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Blimp1 Prevents Methylation of Foxp3 and Loss of Regulatory T Cell Identity at Sites of Inflammation

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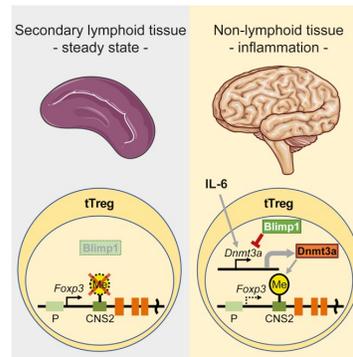
Summary

Foxp3⁺ regulatory T (Treg) cells restrict immune pathology in inflamed tissues; however, an inflammatory environment presents a threat to Treg cell identity and function. Here, we establish a transcriptional signature of central nervous system (CNS) Treg cells that accumulate during experimental autoimmune encephalitis (EAE) and identify a pathway that maintains Treg cell function and identity during severe inflammation. This pathway is dependent on the transcriptional regulator Blimp1, which prevents

downregulation of Foxp3 expression and “toxic” gain-of-function of Treg cells in the inflamed CNS. Blimp1 negatively regulates IL-6- and STAT3-dependent Dnmt3a expression and function restraining methylation of Treg cell-specific conserved non-coding sequence 2 (CNS2) in the *Foxp3* locus. Consequently, CNS2 is heavily methylated when Blimp1 is ablated, leading to a loss of Foxp3 expression and severe disease. These findings identify a Blimp1-dependent pathway that preserves Treg cell stability in inflamed non-lymphoid tissues.

Keywords: regulatory T cells, Blimp1, CNS2, epigenetic regulation, CNS, inflammation, DNA methyltransferases, Foxp3, Interleukin-6

Graphical Abstract



Introduction

Foxp3⁺ Treg cells are either thymus derived (tTreg cells) or induced from conventional CD4⁺ T (Tconv) cells outside the thymus (pTreg cells) or can be generated *in vitro* (iTreg cells) (Abbas et al., 2013). In an inflamed environment, such as the CNS during experimental autoimmune encephalomyelitis (EAE), pTreg cells cannot be generated and tTreg cells migrate to the CNS, become activated in a T cell receptor (TCR)-dependent manner, expand, and acquire suppressive Treg effector functions (Korn et al., 2007, O'Connor et al., 2007). Even though tTreg cells are indispensable to control CNS inflammation (McGeachy et al., 2005), little is known about their resilience to inflammation in the CNS.

Epigenetic DNA marks are crucial determinants of Treg cell identity. Complete demethylation of the conserved non-coding sequence 2 (CNS2), also known as Treg cell-specific demethylated region (TSDR), in the first intron of the *Foxp3* locus is required for optimal expression of Foxp3 (Floess et al., 2007). Conversely, methylation of CNS2 results in the reduced transcription of *Foxp3* and subsequent loss of Treg cell functionality. Notably, less demethylation of CNS2 in iTreg cells causes their instability compared to tTreg cells (Polansky et al., 2008). While epigenetic manipulation is intensely explored to “stabilize” iTreg cells (also for therapeutic use), less is known about modifications of epigenetic DNA marks in tTreg cells. Interestingly, CNS2 demethylation in tTreg cells is already initiated during thymic development (Toker et al., 2013), in a process that appears independent of the induction of Foxp3 (Ohkura et al., 2012). Therefore, impaired DNA demethylation in tTreg cells might compromise their identity in spite of a fully mounted Foxp3-dependent transcriptional program.

Blimp1 is a zinc finger protein, which serves as a transcriptional regulator and is indispensable for the development of plasma cells and fully functioning effector CD8⁺ T cells (Kallies et al., 2009, Rutishauser et al., 2009, Shapiro-Shelef et al., 2003). In CD4⁺ T cells, Blimp1 limits follicular helper T cell differentiation (Choi et al., 2011). Furthermore, Blimp1 transactivates *Il10* and thereby drives the conversion of T helper 1 (Th1) and Th17 cells into type 1 regulatory T (Tr1) cells (Heinemann et al., 2014, Neumann et al., 2014). Finally, Blimp1 has been identified to support a residency program of CD8⁺ T cells

in non-lymphoid tissues ([Mackay et al., 2016](#)). In Treg cells, Blimp1 cooperates with interferon regulatory factor 4 (IRF4) to establish a Treg cell effector program, including the expression of interleukin-10 (IL-10) and granzyme B in particular in non-lymphoid tissues ([Cretney et al., 2011](#), [Vasanthakumar et al., 2015](#)).

Here, we reveal a non-redundant function for Blimp1 in preserving the identity of Treg cells, particularly under conditions of an inflammatory challenge. IL-6 signaling induces and activates the DNA methylating enzyme Dnmt3a, which is mounted to distinct DNA sites in the absence of Blimp1, leading to CNS2 methylation and Foxp3 downregulation. Conversely, Blimp1 inhibits the upregulation of *Dnmt3a*, prevents methylation of CNS2 in the *Foxp3* locus, and thereby maintains Treg cell identity and function. Consequently, Treg cell-specific loss of Blimp1 in an inflammatory environment results in the methylation of CNS2, loss of Foxp3 expression, and the acquisition of a proinflammatory T cell phenotype.

Results

Treg Cells Show Stable Foxp3 Expression in the Inflamed CNS *In Vivo*

Strong inflammation can trigger the loss of Treg cell identity and acquisition of a proinflammatory phenotype in Treg cells ([Zhou et al., 2009](#)). Consistent with earlier reports ([Kim et al., 2017](#), [Korn et al., 2007](#)), CNS Treg cells during EAE did not only express IL-10, but a fraction of them expressed proinflammatory cytokines, including IL-17 and interferon- γ (IFN- γ) ([Figure 1A](#)). In RNA sequencing (RNA-seq) analysis of Foxp3 (GFP)⁺ Treg cells isolated from the spleen and CNS of EAE mice at the peak of disease, CNS Treg cells showed a downregulation of 507 genes and upregulation of 783 genes (>2-fold, $p < 0.05$) as compared with splenic Treg cells, together comprising a transcriptional signature of CNS Treg cells ([Table S1](#)). As expected, principal-component analysis (PCA) showed that splenic and CNS Treg cells grouped together in principal component 2 (PC2) and clearly separated from conventional CD4⁺ T cells isolated from the same organs ([Figure 1B](#)). Interestingly, however, principal component 1 (PC1) separated these populations according to the organ they were isolated from, indicating that the tissue controls significant parts of the transcriptional signature ([Figure 1B](#)). Notably, signature genes of Th1 and Th17 genes were upregulated in CNS Treg cells as compared to splenic Treg cells ([Figures S1A](#) and [S1B](#)). Thus, at the site of inflammation, Treg cells appeared to be poised for proinflammatory effector programs. However, Foxp3 was maintained in CNS Treg cells and expressed in even higher amounts than in splenic Treg cells during disease ([Figure 1C](#)). Thus, we hypothesized that specific mechanisms must exist that preserve Treg cell identity in an inflamed environment.

CNS Treg Cells Express High Amounts of Blimp1 and Display an Effector Treg Cell Signature

Proinflammatory cytokines have been implicated both in the maintenance and loss of Treg cell identity ([Koch et al., 2012](#), [Overacre-Delgoffe et al., 2017](#)). To understand which pathways may have an impact on Treg cells during CNS inflammation, we performed gene set enrichment analyses in CNS versus splenic Treg cells. CNS Treg cells showed pronounced enrichment for IFN- γ -, IL-12-, and IL-27- (but not IL-23, data not shown) induced genes, suggesting that CNS Treg cells can sense multiple inflammatory cytokines during inflammation ([Figure S1C](#)). Notably, *Prdm1* (encoding Blimp1) was common to all three gene sets ([Figures 1D](#) and [1E](#)). Blimp1 expression was higher in CNS Treg cells compared to splenic Treg cells, and effector Treg cell signature genes expressed in Blimp1⁺ versus Blimp1⁻ Treg cells ([Cretney et al., 2011](#)) were highly enriched in the transcriptional profile of CNS as compared to splenic Treg cells ([Figure 1F](#)).

Using a Blimp1 (yellow fluorescent protein [YFP]) reporter mouse ([Rutishauser et al., 2009](#)), we confirmed that the majority of Foxp3⁺ Treg cells were Blimp1 (YFP)⁺ in the inflamed CNS, whereas the fraction of Blimp1 (YFP)⁺ Treg cells was only about 10% in the spleen in steady state ([Figure 1G](#)). Taken together, Treg cells that accumulated in the inflamed CNS displayed a distinct transcriptional signature characterized

by the upregulation of inflammation sensing pathways, high expression of suppressive molecules, and the upregulation of Blimp1.

Blimp1 Is Maintained in CNS Treg Cells in a STAT1-Dependent Manner

TCR-induced IRF4 expression has been shown to drive Blimp1 expression in both Treg cells and conventional T cells (Cretney et al., 2011, Krishnamoorthy et al., 2017, Man et al., 2013). In addition, multiple cytokines in conjunction with TCR signaling have been shown to be able to induce Blimp1 expression through direct binding of signal transducer and activator of transcription (STAT) molecules to the *Prdm1* locus (Cretney et al., 2011, Heinemann et al., 2014, Kwon et al., 2009, Xin et al., 2016). Consistent with this notion, IL-10 and IL-21 (STAT3) and a number of inflammatory cytokines, including IFN- γ (STAT1), IL-12 (STAT4), and IL-27 (STAT3) induced Blimp1 in cultured splenic Treg cells (Figure 1H). To identify inflammatory pathways involved in Blimp1 expression in Treg cells during autoimmune CNS inflammation, we generated mixed bone marrow chimeric mice containing wild-type bone marrow together with either STAT1-deficient, STAT3-deficient (T cell-specific conditional using *Cd4 Cre*) or STAT4-deficient bone marrow. After induction of EAE, Treg cells were sorted from the CNS and spleens of these chimeric mice at the peak of EAE and assessed for expression of *Prdm1* (Blimp1) and its target *Ill10*. When compared to the concomitant wild-type compartment, *Prdm1* was not reduced in STAT3-deficient and STAT4-deficient Treg cells (Figures S2A and S2B); however, it was markedly decreased in STAT1-deficient Treg cells (Figures 1I and 1J). Accordingly, the expression of *Ill10* was lower in STAT1-deficient CNS Treg cells but not in STAT3- and STAT4-deficient Treg cells as compared to wild-type control Treg cells (Figures 1I, 1J, S2A and S2B). Consistent with these findings, *Il27r^{-/-}* and *Il12rb2^{-/-}* Treg cells isolated from mixed bone marrow chimeric mice showed unimpaired expression of *Ill10* (Figure S2C and S2D). Thus, Blimp1 was induced in CNS Treg cells partially in a STAT1-dependent manner.

Blimp1 Is Indispensable for CNS Treg Cell Function

In order to test the function of Blimp1 in CNS Treg cells, we generated Treg cell conditional Blimp1-deficient mice (*Foxp3 Cre* x *Prdm1^{flox/flox}*, termed *Blimp1^{ΔFoxp3}*). After immunization with MOG(35-55)+CFA, *Blimp1^{ΔFoxp3}* mice developed more severe signs of EAE as compared to both “flox” control mice (*Prdm1^{flox/flox}*, termed *Blimp1^{WT}*) and “Cre” control mice (*Foxp3 Cre*), and a proportion succumbed to the disease (Figures 2A, 2B and 2C), indicating that Blimp1 expression in *Foxp3⁺* Treg cells was mandatory for the control of CNS inflammation. Although the loss of Blimp1 in Treg cells did not affect the recruitment of *Foxp3⁺* Treg cells to the CNS in *Blimp1^{ΔFoxp3}* mice (Figure 2D), *Blimp1^{ΔFoxp3}* Treg cells showed reduced expression of *Foxp3*, lost the expression of IL-10, and produced significantly more IL-17 and IFN- γ than their Blimp1-sufficient counterparts (Figure 2E).

Ill10^{-/-} mice develop more severe EAE (Bettelli et al., 1998), and we reasoned that the loss of Blimp1-dependent Treg cell effector functions, including IL-10 production, might be responsible for the failure of *Blimp1^{ΔFoxp3}* Treg cells to control CNS inflammation. However, Treg cell-specific ablation of *Ill10* using *Ill10^{ΔFoxp3}* (*Foxp3 Cre* x *Ill10^{flox/flox}*) mice did not alter EAE disease severity or fraction and number of CNS Treg cells as compared to wild-type control mice (Figure 2F). Notably and in contrast to *Blimp1^{ΔFoxp3}* Treg cells, IL-10-deficient Treg cells isolated from the inflamed CNS did not exhibit a gain of IL-17 production as compared with their wild-type counterparts (Figures 2G and 2H). In summary, the loss of Blimp1 in Treg cells did not only result in a loss of distinct effector functions of Treg cells but appeared to lead to a more global loss of Treg cell identity in the inflamed CNS.

