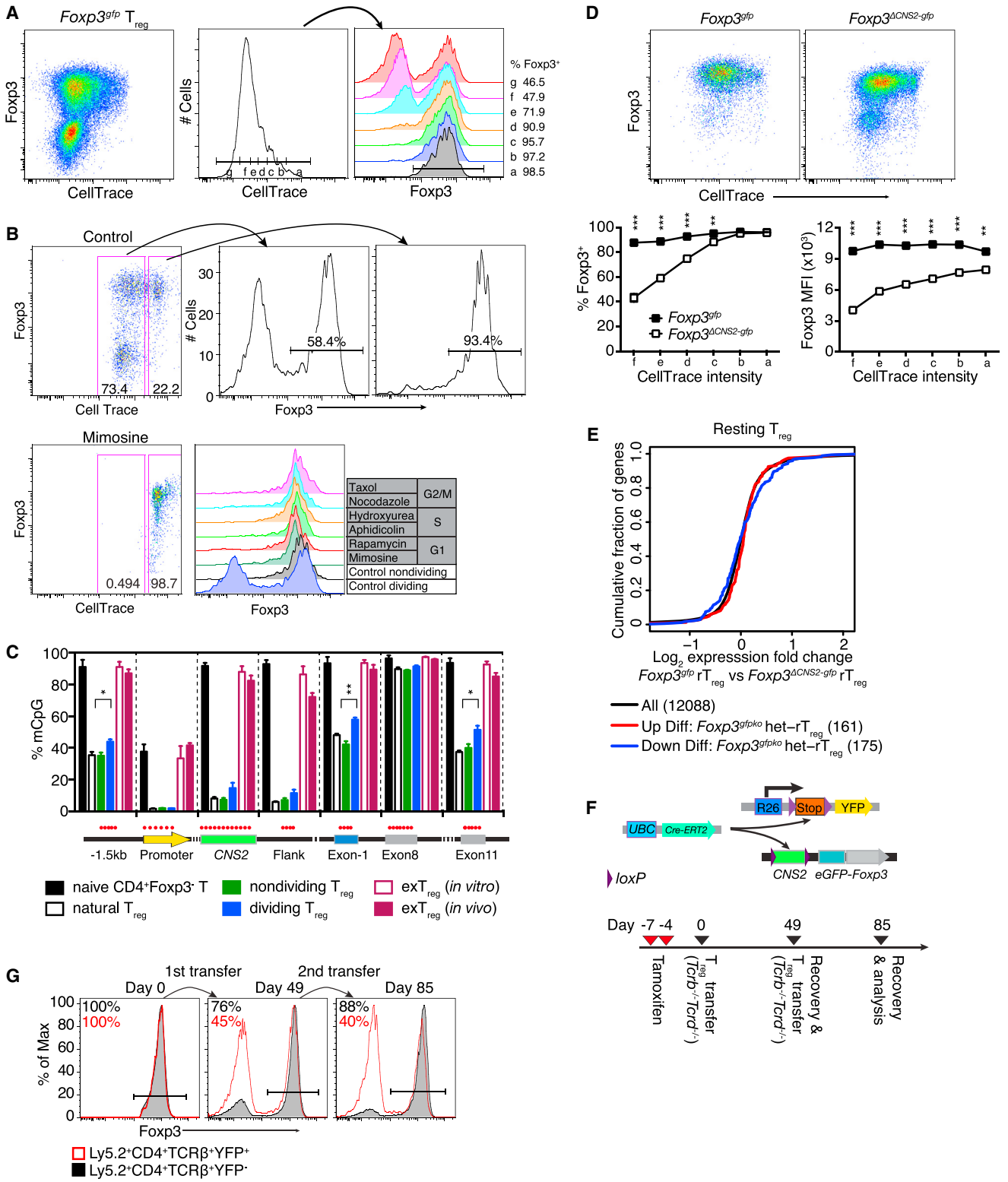


Figure 1. *CNS2* Opposes Cell-Cycle-Dependent Loss of Foxp3 Expression in Mature Treg Cells

(A) Loss of Foxp3 expression during cell division. Highly purified *Foxp3*^{gfp} Treg cells were labeled with CellTrace Violet and were activated by CD3 and CD28 antibody-coated beads in the presence of 200 U/ml IL-2 for 5 days. The data represent one of three independent experiments.

(legend continued on next page)

CNS2 contains a stretch of CpG bases that are fully methylated in precursor cells but undergo demethylation upon Foxp3 expression (Floess et al., 2007; Kim and Leonard, 2007; Polansky et al., 2008; Toker et al., 2013). Previous studies suggested a correlation between the methylated state of *CNS2* and unstable Foxp3 expression (Bailey-Bucktrout et al., 2013; Floess et al., 2007; Polansky et al., 2008). Genetic targeting of the pivotal DNA methyltransferase Dnmt1 or pharmacological inhibition of DNA methyltransferase activity results in a sharp increase in Foxp3 induction efficiency upon activation of naive T cells (Floess et al., 2007; Josefowicz et al., 2009; Kim and Leonard, 2007).

Despite a considerable body of work, the biological role of *CNS2* in the regulation of Foxp3 expression has not been elucidated, and a mechanistic understanding of *CNS2* function and its biological role are lacking. Here, we demonstrated that *CNS2* serves as a sensor of IL-2/STAT5 signaling that prevents Treg conversion into effector T cells upon exposure to proinflammatory cytokines. *CNS2* conferred stable inheritance of Foxp3 expression at limiting amounts of IL-2, which was of particular significance for control of chronic inflammation in a wide range of biological contexts.

RESULTS

Heritability of Foxp3 Expression and CpG Methylation in the *Foxp3* Locus

Lymphopenic or proinflammatory conditions are associated with the instability of Treg cells; however, its cause and consequences have not been examined (Bailey-Bucktrout et al., 2013; Komatsu et al., 2013). We observed accumulation of Foxp3$^-$ cells (exTreg) upon Treg cell division in response to TCR stimulation in vitro. This was unlikely due to the contamination with effector T cells because the starting population contained 99.8% Foxp3$^+$ cells (Figure 1A and data not shown). To demonstrate that the loss of Foxp3 was due to division of Treg cells, we blocked cell-cycle progression using pharmacological inhibitors targeting different stages of the cell cycle. The cell-cycle blockade fully rescued Foxp3 expression (Figure 1B), suggesting that its loss was indeed cell-cycle dependent.

To investigate whether CpG methylation (mCpG) plays a role in the regulation of heritable Foxp3 expression, we examined the mCpG levels across the *Foxp3* locus by bisulfite sequencing in resting and dividing Treg cells as compared to non-Treg cells that do not express Foxp3 or "exTreg" cells that have lost Foxp3 expression either in vitro or in vivo. In agreement with previous reports, −1.5 kb, *CNS2*, and exon −1 were methylated in non-Treg cells and were largely or fully demethylated in Treg cells (Floess et al., 2007; Ohkura et al., 2012) but were remethylated in "exTreg" cells that have lost Foxp3 expression either in vivo or in vitro. Importantly, we found a statistically significant increase in mCpG at sites including the −1.5 kb region, *CNS2*, and exon-1 in dividing Foxp3$^+$ Treg cells (Figure 1C and Figures S1A–S1C available online). Accordingly, low dose of a DNA methyltransferase inhibitor 5-aza-deoxycytidine (5-aza-dC) prevented loss of Foxp3 expression in Treg cells that underwent a comparable number of cell divisions in the presence or absence of the drug (Figure S1D). These findings suggest that CpG methylation might be actively regulated when Treg cells divide and may affect the heritability of Foxp3 expression.

CNS2 Opposes Cell-Cycle-Dependent Loss of Foxp3 Expression in Mature Treg Cells

Studies of *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice on a mixed genetic background showed that *CNS2* maintains Foxp3 expression in Treg cells (Zheng et al., 2010). To explore the biological role of *CNS2* and the molecular mechanisms of its function in depth, we backcrossed *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice onto a C57BL/6 (B6) background (n > 10) and bred them to *CNS2*-sufficient *Foxp3*^{gfp} B6 mice expressing the exact same *Foxp3* reporter allele, which serves as an ideal control when segregated into male littermates (Bettini et al., 2012; Darce et al., 2012). In agreement with previous results on a mixed genetic background (Zheng et al., 2010), significantly more Treg cells isolated from *Foxp3*$^{\Delta CNS2\text{-}gfp}$ B6 mice with the germline *CNS2* deficiency lost Foxp3 expression upon division in comparison to *CNS2*-sufficient *Foxp3*^{gfp} B6

(B) Blockage of cell division prevents loss of Foxp3 expression. Flow cytometric analysis of Foxp3 expression in Treg cells treated with indicated inhibitors in comparison to control nondividing and dividing Treg cells. The data represent one of three independent experiments.

(C) CpG methylation (mCpG) levels in the *Foxp3* locus during Treg cell division in vitro and after loss of Foxp3 expression (exTreg) in vitro and in vivo. mCpG levels in biological replicate samples were averaged at each site of the *Foxp3* locus from >5,000 reads in naive CD4$^+$Foxp3$^-$ T cells (n = 2), ex vivo isolated natural Treg cells (n = 3), nondividing (n = 3) and dividing (n = 3) Treg cells, and in vitro exTreg (n = 4) and in vivo exTreg (n = 2) cells. Red dots represent individual CpG sites. Mean ± SEM; t test comparisons of mCpG in nondividing and dividing Treg cells are shown (see also Figures S1A–S1C).

(D) *CNS2* sustains heritable Foxp3 expression in dividing mature Treg cells. Highly purified Treg cells from *Foxp3*^{gfp} and *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice were cocultured in the presence of CD3 and CD28 antibody-coated beads and 500 U/ml IL-2 for 4–5 days. The data represent one of more than four independent experiments. Gating on CellTrace intensity is similar to (A). Mean ± SEM.

