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Interleukin-23-induced transcription factor Blimp-1 promotes pathogenicity of T helper 17 cells

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SUMMARY

Interleukin-23 (IL-23) is a pro-inflammatory cytokine required for the pathogenicity of T helper 17 (Th17) cells but the molecular mechanisms governing this process remain unclear. We identified the transcription factor Blimp-1 (*Prdm1*) as a key IL-23-induced factor that drove the inflammatory function of Th17 cells. In contrast to thymic deletion of Blimp-1, which causes T cell development defects and spontaneous autoimmunity, peripheral deletion of this transcription factor resulted in reduced Th17 activation and reduced severity of autoimmune encephalomyelitis. Furthermore, genome wide occupancy and overexpression studies in Th17 cells revealed that Blimp-1 co-localized with transcription factors RORγt, STAT-3 and p300 at the *Il23r*, *Il17a/f* and *Csf2* cytokine loci to enhance their expression. Blimp-1 also directly bound to and repressed cytokine loci *Il2* and *Bcl6*. Taken together, our results demonstrate that Blimp-1 is an essential transcription factor downstream of IL-23 that acts in concert with RORγt to activate the Th17 inflammatory program.

INTRODUCTION

Interleukin-17 (IL-17)-producing $CD4^+$ T helper 17 (Th17) cells are necessary for host defense against extracellular pathogens, and when dysregulated, can promote human inflammatory diseases including multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, psoriasis, and asthma (Annunziato et al., 2007; Chabaud et al., 2001; Kebir et al., 2009; Wilson et al., 2007). The early development of Th17 cells is initiated by TGFβ and IL-6 cytokine signaling (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al.,

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2006), which induces expression of the lineage-specific transcription factor RORγt (Ivanov et al., 2006), transcription factor STAT-3, and IL-23 receptor (IL-23R) (Yang et al., 2007). However, TGFβ and IL-6 driven Th17 cells are non-pathogenic and subsequent signaling through IL-23R is essential for further maturation of inflammatory Th17 cells (Ghoreschi et al., 2010; Hirota et al., 2011; Langrish et al., 2005; McGeachy et al., 2007; McGeachy et al., 2009). In the absence of IL-23R, Th17 cells are unable to drive autoimmune inflammatory responses. Human genome wide association studies (GWAS) have clearly demonstrated that *IL23R* gene polymorphisms are linked to the progression of many immune disorders (Duerr et al., 2006).

By using an approach that restricts IL-23R deficiency to T cells, we have previously shown that Th17 cells are arrested at an early activation stage and are unable to mediate autoimmune encephalomyelitis (McGeachy et al., 2009). Recent findings suggest that IL-23 operates through multiple pathways to generate mature pathogenic Th17 cells including maintenance of Th17 signature genes (*Rorc* and *Il17*), induction of effector genes (*Il22*, *Csf2* and *Ifng*), suppression of repressive factors (Ahr, c-maf) and most importantly by enhancing its own signal strength through upregulation of *Il23r* expression (Codarri et al., 2011; El-Behi et al., 2011; Ghoreschi et al., 2010; Hirota et al., 2011; Lee et al., 2012). Despite these important insights, the molecular mechanisms linking IL-23 to Th17 pathogenic function remain undefined. IL-23 activates STAT-3, through adapters JAK2 and TYK2 to mediate its effect (McGeachy et al., 2009; Parham et al., 2002). However, as Th17 cell regulating cytokines IL-6 and IL-21 also activate STAT-3, STAT-3 activation alone cannot explain the requirement of IL-23 in generation of pathogenic Th17 cell and it is likely that additional regulators are involved that cooperate with STAT-3 and ROR©t to promote Th17 inflammatory function. In this study, we have identified a critical IL-23-dependent transcription regulator, Blimp-1, that activates Th17-specific inflammatory genes while simultaneously repressing genes that destabilize the Th17 program.

Blimp-1 is a key regulator of terminal differentiation in several cell types and was originally identified in B cells where it promotes differentiation to antibody secreting plasma cells (Shapiro-Shelef et al., 2003). Studies in T cells have shown that Blimp-1 regulates differentiation of cytotoxic T lymphocytes (CTLs) (Kallies et al., 2009; Rutishauser et al., 2009), IL-10-expressing regulatory T (Treg) cells (Cretney et al., 2011) and T follicular helper (Tfh) cells (Johnston et al., 2009; Nurieva et al., 2009). Mice with Blimp-1 deficiency restricted to T cells develop spontaneous colitis suggesting its involvement in T cell homeostasis (Martins et al., 2006). However, a major caveat with this study is the use of *Cd4*-Cre (*Cd4*^{cre}) or proximal-*Lck*-Cre (*Lck*^{cre}) deletion systems that result in global T cell defects due to deletion of Blimp1 during early thymic development (transition from double negative to double positive stage). Indeed, Blimp-1 deficient (*Prdm1*$^{fl/fl}$*Cd4*^{cre}) mice had dramatically reduced thymocyte numbers and defective Th1, Th2, and Treg cell populations. Two more recent follow up studies using the same *Cd4*^{cre} and *Lck*^{cre} system suggested that Blimp-1 also regulates autoimmune inflammation by suppressing Th17 cells (Lin et al., 2013; Lin et al., 2014; Salehi et al., 2012). However, in the same study, retrovirus-mediated Blimp-1 overexpression in polarized Th17 cells did not suppress IL-17 production making it unclear as to whether or not Blimp-1 directly affects Th17 cells. Thus, in order to assess the mechanism of how Blimp-1 regulates Th17-mediated immunopathology in a system that is