Blimp1 Controls Treg Cell Identity in the Inflamed CNS

In order to test whether Blimp1 controlled Treg cell identity in the inflamed CNS in a Treg cell-intrinsic manner, we generated mixed bone marrow chimeric mice by reconstituting Rag1-deficient recipient mice with a 1:1 mixture of congenically marked wild-type and *Blimp1*^{ΔFoxp3} bone marrow. At the peak of EAE, the CNS Treg cell compartment composed of wild-type (CD45.1) and *Blimp1*^{ΔFoxp3} (CD45.2) Treg cells was analyzed. *Blimp1*^{ΔFoxp3} CNS Treg cells showed a significant downregulation of Foxp3 as compared to their wild-type counterparts (Figure 3A). Furthermore, they had lost IL-10 expression and acquired IL-17 production (Figures 3B and 3C). RNA-seq and subsequent PCA of wild-type (WT) versus *Blimp1*^{ΔFoxp3} CNS Treg cells revealed that Blimp1-deficient Treg cells clustered consistently away from wild-type Treg cells, indicating Blimp1 regulated gene expression both in splenic and CNS Treg cells (Figure 3D). In total, we identified 342 genes upregulated (>2-fold, $p < 0.05$) and 409 genes downregulated (>2-fold, $p > 0.05$) in *Blimp1*^{ΔFoxp3} CNS Treg cells, including reduced expression of known Blimp1 target genes, such as *Il10* and *Gzmb* (Figure 3E; Table S2). Consistent with their increased production of IFN- γ , Blimp1-deficient CNS Treg cells expressed higher amounts of *Tbx21* (T-bet) (Figures S3A and S3B). In line with the reduced expression of Foxp3, genes normally suppressed by Foxp3 showed significant derepression in *Blimp1*^{ΔFoxp3} CNS Treg cells (Figure 3F). In summary, Blimp1 was required to maintain Foxp3 expression and a Treg cell-specific transcriptional profile in CNS Treg cells.

Blimp1 Controls Foxp3 in an Indirect Manner in Treg Cells

In order to address how Blimp1 controlled the transcriptional profile of CNS Treg cells, we performed assay for transposase-accessible chromatin using sequencing (ATAC-seq) in splenic and CNS Treg cells of mixed bone marrow chimeric mice (wild-type plus *Blimp1*^{ΔFoxp3}) at the peak of EAE. We then correlated the information about differentially accessible gene loci, including transcription factor-binding motifs with differences in gene expression between wild-type and *Blimp1*^{ΔFoxp3} CD4⁺ T cells. In conventional T cells derived from the spleen of wild-type plus *Blimp1*^{ΔFoxp3} mixed bone marrow chimeras, no difference in ATAC peaks was detected as expected (Figure 4A). In splenic Treg cells, we identified 2,337 upregulated ATAC peaks in *Blimp1*^{ΔFoxp3} Treg cells, while 1,958 ATAC peaks were higher in wild-type Treg cells (Figure 4A). When we then interrogated these differential ATAC peaks for transcription factor-binding motifs, we identified a panel of transcription factors, including Ets, Runx1, and ROR γ t whose binding motifs were significantly enriched in splenic *Blimp1*^{ΔFoxp3} Treg cells as compared to wild-type Treg cells (Figures 4B and 4C). Conversely, motifs for nuclear factor κ B (NF- κ B) (p65), Erg, and AP1 transcription factors were prominent in ATAC peaks which were reduced in *Blimp1*^{ΔFoxp3} as compared to wild-type Treg cells (Figures 4B and 4C). Thus, the lack of Blimp1 prompted an altered transcriptional network to become operational in Foxp3⁺ Treg cells under inflammatory conditions. Next, we asked to which extent Blimp1 was directly responsible for transcriptional changes in CNS Treg cells. Using a published Blimp1 chromatin immunoprecipitation sequencing (ChIP-seq) dataset (Mackay et al., 2016), we compared Blimp1 binding and ATAC peaks in the top 75 genes differentially expressed in *Blimp1*^{ΔFoxp3} versus wild-type CNS Treg cells. We identified 23 genes with Blimp1 binding sites within 50 kb of the promoter region, suggesting a direct role of Blimp1 in regulating their expression. Due to the different activity of Blimp1 as a repressor or promoter of transcription (Martins and Calame, 2008), we found that some of these genes were upregulated (e.g., *Crtam*), whereas others (such as *Il10*) were downregulated in *Blimp1*^{ΔFoxp3} CNS Treg cells as compared to wild-type CNS Treg cells (Figures 4D and 4E). In contrast, most of the top regulated genes did not show apparent Blimp1 binding despite changes in ATAC peaks, suggesting that their changed expression was not directly regulated by Blimp1. Importantly, in this category of genes, we observed reduced ATAC peaks within genes critical for Treg cell identity (such as the intron 1 enhancer of *Foxp3*) in *Blimp1*^{ΔFoxp3} versus wild-type CNS Treg cells (Figures 4D and 4E), suggesting that the regulation of Foxp3 in *Blimp1*^{ΔFoxp3} Treg cells was dependent on Blimp1 but likely not controlled by a direct transactivation activity of Blimp1 in the *Foxp3* locus.

Loss of Blimp1 Is Associated with Impaired Epigenetic Imprinting of the *Foxp3* Locus

Foxp3 expression is controlled by multiple mechanisms (Feng et al., 2014, Huehn et al., 2009). As we found no evidence for the direct control of the *Foxp3* locus by Blimp1, we explored the possibility of an epigenetic mechanism of *Foxp3* regulation by Blimp1. Pyrosequencing analysis revealed a fully methylated CNS2 in conventional CD4⁺ T cells derived from the CNS of *Blimp1*^{WT} plus *Blimp1*^{ΔFoxp3} mixed bone marrow chimeric mice as expected (Figure 5). In contrast, *Foxp3*⁺ T cells were demethylated at CNS2, identifying them as bona fide Treg cells (Figure 5). However, both in the spleen and (in an even more pronounced manner) in the CNS, *Blimp1*^{ΔFoxp3} Treg cells showed a partial loss of the demethylated state of CNS2 as compared to wild-type Treg cells (Figure 5).

Because *Foxp3* was downregulated in Blimp1-deficient CNS Treg cells, it was possible that our analyses missed Treg cells that had substantially downregulated or lost *Foxp3* (exTreg cells). We, therefore, performed a “fate-mapping” experiment by using an adoptive transfer approach. Bone marrow chimeric mice (wild-type [or *Foxp3 Cre* control] plus *Blimp1*^{ΔFoxp3}) were immunized to increase the fraction of Blimp1-expressing Treg cells in the spleen, and the total Treg cell compartment from these donor mice comprising a mixture of wild-type (or *Foxp3 Cre* control) and *Blimp1*^{ΔFoxp3} Treg cells were transferred into recipient mice together with congenically marked (Thy1.1) conventional CD4⁺ T cells (Figure 6A). After immunization with MOG(35-55) in CFA, Blimp1 sufficient control Treg cells and *Blimp1*^{ΔFoxp3} Treg cells were re-isolated from secondary hosts based on congenic markers (Figure 6B). Despite similar Ki67⁺ fractions in wild-type and *Blimp1*^{ΔFoxp3} Treg cells (Figure 6C), the majority of *Blimp1*^{ΔFoxp3} Treg cells had lost *Foxp3* expression, whereas wild-type Treg cells remained *Foxp3* positive (Figure 6D). Accordingly, CNS2 as well as CpG islands of other key Treg cell loci, including the *Foxp3* promoter region and *Ctla4* in exTreg cells derived from *Blimp1*^{ΔFoxp3} Treg cells, were almost fully methylated (Figure 6E). In contrast, wild-type Treg cells kept the demethylated state of CNS2 in the *Foxp3* locus (Figure 6E).

Antagonistic Control of CNS2 (TSDR) Methylation by Blimp1 and IL-6-STAT3 through *Dnmt3a*

The methylation status of the *Foxp3* CNS2 has previously been shown to be controlled by the ten-eleven-translocation (Tet) family of enzymes, which contribute to its demethylation (Yue et al., 2016), and DNA-methyltransferases (Dnmts), which oppose the activity of Tet enzymes and methylate CpG islands in the CNS2 (Feng et al., 2014, Josefowicz et al., 2009). Although genes encoding Tet molecules were not substantially de-regulated in *Blimp1*^{ΔFoxp3} Treg cells (Figure S4), we found significantly increased expression of *Dnmt3a* (but not *Dnmt1*) in Blimp1-deficient exTreg cells (Figure 6F). Notably, ATAC-seq analysis in CNS Treg cells isolated from mixed bone marrow chimeric mice at peak EAE showed an increase in ATAC peaks in *Dnmt3a* in *Blimp1*^{ΔFoxp3} CNS Treg cells as compared to wild-type CNS Treg cells, and re-analysis of Blimp1 ChIP-seq data (Mackay et al., 2016) confirmed Blimp1 binding in the *Dnmt3a* locus (Figure S4). In contrast, *Dnmt1* lacked ATAC peaks and Blimp1 binding in wild-type and *Blimp1*^{ΔFoxp3} CNS Treg cells, and the *Tet* loci either lacked or had decreased ATAC peaks in *Blimp1*^{ΔFoxp3} versus wild-type CNS Treg cells (Figure S4). Consistent with higher *Dnmt3a* expression in Blimp1-deficient versus control Treg cells, we found increased methylation of other known *Dnmt3a* target genes, including *Coro2a*, *Nfkbiz*, and *Ikzf3* (Kramer et al., 2017), in *Blimp1*^{ΔFoxp3} Treg cells re-isolated from MOG(35-55)+CFA-immunized secondary host mice compared to wild-type Treg cells (Figure 6G). Thus, our data indicated that in an inflammatory environment, *Dnmt3a* was suppressed by Blimp1.

Inflammatory signals have been associated with the loss of Treg cell identity (Zhou et al., 2009), and IL-6 in particular has been shown to drive the expression of inflammatory cytokines in *Foxp3*⁺ Treg cells (Yang et al., 2008). We hypothesized that IL-6 may contribute to the loss of *Foxp3* observed in *Blimp1*^{ΔFoxp3} Treg cells. Therefore, we interrogated the transcriptome of CNS Treg cells versus splenic Treg cells of EAE

mice for the enrichment of IL-6 target genes. We found that IL-6 target genes were enriched in the transcriptome of CNS Treg cells as compared to splenic Treg cells (Figure 7A), suggesting that CNS Treg cells indeed sensed IL-6. We also hypothesized that IL-6 induced the expression of Dnmt3a, which would contribute to the methylation of the *Foxp3* locus. Notably, the *Dnmt3a* locus was more accessible in *Blimp1*^{ΔFoxp3} CNS Treg cells than in wild-type CNS Treg cells in our ATAC-seq analysis (Figure S4), and some genomic regions of *Dnmt3a* covered by ATAC peaks comprised STAT3 binding sites according to previously published STAT3 ChIP-seq data (Hirahara et al., 2015) in CD4⁺ T cells (Figure S5A). In order to analyze the role of Blimp1 in the IL-6-mediated induction of Dnmt3a in wild-type Treg cells, we made use of the observation that KLRG1⁺ Treg cells expressed high amounts of Blimp1, whereas KLRG1⁻ Treg cells essentially lacked Blimp1 (Figure 7B) and tested the expression of Dnmt3 in KLRG1⁻ Treg cells of wild-type and *Il6*^{-/-} mice. Dnmt3a expression in sorted KLRG1⁻ Foxp3 (GFP)⁺ Treg cells in the spleen of *Il6*^{-/-} mice was reduced as compared to wild-type littermates (Figure 7B), suggesting that IL-6 was required for the expression of Dnmt3a in KLRG1⁻ Treg cells. Although the capacity to sense IL-6 was similar in Blimp1 (YFP)⁻ and Blimp1 (YFP)⁺ Treg cells (Figure S5B), IL-6 induced the downregulation of Foxp3 in Blimp1 (YFP)⁻ but not Blimp1 (YFP)⁺ Treg cells *in vitro* (Figure 7C). Interestingly, Blimp1 did not only bind directly to the *Dnmt3a* locus but also to the *Stat3* locus, where the binding of Blimp1 was associated with reduced ATAC peaks in wild-type as compared to *Blimp1*^{ΔFoxp3} Treg cells isolated from the CNS of mixed bone marrow chimeras (Figure S5A), consistent with the reduced expression of STAT3 in wild-type Treg cells as compared with *Blimp1*^{ΔFoxp3} Treg cells (see Figure S3B), indicating that Blimp1 might also suppress the expression of STAT3.