(E) Similar gene expression profiles of *CNS2*-deficient and -sufficient resting Treg cells (rTreg). Poly(A) RNA libraries were generated using FACS-sorted CD44^{low}CD62L^{high} rTreg cells from male *Foxp3*^{gfp} and *Foxp3*$^{\Delta CNS2\text{-}gfp}$ littermates. Relative gene expression levels (cumulative fraction of genes) in *CNS2*-sufficient and -deficient rTreg cells were compared to those up- and downregulated in GFP$^+$ cells ("Treg wannabes") from *Foxp3*$^{gfpko/WT}$ heterozygous females and wild-type rTreg cells (n = 3 each). The numbers of genes in each comparison group are indicated in parentheses.

(F) Acute deletion of *CNS2* in mature Treg cells was induced upon tamoxifen treatment of *Foxp3*$^{CNS2fl\text{-}gfp}$ UBC$^{Cre\text{-}ERT2}$ R26Y male mice 7 days before the adoptive cell transfer. Treg (GFP$^+$) cells were sorted by FACS and were transferred together with allelically marked naive CD4$^+$Foxp3$^-$ T cells into T-cell-deficient hosts (*Tcrb*$^{-/-}$ *Tcrd*$^{-/-}$). At 7 weeks after transfer, Foxp3 expression was analyzed and the remaining Foxp3$^+$ (GFP$^+$) Treg cells were purified by FACS and cotransferred with newly isolated naive CD4$^+$Foxp3$^-$ T cells into secondary *Tcrb*$^{-/-}$ *Tcrd*$^{-/-}$ recipients. Foxp3 expression was assessed 5 weeks later.

(G) Flow cytometric analysis of Foxp3 expression before cell transfer and after recovery. Transferred YFP$^+$ and YFP$^-$ Treg cells were originally isolated from tamoxifen-treated *Foxp3*$^{CNS2fl\text{-}gfp}$ UBC$^{Cre\text{-}ERT2}$ R26Y and *Foxp3*$^{CNS2fl\text{-}gfp}$ R26Y mice, respectively. The data represent one of more than three independent experiments.

See also Figure S1.

littermates (Figure 1D). To exclude a possibility that *CNS2* may have a direct impact on the expression of genes other than *Foxp3* through long-range chromatin interactions that could contribute to impaired Foxp3 maintenance, we performed RNA-seq gene expression analysis of *CNS2*-sufficient and -deficient resting Treg cells. Comparable gene expression observed in these *CNS2*-sufficient and -deficient Treg cell populations (Figure 1E) indicated that *CNS2* has a direct and specific role in the maintenance of *Foxp3* expression and Treg cell fate in dividing activated Treg cells.

These findings raised the question of at which stage of Treg cell differentiation *CNS2* exerts its function. To address this, we deleted a conditional $CNS2^{fl}$ allele in mature Treg cells. Treg cells expressing $Foxp3^{gfp}$ reporter that underwent tamoxifen-induced Cre-ER ($UBC^{Cre\text{-}ERT2}$)-mediated *CNS2* deletion were tagged by YFP through the concomitant activation of R26Y recombination reporter allele (Figure 1F). YFP expression was highly correlated with *CNS2* deletion, with only 4.7% ± 0.6% YFP-tagged cells retaining the unrecombined allele as measured by quantitative PCR (qPCR) (data not shown). To test the role of *CNS2* in the maintenance of Foxp3 expression in mature Treg cells in vivo, we transferred highly purified YFP^+GFP^+ Treg cells (>99.8% purity) from $Foxp3^{CNS2fl\text{-}gfp}$ $UBC^{Cre\text{-}ERT2}$ R26Y mice into T-cell-deficient $Tcrb^{-/-}Tcrd^{-/-}$ recipients together with allelically marked naive $Ly5.1^+$ $CD4^+Foxp3^-$ T cells and assessed Foxp3 expression 7 weeks after transfer. Because ~50% GFP^+YFP^- Treg cells in tamoxifen-treated $Foxp3^{CNS2fl\text{-}gfp}$ $UBC^{Cre\text{-}ERT2}$ R26Y mice lost *CNS2* and the R26Y underreported recombination at the *Foxp3* locus (data not shown), we used YFP^-GFP^+ Treg cells sorted from untreated $Foxp3^{CNS2fl\text{-}gfp}$ $UBC^{Cre\text{-}ERT2}$ R26Y mice or from tamoxifen-treated $Foxp3^{CNS2fl\text{-}gfp}$ R26Y mice as negative controls. Consistent with our previous findings, only a minor portion of control Treg cells lost Foxp3 expression when analyzed 7 weeks after transfer (Figures 1G, S1E, and S1F) (Rubtsov et al., 2010). In contrast, >55% of the progeny of Treg cells subjected to acute *CNS2* ablation lost Foxp3 expression. Because we transferred the majority of Treg cells with unrecombined $CNS2^{fl}$ and the minority with ablated $CNS2^{fl}$ cells, or control $Foxp3^{gfp}$ cells, the experimental and control groups of recipient mice remained healthy. Thus, *CNS2* maintains Foxp3 expression in mature Treg cells.

The observation that only some mature Treg cells lost Foxp3 expression raised a question of whether the Treg cell population is heterogeneous in its reliance on *CNS2* for Foxp3 maintenance, with subsets of cells being dependent or independent of *CNS2*. It was possible, for example, that the loss of *CNS2* affected only $CD25^{low}$ Treg cells prone to Foxp3 loss (Komatsu et al., 2009; Komatsu et al., 2013). Such a scenario implied that *CNS2* acted in a deterministic manner to maintain Foxp3 expression in a *CNS2*-dependent Treg cell subset and that *CNS2*-deficient Treg cells that retained Foxp3 expression after homeostatic expansion were independent of *CNS2* for Foxp3 maintenance. Alternatively, it was possible that *CNS2* was required for the maintenance of Foxp3 expression in the entire population of dividing Treg cells, but Foxp3 was lost in a stochastic manner. To explore these alternatives, we isolated *CNS2*-sufficient and -deficient YFP^+GFP^+ Treg cells 7 weeks after the first transfer and transferred them again into T cell-deficient recipients. Five weeks after secondary transfer, we observed an essentially identical pattern to that of the primary transfer, i.e., ~60% loss of Foxp3 expression in the absence of *CNS2* (Figures 1F and 1G). These results suggested that the loss of Foxp3 expression in mature *CNS2*-deficient Treg cells was an apparently stochastic event associated with their division.

CNS2 Sustains Heritable Foxp3 Expression in Mature Treg Cells at Limiting Amounts of IL-2

We reasoned that the observed loss of Foxp3 by some Treg cells was either due to the deprivation of a factor(s) that is required for the maintenance of Foxp3 expression and is present in limiting amounts or due to the exposure to a limiting factor(s) that represses Foxp3 expression. IL-2 and STAT5 signaling plays an important role in the regulation of Foxp3 expression (Fontenot et al., 2005; Sakaguchi et al., 1995). Our recent cell fate mapping studies showed that IL-2 deprivation caused by administration of IL-2-neutralizing antibody results in a loss of Foxp3 expression by only a minor subset (~15%) of YFP-tagged Treg cells (Rubtsov et al., 2010), though this may be an underestimate because IL-2/STAT5 signaling facilitates Treg cell viability. Thus, IL-2 could be a factor that "positively" acts via *CNS2* and whose deprivation leads to a pronounced loss of Foxp3 expression in the absence of *CNS2*. Indeed, *CNS2* contains multiple STAT5-binding motifs and can be bound by STAT5 (Figure S1A) (Ogawa et al., 2013; Yao et al., 2007).

Previous studies suggested that Treg cells expressing low amounts of CD25, a subunit of high-affinity IL-2 receptor, are unstable, whereas $CD25^{high}$ Treg cells faithfully maintain Foxp3 expression. Thus, we sought to examine whether *CNS2* is required for the maintenance of Foxp3 expression only in $CD25^{low}$ or in both $CD25^{low}$ and $CD25^{high}$ Treg cells in vivo that are less or more efficient in IL-2 capture and signaling, respectively, as assessed by STAT5 phosphorylation (Figure S2A). Upon transfer into lymphopenic host, both $CD25^{high}$ and $CD25^{low}$ *CNS2*-deficient Treg cells showed markedly reduced ability to maintain Foxp3 expression upon cell division, compared to their wild-type counterparts, with $CD25^{low}$ *CNS2*-deficient Treg cells exhibiting the most pronounced Foxp3 loss (Figures 2A and S2B). These results raised the possibility that increased IL-2 signaling may rescue, at least in part, the loss of Foxp3 expression.

We directly tested this idea by asking whether provision of high amounts of IL-2 can rescue, at least in part, the loss of Foxp3 in *CNS2*-deficient Treg cells. Upon TCR engagement, *CNS2*-deficient Treg cells that divided multiple times ($\geq$3, denoted as "dividing") largely maintained Foxp3 expression in the presence of high amounts of IL-2, whereas at lower IL-2 levels, a marked reduction in Foxp3 expression was observed on a per cell basis as well as in the percentage of $Foxp3^+$ cells (Figures 2B and 2C). Expectedly, *CNS2*-deficient Treg cells that did not divide or divided less than three times ("nondividing") did not significantly change Foxp3 expression in comparison to control Treg cells. Reduced Foxp3 amounts on a per cell basis observed in *CNS2*-deficient Treg cells in vivo and upon their division in vitro were likely due to an incomplete Foxp3 protein turnover in Treg cells with the silenced *Foxp3* locus (Figures 1D, 2B, and 2C). Importantly, similar analysis of mature Treg cells