not complicated by thymic T cell developmental defects, we took advantage of a distal-*Lck*-Cre (*Dlck*^{cre}) system that promotes deletion of genes during the late single positive (SP) thymic development stage. (Zhang et al., 2005; Zhang and Bevan, 2012). In contrast to the *Prdm1*$^{fl/fl}$*Cd4*^{cre} mice, our Blimp-1 CKO (*Prdm1*$^{fl/fl}$*Rosa-YFP*$^{fl/fl}$*Dlck*^{cre}) mice had normal number of thymocytes and did not exhibit any signs of spontaneous autoimmunity. Using this mouse system and retrovirus mediated overexpression approaches we demonstrated that Blimp-1 was a direct target gene of IL-23 that plays a critical role in the generation of pathogenic Th17 cells by enhancing expression of IL-23R, GM-CSF and interferon γ (IFNγ) while repressing IL-2. Furthermore, our ChIP-Seq data showed that Blimp-1 directly bound and co-localized with STAT-3, p300 transcription co-activator, and RORγt on the *Il23r*, *Il17f* and *Csf2* loci to promote pathogenic function of Th17 cells. transcription factor downstream of IL-23 that synergizes with RORγt to drive the Th17 inflammatory program

RESULTS

IL-23 induces *Prdm1* in Th17 cells

An accumulating body of data clearly indicates that exposure to IL-23 is pivotal for Th17 cells to attain effector function. However, the molecular mechanism behind this phenomenon remains unclear. To characterize the molecular pathway initiated by IL-23 *in vivo*, we transferred a relatively low number of congenically marked *Il23r*$^{-/-}$ or wild-type (WT) OT-II T cells (specific for the ovalbumin) into C57BL/6 mice and investigated the differential gene expression in the absence or presence of IL-23R signaling. Affymetrix analysis followed by taqman analysis was performed on IL-17^{+} and IL-17^{-} OT-II cells from ovalbumin (OVA)$_{323–339}$ peptide immunized mice (Fig. 1A). Both WT and *Il23r*$^{-/-}$ IL-17-producing cells expressed high mRNA transcription for *Il17a* and *Rorc* whereas *Il23r* mRNA expression was greatly diminished in IL-23R deficient IL-17-producing cells. WT IL-17^{+} OT-II cells expressed significantly higher mRNA transcription of *Prdm1* (Blimp-1), *Csf2* and *Tbx21* compared to IL-17− cells and this expression was markedly reduced in the absence of IL-23 signaling (Fig. 1A). To determine whether Blimp-1 was expressed specifically in Th17 cells *in vivo*, we performed gene expression analysis of sorted IL-17 and IFNγ producing cells from the draining lymph node (dLN) of MOG$_{35–55}$ (myelin oligodendrocyte glycoprotein) Complete Freund's Adjuvant (CFA) immunized mice. As expected, prototypical transcription of Th17 signature genes, such as *Il17a*, *Il23r*, and *Rorc* was higher in Th17 cells (RORγt^{+}IL-17^{+} IFNγ-) whereas the transcription of bona fide Th1 genes *Ifng* and *Tbx21* was enhanced in Th1 cells (RORγt^{-}IFNγ^{+}IL-17^{-}) (Fig. 1B). *Prdm1* transcription was markedly higher (~4–8 fold) in Th17 as well as inflammatory Th17 cells that coexpressed IL-17 and IFNγ (RORγt^{+} IL-17^{+} IFNγ^{+}) cells as compared to Th1 cells (Fig. 1B). To further confirm Th17-specific Blimp-1 expression, Blimp-1-YFP reporter (*Prdm1-YFP*) mice were immunized with CFA-MOG peptide. Cellular analysis revealed Blimp-1 expression to be exclusively restricted to Th17 and not Th1 cells in the experimental autoimmune encephalomyelitis (EAE) model (Fig. 1C). Moreover, Blimp-1^{+} Th17 cells downregulated IL-2, indicative of a more mature Th17 population. These results suggest that IL-23 signaling promotes *Prdm1* expression to drive functional maturation of Th17 cells.