Finally, when KLRG1⁻ Treg cells isolated from *Dnmt3a*^{-/-} mice (*Dnmt3a*^{flx/flx} × *Mx1 Cre*; *Dnmt3a*^{ΔMx1}) were exposed to IL-6 *in vitro*, the downregulation of Foxp3 was significantly less pronounced in *Dnmt3a*^{-/-} Treg cells as compared to control Treg cells (*Dnmt3a*^{wt/wt} × *Mx1 Cre*) (Figure 7D). In addition, the IL-6-induced loss of Foxp3 in Blimp1⁻ Treg cells was rescued when Dnmt3a was pharmacologically blocked using the inhibitor SGI-1027 (Figures 7E and S5C). Thus, the loss of Dnmt3a by genetic ablation or pharmacologic inhibition protected Blimp1⁻ Treg cells from the loss of Foxp3. In summary, our data indicated that Blimp1 controlled the stability of Treg cells by suppressing the expression and function of Dnmt3a, which was induced in an inflammatory environment in an IL-6-STAT3-dependent manner.

Discussion

Treg cells have been shown to be remarkably stable and can preserve their identity and function in a range of conditions both during steady state and in inflammation (Rubtsov et al., 2010). A variety of signals, including costimulatory signals, were identified to contribute to the stability of Treg cells (DuPage et al., 2015). However, Treg cells lose their identity upon the loss of Foxp3 expression (Williams and Rudensky, 2007), and the idea of lineage stability of Treg cells has been challenged in severe inflammation (Zhou et al., 2009). On the other hand, proinflammatory cytokines, including IL-12, IL-27, and IFN-γ, have been shown to shape Treg cell responses. Although IL-12 and IL-27 are considered to adapt Treg cells to suppress the corresponding (Th1) effector responses (Hall et al., 2012, Koch et al., 2009), IFN-γ has been reported to induce an intrinsic “fragility of Treg cells,” even though it does not induce a loss of Foxp3 (Overacre-Delgoffe et al., 2017). Yet, sensing of IFN-γ by Treg cells is also important for their IL-10 production in the context of Th1 responses (Koch et al., 2012). This is consistent with our data, which show that STAT1 signaling in Treg cells is required for the expression of Blimp1, a major regulator of IL-10 in Treg cells (Cretney et al., 2011), and is a factor required to maintain Foxp3 expression as we show here. Although STAT3, STAT4, and STAT5 regulate Blimp1 expression in conventional T cells (Heinemann et al., 2014, Kwon et al., 2009, Neumann et al., 2014, Xin et al., 2016), STAT1 has been shown to directly transactivate the promoter of *Blimp1* in B cells (Chen et al., 2016). Whether STAT1 activation in Treg cells

is dependent on the canonical interferon-induced pathway or non-canonical STAT1 activation by TLR signals (Luu et al., 2014) remains to be determined. Notably, although IL-10 derived from conventional T cells is required to induce full suppressive effector functions in Treg cells (Chaudhry et al., 2011, Heinemann et al., 2014), genetic ablation of the IL-10 receptor (*Il10ra*) in Treg cells is not associated with a loss of Foxp3 expression (Chaudhry et al., 2011). Therefore, our data support the idea that proinflammatory (rather than anti-inflammatory) cytokine signals appear to initiate a feedback loop that maintains Treg cell identity and function in an inflammatory environment.

Blimp1-mediated maintenance of Treg cell stability becomes indispensable under “inflammatory pressure.” Interestingly, it has previously been reported in a fate mapping system that a subset of Foxp3⁺ Treg cells loses Foxp3 expression and becomes proinflammatory (Bailey-Bucktrout et al., 2013), and it is intriguing to speculate that these are Foxp3⁺ Treg cells that fail to sufficiently upregulate Blimp1. In fact, in two recently published studies, lower expression of Foxp3 in Blimp1-deficient Treg cells was measured but not further explored (Bankoti et al., 2017, Ogawa et al., 2018). Yet, a stronger proliferation of Blimp1-deficient as compared to Blimp1-sufficient Treg cells was observed, which is consistent with lower levels of Foxp3 in Blimp1-deficient Treg cells (Bankoti et al., 2017). Here, our data provide a mechanistic underpinning for cytokine-guided stability or plasticity of Treg cells by linking cytokine signaling pathways to the Blimp1-mediated transcriptional modulation of the enzymatic machinery that controls DNA methylation in *cis* regulatory elements of the *Foxp3* locus. Tet family enzymes and Dnmts are opposing players in demethylating or methylating CpG islands within DNA elements in immune cells and non-immune cells (Tang et al., 2015). Unmethylated DNA is *de novo* methylated by Dnmt3a, whereas Dnmt1 is responsible for the maintenance of pre-existing methylation (Lyko, 2018). Consistent with this model, under conditions of inflammation, Dnmt3a might be more important for the *de novo* methylation of the usually fully demethylated CNS2 of Treg cells. We observed an upregulation of Dnmt3a in Blimp1-deficient Treg cells. Together with a guided recruitment of Dnmt3a to DNA sites (Hervouet et al., 2009) perhaps facilitated by transcription factors such as Ets1, which binds to CNS2 (Polansky et al., 2010) and whose binding motif is overrepresented in the ATAC peaks of *Blimp1*^{ΔFoxp3} Treg cells, Dnmt3a might not only be more abundant but also more active in *Blimp1*^{ΔFoxp3} Treg cells as compared to wild-type Treg cells, resulting in methylation of distinct target loci.

In Treg cells, CNS2 acts as a sensor for IL-2 whose downstream signal transducer STAT5 binds to CNS2. The susceptibility of this sensor to STAT5 binding is, in turn, controlled by its methylation status (Feng et al., 2014). IL-6-STAT3-mediated Dnmt3a activation promotes CNS2 methylation and, thus, likely abrogates STAT5 binding, resulting in reduced Foxp3 transcription under limiting IL-2 conditions (Feng et al., 2014). Our data suggest that there might be a direct antagonistic control of *Dnmt3a* by STAT3 versus Blimp1 (see Figure S5D). Our model is not mutually exclusive with the idea that some genes that are suppressed by Foxp3 (Zhou et al., 2008) are also directly (transcriptionally) suppressed by Blimp1. For instance, direct suppression of *Il17a* has recently been described in Helios⁻RORγt⁺ pTregs in the intestine (Ogawa et al., 2018). However, in autoimmune CNS inflammation, pTreg cells are not induced (Josefowicz et al., 2012, Korn et al., 2007), and the “toxic gain” of IL-17 production, which we observed in Blimp1-deficient CNS Treg cells, only represents a minor contribution to the overall production of IL-17 in the CNS of EAE mice. Therefore, we conclude that the global loss of Treg cell identity through the loss of Foxp3 expression as a consequence of a lack of Blimp1 in CNS Treg cells is responsible for the uncontrolled inflammatory phenotype that we noticed in *Blimp1*^{ΔFoxp3} mice. In future studies, it will have to be seen how the Blimp1-mediated transcriptional regulation of Dnmt3a and its recruitment to DNA loci determine a hierarchy of epigenetic regulation of distinct genetic loci in Treg cells. In addition, the activation of the IL-6-STAT3 pathway has many downstream targets, and specific modulators of that pathway that result in enhanced expression and function of Dnmt3a in Treg cells need to be identified.

In summary, we have identified a mechanism that antagonizes IL-6-STAT3-induced abrogation of Foxp3 in

tTreg cells. This pathway is Blimp1 dependent and supports Treg cell identity at sites of inflammation by keeping CNS2 in a demethylated state, thus allowing the continuous expression of Foxp3 and maintenance of suppressive function. The opposing activities of STAT3 and Blimp1 converging on Dnmt3a will be interesting to further explore for druggable targets that could be used to stabilize Treg cells in autoimmunity and destabilize them for the treatment of cancer.

STAR★Methods

Key Resources Table

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Thomas Korn (thomas.korn@tum.de).

Experimental Models and Subject Details

Animals *Prdm1.yfp* (Blimp1 (YFP)) reporter mice ([Rutishauser et al., 2009](#)), *Rag1*^{-/-} mice ([Mombaerts et al., 1992](#)), *Il12rb2*^{-/-} mice ([Wu et al., 2000](#)), *WSX (Il27r)*^{-/-} mice ([Yoshida et al., 2001](#)), *Prdm1*^{flox/flox} (*Blimp1*^{flox/flox}) ([Shapiro-Shelef et al., 2003](#)), *Il6*^{-/-} mice ([Kopf et al., 1994](#)), and congenic CD45.1 mice ([Shen et al., 1985](#)) were obtained from Jackson Laboratory. Treg conditional Blimp1-deficient mice did not develop spontaneous autoimmunity. DEREK mice ([Lahl et al., 2007](#)) were kindly provided by T. Sparwasser (Twincore, Hannover Medical School, Hannover, Germany). *Stat3*^{flox/flox} (*Stat3*^{tm2Aki}) mice ([Takeda et al., 1998](#)) were a gift of F. Greten (Institute for Tumor Biology and Experimental Therapy, Georg-Speyer Haus, Frankfurt). *Stat1*^{-/-} mice ([Durbin et al., 1996](#)) were kindly provided by S. Kemmner (U. Heemann, Department of Nephrology, Technical University (TU) Munich). *Stat4*^{-/-} mice ([Kaplan et al., 1996](#)) were obtained from M. Löhning (Department of Rheumatology and Clinical Immunology, Charité-Universitätsmedizin Berlin). To generate mice with cell type-specific excision of loxP-flanked cassettes, mice with loxP-flanked alleles were bred with CD4 Cre (Tg(*Cd4-cre*)^{1Cwi}) mice ([Lee et al., 2001](#)) (from Jackson Laboratory) or with *Foxp3 Cre* mice ([Wing et al., 2008](#)) obtained on the C57BL/6 background from M. Oukka (Department of Pediatrics, University of Washington, USA). *Dnmt3a*^{flox/flox} mice were originally obtained from Dr. En Li (Novartis) ([Okano et al., 1999](#)). *Dnmt3a*^{-/-} mice were generated by breeding *Dnmt3a*^{flox/flox} mice with Mx1-Cre mice (from The Jackson Laboratory). Deletion of the floxed *Dnmt3a* allele was induced by intraperitoneal injection of 250 µg polyI:C (Sigma, P1530) four times every other day. CD90.1 mice were provided by V. Buchholz (Institute for Medical Microbiology, Immunology and Hygiene, TU Munich). Rosa26 RFP ([Luche et al., 2007](#)) x *Foxp3 Cre* mice were kindly provided by Tobias Bopp (Institute of Immunology, Johannes Gutenberg University, Mainz). *Il10*^{flox/flox} x *Foxp3 Cre* mice were generated in WEHI by crossing *Il10*^{flox/flox} mice ([Roers et al., 2004](#)) to *Foxp3 Cre* mice ([Rubtsov et al., 2008](#)), both obtained from Jackson Laboratory. All mouse strains were on pure C57BL/6 background. Animals were kept in a specific pathogen-free facility at the Technical University of Munich or at the Walter and Eliza Hall Institute of Medical Research, Parkville, Australia. All experimental protocols were approved by the standing committee for experimentation with laboratory animals of the Bavarian state authorities or the animal ethics committee of the Walter and Eliza Hall Institute and carried out in accordance with the corresponding guidelines. 6-10 week old age and sex-matched mice were used for all experiments, except for those involving methylation analysis (see [Method details](#)), in which only male mice were used.

Method Details

Generation of mixed bone marrow chimeras For generation of mixed bone marrow chimeras, *Rag1*^{-/-}

mice at 6-8 weeks of age were used as recipients. The mice were irradiated at a total dose of 7 Gray with two times 3.5 Gray 3h apart. $5-10 \times 10^6$ donor bone marrow cells depleted of CD90.2⁺ cells were injected *i.v.* into irradiated *Rag1*^{-/-} recipients within 16-20 h post irradiation. The reconstituted mice were maintained on antibiotic water (Enrofloxacin, Bayer, 0.1 mg/ml) for 2 weeks after transplantation. The reconstitution of the hematopoietic compartment was checked 5-6 weeks post cell transfer in peripheral blood.

Induction of EAE EAE was induced by subcutaneous immunization in the base of tail with 200 µg of MOG35-55 (MEVGWYRSPFSRVVHLYRNGK; Auspep, Tullamarine, Australia) in complete Freund's adjuvant containing 500 µg Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) per mouse plus intravenous injection (*i.v.*) of 200 ng pertussis toxin (Sigma-Aldrich) on days 0 and 2 after immunization. Disease progress and severity were assessed as described before ([Korn et al., 2008](#)). The peak of disease was typically between 15 and 20 days post immunization.