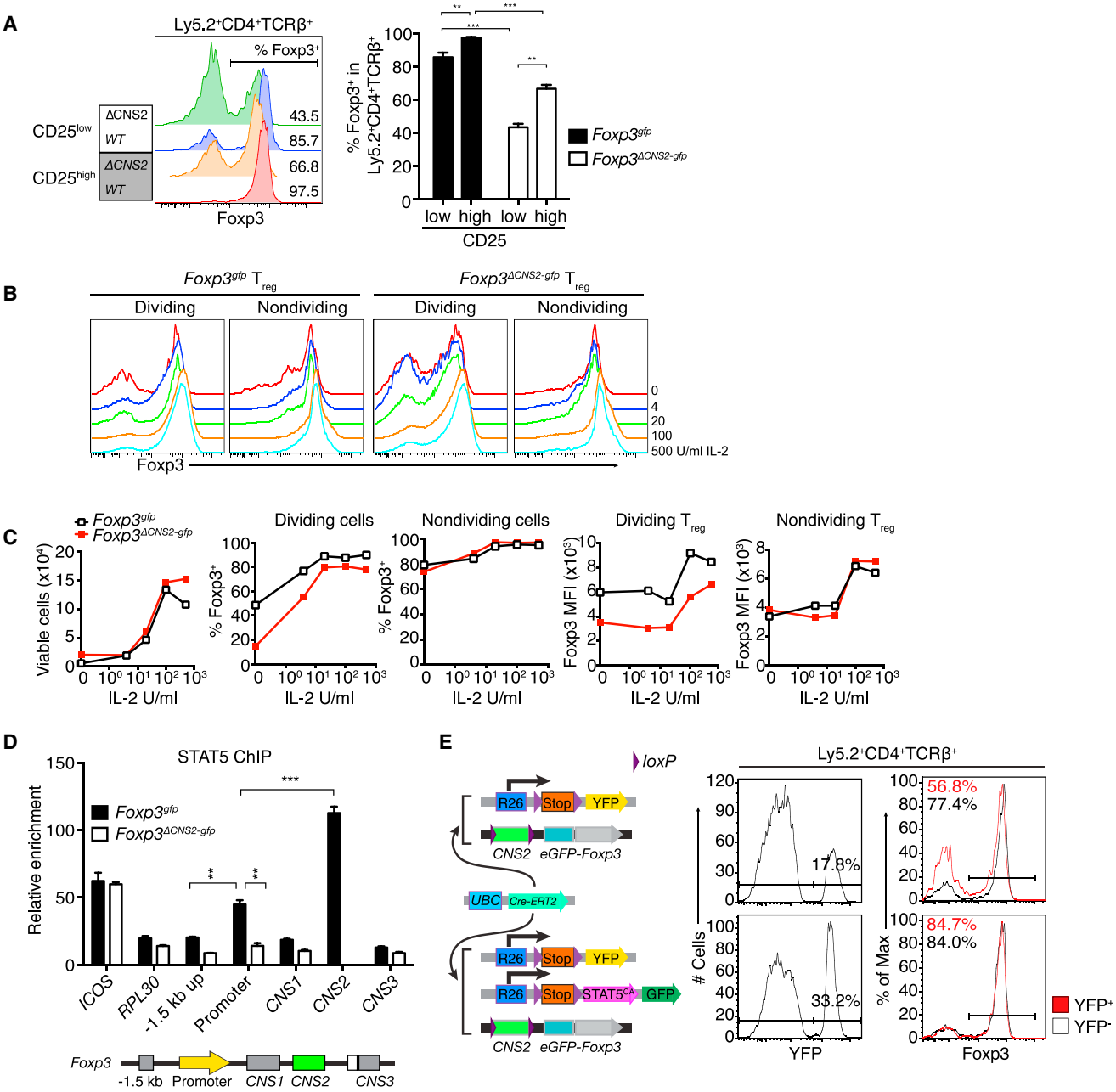


Figure 2. *CNS2* Sustains Heritable Foxp3 Expression in Mature Treg Cells in the Presence of Limiting Amounts of IL-2

(A) The stability of Foxp3 expression is more sensitive to low levels of CD25 expression in *CNS2*-deficient Treg cells. Doubly sorted Ly5.2 *CNS2*-sufficient (*Foxp3*^{gfp}) and -deficient (*Foxp3*$^{\Delta CNS2\text{-}gfp}$) Treg cells were cotransferred with Ly5.1 naive CD4$^+$Foxp3$^-$ T cells into T-cell-deficient *Tcrb*$^{-/-}$ *Tcrd*$^{-/-}$ mice and were recovered for analysis 5 weeks later. *CNS2*-sufficient (n = 5 per group), *CNS2*-deficient (n = 3 per group). Mean ± SEM.

(B and C) *CNS2*-deficient Treg cells are susceptible to low levels of IL-2. Treg cells were doubly sorted by FACS and were activated with plate-coated CD3 and CD28 antibodies in the presence of titrated amounts of IL-2 for 4 days. Foxp3 expression in the dividing (≥3 cell divisions) and nondividing (<3 cell divisions) cells was monitored by flow cytometry. The data represent one of more than three independent experiments.

(D) ChIP-qPCR analysis of STAT5 binding to the *Foxp3* locus in *CNS2*-sufficient and -deficient Treg cells. In-vitro-expanded FACS-purified Treg cells from *Foxp3*^{gfp} or *Foxp3*$^{\Delta CNS2\text{-}gfp}$ male mice were stimulated with 500 U/ml IL-2 for 30 min. Relative enrichment was calculated by normalizing to background STAT5 binding to control region (GM5069). The data are shown as means ± SEMs of triplicates and represent one of three independent experiments.

(E) Expression of hypermorphic STAT5 (STAT5^{CA}) rescues unstable Foxp3 expression in *CNS2*-deficient Treg cells in vivo. Treg cells from Ly5.2 UBC$^{Cre\text{-}ERT2}$ R26Y *Foxp3*$^{CNS2fl\text{-}gfp}$ and UBC$^{Cre\text{-}ERT2}$ R26Y/ R26-Stop^{fl}-STAT5^{CA} *Foxp3*$^{CNS2fl\text{-}gfp}$ were doubly sorted by FACS and cotransferred with naive Ly5.1 CD4$^+$Foxp3$^-$ T cells into *Tcrb*$^{-/-}$ *Tcrd*$^{-/-}$ mice. At 4 weeks after tamoxifen gavage, YFP$^+$ or YFP$^-$ Ly5.2$^+$ CD4$^+$TCRβ$^+$ cells were isolated and analyzed for Foxp3 expression (six mice per group). The data represent one of more than three independent experiments.

See also Figure S2.

subjected to tamoxifen-induced deletion of a "floxed" *CNS2* allele showed similar sensitivity of heritable Foxp3 expression to low levels of IL-2 (Figure S2C). The observed effect was unlikely due to a differential effect of IL-2 on the survival of *CNS2*-sufficient versus -deficient Treg cells because provision of IL-2 similarly increased the viability of both *CNS2*-sufficient and -deficient Treg cells in vitro (Figure 2C). These results suggested that *CNS2* confers the ability of dividing Treg cells to sustain Foxp3 expression upon IL-2 deprivation.

These findings raised the possibility that the observed rescue of *CNS2* deficiency was due to increased STAT5 activation in response to high amounts of IL-2. Previous studies showed binding of STAT5 to *CNS2* (Ogawa et al., 2013; Yao et al., 2007). In agreement with these data, we found that, within the *Foxp3* locus, STAT5 binds predominantly to *CNS2* and, to a markedly lesser extent, to the *Foxp3* promoter (Figure 2D). Interestingly, in the absence of *CNS2*, binding of STAT5 was noticeably reduced in the *Foxp3* promoter and in its −1.5 kb upstream region, suggesting potential interaction between *CNS2* and these sites. This observation was consistent with the idea that the partial rescue of the Foxp3 loss associated with *CNS2* deficiency in the presence of high amounts of IL-2 was due to heightened STAT5 activation. To address this possibility, we took advantage of a conditional R26-STOP^{fl}-STAT5$^{CA/gfp}$ transgene encoding a constitutively active form of STAT5 (STAT5^{CA}) and a GFP reporter preceded by loxP site flanked STOP cassette inserted into the *Rosa26* locus (T.C. and A.Y.R., unpublished data). Forced expression of STAT5^{CA} transgene in mature Treg cells upon deletion of a conditional *CNS2* allele mediated by tamoxifen-induced Cre rescued unstable Foxp3 expression and restored Treg cell stability to a level observed in wild-type Treg cells (>90% YFP^{+} cells were *CNS2* deficient) (Figure 2E). These results suggested that enhanced IL-2 signaling through activation of STAT5 was able to compensate for the absence of *CNS2* and that *CNS2* affords Treg cells the ability to effectively sustain Treg identity at low levels of IL-2.

CNS2 Stabilizes Foxp3 Expression in the Presence of Proinflammatory Cytokines

In contrast to the positive role of IL-2 signaling in stabilizing heritable Foxp3 expression, proinflammatory cytokines IL-6, IFNγ, IL-12, and IL-4 even in the presence of optimal amounts of IL-2 severely compromised Foxp3 expression in dividing *CNS2*-deficient Treg cells, whereas in *CNS2*-sufficient Treg cells, only a fairly small loss of Foxp3 was observed (Figures 3A and S3A and data not shown). Because in vitro IL-4 exposure caused particularly severe loss of Foxp3 expression in dividing Treg cells, for further investigation of this phenomenon, we focused on IL-4 as an example of proinflammatory cytokines. Notably, the loss of Foxp3 expression induced by IL-4 in *CNS2*-deficient Treg cells was also observed at the mRNA level and was cell-cycle dependent (Figures S3A and S3B).

To explore whether STAT family members activated downstream of proinflammatory cytokine signaling bind to the *Foxp3* locus and possibly compete with STAT5, we employed ChIP-qPCR to examine STAT6- and STAT5-binding sites at the *Foxp3* locus in Treg cells stimulated with IL-2 and IL-4 or with IL-2 alone. We found that, upon combined IL-2 and IL-4 stimulation, STAT6 and STAT5 bind to similar sites—namely, *CNS2*, the *Foxp3* promoter, and its −1.5 kb upstream region (Figure 3B). Concomitant with STAT6 binding, STAT5 binding was markedly reduced at the three main sites despite the presence of the same amounts of IL-2 that was used in combination with IL-4 or alone (Figure 3B). Of note, IL-2-dependent STAT5 phosphorylation was comparable when Treg cells were stimulated with IL-2 in the presence or absence of IL-4 (Figure S3C).