Prdm1 expression in Th17 cells is STAT-3 dependent

We next evaluated whether IL-23 mediated Blimp-1 induction in Th17 cells was STAT-3 dependent. Genome wide STAT-3 occupancy studies (ChIP-Seq) in IL-23 driven Th17 cells demonstrated direct binding of STAT-3 to *Prdm1* locus at promoter, intronic and 3-downstream regions (Fig. 1D). To further explore whether IL-23 mediated STAT-3 or STAT-1 activation signal was required for *Prdm1* expression inTh17 cells (Parham et al.,2002), we polarized STAT-1 KO (*Stat3*$^{fl/fl}$*Cd4*^{cre}) and STAT-1 KO (*Stat1*$^{-/-}$) CD4^{+} T cells to Th17 cells and assessed *Prdm1* expression (Fig. 1E). Consistent with the previously recognized importance of STAT3 in Th17 differentiation, IL-23-dependent induction of *Prdm1* was STAT3 dependent. In contrast, the presence or absence of STAT1 had no effect. As a control, we also assessed the induction of *Il23r* and *Il17a*, which was also STAT3-dependent whereas *Tbx21*, a recently recognized target gene of IL-23 (Hirota et al., 2011; Lee et al.) was STAT-1 but not STAT-3 dependent. Additionally, we treated the dLN cells from MOG immunized “IL-17-EGFP reporter (*Il17a-GFP*) mice” with IL-23 in the presence of a STAT-3 pharmacological inhibitor, Stattic and similar to *Il23r* and *Il22*, *Prdm1* gene transcription was completely downregulated on sorted IL-17.EGFP^{+} cells (Suppl.Fig. 1). Collectively, these results suggest that IL-23 can directly induce Blimp-1 expression in Th17 cells in a STAT-3 dependent manner.

Blimp-1 deficiency inhibits development of inflammatory Th17 cells

Previous studies using *Cd4*-Cre mediated deletion of Blimp1 (*Prdm1*$^{fl/fl}$*Cd4*^{cre}) suggested that Blimp-1 may regulate Th17 development and function. However, these mice are autoimmune prone and have multiple T cell defects associated with dysregulated Treg and Th1 cells (Martins et al., 2006). The lack of anti-IL-17 treatment studies along with the defective thymic development of the *Prdm1*$^{fl/fl}$*Cd4*^{cre} mice made it difficult to demonstrate a direct role of Blimp-1 in Th17 cells. To assess the function of Blimp-1 in normally developing Th17 cells during an immune response, we generated Blimp-1 CKO mice by crossing *Prdm1* floxed (*Prdm1*$^{fl/fl}$) mice (Martins et al., 2006) to distal-*lck*-Cre mice (*Dlck*^{cre}). The distal-*lck* promoter is turned on after positive selection of thymocytes and drives Cre-mediated recombination specifically in the peripheral mature T cell compartment thereby avoiding any thymic T cell development defects due to *Prdm1* deficiency. Furthermore, *Prdm1*$^{fl/fl}$*Dlck*^{cre} mice were bred to *ROSA*-flox-STOP-flox-YFP (*Prdm1*$^{fl/fl}$*Rosa-YFP*$^{fl/fl}$*Dlck*^{cre}) mice to allow tracking of Blimp-1 deleted cells. *Dlck*-Cre deletion resulted in YFP expression in some mature single positive CD4^{+} T cells in the thymus (6%). However, the deletion continued in the periphery and about 60% of CD4^{+} T cells in lymph nodes and 72% of CD4^{+} T cells in colons expressed YFP (Suppl. Fig. 2A). Furthermore, to test the deletion efficiency, *Prdm1* mRNA expression was measured in sorted CD4^{+}YFP^{+} effector T cells (CD44^{hi}CD62L^{lo}). *Prdm1* mRNA expression was higher in effector (CD44^{hi}CD62L^{lo}) versus naïve cells (CD44^{lo}CD62L^{hi}) (Suppl. Fig. 2B). However, in CD4^{+}YFP^{+} effector T cells from Blimp-1 CKO mice, *Prdm1* expression was completely eliminated (Suppl. Fig. 2B). To account for potential differences in deletion of floxed *Prdm1* versus ROSA-flox-STOP-flox-YFP, YFP^{+} cells from Blimp-1CKO were compared to YFP^{+} cells from either Blimp-1 heterozygous (*Prdm1*$^{fl/+}$*Rosa-YFP*$^{fl/fl}$*Dlck*^{cre}) mice or Blimp-1 wild type (*Prdm1*$^{wt/wt}$*Rosa-YFP*$^{fl/fl}$*Dlck*^{cre}) mice.

As compared to *Cd4*-Cre or *Lck*-Cre, thymocyte development analysis of *Dlck*-Cre mediated Blimp-1 CKO mice revealed no differences in total thymocyte numbers or in various thymic subpopulations (DN, DP, SP), indicating that the thymic development process is unaffected in these mice (Suppl. Fig. 2C). Next, we assessed the colons from these mice to test for any signs of spontaneous autoimmune inflammation. Histological analysis of colons showed no signs of colitis (Fig. 2A). Moreover, activation status analysis showed no differences in the frequency of naïve, effector and memory T cell populations in the spleen and colonic lamina propria (cLP) of Blimp-1 CKO mice (Fig. 2B). As previous studies have reported defective IL-17 and IFNγ expression by Blimp-1-deficient cells from *Cd4*-Cre mice, next we examined the production of cytokines by Blimp-1 deficient cells from *Dlck*-Cre mice. No obvious differences in IL-17 expression by splenic or cLP cells were observed (Fig. 2B). We also evaluated the frequency and functionality of Blimp-1 deficient $CD4^+YFP^+Foxp3^+$ Treg cells in the spleen and colon of Blimp-1 CKO mice (Fig 2C). No difference was observed in the percent of Treg population in the spleen or colon of Blimp-1 CKO mice. Additionally, we evaluated the expression of functional molecules CTLA-4, ICOS and CD103 on Blimp-1 deficient Treg cells and again no significant differences were detected (Fig 2C). Collectively, these results indicate that Blimp-1 CKO mice are developmentally normal mice that are completely free from spontaneous autoimmune inflammation.