Adoptive transfer of Treg cells 1.0×10^5 - 3.0×10^5 FACS-sorted Treg cells (CD4⁺Foxp3 (GFP)⁺) from mixed bone marrow chimeras were transferred into *Rag1*^{-/-} recipients along with 0.5×10^6 - 1.5×10^6 naive conventional T cells (CD4⁺CD25⁻CD44⁻).

Preparation of mononuclear cells from the central nervous system (CNS) Mice were perfused through the left cardiac ventricle with phosphate-buffered saline. The forebrain and cerebellum were dissected and spinal cords flushed out with phosphate-buffered saline by hydrostatic pressure. CNS tissue was cut into pieces and digested with 2.5 mg/ml collagenase D (Roche Diagnostics) and 1 mg/ml DNaseI (Roche Diagnostics) at 37°C for 45 min. Mononuclear cells were isolated by passing the CNS tissue through a 70 µm cell strainer (BD Biosciences), followed by a percoll gradient (70%/37%) centrifugation. The cells were removed from the interphase, washed and resuspended in culture medium for further analysis.

Preparation of peripheral T cells Peripheral CD4⁺ T cells were enriched from pooled spleen and lymph node cells using anti-CD4 microbeads (Miltenyi Biotec) followed by magnetic separation. Subsequently, Blimp1 (YFP)⁻ Treg cells and Blimp1 (YFP)⁺ Treg cells were sorted from Blimp1 (YFP) reporter mice as CD4⁺YFP⁻CD25⁺GITR⁺ and CD4⁺YFP⁺CD25⁺GITR⁺, respectively. Naive CD4⁺ T cells (CD4⁺CD25⁻CD44⁻) or (CD4⁺Foxp3 (GFP)⁻CD44⁻) were sorted on a FACS Aria III machine (B D Biosciences).

Cell culture 10% FCS containing DMEM supplemented with 5×10^{-5} M β-mercaptoethanol, 1 mM sodium pyruvate, non-essential amino acids, L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin was used as culture medium. Cells were cultured in 96 well U bottom plates (Corning) at 37°C/5% CO₂. 0.5×10^5 or 1.0×10^5 FACS-sorted Treg cells (CD4⁺CD25⁺GITR⁺ or CD4⁺Foxp3 (GFP)⁺) were stimulated with anti-CD3 and anti-CD28 dynabeads (Invitrogen) for four days in the presence of either recombinant murine IL-2 (100 ng/ml) alone or with individual cytokines (recombinant murine); IL-6 (50 ng/ml) or IL-1β, IL-4, IL-10, IL-12, IL-17, IL-21, IL-23, IL-24, IL-27, IL-33 and IFN-γ (100 ng/ml). All cytokines were purchased from Miltenyi Biotec and R & D Systems. In some experiments, Treg cell cultures were treated with SGI-1027 (indicated concentrations) (Sigma Aldrich). For iTreg cell cultures, naive T cells (CD4⁺Foxp3 (GFP)⁻CD62L⁺CD44⁻) were sorted from Foxp3 (GFP) reporter mice and cultured with anti-CD3 and anti-CD28 dynabeads for five days in the presence of IL-2 (10 ng/ml) and TGF-β (5 ng/ml; R & D Systems).

Antibodies and flow cytometry Cell suspensions from lymphoid organs were stained with fluorochrome-conjugated anti-mouse CD4 (RM4-5), CD25 (PC61.5), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD90.1 (OX-7), GITR (CD357) (DTA-1), Ki67 (16A8), CD126 (D7715A7) and KLRG1 (2F1), which were purchased from either Biolegend, eBioscience or BD Biosciences. For dead cell exclusion, LIVE/DEAD[®] fixable Near-IR stain kit (Invitrogen) was used. For intracellular staining, Foxp3 staining kit (eBioscience) was used for Foxp3 (FKJ-16 s), IL-10 (JES5-16E3), IL-17 (TC11-18H10), IFN-γ (XMG1.2).

For cytokine stainings, cells were stimulated in culture medium containing phorbol 12-myristate 13-acetate (PMA, 20 ng/ml, Sigma), ionomycin (1 µg/ml, Sigma), and monensin (GolgiStop 1 µl/ml, BD Biosciences) at 37°C/5% CO₂ for 4 h. Flow cytometric analysis was performed on a CyAn ADP flow cytometer (Beckman Coulter) or a FACS Aria III (BD Biosciences), and flow cytometric data were analyzed using FlowJo software (Tree Star).

Quantitative PCR Total RNA was isolated from purified Treg cells and Tconv cells with RNAeasy Plus micro kit (QIAGEN). The isolated RNA was transcribed into cDNA using the TaqMan Reverse Transcription Reagents Kit (Life Technologies) according to the manufacturer's instructions. Probes were purchased from Life Technologies and the assays were performed on 96-well reaction plates (Life Technologies). The real time PCR was performed on StepOnePlus system (Life Technologies). In all experiments *Actb* was used as reference gene to normalize gene expression.

RNA-seq Total RNA was isolated from purified Treg cells and Tconv cells with RNAeasy Plus micro kit (QIAGEN). Quality and integrity of total RNA was controlled on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies). RNaseq analysis: library preparation for bulk 3'-sequencing of poly(A)-RNA was done as described previously ([Parekh et al., 2016](#)). Briefly, barcoded cDNA of each sample was generated with a Maxima RT polymerase (Thermo Fisher) using oligo-dT primer containing barcodes, unique molecular identifiers (UMIs) and an adaptor. 5' ends of the cDNAs were extended by a template switch oligo (TSO) and full-length cDNA was amplified with primers binding to the TSO-site and the adaptor. cDNA was tagmented with the Nextera XT kit (Illumina) and 3' end-fragments finally amplified using primers with Illumina P5 and P7 overhangs. In comparison to [Parekh et al. \(2016\)](#), the P5 and P7 sites were exchanged to allow sequencing of the cDNA in read1 and barcodes and UMIs in read2 to achieve a better cluster recognition. The library was sequenced on a NextSeq 500 (Illumina) with 75 cycles for the cDNA in read1 and 16 cycles for the barcodes and UMIs in read2. Data was processed using the published Drop-seq pipeline (v1.0) to generate sample- and gene-wise UMI tables ([Macosko et al., 2015](#)). Reference genome (GRCm38) was used for alignment. Transcript and gene definitions were used according to the ENSEMBL annotation release 75. Differential gene expression was calculated using the DESeq2 package (v1.18.0) ([Love et al., 2014](#)). We focused on genes with less than 5% probability to be false positive (p-adjusted < 0.05). When necessary, log₂ transformation was realized using "rlog" function from Deseq2.

For gene set enrichment analysis (GSEA) a ranked list of the fold change of RNA-seq read values of the samples to be compared was calculated. The java GSEA Desktop Application v2.2.1, was used in conjunction with the Molecular Signatures Database v45.1 to run the analysis. We always used sample label (phenotype) permutation and thus applied an FDR of 0.25.

ChIP-seq To assess Blimp1 binding at specific gene loci, we interrogated the ChIP-seq data published earlier ([Mackay et al., 2016](#)). Here, CD8⁺ T cells from *Blimp1*-Bio mice carrying a biotin acceptor sequence at the carboxyl terminus of Blimp1, which was biotinylated *in vivo* by coexpression of the *Escherichia coli* biotin ligase BirA from the Rosa26^{BirA} allele, were stimulated *in vitro* with anti-CD3 and anti-CD28 in the presence of IL-2 and IL-12 ([Mackay et al., 2016](#)). To obtain STAT3 ChIP-seq data, murine naive CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of IL-6 ([Hirahara et al., 2015](#)). ChIP-seq tracks were generated using Integrated genome browser ([Robinson et al., 2011](#)).

ATAC-seq ATAC-seq was done as described by [Corces et al., \(2017\)](#). Briefly, 50000 cells (viability > 90%) were pelleted (500 *rcf.*, 4°C, 5 min) and re-suspended in ATAC Resuspension Buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM MgCl₂) supplemented with 0.1% NP40, 0.1% Tween-20 and 0.01% digitonin for lysis, incubated on ice for 3 min and then 1ml of ATAC Resuspension Buffer supplemented only with 0.1% Tween-20 was added and spun at 500 g for 10 min at 4°C to collect nuclei. Nuclei were subsequently re-suspended in 50 µl Transposase reaction containing 25µL 2x tagmentation buffer, 5.25 µl water, 2.5 µl Tn5 Transposase (Illumina Nextera DNA Library Preparation Kit, cat. FC-121-1030), 16.5 µl PBS, 0.25 µl of

2% digitonin (Promega, G9441), 0.5 µl of 10% Tween-20. Reactions were incubated for 30 min at 37°C in a thermomixer shaking at 900 rpm and DNA purified using QIAGEN PCR clean-up MinElute kit (QIAGEN). The transposed DNA was subsequently amplified in 50µl reactions with custom primers as described ([Buenrostro et al., 2013](#)). After 4 cycles libraries were then monitored with qPCR: 5 µl PCR sample in a 15 µl reaction with the same primers. qPCR output was monitored for the ΔRN; 0.25 ΔRN cycle number was used to estimate the number of additional cycles of the PCR reaction needed for the remaining PCR samples. Amplified libraries were purified with the QIAGEN PCR clean-up MinElute kit (QIAGEN) and size selected for fragments less than 600 bp using the Agencourt AMPure XP beads (Beckman Coulter). Libraries were quality controlled by Qubit and Agilent DNA Bioanalyzer analysis. Deep sequencing was performed on a HiSeq 1500 system according to the standard Illumina protocol for 50bp single-end reads.

ATAC-seq reads were aligned to the mouse genome mm10 using Bowtie ([Langmead, 2010](#)) with options “-q -n 2 -best -chunkmbs 2000 -p 32 -S.” ATAC peaks over Input background were identified using Homer ([Heinz et al., 2010](#)) findPeaks.pl with option “-style factor.” Peaks from all samples were merged using mergePeaks resulting in a unified Peak set. The peak list was filtered for promoter-associated peaks (distance to TSS < 1000bp) with bedtools. Raw ATAC coverage counts were then calculated with annotatePeaks within 500bp across the peak centers. Differential ATAC peaks were determined with the DESeq2 result function and filtered for padj < 0.05. Genomic feature annotation of ATAC-seq peaks was done using annotatePeaks. Transcription factor motif prediction was done with Homer findMotifsGenome.pl.

Methylation analysis For all methylation analyses, cells from male mice were used. Bisulfite-converted DNA was prepared from FACS-sorted Treg cells and Tconv cells according to the manufacturer’s instructions (EZ DNA Methylation-Direct Kit, Zymo Research). Pyrosequencing was performed as described previously ([Yang et al., 2016](#)). Amplification and sequencing of the Treg cell-specific demethylated region (TSDR) (chromosome position X:7583986-7584149), the *Foxp3* promoter (chromosome position X:7579550-7579731), a region in *Ctla4* (chromosome position 1:60912472-60912685), *Coro2a* (chromosome position 4:46567312-46567503), *Nfkbiz* (chromosome position 16:55836298-55836608) and *Ikzf3* (chromosome position 11:98476715-98477042) was performed with the following amplification/sequencing primer sets: mTSDR-for, biotinylated (5'-TAAGGGGGTTTAAATATTTATGAGGTTT-3'), mTSDR-rev (5'-CCTAAACTTAACCAAATTTTCTACC-3'), mTSDR-S1 (5'-ACCCAAATAAAATAATATAAATACT-3'), mTSDR-S2 (5'-ATCTACCCACAAATTT-3'), mTSDR-S3 (5'-AACCAAATTTTCTACCATT-3'), mFoxp3Pr-for (5'-GTTTATTTTGGTGAAGTGGATTGTTAGAGG-3'), mFoxp3Pr-rev, biotinylated (5'-CCCTACAATTATCAACACACACTCAT-3'), mFoxp3Pr-S1 (5'-TTTGTGGGAAATTGTTATATTATTA-3'), mCTLA4-for (5'-AGTGGTGTGGTTAGTAGTTATGG-3'), mCTLA4-rev, biotinylated (5'-ACATAAATTCCACCTTACAAAATACAAT-3'), mCTLA4-S1 (5'-GTTGGTTAGTAGTTATGGT-3'), mCTLA4-S2 (5'-TTATTATTATATAATATTGATGAGG-3'), mCTLA4-S3 (5'-GATAAATGATTAATGATTGAGG-3'), mCTLA4-S4 (5'-ATTTTATTATTAAGGATTGAGAG-3'), mCoro2a-for (5'-GGTTTTTTGAATTATTTGAGTTGTTAGAA-3'), mCoro2a-rev, biotinylated (5'-CCTACTCCTTACAAAATAAACCTTACTA-3'), mCoro2a-S1 (5'-TTTGAGTTGTTAGAATTTATAAAA-3'), mNfkbiz-for (5'-TGAAGGAAAAGGGGATAAATTTGT), mNfkbiz-rev, biotinylated (5'-CTTAAAACATTCACCCACTTACC-3'), mNfkbiz-S1 (5'-GGATTTTGATTTTGGGATG-3'), mNfkbiz-S2 (5'-ATTTTGAGGTTTATGTTGAAT-3'), mIkzf3-Ex7-for (5'-AAGTGTGGAGGTAAGATATATTAAGT-3'), mIkzf3-Ex7-rev, biotinylated (5'-ACCAAATACTCCCCTATTCTTTAACTA-3'), mIkzf3-Ex7-S1 (5'-

GGATAGATTAGTAAGTAATGTGGTTA-3'), mIkzf3-Ex7-S2 (5'-AGTAGATATTTTGGATGAGATAATATAG-3'). The indicated chromosome positions refer to genome assembly GRCm38.p5.