Because STAT5 and STAT6 bind to the same sites within the *Foxp3* locus and promote the maintenance and loss of Foxp3 expression, respectively, we asked what protein factors might associate with STAT5 and STAT6 and might contribute to their opposing activity. In light of the observed connection between CpG methylation in the *Foxp3* locus, cell division, and the loss of Foxp3 expression (Figure 1C), we tested whether STAT5 or STAT6 interact with DNA methyltransferases Dnmt1 and Dnmt3a, responsible for maintenance and de novo DNA methylation, respectively (Wu and Zhang, 2010). We found that STAT6, but not STAT5, coprecipitates with Dnmt1 and, to a lesser degree, with Dnmt3a (Figure 3C). In agreement with a previous report (Zhang et al., 2005), we also observed Dnmt1 association with STAT3 (data not shown) and STAT3 binding to the aforementioned STAT5-binding sites in the *Foxp3* locus (Figure S3D).

We previously reported that, after transient depletion of Treg cells in *Foxp3^{DTR}* mice, recovering Treg cells rapidly divide in the presence of high amounts of Th2, Th1, and Th17 cytokines (Arvey et al., 2014; Kim et al., 2007). Under these conditions, we observed sharply increased CpG methylation at the −1.5 kb region and exon-1 in dividing activated Treg cells (Figure 3D). Furthermore, blockage of Dnmt activity with 5-aza-dC resulted in considerable albeit partial rescue of impaired Foxp3 maintenance by dividing *CNS2*-deficient Treg cells in the presence of IL-4 in vitro (Figure 3E). Together, our data suggest that STAT5 competes with STAT6 for the binding to the *Foxp3* locus and prevents silencing of Foxp3 expression by opposing STAT6-mediated recruitment of Dnmt1 to *Foxp3* regulatory elements that are partially remethylated during Treg cell division.

CNS2-Dependent Heritable Foxp3 Expression and Maintenance of Treg Cells in the Steady State

Next, we investigated the biological significance of heritable Foxp3 expression in differentiated Treg cells. In agreement with previous analysis of mice on a mixed genetic background, *Foxp3$^{\Delta CNS2\text{-}gfp}$* B6 mice displayed reduced Treg cell frequency in the peripheral lymphoid organs and heightened activation of effector T cells and proinflammatory cytokine production in comparison to their *Foxp3^{gfp}* littermates (Figures 6C and 6D). Because Treg cell frequency can be altered in response to inflammation, we wanted to assess the impact of *CNS2* deficiency on Treg cell frequency in noninflammatory settings. Therefore, we generated mixed bone marrow (BM) chimeras by transferring allelically marked BM cells from *CNS2*-deficient Ly5.2 *Foxp3$^{\Delta CNS2\text{-}gfp}$* and *CNS2*-sufficient Ly5.1 *Foxp3^{gfp}* male mice into irradiated T cell-deficient recipients (Figure 4A). Analyses of the ratios of *CNS2*-deficient Ly5.2^{+} and -sufficient Ly5.1^{+} Treg cells showed that *CNS2*-deficient and -sufficient Treg cells were equally represented in the

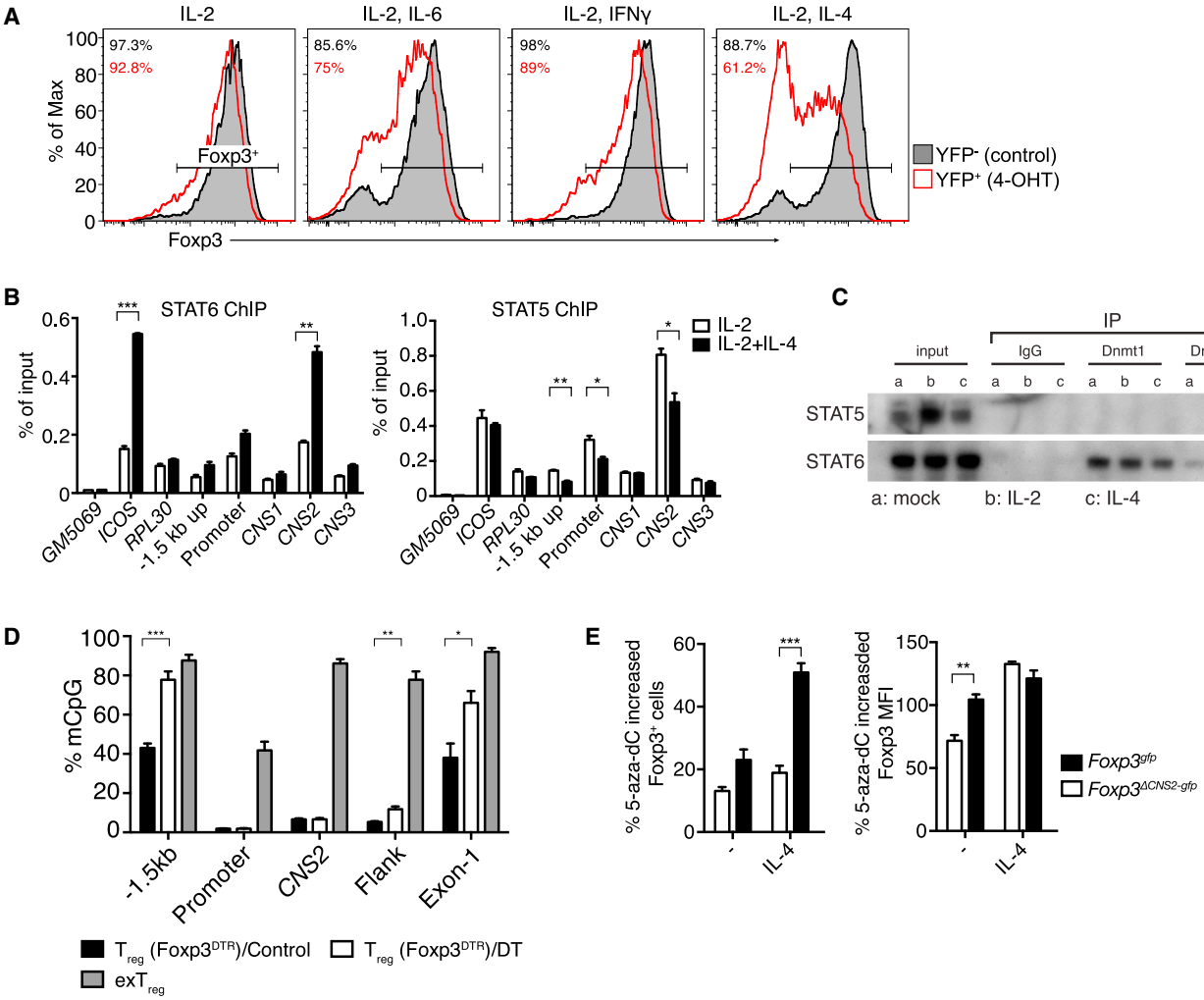


Figure 3. IL-2-STAT5-*CNS2* Axis Stabilizes Foxp3 Expression in the Presence of Proinflammatory Cytokines

(A) Dividing *Foxp3*$^{\Delta CNS2\text{-}gfp}$ Treg cells lose Foxp3 expression upon exposure to proinflammatory cytokines. Doubly sorted Treg cells from UBC$^{Cre\text{-}ERT2}$ R26Y *Foxp3*$^{CNS2fl\text{-}gfp}$ mice were activated in vitro with plate-coated CD3 and CD28 antibodies in the presence of IL-2 (500 U/ml) and proinflammatory cytokines. Deletion of *CNS2* was induced upon treatment with 4-hydroxytamoxifen (4-OHT). Histograms show only the dividing cells (≥3 divisions). The data represent one of more than three independent experiments.

(B) ChIP-qPCR analysis of STAT5 and STAT6 binding to the *Foxp3* locus in in-vitro-expanded FACS-purified Treg cells from *Foxp3*^{gfp} mice and stimulated with IL-2 and IL-4 for 30 min. Relative enrichment was calculated by normalizing to the input of ChIP. The data are shown as means ± SEMs of triplicates and represent one of three independent experiments.

(C) STAT6, but not STAT5, is associated with DNA methyltransferases. Dnmt1 and Dnmt3a coprecipitation with STAT5 and STAT6 was performed using nuclear extracts from in-vitro-expanded FACS-purified Treg cells from *Foxp3*^{gfp} mice as in (B). The data represent one of more than three independent experiments.

(D) Elevated CpG methylation in the *Foxp3* locus in Treg cells expanding under inflammatory conditions in vivo. Activated Treg cells were FACS purified from *Foxp3*^{DTR} mice on day 10 after diphtheria toxin injection (see Extended Experimental Procedures). CpG methylation levels were averaged from >5,000 reads in each site of the *Foxp3* locus and were compared to control Treg cells and ex vivo isolated exTreg cells (see also Figure S1B). The data represent two independent experiments. Mean ± SEM.

(E) Blockage of Dnmt activity with 5-aza-deoxycytidine (5-aza-dC) partially restores heritable Foxp3 expression in Treg cells exposed to IL-4. Doubly sorted Treg cells from Ly5.1 *Foxp3*^{gfp} and Ly5.2 *Foxp3*$^{\Delta CNS2\text{-}gfp}$ male mice were cocultured with CD3 and CD28 antibody-coated beads for 7 days in the presence of 500 U/ml IL-2 with or without IL-4 or 0.1μM 5-aza-dC. Relative increases in the percentages of Foxp3$^+$ cells and Foxp3 expression (MFI) on a per cell basis were calculated. The data represent one of more than three independent experiments. Mean ± SEM.