To test whether Blimp-1 deletion affects Th17 differentiation, we assessed cytokine production by activated T cells from the dLNs of mice immunized with $MOG_{35–55}$ in CFA. The proportion of YFP^+ CD4 T cells producing IL-17 was marginally reduced in the absence of *Prdm1* (Fig. 2D). But more importantly, the *Prdm1* deficient IL-17 producers were not able to co-express GM-CSF and IFNγ (Fig. 2E), factors that have been shown to be critical for the inflammatory function of Th17 cells (Codarri et al., 2011; El-Behi et al., 2011; Ghoreschi et al., 2010; Hirota et al., 2011). These results suggest that in the absence of Blimp-1 Th17 cells are unable to attain complete functional maturation. Another factor linked to Th17 cell maturation is downregulation of IL-2 (McGeachy et al., 2009). We found that about 60% of the Th17 cells were IL-2 co-producers and the remaining cells became mature IL-17 single producers (Fig. 2F). But in the absence of Blimp-1, all of the Th17 cells were arrested at the early activation stage and were unable to downregulate IL-2 expression. Taken together, these results indicate that Blimp-1 plays a critical role in full effector differentiation of Th17 cells.

Blimp-1 CKO mice have reduced EAE incidence and severity

As GM-CSF and IFNγ expression has been linked to the encephalitogenic potential of Th17 cells, we assessed EAE development, a Th17 cell-dependent mouse model of human multiple sclerosis in Blimp-1-deficient mice. Blimp-1 CKO mice had reduced EAE (Fig. 3A, Table 1). In the central nervous system (CNS) (spinal cord and brain), (Fig. 3B) Blimp-1 CKO mice had markedly fewer $CD4^+$ T cells compared to heterozygous and wild type mice. The reduced absolute numbers of CD4 T cells (~90% reduction) further emphasized the defect in the ability of Blimp-1 CKO $CD4^+$ T cells to infiltrate the CNS (Fig. 3B). Furthermore, the few Blimp-1 CKO YFP^+ $CD4^+$ T cells that were able to infiltrate the CNS were functionally defective and had reduced IL-17 production (Fig. 3C). More

importantly, there was a significant defect in the ability of Blimp-1 deficient Th17 cells to co-express GM-CSF and IFNγ (Fig. 3D). IFNγ-producing RORγt$^+$ Th17 cells are known to be present in the lesional tissue in EAE and preferentially accumulate in the CNS of patients with multiple sclerosis (Kebir et al., 2009), highlighting their importance in promoting inflammation.

Since there is reduced accumulation of inflammatory cells in the CNS in the absence of Blimp-1, we tested whether Blimp-1 controls the ability of Th17 cells to migrate, expand, survive, or infiltrate into the CNS. There was a significant reduction in the proportion of cells that expressed CCR6 and CXCR3 in the absence of Blimp-1 (Suppl.Fig. 3A) suggesting that Blimp-1 regulated expression of chemokine receptors on Th17 cells for trafficking to sites of inflammation. Also, in the absence of Blimp-1, fewer Th17 cells were Ki67 positive (a cellular proliferation marker) (Suppl.Fig. 3B), indicating that Blimp-1 also promotes optimal expansion of Th17 cells. However, the proportion of CD4 T cells and specifically Th17 cells expressing Bcl-2 (a factor associated with cell survival) did not differ between Blimp-1 CKO and heterozygous mice (Suppl.Fig. 3C). Together, these results demonstrate that Blimp-1 is required for optimal Th17 cell differentiation, thus allowing Th17 cells to migrate to the site of inflammation and mediate pathogenic functions.

Blimp-1 regulates inflammatory Th17 cells in gut

Next, to assess whether Blimp-1 is essential in regulating the pathogenic potential of Th17 cells in other inflammatory tissue sites as well, we used *Citrobacter rodentium* infection induced gut inflammation model that is IL-23 dependent. Similar to EAE, at d11 post infection there was a significant defect in the ability of Blimp-1 deficient Th17 cells to express GM-CSF, IFNγ and to downregulate IL-2 (Fig. 4A). However, single IFNγ producers were not affected by Blimp-1 deficiency (Fig. 4A, B). These results further confirm that Blimp-1 preferentially regulates inflammatory Th17 cells.

Molecular mechanism of Blimp-1-mediated Th17 pathogenicity

Although well accepted as a transcriptional repressor, our findings suggest that Blimp-1 can also serve as an activator in Th17 cells. Blimp-1-mediated Th17 cell regulation could be due to direct activation of target genes or via indirect downstream effects on Th17 cells. To identify the transcriptional targets of Blimp-1 in Th17 cells, a genome wide Blimp-1 occupancy study (ChIP-Seq) was conducted on purified IL-17.EGFP$^+$ cells differentiated in the presence of TGFβ, IL-6 and IL-23 (Fig. 5). In addition, purified Foxp3.EGFP$^+$ cells were used as controls (Suppl.Fig. 4). This study revealed that Blimp-1 directly bound to *Il23r*, *Il17* and *Csf2* regulatory regions (Fig. 5). Moreover, Blimp-1 co-localized within close proximity of p300, STAT-3 and RORγt (Fig. 5). p300 is a histone acetyltransferase that marks the active transcription regulatory domains while STAT-3 is a known co-regulator of Th17-specific target genes. The fact that Blimp-1 bound in close proximity to p300 and STAT-3 (Suppl.Fig. 5) suggests that these DNA elements are important regulatory domains for driving Th17 differentiation. In contrast to Th17 cells, Blimp-1 ChIP-Seq analysis of purified Foxp3.EGFP$^+$ cells revealed no direct binding on *Il23r* or *Il17f* regulatory regions suggesting that Blimp-1 specifically regulates these genes in Th17 cells (Suppl.Fig. 4). Based on these results, we hypothesized that