Quantification and Statistical Analysis

Statistical evaluations of cell frequency measurements, cell numbers, mRNA amounts, and protein levels were performed with the unpaired Student's t test when two populations were compared. Two-tailed p values < 0.05 were considered significant. Multiple comparisons were performed with one-way ANOVA or two-way ANOVA followed by post hoc multiple comparisons tests as indicated in the legends to the figures. EAE scores between groups were analyzed as disease burden per individual day with one-way ANOVA and post hoc test as indicated. Survival curve was calculated using Kaplan-Meier analysis and the p values were analyzed with log-rank test (Mantel-Cox). A p value of p < 0.05 was considered significant. Calculations were performed using Graph Pad Prism v5.0 (Graph-Pad software).

Data Availability

The data that support the findings of this study are available from the corresponding author upon request. Accession codes: The RNA-seq data are available at <https://www.ebi.ac.uk/ena> under the accession code ENA: PRJEB30787. The ATAC-seq data are available at <https://www.ncbi.nlm.nih.gov/geo> under the accession code GEO: [GSE121764](https://www.ncbi.nlm.nih.gov/geo/acc/GSE121764).

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Author Contributions

G.G. designed and performed most experiments and drafted the manuscript. A.M., A.V., Y.Z., T.R., M.H., C.P., L.A., and B.K. performed or contributed to specific experiments. G.L. performed RNA-seq data analysis. H.M. and G.S. generated and analyzed ATAC-seq data. S.F. and J.H. performed bisulfite pyrosequencing and interpreted the methylation data. R.O. and R.R. generated RNA-seq data. L.P., R.K., and M.K. provided reagents and analyzed data. A.W. supervised and analyzed EAE (*Foxp3 Cre*) data. A.K. supervised experiments, analyzed data, and edited the manuscript. T.K. conceptualized the study, supervised the experiments, analyzed data, and wrote the manuscript.

Declaration of Interests

The authors declare no competing financial interests.

Notes

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Footnotes

Supplemental Information includes five figures and two tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2019.01.070>.

Supplemental Information

Document S1. Figures S1–S5:

Table S1. Differentially Expressed Genes in Wild-Type Foxp3⁺ Treg Cells Isolated from the CNS or the Spleen of Mice at the Peak of EAE, Related to Figure 1:

Table S2. Differentially Expressed Genes in Wild-Type Foxp3⁺ Treg Cells Versus Blimp1-Deficient Foxp3⁺ Treg Cells Isolated from the CNS of Mixed Bone Marrow Chimeric Mice (Wild-Type [CD45.1] Plus *Blimp1*^{ΔFoxp3} [CD45.2]) at the Peak of EAE, Related to Figure 3:

Document S2. Article plus Supplemental Information:

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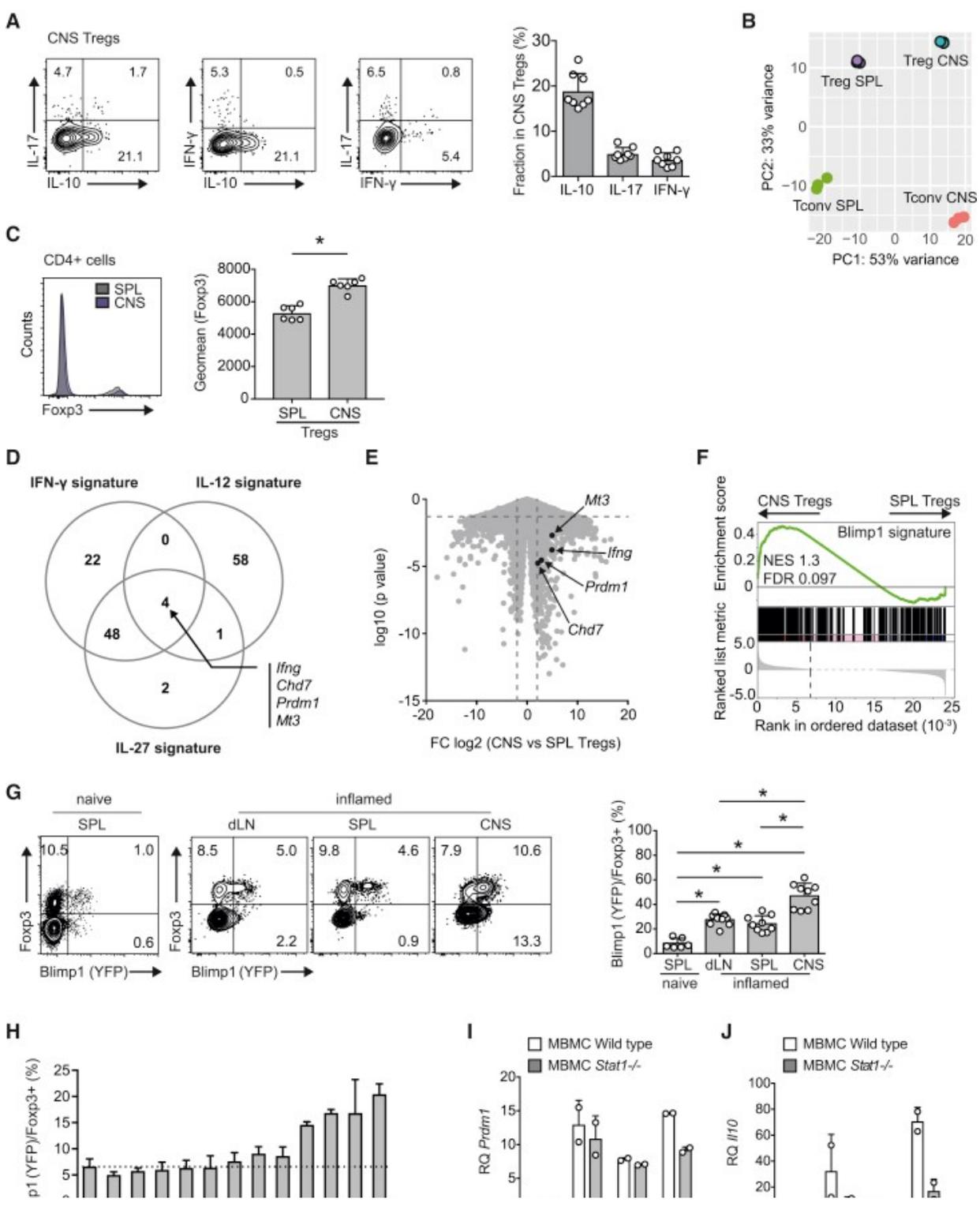
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Figures and Tables

Figure 1



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CNS Treg Cells are Stable and Express Blimp1 in Response to Proinflammatory Cytokines

(A) Mononuclear cells were isolated from the CNS of EAE mice at the peak of disease and were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin to analyze the expression of IL-10, IL-17, and IFN- γ in Treg cells by flow cytometry. Mean of eight biological replicates \pm SD, derived from three independent experiments. Symbols depict individual mice (bars, mean \pm SD).

(B) CD4⁺Foxp3⁺ Treg cells were sorted from the CNS and spleen (SPL) of Foxp3 (GFP) reporter mice at the peak of EAE and subjected to RNA-seq. Principal-component analysis.

(C) Foxp3 expression by intracellular staining of splenic Treg cells and CNS Treg cells at the peak of EAE. Mean of six biological replicates ± SD, derived from two independent experiments. Symbols depict individual mice (bars, mean ± SD), t test, p < 0.00005.

(D and E) Enrichment of CNS Treg “signature” genes in gene sets upregulated in T cells in response to IL-12 ([Agarwal et al., 2009](#)) or IL-27 or IFN-γ ([Hall et al., 2012](#)). Overlap of core enriched genes of the IL-12, IL-27, and IFN-γ signatures in CNS Treg cells as venn diagram (D) and volcano plot (E).

(F) Enrichment of Blimp1 signature genes ([Cretney et al., 2011](#)) within CNS Treg cells and spleen Treg cells.

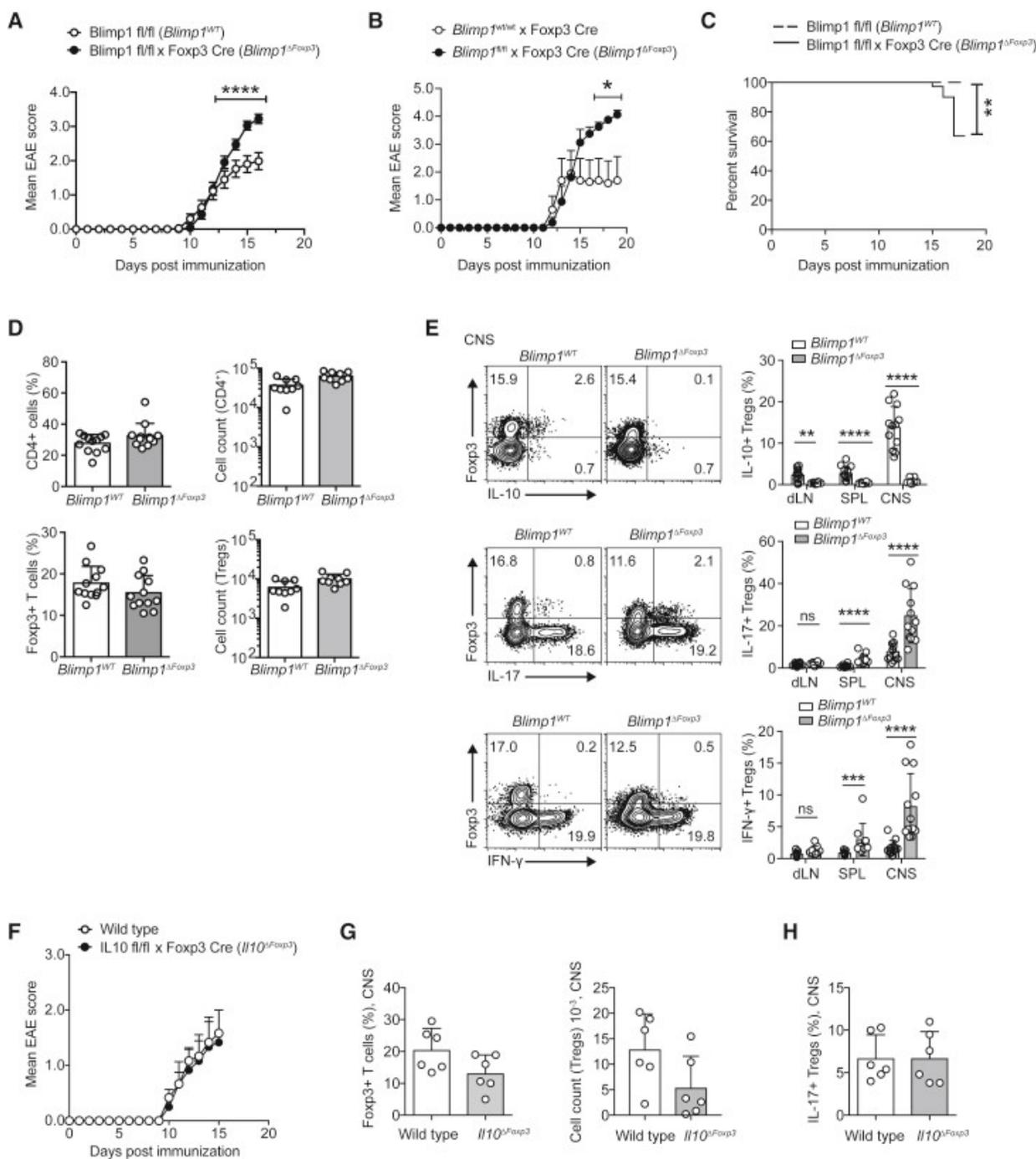
(G) Blimp1 expression was analyzed within Foxp3⁺ cells in naive spleen and in indicated compartments at the peak of EAE in Blimp1 (YFP) reporter mice. Fraction of Blimp1 (YFP)⁺ cells in Treg cells, mean of nine biological replicates ± SD, derived from three independent experiments. Symbols depict individual mice (bars, mean ± SD). Significance was calculated using ANOVA plus Tukey’s post test, p < 0.05.