See also Figure S3.

thymus (Ly5.2$^+$/Ly5.1$^+$ ratio 0.93) and that *CNS2*-deficient Treg cells were only moderately underrepresented in the secondary lymphoid organs (Ly5.2$^+$/Ly5.1$^+$ ratio 0.42-0.64) (Figure 4B). In contrast, in nonlymphoid organs of these mice, we observed a pronounced skewing toward *CNS2*-sufficient Treg cells (Ly5.2$^+$/Ly5.1$^+$ ratio 0.15-0.36) and a marked decrease in the level of Foxp3 protein expression on a per cell basis (Figure 4C). Furthermore, *CNS2*-deficient Treg cells were underrepresented

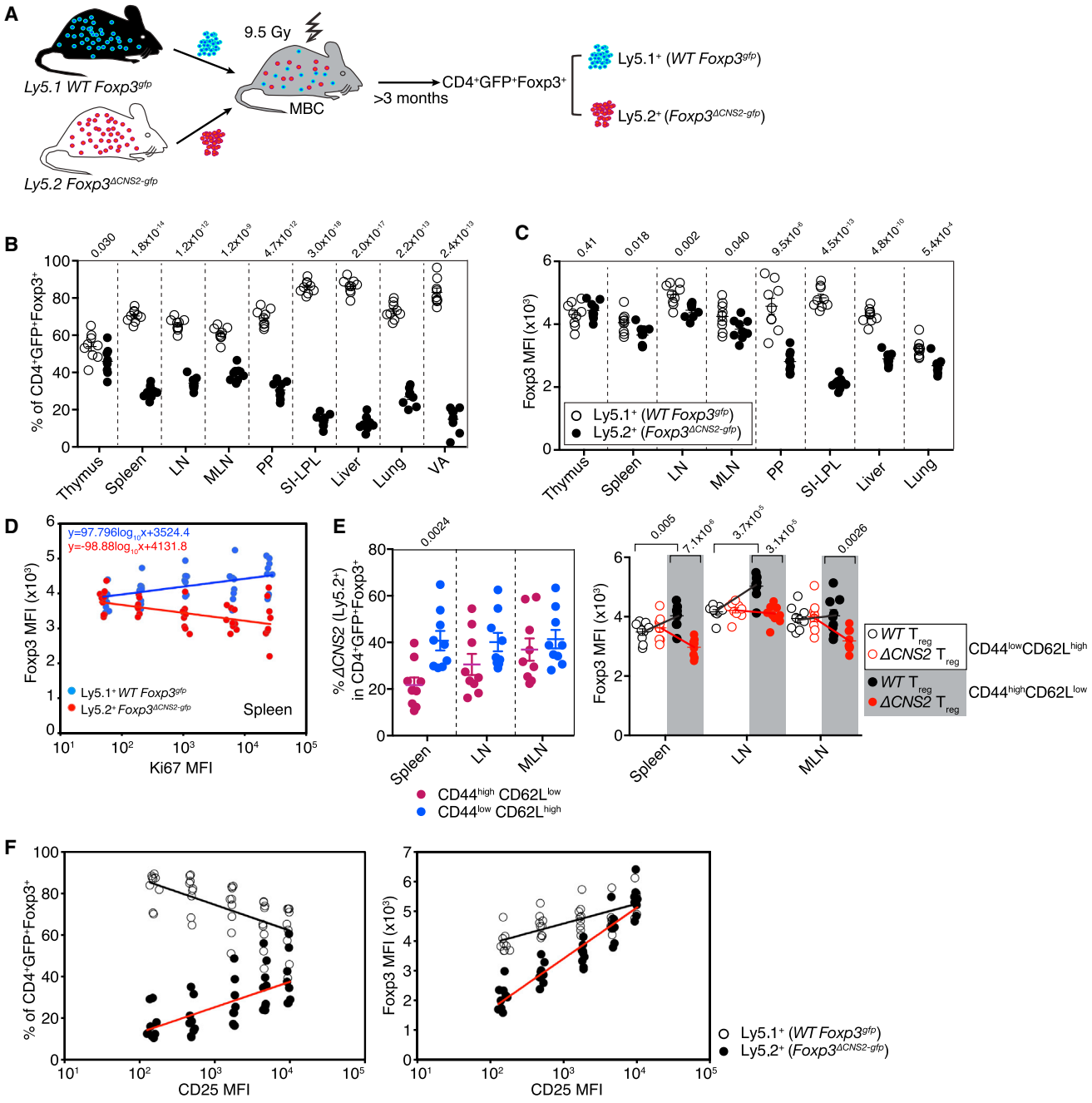


Figure 4. *CNS2*-Dependent Maintenance of Heritable Foxp3 Expression and Treg Cell Numbers in the Steady State

(A) Schematic of the generation of mixed bone marrow chimeras.

(B and C) Treg cell frequency and Foxp3 expression level in Ly5.1$^+$ *Foxp3^{gfp}* and Ly5.2$^+$ *Foxp3$^{\Delta CNS2\text{-}gfp}$* cells in the thymus, spleen, lymph nodes (LN), mesenteric lymph nodes (MLN), Peyer's patch (PP), small intestine lamina propria (SI-LPL), liver, lungs, and visceral adipose tissue (VA) of the mixed BM chimeras. Percentages of Ly5.1$^+$ and Ly5.2$^+$ cells in the reconstituted Treg (CD4$^+$GFP$^+$Foxp3$^+$) cells were normalized based on the reconstitution efficiency of total Ly5.1$^+$ and Ly5.2$^+$ lymphocytes in the thymus. Mean ± SEM n = 9 per group; the data represent one of three independent experiments.

(D) Correlation of Ki67 expression with the difference in Foxp3 MFI between *CNS2*-sufficient and -deficient Treg cells (spleen).

(E) Reduced frequency of and lower Foxp3 expression level in *Foxp3$^{\Delta CNS2\text{-}gfp}$* Treg cells in activated Treg cell population (CD44^{high}CD62L^{low}). Mean ± SEM.

(F) CD25 expression level was correlated with the frequency of *CNS2*-deficient Treg cells in the mixed population and was anticorrelated with the difference in Foxp3 expression levels between *CNS2*-sufficient and -deficient Treg cells (spleen).

See also Figure S4.

among dividing Ki67$^+$ Treg cells, which was associated with reduced Foxp3 expression level at the single-cell level (Figures 4D and S4A–S4C). Consistently, we observed reduced numbers of *CNS2*-deficient Treg cells and reduced levels of Foxp3 in activated (CD44^{high}CD62L^{low}) dividing *CNS2*-deficient Treg cells (Figures 4E, S4D, and S4E). In further support for the aforementioned notion that higher IL-2 signaling in Treg cells could partially compensate for *CNS2* deficiency, we noticed a marked enrichment of cells expressing higher CD25 in *CNS2*-deficient Treg cells and a correlation of the level of CD25 expression with the frequency and the amount of Foxp3 expression in *CNS2*-deficient Treg cells (Figure 4F and data not shown). These observations suggested that, under noninflammatory conditions, a key function of *CNS2* is to maintain Foxp3 expression in dividing Treg cells in nonlymphoid tissues.

CNS2 Prevents Differentiation of Treg Cells into Effector Cells

Next, we asked whether *CNS2* is required to prevent acquisition of alternative cell fates, i.e., differentiation into effector cells, by mature Treg cells under the condition of autoimmune inflammation. Previous studies demonstrated a prominent capacity of Treg cells to limit inflammation in the brain and the spinal cord and limb paralysis resulting from experimental autoimmune encephalomyelitis (EAE). EAE serves as an animal model for multiple sclerosis and is induced upon immunization of mice with a peptide derived from myelin oligodendrocyte glycoprotein (MOG35-55) in complete Freund's adjuvant (CFA) (Pachner, 2011; Stromnes and Goverman, 2006; Webster et al., 2009). Furthermore, a recent study suggested that some Treg cells—in particular, those specific for MOG35-55 peptide—lose Foxp3 expression and become effector T cells in the course of EAE (Bailey-Bucktrout et al., 2013). These observations raised the question of whether *CNS2* was dispensable under autoimmune inflammatory conditions because its function in the maintenance of Foxp3 expression was abolished. Alternatively, it was possible that the requirement for *CNS2* was heightened and that Foxp3 prevented deviation of Treg cells into proinflammatory effector T cells under inflammatory conditions. To answer these questions, we assessed Foxp3 and proinflammatory cytokine expression by *CNS2*-deficient and -sufficient Treg cells present in the same tissue inflammatory settings during the course of EAE. To track the dynamics of cell populations in these experiments, we adoptively transferred Treg cells double sorted from Ly5.2 UBC$^{Cre\text{-}ERT2}$ *Foxp3*$^{CNS2fl\text{-}gfp}$ R26Y together with naive Ly5.1$^+$ CD4$^+$Foxp3$^-$ T cells into lymphopenic recipients (Figure 5A). Mice were immunized with MOG peptide in CFA immediately after acute depletion of *CNS2* induced with tamoxifen in ~30% of transferred Treg cells. Due to limited numbers of effector T cells and ~70% of Treg cells with the unrecombined *CNS2*^{fl} allele, we observed very mild disease (clinical score ~1.5–2) in these recipients. We found that almost all (>95%) *CNS2*-deficient Treg cells lost Foxp3 expression in the central nervous system (brain and spinal cord); in contrast, around one-third of *CNS2*-sufficient Treg cells retained Foxp3 expression (Figures 5B and 5C). Thus, *CNS2*-dependent stable inheritance of Foxp3 expression plays a crucial role in maintaining Treg cell numbers under local inflammatory conditions in vivo.

Loss of Foxp3 expression by a cohort of Ly5.2$^+$ *CNS2*-deficient Treg cells was associated with acquisition of the production of IL-2 and proinflammatory cytokines IFNγ and IL-17 (Figure 5B and C). Thus, under inflammatory conditions, *CNS2* prevents the loss of Foxp3 by dividing Treg cells, as well as their differentiation into effector T cells. As a consequence of sharply reduced Foxp3 levels, *CNS2*-deficient Treg cells displayed lower amounts of CTLA4 and failed to effectively restrain autoimmune disease upon cotransfer into T cell-deficient hosts with effector CD4$^+$ T cells isolated from *Foxp3*-deficient mice (Figures S5A and S5B).