Blimp-1 regulates IL-23-mediated Th17 effector function primarily by amplifying IL-23 signaling via induction of *Il23r* expression and simultaneously enhances *Il17* and *Csf2* transcription. To test this, we ectopically expressed Blimp-1 in activated Th17 cells and enforced expression of Blimp-1 strongly upregulated *Il23r* expression in Th17 cells (Fig. 6A). To further confirm Blimp-1 mediated *Il23r* regulation, "IL-23R.mCherry reporter (*Il23r-mCherry*) mice" were transduced with Blimp-1.EGFP retrovirus and again Blimp-1 overexpression markedly enhanced IL-23R.mCherry expression in Th17 cells (Fig. 6B). Consistent with previous studies Blimp-1 overexpression did not alter IL-17 expression indicating that Blimp-1 cannot directly repress IL-17 (Salehi et al., 2012). However, enforced expression of RORγt further enhanced IL-17 expression (data not shown). Ectopic expression of Blimp1—but not RORγt—strongly induced GM-CSF production in Th17 cells (Fig. 6C). Collectively, these results validate that Blimp-1 is a major regulator of IL-23-dependent Th17 effector function. To further understand how Blimp-1 globally regulates Th17 cells, we performed gene expression analysis of Blimp-1 and RORγt retrovirus-transduced Th17 cells as well as activated but non-polarized Th0 cells. Blimp-1 over-expression maintained mRNA transcription of Th17 signature genes including *Rorc, Il6R* and *Il7R*, while further enhanced *Il17a*, *Il23R* and *Il1r1* (Fig. 6D heat map; Suppl. Table 1 normalized values). Importantly, Blimp-1 up-regulated Th17 effector genes such as *Ifng*, and *Csf2*. Concomitantly, Blimp-1 served as a repressor by downregulating multiple T cell lineage-specification genes including *Bcl6*, *Il2, Foxp3, Gata3*, and *Il27ra*, which are known to destabilize Th17 cells. Based on these findings, we propose that IL-23 induces Blimp-1 to establish an inflammatory Th17 phenotype. A recent paper has shown that Blimp-1 together with IRF4 promotes IL-10 production in Treg cells (Cretney et al., 2011). However, over-expression of Blimp-1 did not enhance IL-10 production in Th17 cells (Fig. 6E).

Ectopic expression of Blimp-1 drives Th17 signature genes exclusively in RORγt^{+} Th17 cells and not in Th0 cells, suggesting that presence of RORγt is essential for Blimp-1-mediated function in Th17 cells (Fig. 6D). To further confirm this, we generated a RORγ inducible knockout (RORγ CKO) mouse by crossing *Rorc* floxed (*Rorc*$^{fl/fl}$) mice to *Rosa*-CreERT2 mice (*Rosa*$^{cre/ERT2}$) where the Cre recombinase activity is inducible following Tamoxifen administration. Naïve T cells from RORγ CKO mice were first stimulated with TGF-β plus IL-6 to initiate RORγt-dependent Th17 differentiation. Following this, cells were rested and reactivated with IL-23 in the absence of TGFβ to induce pathogenic Th17 cells. Activation with IL-23 induced robust GM-CSF expression in Th17 cells (Fig. 6D top panel). Furthermore, Tamoxifen mediated *Rorc* deletion at priming stage significantly blocked IL-17. In comparison, *Rorc* deletion at reactivation stage not only reduced IL-17 but also completely inhibited GM-CSF expression by Th17 cells (Fig. 6D top panel). Blimp-1 overexpression was sufficient to induce optimal GM-CSF in Th17 cells even in the absence of any added IL-23 (Fig. 6D bottom panel). Most importantly, deletion of *Rorc* in Blimp-1 transduced Th17 cells completely blocked GM-CSF expression and inhibited pathogenic Th17 differentiation. Collectively, this data suggests that Blimp1 and RORγt act in concert to stabilize the Th17 inflammatory differentiation program.