(H) Cytokines inducing Blimp1 in Treg cells *in vitro*. CD4⁺CD25⁺GITR⁺Blimp1 (YFP)⁻ Treg cells were sorted from the spleen and lymph nodes (LNs) of naive Blimp1 (YFP) reporter mice and cultured with anti-CD3 and anti-CD28 dynabeads in the presence of the indicated cytokines. On day 4, Blimp1 (YFP) expression was analyzed by flow cytometry. Data are summarized from two independent experiments (three technical replicates per experiment) (mean ± SD).

(I and J) Blimp1 is induced in a STAT1-dependent manner *in vivo*. Tconv and Treg cells were fluorescence-activated cell sorting (FACS) sorted from the spleen and CNS of immunized mixed bone marrow chimeras generated with wild-type plus *Stat1*^{-/-} bone marrow. The expressions of *Prdm1* (encoding Blimp1) (I) and *Il10* (J) in Tconv and Treg cells were analyzed by qPCR of re-sorted congenically marked control and knockout cells. Data were summarized from two independent biological replicates. Symbols depict individual biological replicates (bars, mean ± SD).

See also [Figures S1](#) and [S2](#) and [Table S1](#).

Figure 2



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Ablation of Blimp1 in Treg Cells Results in Failure to Control CNS Inflammation

Blimp1^{fllox/fllox} (*Blimp1*^{WT}) or *Foxp3* Cre mice and *Blimp1*^{fllox/fllox} × *Foxp3* Cre (*Blimp1*^{ΔFoxp3}) were immunized with MOG(35-55) in CFA to induce EAE. Mononuclear cells were isolated at the peak of EAE.

(A and B) EAE scores of *Blimp1*^{ΔFoxp3} mice in comparison to *Blimp1*^{WT} controls (A) and *Blimp1*^{ΔFoxp3} mice in comparison to Cre control mice (*Blimp1*^{wt/wt} × *Foxp3* Cre) (B). Data were summarized from three independent experiments. (*Blimp1*^{WT}, n = 20; *Foxp3* Cre, n = 4; *Blimp1*^{ΔFoxp3}, n = 31) (mean ± SEM). (two-way ANOVA, Sidak post test, p < 0.05).

(C) Survival curve. Data were summarized from three independent experiments (*Blimp1*^{WT}, n = 8; *Blimp1*^{ΔFoxp3}, n = 10).

Log-rank test (Mantel-Cox) (** $p < 0.01$).

(D) Frequencies and absolute cell numbers of live CD4⁺ T cells and CD4⁺Foxp3⁺ Treg cells isolated from the CNS at the peak of EAE. Data were summarized from three independent experiments (mean \pm SD).

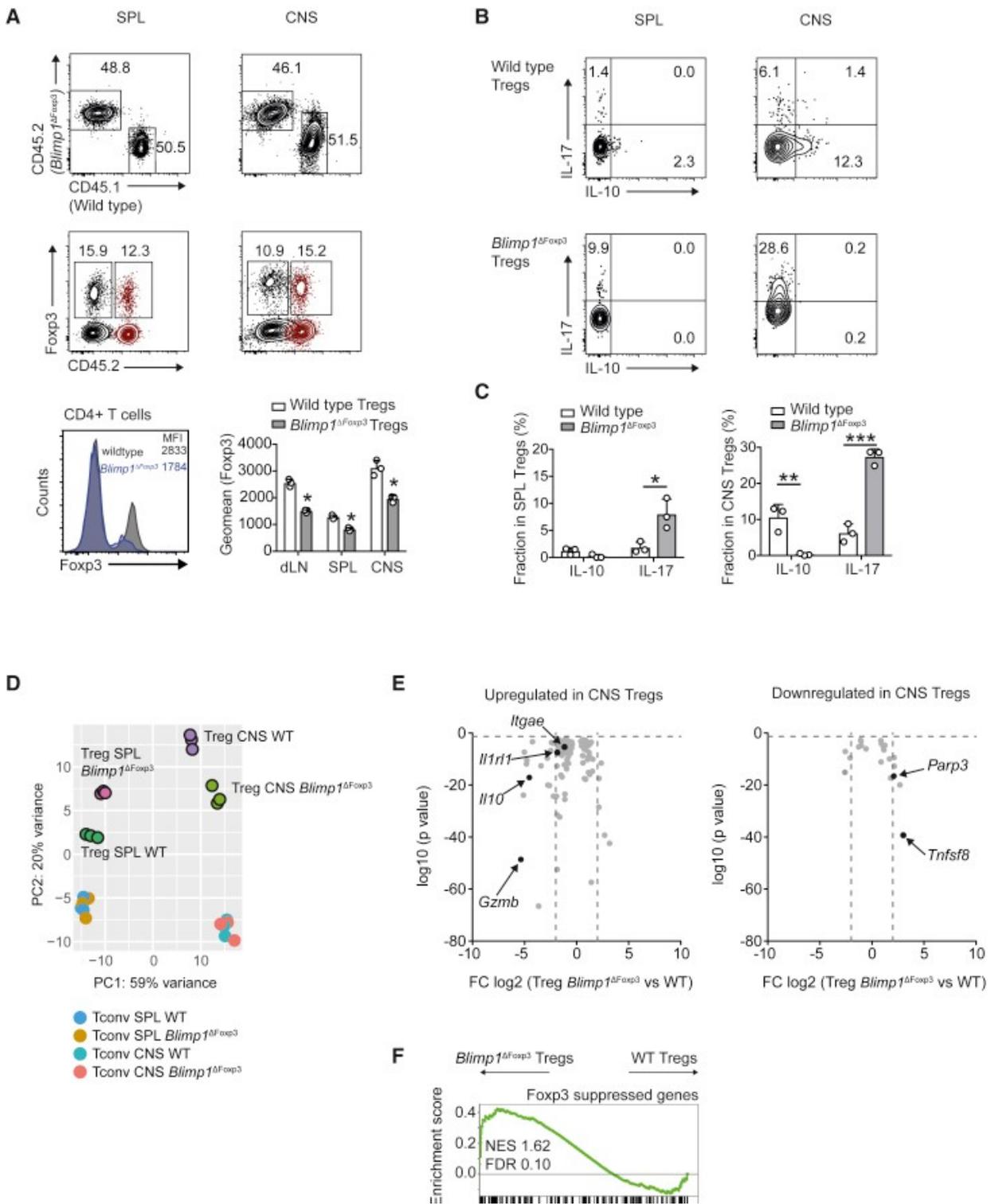
(E) Mononuclear cells were isolated from the draining lymph node (dLN), spleen, and CNS and stimulated with PMA and ionomycin to analyze IL-10, IL-17, and IFN- γ in Treg cells by flow cytometry. Data are summarized from twelve biological replicates, derived from three independent experiments. Symbols depict individual mice (bars, mean \pm SD). Mann-Whitney test (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

(F–H) Wild-type and *Il10*^{fllox/fllox} \times *Foxp3* *Cre* (*Il10* ^{Δ Foxp3}) mice were immunized with MOG(35-55) in CFA to induce EAE. Wild-type, $n = 6$; *Il10* ^{Δ Foxp3}, $n = 6$ (mean \pm SD) (F).

(G) Mononuclear cells were isolated from the CNS and analyzed for Foxp3 expression by flow cytometry. The graph represents the frequency and absolute cell numbers of Treg cells. Symbols depict individual mice (bars, mean \pm SD).

(H) The isolated mononuclear cells from the CNS were stimulated with PMA and ionomycin to analyze IL-17 expression in Foxp3⁺ Treg cells by flow cytometry. Data are summarized from six biological replicates. Symbols depict individual mice (bars, mean \pm SD).

Figure 3



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Blimp1 Governs Treg Cell Identity in CNS Treg Cells

Mixed bone marrow chimeras (MBMCs) were generated by reconstituting *Rag1*^{-/-} hosts with (CD45.1, wild-type) and *Blimp1* Δ Foxp3 (CD45.2) bone marrow at a ratio of 1:1. The mixed bone marrow chimeras were immunized with MOG(35-55) in CFA to induce EAE. The mice were analyzed at the peak of EAE.

(A) Chimerism between wild-type and *Blimp1* Δ Foxp3 cells in the live CD4⁺ gate of spleen and CNS mononuclear cells and

analysis of Foxp3 expression by flow cytometry in control (wild-type) Treg cells and *Blimp1*^{ΔFoxp3} Treg cells isolated from the mixed bone marrow chimeras at the peak of EAE. Data are representative and summarized from three biological replicates. Symbols depict individual mice (bars, mean ± SD). Student's t test (*p < 0.05).

(B) Expression of IL-10 and IL-17 in Foxp3⁺ Treg cells after *ex vivo* stimulation with PMA and ionomycin.

(C) Data were summarized from three biological replicates. Symbols depict individual mice (bars, mean ± SD). Student's t test (*p < 0.05).

(D–F) Treg cells and conventional CD4⁺ T cells (Tconv) were sorted at the peak of EAE from the spleen and CNS of immunized mixed bone marrow chimeras (CD45.1 × *Foxp3*^{GFP}, wild-type; CD45.2, *Blimp1*^{ΔFoxp3} × *Foxp3*^{GFP}) and subjected to RNA-seq.

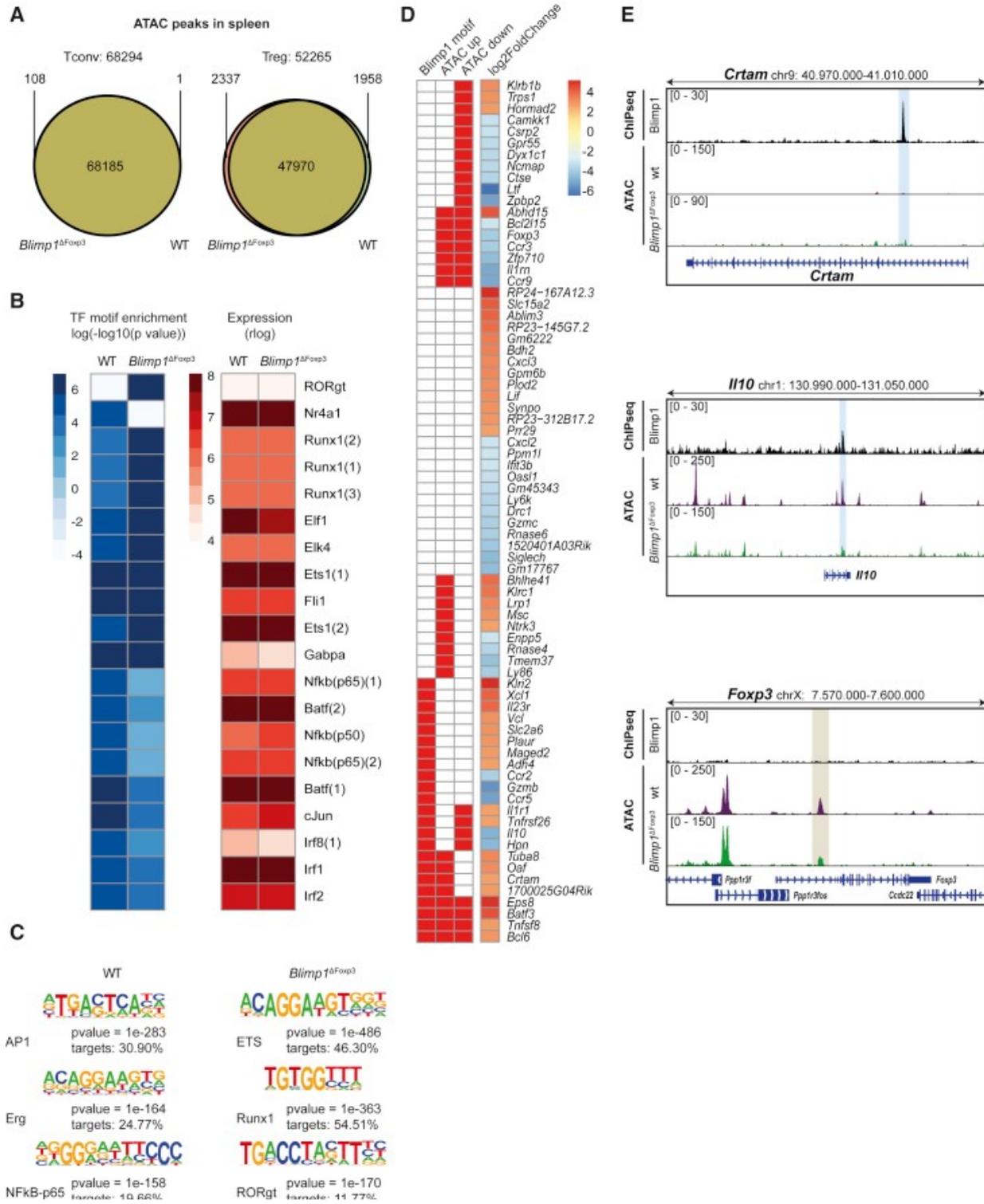
(D) Principal-component analysis of control Treg cells (wild-type), *Blimp1*^{ΔFoxp3} Treg cells, and Tconv cells isolated from mixed bone marrow chimeras, calculated for all genes with greatest difference according to gene expression, with log₂ transformed row and column standardized data.