CNS2-Dependent Maintenance of Foxp3 Expression Is Essential for Suppressing Chronic Inflammation

These results suggested that the failure to maintain the Treg cell fate in the absence of *CNS2* would result in heightened inflammation and disease in a variety of settings due to a reduced suppressor function of Treg cells and their increased conversion into effector T cells (Figures 5 and S5). To test this idea, we evaluated histopathology in aged 12- to 15-month-old *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice. We found that *CNS2* deficiency resulted in severe inflammatory lesions in numerous nonlymphoid organs, including lung, liver, stomach, and small and large intestine (Figure 6E and data not shown). In addition, *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice exhibited decreased body weight, elevated circulating immunoglobulin (Ig) levels, and activated effector T cells (Figures 6A–6C). Thus, *CNS2* deficiency results in spontaneous age-dependent inflammatory lesions in nonlymphoid organs.

As a consequence of inflammation, *CNS2*-deficient Treg cell may lose more functional Treg cells due to cell division, proinflammatory cytokine exposure, and/or IL-2 deprivation (Figures 3A, 2B, and 2C). To directly test the impact of inflammation on *CNS2*-deficient Treg stability in vivo without manipulation of Treg cells in vitro, we immunized mixed bone marrow chimeras of Ly5.1 *Foxp3*^{gfp} and Ly5.2 *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice with MOG peptide and found that, in contrast to *CNS2*-sufficient Treg cells, *CNS2*-deficient Treg frequency was remarkably lower in the central nervous system, which was coupled with a dramatically diminished Foxp3 protein level (Figure 7A). We also examined the course of MOG peptide-induced EAE in *Foxp3*$^{\Delta CNS2\text{-}gfp}$ and *Foxp3*^{gfp} mice. Although *CNS2* deficiency did not impact the onset of the disease or its severity during the initial progressive phase, the disease in *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice failed to remit and exhibited a chronic, relentless course after reaching its peak in contrast to the remitting-relapsing disease observed in control *Foxp3*^{gfp} mice (Figures 7B and S6A). The heightened clinical manifestations, including limb paralysis and weight loss, correlated with a diminished Treg cell frequency among lymphocytes present in the brain and the spinal cord (Figures S6B–S6D and data not shown). Unlike other nonlymphoid tissues, Treg and effector T cells were barely detectable in the brain and spinal cord of unchallenged control and mutant animals (data not shown). Thus, the more severe, relapse-free disease course is unlikely due to a diminished pre-existing pool of Treg cells in *CNS2*-deficient mice but is rather a consequence of a loss of Foxp3 expression by *CNS2*-deficient Treg cells forced into the cell cycle in the setting of a localized chronic autoimmune inflammation (Figures 5 and 7A).

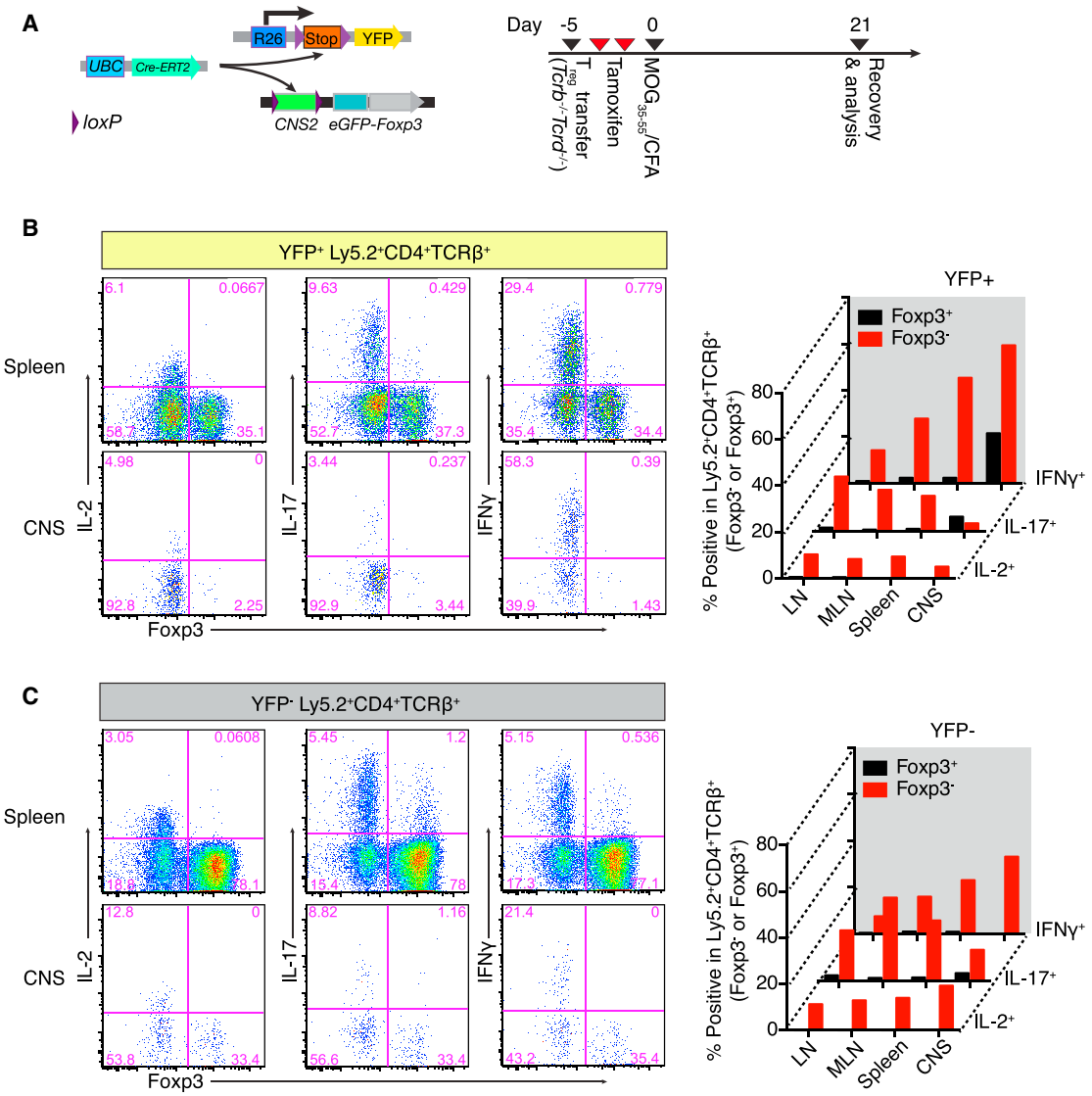


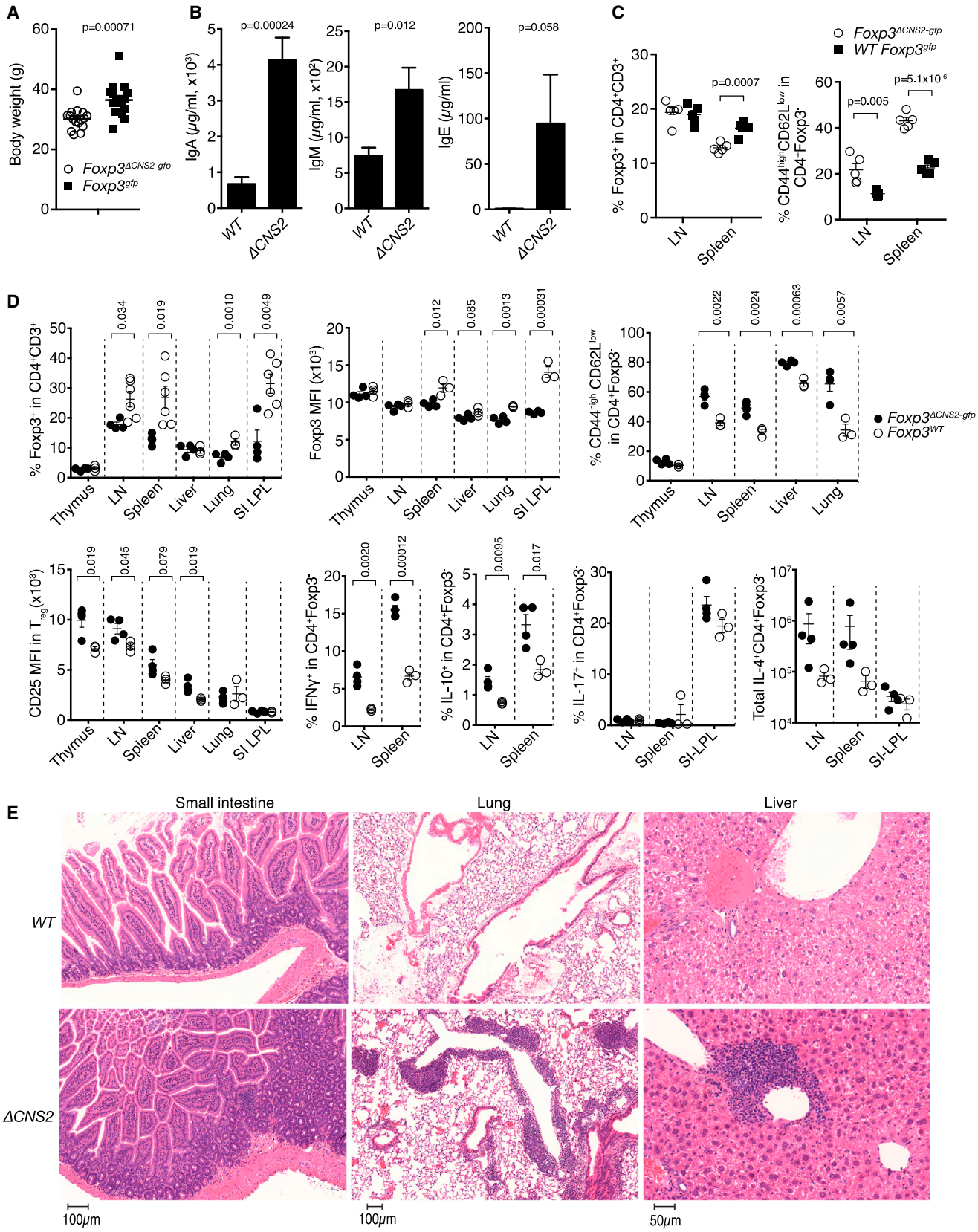
Figure 5. *CNS2*-Dependent Inheritance of Foxp3 Expression Prevents Treg Cell Conversion to Effector Cells

(A) Schematic of the experimental design. Treg cells doubly sorted from Ly5.2 *Foxp3*$^{CNS2fl\text{-}gfp}$ UBC$^{Cre\text{-}ERT2}$ R26Y male mice were cotransferred with Ly5.1 naive CD4$^+$Foxp3$^-$ T cells into *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ recipients. Acute ablation of *CNS2* was induced by tamoxifen gavage, and Ly5.2$^+$CD4$^+$TCRβ$^+$ T cells were analyzed 21 days after immunization with MOG35-55/CFA.