DISCUSSION

IL-17 producing $CD4^+$ T helper cells driven by TGFβ and IL-6 have limited inflammatory functions. It is now well established that IL-23-mediated signaling is required for induction of IL-22, GM-CSF, and IFNγ, which are essential Th17 inflammatory genes. Th17 cells are also known to be phenotypically unstable and the expression of the master regulator – RORγt – does not guarantee functional stability. Factors such as IL-2, IL-12, IL-27, and IL-4 that stimulate Treg, Th1, and Th2 subsets are potent inhibitors of the Th17 developmental pathway. Under *in vivo* conditions, the presence of IL-23 is important to maintain and stabilize the expression of RORγt, IL-23R, IL-17, IL-22 and GM-CSF. Until now, limited mechanistic data was available to explain how IL-23 stabilizes the inflammatory phenotype of Th17 cells. IL-23 primarily activates STAT3, which maintains *Rorc* expression; but both IL-6 and IL-21 are potent STAT3 activators, therefore STAT3 activation alone cannot explain the requirement for IL-23. Our current study showed that IL-23 directly upregulated Blimp-1 in a STAT3-dependent manner, which was essential for down regulating IL-2 and maintaining the stability of the pathogenic Th17 cells. In the absence of Blimp-1, IL-17-producing cells did not produce GM-CSF and IFNγ and could not promote chronic inflammatory responses such as EAE. This phenotype could also be impacted by Blimp-1 deficiency in CD8 T cells. Based on published studies, it is known that Blimp-1 is essential for terminal differentiation of $CD8^+$ effector T cells (Rutishauser et al., 2009; Shin et al., 2009). These studies utilized the Granzyme-Cre system causing Blimp-1 deletion in activated $CD8^+$ T cells, and, similar to *Dlck*-Cre, thymic T cell development is left intact. However, to assess the role of Blimp-1 in Th17 development we have utilized autoimmune inflammatory models (EAE and Citrobacter) that are known to 'primarily' depend on CD4 T cells. Therefore, the impact of Blimp deficiency in CD8 T cells in our system is likely to be negligible.

It is well established that constitutive Blimp-1−/− mice are embryonically lethal and therefore, not useful for T cell studies. *Cd4*-Cre-mediated deletion of Blimp-1 or other T cell genes often leads to profound immune dysregulation, including decrease thymocyte survival, skewing of TCR repertoire that can be associated with defective NKT, Treg, and multiple $CD4^+$ helper subsets. *Lck*-cre-mediated deletion of Blimp-1 has similar issues due to defects in early thymocyte development. Such T cell development defects could then lead to increased autoimmunity in *Cd4*- or *Lck*-cre-mediated Blimp1-deficient mice (Martins et al., 2006). Therefore, in order to study the role of Blimp1 in normally developing Th17 cells, we used Distal *Lck*-Cre system that promotes deletion of genes during the late single positive (SP) thymic development stage and continues in peripheral T cells, thus avoiding global T cell defects due to deletion of Blimp1 in thymus (Zhang et al., 2005). In fact, a recent study comparing the effects of Distal *Lck*- Cre- versus *Cd4*-Cre-mediated TGF-βRII deficiency show profound differences where Distal *Lck*-Cre deletion model is completely devoid of any signs of autoimmunity in contrast to the autoimmune prone *Cd4*-Cre deletion model (Zhang and Bevan, 2012). Using the Distal-*Lck*-Cre-mediated genetic deletion and retrovirus-mediated overexpression approaches, we demonstrated that Blimp-1 is an IL-23 target gene that plays a critical role in the generation of pathogenic Th17 cells by enhancing expression of IL-23R, GM-CSF and IFNγ while repressing IL-2 in Th17 cells.

Becher and colleagues reported that the presence of RORγt is necessary for Th17 cell production of GM-CSF (Codarri et al., 2011). This is likely due to direct engagement of RORγt on a *Csf2* enhancer element. However, enforced expression of RORγt alone does not increase GM-CSF expression in Th17 cells suggesting additional enhancer factors are required. Enhancer activity is measured by histone modification marks such as p300, a histone acetyltransferase for which occupancy is indicative of an active regulatory domain. In Th17 cells, BATF, IRF4 and STAT-3 deficiency significantly reduces p300 binding but the lack of RORγt (master regulator) has almost no effect on the enhancer landscape (Ciofani et al., 2012). Furthermore, RORγt seems to serve as a modulator that fine tunes the pre-established Th17 lineage program by turning on Th17-specific genes while simultaneously repressing genes associated with alternate lineage fates (Ciofani et al., 2012). In this way, RORγt exploits the enhancer landscape created by BATF, IRF4 (TCR induced) and STAT-3 (cytokine induced) to control the developmental path of Th17 cells. Our finding that the Blimp-1 enhancer engages *Il23r, Il17f,* and *csf2* loci in close proximity to p300, STAT3, and RORγt is highly significant. It predicts that Blimp-1 is one of the transcription regulators sequentially recruited to Th17-specifying genes that control the pathogenic function of Th17 cells. Our results further argue against the notion of a single factor (e.g. RORγt or STAT3) governing lineage specificity and function. Taken together, these data are consistent with the notion that Blimp-1, as a single factor, does not govern any given T cell effector phenotype. Rather, Blimp-1 likely has distinct gene-regulating activities depending on the inflammatory or anti-inflammatory enhancer landscape created by the multi-factorial transcriptional complex—composed of BATF, IRF4, c-MAF, STAT-3, STAT-5, RORγt, or Foxp3.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J mice were obtained from Jackson Research Labs. OT-II (ovalbumin peptide$_{323–339}$-TCR-tg) and CD45.1^{+} mice were obtained from Jackson Research Labs and interbred at Merck. The generation of *Il23ra*$^{-/-}$ mice has been described (Chan et al., 2006). *Il23ra*$^{-/-}$ OT-II CD45.1^{+} mice were generated at Merck. IL-17-EGFP Knockin reporter mice were generated and provided by Biocytogen. *Stat3*$^{fl/fl}$ mice were from D. Levy (Lee et al., 2002) and bred with *Cd4*-Cre Tg mice. STAT-1 KO (*Stat1*$^{-/-}$) mice were also from D. Levy (Durbin et al., 1996). STAT-3 CKO (*Stat3*$^{fl/fl}$*Cd4*^{cre}) and STAT-1 KO mice were bred and maintained at NIH and studies were performed according to the NIH guidelines for the use and care of live animals and were approved by the NIAMS IACUC committee. *Prdm1*$^{fl/fl}$ (Martins et al., 2006) were purchased from Jackson Research Labs and *Rosa-YFPflDlckcre* mice were provided by Pamela J. Fink (University of Washington) (originally from Nigel Killeen) and interbred at Merck. *Prdm1-YFP* mice were provided by Chris Hunter (University of Pennsylvania). RORγ CKO (*Rorc*$^{fl/fl}$*Rosa*$^{cre/ERT2}$) and IL-23R-mCherry (*Il23r-mCherry*) mice were generated at Merck. Foxp3-GDL (*Foxp3-GFP-DTR-Luci*) mice were generated and provided by G. Hammerling, DKFZ, Heidelberg, Germany. Animals were maintained in a barrier SPF facility and all animal procedures were approved by the Merck IACUC committee, in accordance with AAALAC guidelines.