(E) Genes upregulated (top) or downregulated (bottom) in CNS Treg cells as compared to spleen Treg cells (see [Figure 1](#)) were assessed as to their expression in *Blimp1*^{ΔFoxp3} versus wild-type Treg cells and displayed in volcano plots.

(F) Enrichment of genes that are normally suppressed by Foxp3 ([Williams and Rudensky, 2007](#)) in *Blimp1*^{ΔFoxp3} CNS Treg cells as compared to wild-type Treg cells.

See also [Figure S3](#) and [Table S2](#).

Figure 4



[Open in a separate window](#)

Blimp1 Regulates Genes in CNS Treg Cells in a Direct and Indirect Manner

(A) ATAC peaks in splenic wild-type and *Blimp1*^{ΔFoxp3} Tconv and Treg cells were quantified. Very few peaks were differentially enriched in Tconv cells (left), whereas substantial dysregulation could be identified in Treg cells (right).

(B) Transcription factor motif prediction in differential ATAC peaks of splenic wild-type versus *Blimp1*^{ΔFoxp3} Treg cells was performed with Homer by using known transcription factor motifs. Heatmap displays the log of the transcription

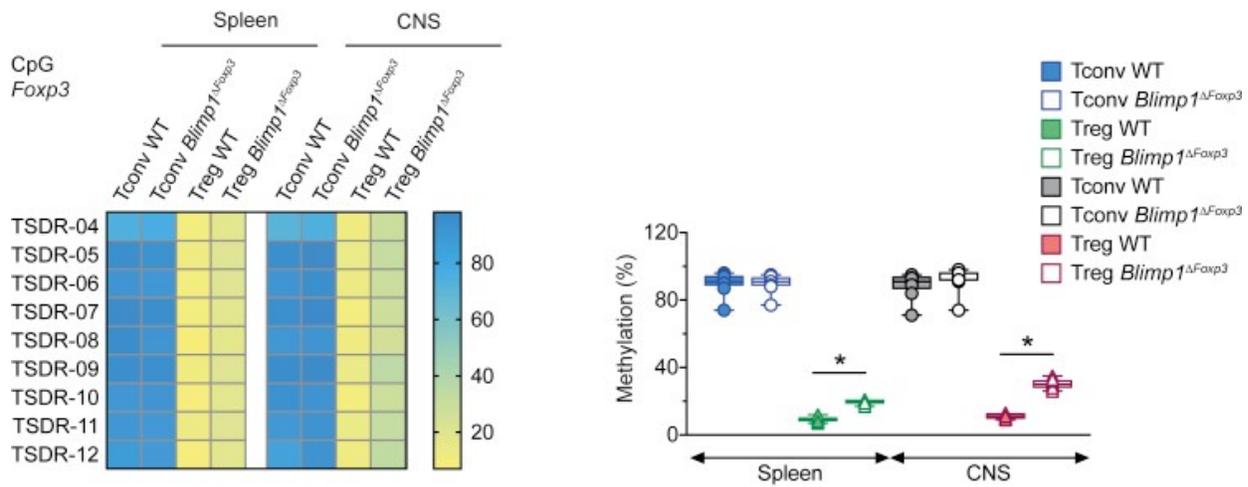
factor (TF) prediction p value (left) and the expression of the respective transcription factors (right). For some transcription factors, multiple motifs were tested (denoted by numbers after the transcription factor name).

(C) Homer was used for *de novo* motif prediction in differential ATAC peaks of splenic wild-type versus *Blimp1*^{ΔFoxp3} Treg cells. The top 3 motifs of this analysis are shown.

(D) Blimp1 peaks and differential ATAC peaks between wild-type and *Blimp1*^{ΔFoxp3} Treg cells in CNS were identified within 50 kb of the transcriptional start sites of differentially expressed genes. Heatmap displays Blimp1 binding and ATAC up- or downregulated peaks in the top 75 differentially expressed genes between wild-type and *Blimp1*^{ΔFoxp3} Treg cells in CNS (red bars).

(E) Integrative genomics viewer (IGV) screenshots of Blimp1 ChIP-seq ([Mackay et al., 2016](#)) and ATAC-seq tracks for CNS wild-type and *Blimp1*^{ΔFoxp3} Treg cells. The scale of the *Blimp1*^{ΔFoxp3} Treg ATAC-seq track was adjusted to display comparable intensities for non-changed ATAC peaks due to the overall reduced signal intensity in this dataset.

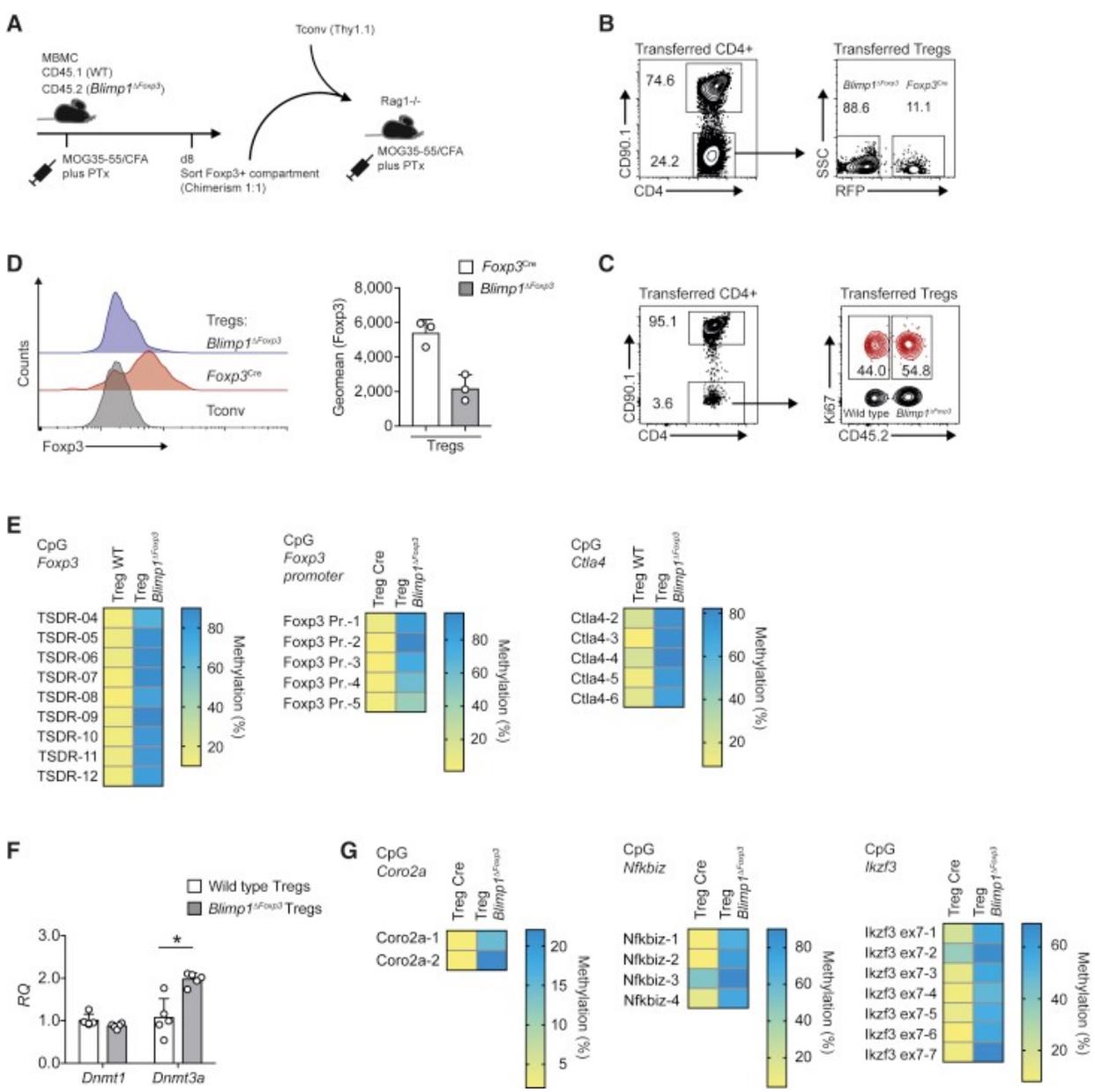
Figure 5



Blimp1 in Treg Cells Is Required to Maintain the Demethylated State of CNS2

Treg cells and Tconv cells from both the wild-type and *Blimp1*^{ΔFoxp3} compartments of mixed bone marrow chimeras were sorted from the spleen and the CNS according to experimental set up of Figure 3, and their genomic DNA was analyzed for the methylation status of CNS2 (TSDR). The data are representative of three independent biological replicates. Each line represents one CpG motif (left). The degree of methylation at each CpG motif is represented according to the color code. Cumulative quantification of the methylation of all CpG islands in CNS2 (TSDR) in Tconv and Treg cells as indicated (right). One-way ANOVA with Holm-Sidak's post-test (*p < 0.0001).

Figure 6



Lack of Blimp1 in Treg Cells Results in Instability of Treg Cells through Epigenetic Changes

(A) Schematic scheme of experimental set up.

(B and C) Total Treg cells, namely, both control Foxp3 Treg cells (*Foxp3 Cre*) (B) or wild-type (C) and *Blimp1*^{ΔFoxp3} Treg cells together, were sorted from spleen and draining lymph node of immunized mixed bone marrow chimera on day 8 post-immunization and were transferred into *Rag1*^{-/-} recipient mice along with congenically marked CD25⁻ CD44⁻ CD90.1⁺ naive conventional CD4⁺ T cells (Thy 1.1 Tconv). On day 1 post-transfer, the recipient mice were immunized with MOG(35-55) and CFA. Mononuclear cells were isolated from the spleen and draining lymph node for analysis 8 days post-immunization.

(C) Ki67 staining in wild-type and *Blimp1*^{ΔFoxp3} Treg cells re-isolated from the spleen of the secondary hosts. Representative of three independent biological replicates.