(B and C) YFP$^+$ and YFP$^-$ of Ly5.2$^+$CD4$^+$TCRβ$^+$ T cells were separated by FACS and stimulated with PMA and ionomycin, and cytokine production of the cells was analyzed by flow cytometry. The data represent one of three independent experiments. CNS, combined infiltrated lymphocytes from the central nervous system (brains and spinal cords).

See also Figure S5.

In addition to spontaneous autoimmune lesions in nonlymphoid organs and autoantigen-driven focal autoimmunity, we explored the role of *CNS2*-dependent maintenance of Treg cell fate during sterile inflammation associated with obesity. Because metabolic perturbation induced by a high-fat diet (HFD) causes mild chronic inflammation (Feuerer et al., 2009), we first investigated Treg cell frequency and effector T cell phenotypes in wild-type *Foxp3*^{gfp} and *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice that were fed with 60 kcal % high-fat diet (>10 weeks). We found that the frequency of *CNS2*-sufficient Treg cells was increased in spleen and liver but was decreased in visceral adipose tissue in mice on a HFD. In contrast, the frequency of *CNS2*-deficient Treg cells was not increased by HFD in comparison to regular diet despite a marked increase in CD25$^+$ cells in *CNS2*-deficient mice (Figures 7C, 7D, and S7A–S7C). The failure to increase Treg cell numbers was associated with a dramatic increase in the numbers of activated effector CD44^{high}CD62L^{low} Foxp3$^-$CD4$^+$ T cells in these mice (Figure 7C, right). These results suggest that chronic inflammation resulting from a metabolic disorder requires continuous maintenance of Foxp3



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expression when Treg cells divide in response to inflammatory cues.

Finally, we assessed the role of *CNS2* during chronic viral infection. *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice infected with chronic lymphocytic choriomeningitis virus (LCMV) clone 13 showed a severe progression of the wasting disease during the chronic phase of infection, which was less pronounced in control *Foxp3*^{gfp} mice. Interestingly, the weight loss of *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice was comparable to control *Foxp3*^{gfp} mice during the acute phase of infection (d7) but was severely increased during the chronic phase, when Treg cells are massively expanding (Punkosdy et al., 2011) (Figure 7E), suggesting that *CNS2*-dependent inheritable maintenance of Treg cells is critical for limiting inflammation during chronic viral infection.

Together, these data demonstrate the crucial role of *CNS2*-dependent inheritance of Foxp3 expression and highlight a mechanism by which a dedicated *cis*-element serves to maintain cellular identity during the vulnerable stage of cell division, a prerequisite for Treg cell function during immune homeostasis and acute and chronic inflammation.

DISCUSSION

We found that Treg cell proliferation was a prerequisite for silencing of the *Foxp3* locus and that *CNS2* opposed the shutdown of *Foxp3* expression. Previous studies showed that *CNS2* CpGs are methylated in immature Treg cells and are demethylated in mature Treg cells and that a demethylated state of *CNS2* was correlated with stable Foxp3 expression (Floess et al., 2007; Polansky et al., 2008; Toker et al., 2013). Our analysis of stage-specific deletion of *CNS2* revealed that its functionality is conditional upon its demethylation in mature Treg cells (data not shown). Thus, demethylated *CNS2* opposed *Foxp3* silencing in fully differentiated mature Treg cells in an apparently stochastic manner, and the activity of *CNS2* was continuously required for the maintenance of heritable Foxp3 expression.

In search of factors that converge on *CNS2* to maintain Foxp3 expression during cell division, we uncovered a key role of IL-2-signaling-induced STAT5 activation, which was supported by previous studies of IL-2 signaling in the differentiation and maintenance of Treg cells (Fontenot et al., 2005; Setoguchi et al., 2005). Our results suggest that, at physiologic levels, IL-2 signaling stabilizes heritable Foxp3 expression in dividing mature Treg cells to a large degree through *CNS2*. Consistent with this notion and previous work, we found that STAT5 binds to *CNS2* and, to a lesser degree, to the *Foxp3* promoter (Ogawa et al., 2013; Yao et al., 2007). Importantly, in the absence of *CNS2*, STAT5 binding to the *Foxp3* promoter was markedly reduced, suggesting that *CNS2* may interact with the promoter through a short-distance loop. Consistent with these results, heightened IL-2 signaling or induced expression of STAT5^{CA} restored stable Foxp3 expression in *CNS2*-deficient Treg cells. On the other hand, exposure to proinflammatory cytokines, including IL-4, IL-6, and IFNγ, resulted in a markedly increased loss of Foxp3 in dividing Treg cells in the absence of *CNS2* despite the presence of optimal amounts of IL-2. IL-4-activated STAT6 competes with STAT5 for the binding to similar sites in the *Foxp3* locus and counteracts STAT5 activity. Unlike STAT5, STAT6 interacted with Dnmt1; CpG methylation at the *Foxp3* locus was increased during Treg cell division and, upon loss of Foxp3 expression, was fully returned to the high methylation state of effector T cells. Of note, IL-6-activated STAT3 also binds to the aforementioned sites within the *Foxp3* locus and associates with Dnmt1 (data not shown) (Zhang et al., 2005). Together, these observations suggest that, in the absence of *CNS2*, STAT proteins activated downstream of proinflammatory cytokine signaling may effectively compete with STAT5 for binding to the *Foxp3* locus and may bring DNA methyltransferases and thereby silence Foxp3 transcription. Our experiments indicate that *CNS2* likely increases STAT5 occupancy at the *Foxp3* promoter prior to inflammation and hence opposes proinflammatory cytokine-driven silencing of the *Foxp3* locus. The sum of the opposing effects of IL-2 and proinflammatory cytokine signaling determines the inheritance of Foxp3 expression and the fate of dividing Treg cells. Although in-depth mechanistic understanding of this phenomenon would require careful quantification of active STAT proteins and their affinities for *Foxp3*-binding sites in Treg cells, our results suggest that *CNS2* skews this balance by recruiting STAT5 to the *Foxp3* promoter and enabling Treg cells to withstand diminished IL-2 and heightened inflammatory cytokine signaling. Thereby, *CNS2* serves as a critical determinant of Treg cell lineage stability upon cell division in inflammatory environments in which IL-2 amounts are limiting. Under physiological conditions, *CNS2* maintains the Treg cell population and thereby prevents spontaneous age-dependent inflammation in nonlymphoid organs. Furthermore, *CNS2*-dependent maintenance of Foxp3 expression limits the chronic, but not acute, phase of organ-specific autoimmune inflammation and precludes the acquisition of effector functions by Treg cells in response to inflammatory cues. Similarly, compromised inheritance of Foxp3 expression in Treg cells in the absence of *CNS2* resulted in diminished health status in the course of chronic viral infection as well as increased immune cell activation during sterile metabolic inflammation.

It seems likely that the observed features of regulation of the inheritance of Treg cell identity upon integration of extracellular cues by a *cis*-regulatory element dedicated to cell fate maintenance may be operational in other dividing cell types. Susceptibility to a stochastic downregulation of the expression of a lineage specification factor such as Foxp3 and the need for a

Figure 6. *CNS2*-Dependent Inheritance of Treg Cell Identity Prevents Spontaneous Age-Dependent Autoimmunity

(A) Diminished body weight of *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice (~12 months old) in comparison to wild-type littermates. WT 36.45 ± 1.37 g, *ΔCNS2* 30.18 ± 0.88 g. n = 16 per group. Mean ± SEM.

(B) Serum Ig levels in *Foxp3*$^{\Delta CNS2\text{-}gfp}$ and littermate control mice (~12 months old) determined by ELISA. WT n = 6, *Foxp3*$^{\Delta CNS2\text{-}gfp}$ n = 4. Mean ± SEM.

(C) Frequencies of Treg cells and activated CD4^{+}Foxp3^{-} T cells (CD44^{high}CD62L^{low}) in *Foxp3*$^{\Delta CNS2\text{-}gfp}$ and control mice (~3 months old; n = 5 per group). Mean ± SEM.

(D and E) Flow cytometric analyses of Foxp3 and CD25 expression, activation of, and cytokine production by CD4^{+} Foxp3^{-} T cells and tissue histopathology in 12- to 15-month-old *Foxp3*$^{\Delta CNS2\text{-}gfp}$ mice. Data represent one of two or more independent experiment. Mean ± SEM.