Reagents

The following flow cytometry antibodies were purchased from BD Biosciences: CD4 (RM4–5), CD45.1 (A20), CD44 (IM7), IFN-γ (XMG1.2), IL-17 (TC11–18H10), IL-2 (JES6–5H4), CCR6 (140706), Ki-67 (B56), Bcl-2 (Bcl-2/100) and pSTAT3 (pS727). GM-CSF (MP1–22E9), T-bet (4B10), and CXCR3 (173) antibodies were purchased from eBioscience. Intracellular cytokine staining was performed with Cytofix-cytoperm kit from BD according to the manufacturer's instructions. pSTAT3 staining was performed with BD Phosflow kit according to the manufacturer's instructions. OVA(323–339) was obtained from Biosynthesis Inc (Lewisville, TX). Neutralizing anti-IL-2 antibody (S4B6–1) was purchased from BioXcell. Anti-IFNγ (XMG1.2) and anti-IL-4 (11B11) antibodies were produced internally. Tamoxifen and Stattic were purchased from Sigma.

Retroviral vectors and transductions

pMIG-GFP and pMIG-Blimp-1-GFP was a kind gift from Dr. Shane Crotty (la Jolla Institute for Allergy and Immunology, La Jolla, CA). The Rorγt cDNA was PCR amplified and cloned into pMIG to generate pMIG-Rorγt-GFP. Virions were produced by transfecting Plat-E cell line with the indicated plasmids using Fugene HD (Promega). Viral supernatant were collected and supplemented with 8μg/ml of polybrene (Sigma) before spinfection.

The T cell culture media used was complete IMDM (IMDM medium containing 10% (vol/vol) FCS, supplemented with penicillin and streptomycin, L-glutamine, HEPES, pH 7.2, sodium pyruvate and 2-mercaptoethanol). CD45.1^{+}OT-II cells were polarized to Th17 cells with $OVA_{323–339}$ (10μg/ml) and anti-CD28 (1μg/ml) in the presence of TGF-β (10ng/ml) plus IL-6 (100ng/ml), anti-IFN-γ (10μg/ml) and anti-IL-4 (10μg/ml) (1st stimulation). *Rorc*$^{fl/fl}$*Rosa*$^{cre/ERT2}$ and IL-23RmCherry mice were also polarized to Th17 cells with anti-CD3 (2.5μg/ml) and anti-CD28 (1μg/ml) in the presence of TGF-β, IL-6, anti-IFN-γ and anti-IL-4. At 48h following activation, fresh retrovirus supernatant was added and the cells were spinfected at 2000 rpm for 2hr at 30°C. Cells were allowed to rest for 1 d, followed by reactivation for 72 h with OVA /anti-CD3 and anti-CD28 (2nd stimulation) in the presence or absence of IL-23 (Th17) or anti-IFN-γ and anti-IL-4 (Th0), then stimulated with the phorbol ester PMA and ionomycin in the presence of GolgiPlug for the final 3 h and stained intracellularly for FACS analysis.

EAE

C57BL/6 mice (including IL-17-EGFP Knockin reporter mice and Blimp-1 CKO were immunized in four sites on the back with 100 μg $MOG_{35–55}$ in 200 ml CFA containing 100 μg M. tuberculosis strain H37Ra. All mice also received 100 ng pertussis toxin (List Biological Laboratories) intraperitoneally on days 0 and 2. Mice were assigned clinical scores for EAE according to the following scale: 1, flaccid tail; 2, impaired righting reflex and hindlimb weakness; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, hindlimb paralysis with partial forelimb paralysis; 6, moribund.

Citrobacter infection

Mice were infected orally with 10^9 colony-forming units (CFU) of *Citrobacter rodentium* strain DBS100 (ATCC, catalog number 51459) and colons were analyzed.