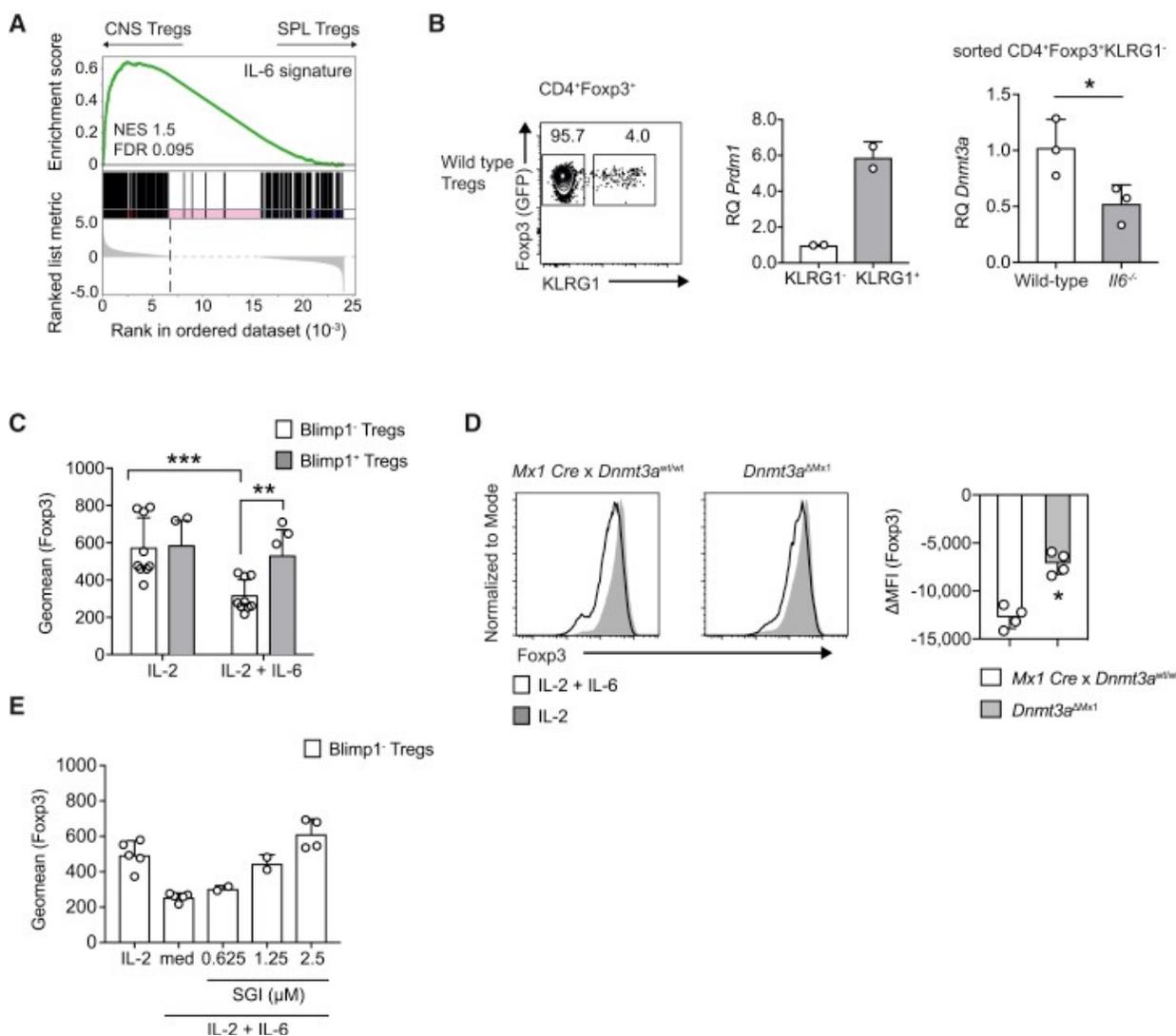
(D) Foxp3 expression was analyzed by intracellular staining. Frequency and geomean of Foxp3⁺ cells within the transferred *Foxp3 Cre* control Treg cells and *Blimp1*^{ΔFoxp3} Treg cells isolated from the spleen of secondary hosts. Cumulative data of five biological replicates derived from two independent experiments. Symbols depict individual mice (bars, mean ± SD). Student's t test (*p < 0.05).

(E) Wild-type and *Blimp1*^{ΔFoxp3} Treg cells were sorted from the secondary hosts, and genomic DNA was analyzed for methylation status of CNS2 (TSDR), *Foxp3* promoter, and *Ctla4*. Representative of three independent biological replicates. Each line represents one CpG motif. The degree of methylation at each CpG motif is depicted according to the color code.

(F) Gene expression of *Dnmt1* and *Dnmt3a* by qPCR in wild-type and *Blimp1*^{ΔFoxp3} Treg cells sorted from the spleen of secondary host mice. The expression of each gene was normalized to the control gene *Actb* and was plotted relative to the mean expression value of replicates for each gene. Cumulative data from five biological replicates, derived from two independent experiments. Symbols depict individual biological replicates (bars, mean ± SD). Student's t test (*p < 0.05).

(G) Methylation status of known *Dnmt3a* target genes in control Treg cells (*Foxp3 Cre* × *Blimp1*^{wt/wt}) and *Blimp1*^{ΔFoxp3} Treg cells (*Foxp3 Cre* × *Blimp1*^{flox/flox}) re-isolated from secondary hosts. Representative of three independent biological replicates. The degree of methylation at each CpG motif is depicted according to the color code.

See also [Figure S4](#).

Figure 7

IL-6 Promotes the Loss of Foxp3 in Treg Cells in the Absence of Blimp1 by Inducing DNA Methylation

(A) Treg cells sense IL-6 in the inflamed CNS. The CNS Treg cell transcriptome (see [Figure 1](#)) is enriched for IL-6 signature genes as assessed by gene set enrichment analysis (GSEA).

(B) KLRG1⁻Foxp3 (GFP)⁺ and KLRG1⁺Foxp3 (GFP)⁺ Treg cells were sorted from spleen by flow cytometry of wild-type mice and tested for *Prdm1* (encoding Blimp1) expression by qPCR. Data are summarized from two biological replicates. KLRG1⁻Blimp1⁻Foxp3 (GFP)⁺ Treg cells were sorted from spleen and lymph nodes of wild-type and *Il6*^{-/-} mice and tested for *Dnmt3a* expression by qPCR. Data are summarized from three biological replicates. Symbols depict individual biological replicates (bars, mean ± SD). Student's t test (*p < 0.05).

(C) Blimp1 (YFP)⁻ and Blimp1 (YFP)⁺ Treg cells were sorted from the spleen and LNs of unmanipulated Blimp1 (YFP) reporter mice and stimulated with anti-CD3 and anti-CD28 dynabeads in the presence of either IL-2 alone or IL-2 and IL-6. On day 4, cultured Treg cells were analyzed for Foxp3 expression by intracellular staining. Cumulative data of three independent experiments, (mean ± SD). Two-way ANOVA (Sidak's multiple comparison test), p < 0.05.

(D) Lack of *Dnmt3a* reduces loss of Foxp3 in Treg cells in response to IL-6. KLRG1⁻ (Blimp1⁻) Foxp3⁺ Treg cells were purified from the spleen and LNs of control mice or inducible *Dnmt3a*-deficient animals (*Dnmt3a*^{ΔMx1}), cultured in the absence or presence of IL-6, and assessed for Foxp3 expression by flow cytometry after 4 days. Representative histograms of Foxp3 expression in IL-2 alone or IL-2 and IL-6 cultured control or *Dnmt3a*^{ΔMx1} Treg cells. ΔMFI (mean fluorescence intensity; Foxp3) in individual mice (bars, mean ± SD). Student's t test (*p < 0.05).

(E) In some cultures, the Blimp1 (YFP)⁻ Treg cells were additionally treated with SGI-1027 at indicated concentrations to

block Dnmt3a activity and analyzed for Foxp3 expression level on day 5 of culture. Cumulative data from two independent experiments.

See also [Figure S5](#).

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD4-PerCP-eF710 (RM4-5)	eBioscience	Cat#46-0042; RRID: AB_1834431
CD4-BV421 (GK1.5)	Biolegend	Cat#100437; RRID: AB_100437
KLRG1-PE-Cy7 (2F1)	eBioscience	Cat#25-5893; RRID: AB_1518768
GITR (CD357)-PE (DTA-1)	BD	Cat#558119; RRID: AB_647208
CD25-PB (PC61 5.3)	eBioscience	Cat#RM6028; RRID: AB_2556574
CD44-BV421 (IM7)	BD	Cat#563970; RRID: AB_2738517
CD62L-APC (MEL-14)	BD	Cat#553152; RRID: AB_398533
CD126-APC (D7715A7)	Biolegend	Cat#115811; RRID: AB_2296238
CD45.1-PE-Cy7 (A20)	eBioscience	Cat#25-0453; RRID: AB_469629
CD45.1-APC-eF780 (A20)	eBioscience	Cat#47-0453; RRID: AB_1582229
CD45.2-APC-eF780 (104)	eBioscience	Cat#47-0454; RRID: AB_1272175
CD45.2-BV421 (104)	Biolegend	Cat#109832; RRID: AB_2565511
CD90.1-AF647 (OX-7)	Biolegend	Cat#202508; RRID: AB_492884
Foxp3-eF450 (FJK-16 s)	eBioscience	Cat#48-5773; RRID: AB_1518813
Foxp3-PE (FJK-16 s)	eBioscience	Cat#72-5775; RRID: AB_469978
IL-10-APC (JES5-16E3)	BD	Cat#554468; RRID: AB_398558
IFN- γ -PE (XMG1.2)	BD	Cat#554412; RRID: AB_395376
IFN- γ -PECy7 (XMG1.2)	eBioscience	Cat#25-7311; RRID: AB_469680
IL-17A-PerCP-Cy5.5 (TC11-18H10)	BD	Cat#560666; RRID: AB_1937311

REAGENT or RESOURCE	SOURCE	IDENTIFIER
IL-17A-PE (TC11-18H10)	BD	Cat#559502; RRID: AB_397256
Ki67-AF647 (16A8)	Biolegend	Cat#652408; RRID: AB_2562139
Chemicals, Peptides, and Recombinant Proteins		
MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK)	Auspep	N/A
SGI-1027	Sigma	Cat#SML1358
Deposited Data		
ATAC-seq	This paper	GEO: GSE121764
RNA-seq	This paper	ENA: PRJEB30787
Experimental Models: Organisms/Strains		
Mouse: B6.129S7- <i>Rag1</i> ^{tm1Mom} /J	The Jackson Laboratory	JAX, Stock#:002216
Mouse: B6.SJL-Ptprc ^a Pepc ^b /BoyJ	The Jackson Laboratory	JAX, Stock#: 002014
Mouse: B6.PL- <i>Thy1</i> ^a /CyJ (CD90.1)	V. Buchholz (Institute of Medical Microbiology, Immunology and Hygiene, TU Munich)	JAX, Stock#: 000406
Mouse: B6.Cg-Tg(<i>Prdm1</i> -EYFP)1Mnz/J	The Jackson Laboratory	JAX, Stock#:008828
Mouse: B6.129- <i>Prdm1</i> ^{tm1Clme} /J (<i>Prdm1</i> ^{flox/flox})	The Jackson Laboratory	JAX, Stock#:008100
Mouse: B6.129S1- <i>Il12rb2</i> ^{tm1Jm} /J	The Jackson Laboratory	JAX, Stock#:003248
Mouse: B6N.129P2- <i>Il27ra</i> ^{tm1Mak} /J	The Jackson Laboratory	JAX, Stock#:018078
Mouse: B6.129S2- <i>Il6</i> ^{tm1Kopf} /J	The Jackson Laboratory	JAX, Stock#:002650
Mouse: B6.129S(Cg)- <i>Stat1</i> ^{tm1Dlv} /J	S. Kemmner (U. Heemann, Department of Nephrology, Technical University (TU) Munich)	JAX, Stock#: 012606
Mouse: B6.Cg- <i>Stat3</i> ^{tm2Aki} _x CD4 Cre	F. Greten (Institute for Tumor Biology and Experimental Therapy, Georg-Speyer Haus, Frankfurt)	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: C.129S2-Stat4 ^{tm1Gru} /J	M. Löhning (Department of Rheumatology and Clinical Immunology, Charité-Universitätsmedizin Berlin)	JAX, Stock#: 002826
Mouse: C57BL/6-Tg(<i>Foxp3</i> -DTR/EGFP)23.2Spar/Mmjax (DEREG)	T. Sparwasser (Hannover Medical School, Hannover)	JAX, Stock#: 32050
Mouse: B6.129(Cg)- <i>Foxp3</i> ^{tm4(YFP/cre)} Ayr/J	The Jackson Laboratory	JAX, Stock#: 016959
Mouse: B6.Cg-Tg(Mx1-cre)1Cgn/J (Mx1-Cre)	The Jackson Laboratories	JAX, Stock#: 003556
Mouse: B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ (CD4 Cre)	The Jackson Laboratories	JAX, Stock#: 022071
Mouse: <i>Il10</i> ^{tm1.1Roer} (<i>Il10</i> ^{flox/flox})	Roers et al., 2004	MGI: 3521569
Mouse: <i>Il10</i> ^{flox/flox} x B6.129(Cg)- <i>Foxp3</i> ^{tm4(YFP/cre)} Ayr/J (<i>Il10</i> ^{ΔFoxp3})	This paper	N/A
Mouse: <i>Foxp3</i> ^{tm1(cre)} Saka (<i>'Foxp3 Cre'</i>)	M. Oukka (Department of Pediatrics, University of Washington, USA)	MGI: 3812203
Mouse: Rosa26 RFP x <i>Foxp3</i> Cre	T. Bopp (Institute of Immunology, Johannes Gutenberg University, Mainz)	N/A
Mouse: <i>Prdm1</i> ^{flox/flox} x <i>Foxp3</i> Cre (<i>Blimp1</i> ^{ΔFoxp3})	This paper	N/A
Mouse: <i>Dnmt3a</i> ^{flox/flox}	Dr. En Li (Novartis Pharmaceutical Inc.)	N/A
Mouse: <i>Dnmt3a</i> ^{flox/flox} x Mx1-Cre (<i>Dnmt3a</i> ^{ΔMx1})	This paper	N/A

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