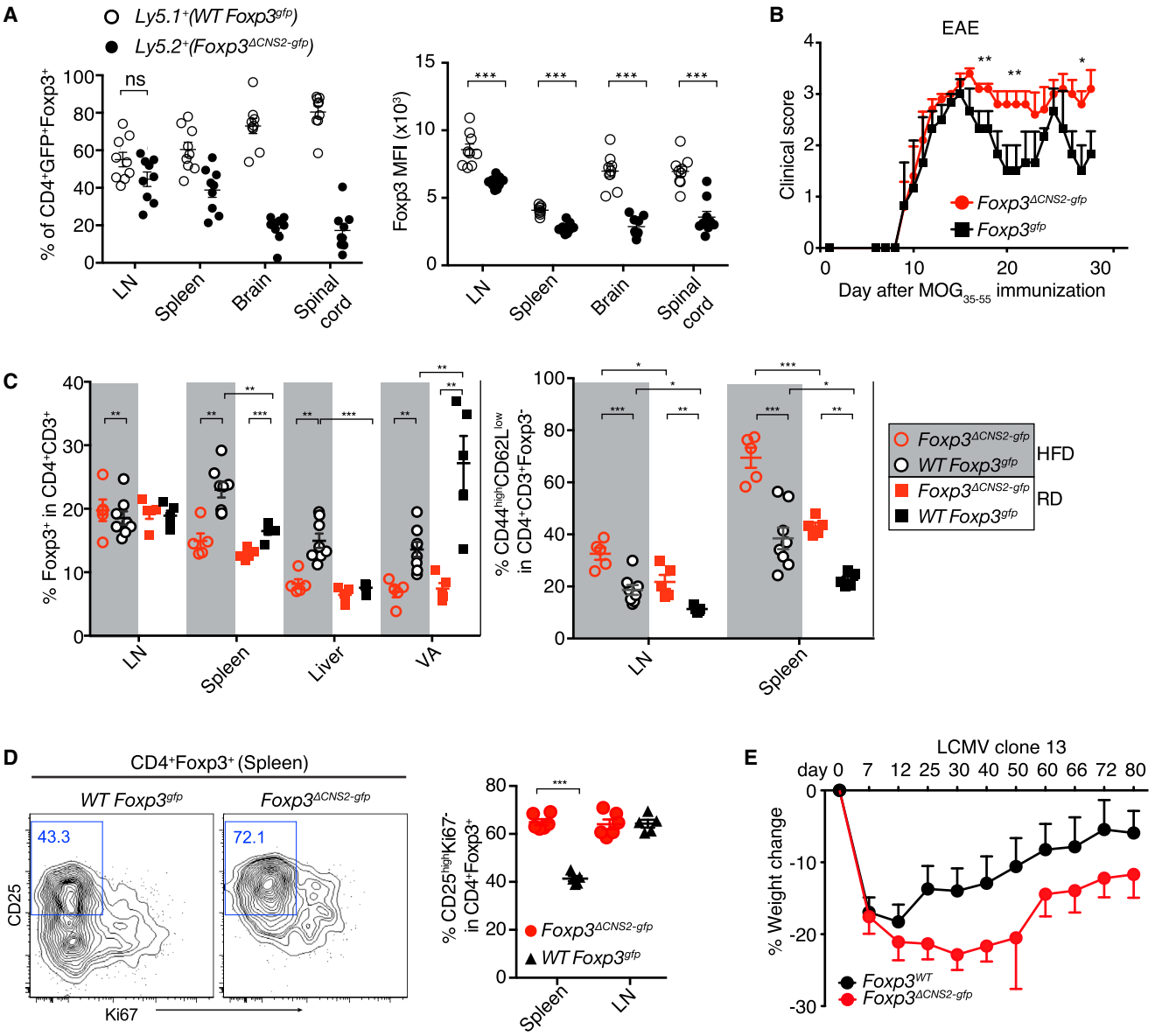

Figure 7. *CNS2*-Dependent Maintenance of Foxp3 Expression Is Critical for Suppressing Chronic Inflammation

(A) Flow cytometric analysis of Foxp3 expression in *CNS2*-deficient Treg cells in the central nervous system after MOG immunization. Chimeric mice transferred with Ly5.1 *Foxp3*^{gfp} and Ly5.2 *Foxp3*$^{\Delta CNS2\text{-}gfp}$ BM (see also Figure 4A) were immunized with MOG35-55 in CFA and were analyzed on day 14. Mean ± SEM, n = 9.

(B) Loss of *CNS2* aggravates the chronic phase of EAE. Male *Foxp3*$^{\Delta CNS2\text{-}gfp}$ and *Foxp3*^{gfp} littermates were immunized with MOG35-55/CFA, and their body weights and clinical disease scores were monitored. Mean + SEM. WT n = 8, *Foxp3*$^{\Delta CNS2\text{-}gfp}$ n = 10. Data represent one of three independent experiments.

(C) Analysis of Treg and activated effector CD4$^+$Foxp3$^-$ T cells in *Foxp3*$^{\Delta CNS2\text{-}gfp}$ and littermate control male mice on a high-fat diet (HFD) and control diet (RD, regular diet). Mice were fed with HFD or RD for 10 weeks before the analysis. Data represent one of more than three independent experiments. Mean ± SEM. WT (RD) n = 5, WT (HFD) n = 8, *Foxp3*$^{\Delta CNS2\text{-}gfp}$ (RD) n = 5, *Foxp3*$^{\Delta CNS2\text{-}gfp}$ (HFD) n = 5.

(D) Flow cytometric analysis of CD25^{high}Ki67$^-$ Treg cells in *Foxp3*$^{\Delta CNS2\text{-}gfp}$ and littermate control mice on a HFD diet.

(E) Body weight of *Foxp3*$^{\Delta CNS2\text{-}gfp}$ and littermate control mice infected with LCMV clone 13 ($p \leq 0.05$). The data represent one of two independent experiments. Mean ± SEM. WT n = 6, *Foxp3*$^{\Delta CNS2\text{-}gfp}$ n = 5.

See also Figures S6 and S7.

dedicated regulatory element to oppose such a loss might be linked to the intrinsic inhibition of transcriptional activity, splitting and dilution of transcription factors, and histone modifications during cell division (Gottesfeld and Forbes, 1997; Mullen et al., 2001; Probst et al., 2009). Thus, the mitosis-related transcriptional fluctuation could increase the probability of complete transcriptional silencing of Foxp3 expression through yet-to-be defined active or passive processes (Losick and Desplan, 2008). Considering the respective positive and negative roles of IL-2 and proinflammatory cytokines in the heritable

expression of Foxp3, it seems likely that the promotion of cell division by these factors may increase the vulnerability of Treg cells to the loss of their identity due to the stochastic silencing of Foxp3 by molecular mechanisms that remain to be explored.

In conclusion, we found that *CNS2*, a *cis*-acting regulatory element dedicated to heritable maintenance of the active state of the *Foxp3* locus, enables dividing Treg cells to maintain their differentiated state upon deprivation of their essential growth factor IL-2 and upon exposure to proinflammatory cues driving alternative effector T cell differentiation. *CNS2*-dependent stability of the Treg cell lineage is critical for preventing spontaneous, age-dependent chronic inflammation in a variety of nonlymphoid organs, for restraining metabolic inflammation associated with diet-induced obesity, and for limiting organ-specific autoimmunity as well as the wasting syndrome during chronic viral infection.

EXPERIMENTAL PROCEDURES

See the Extended Experimental Procedures for further information

Animals

Foxp3$^{\Delta CNS2\text{-}gfp}$ and *Foxp3*$^{CNS2fl\text{-}gfp}$ mice (Zheng et al., 2010) were backcrossed onto C57BL/6 background for more than ten generations. *Foxp3*^{DTR} and *Foxp3*^{gfpko} were described previously (Gavin et al., 2007; Kim et al., 2007). R26-Stop^{fl}-STAT5^{CA} mice were generated by inserting a coding sequence of STAT5^{CA} and a GFP reporter into the *Rosa26* locus (Sasaki et al., 2006) (T.C. and A.Y.R., unpublished results). All animals were maintained in the MSKCC animal facility under SPF conditions, and the experiments were performed according to the institutional guidelines (IACUC 08-10-023).

Generation of Bone Marrow Chimeras and Adoptive T Cell Transfers

For the generation of bone marrow (BM) chimeras, recipient mice were irradiated (9.5 Gy) 24 hr prior to bone marrow stem cell transfer. BM cells were depleted of T cells with Dynabeads FlowComp Mouse Pan T kit (Life Technologies) and were injected i.v. into irradiated recipients. After BM transfer, recipient mice received neomycin with drinking water (2 mg/ml) for 3 weeks and were analyzed 8–12 weeks later.

For adoptive cell transfers, Treg cells were purified from *Foxp3*^{gfp}, *Foxp3*$^{\Delta CNS2\text{-}gfp}$, or *Foxp3*$^{CNS2fl\text{-}gfp}$ males by double-FACS sorting based on GFP expression after enrichment of CD4 T cells with Dynal magnetic beads (Life Technologies) and were cotransferred with congenically labeled (Ly5.1) naive CD4^{+}Foxp3^{-} T cells at a 1:5 ratio (total 2.5 × 10^{6} cells per recipient) into T-cell-deficient *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ recipients. Transferred cell subsets were analyzed by flow cytometry ~4 weeks later.

Statistical Analysis

For statistical analysis, unpaired two-tailed Student's t test was performed using Prism (GraphPad) or Excel (Microsoft). Regression analysis and plotting were performed with Excel. *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001, and **** p $\leq$ 0.0001.

ACCESSION NUMBERS

The GEO accession number of the RNA sequencing data reported in this study is GSE58905.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.07.031.

AUTHOR CONTRIBUTIONS

Y.F. and A.Y.R. conceived of and designed the experiments and interpreted the results. A.A. analyzed RNA sequencing data. T.C. generated STAT5^{CA} mice. G.G. performed LCMV experiments. J.v.d.V. performed pilot ChIP experiments and interpreted the results. Y.F. and A.Y.R. wrote the manuscript.

ACKNOWLEDGMENTS

We thank MSKCC Genomics and Bioinformatics Core facilities for RNA sequencing and Flow Cytometry Core facility for assistance with cell sorting. We thank W. Hu for assistance with the analysis of bisulfite sequencing data and P. DeRoos, S. Hemmers, Q. Li, G. Loeb, and S. Dikiy for technical assistance. MSKCC core facilities are supported by Cancer Center Support Grant CCSG P30 CA008748. Y.F. was supported by a Postdoctoral Fellowship of the Cancer Research Institute. This study was supported by NIH grant AI034206 and the Howard Hughes Medical Institute (A.Y.R.).

Received: April 16, 2014
Revised: June 30, 2014
Accepted: July 25, 2014
Published: August 14, 2014

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