Isolation of lamina propria lymphocytes from colon

Mice were killed and colons were removed opened longitudinally. The colons were then thoroughly washed PBS and cut into1 cm segments. The pieces were incubated twice in 5 ml of dissociation solution (5 mM EDTA in HBSS) for 15 min at 37°C waterbath with shaking. After each incubation, the epithelial cell layer was removed by 15 sec of intensive vortexing. After the second EDTA incubation the pieces were washed in PBS, and placed in 5ml of digestion mix containing 10% FCS, 1 mg/ml of Collagenase D (Sigma), DNase I (Sigma), and 50 U/ml Dispase (BD) followed by chopping into tiny pieces. Digestion was performed by incubating the pieces at 37°C for 20 min in a waterbath with shaking. After the initial 20min, the solution was vortexed intensely and passed through a 40 mm cell strainer. Supernatant was spun down and the pellet was resuspended in 8 ml of the 40% fraction of a 40:80 Percoll (9parts Percoll $^+$ 1part 10X PBS) gradient, and overlaid on 5 ml of the 80% fraction in a 15 ml tube. Percoll gradient separation was performed by centrifugation for 30 min at 2200 rpm at room temperature with no brake. Lamina propria lymphocytes (LPLs) were collected at the interphase of the Percoll gradient, washed once, and resuspended in medium.

OT-II tracking studies

Recipient mice ($CD45.2^+$) received 1×105 $CD45.1^+$ *Il23ra–/–* or *Il23ra*$^+$/$^+$ OT-II $CD4^+$ T cells intravenously 1 d before being immunized subcutaneously in the flank with 100 mg OVA (323–339) in CFA. The phenotype of OT-II^+ cells was assessed by flow cytometry with gating on live $CD4^+CD45.1^+$ cells.

T cell stimulation for cytokine analysis

For stimulation of $CD4^+$ T cells ex vivo for cytokine analysis, draining lymph nodes were collected at various time points after immunization and single-cell suspensions were obtained. Cells were stimulated for 4 h with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml; both from Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences) in complete medium (RPMI medium containing 10% (vol/vol) FCS, supplemented with penicillin and streptomycin, L-glutamine, HEPES, pH 7.2, sodium pyruvate and 2-mercaptoethanol), followed by flow cytometry staining and analysis. For stimulation of $CD4^+$ T cells ex vivo for analysis of STAT3 phosphorylation, suspensions of cells from draining lymph nodes were incubated for 15 min with complete medium alone, IL-23 (20 ng/ml) or IL-6 (100 ng/ml). CNS mononuclear cells were isolated as described(Langrish et al., 2005) from mice with EAE and cells were cultured for 24 h in complete medium with MOG (35–55) (100 ug/ml) in the presence or absence of IL-23. GolgiPlug (BD Biosciences) was added for the final 4 h of culture before flow cytometry.

Cell sorting

To sort cell subsets based on intracellular cytokine expression, cells from draining lymph nodes of $OVA_{323–339}$ or $MOG_{35–55}$ immunized mice were stimulated ex vivo for 4 hours with PMA and ionomycin, surface stained, fixed, permeabilized and stained intracellularly for IL-17 and IFNγ. RVC (Ribonuclease vanadyl complex) RNase inhibitor (Sigma-Aldrich) was added during the fixation, intracellular staining and cell sorting steps to keep the RNA intact. Fixed cells were sorted based on IL-17 and IFNγ protein expression and gene expression analysis was performed. For sorting ex vivo cytokine stimulated IL-17^+ cells, draining lymph node cells were isolated from MOG immunized IL-17.EGFP Knockin mice and rested for 1.5 hours, followed by stimulation with IL-23 or IL-6 for 2 hours in the presence or absence of Stattic (Sigma-Aldrich). Stimulated cells were sorted based on IL-17.EGFP and CD4 expression.

Real-time quantitative PCR for gene expression:

For real-time PCR analysis, total RNA was isolated from cells using Arcturus PicoPure RNA Isolation method, according to manufacturer's protocol (Applied Biosystems, Life Technologies, Foster City, CA).

DNase-treated total RNA was amplified using NuGen WT-Ovation Pico RNA Amplification System per manufacturer's instructions (NuGen Technologies Inc., San Carlos, CA). Primers were designed using Primer Express (Applied Biosystems, Life Technologies, Foster City, CA), or obtained commercially from Applied Biosystems (Foster City, CA). For Prdm1 primers the assay # is Mm00476128_m1. The primers are in the coding region and designed at the exon boundary of 2–3. Real-time quantitative PCR on 10 ng of amplified cDNA from each sample was performed using either two gene-specific unlabelled primers utilized at 400 nM in a Applied Biosystems SYBR green real-time quantitative PCR assay or two unlabelled primers at 900 nM each were used with 250 nM of FAM-labeled probe (Applied Biosystems, Life Technologies, Foster City, CA) in a TAQMAN™ real-time quantitative PCR reaction. Either of the following technologies was used to collect data; an ABI 7300 or 7900 sequence detection system or the Fluidigm Biomark (Fluidigm, Foster City). The absence of genomic DNA contamination was confirmed using primers that recognize genomic region of the CD4 promoter. Ubiquitin mRNA transcription was measured in a separate reaction and used to normalize the data by the Δ-Δ Ct method. (Using the mean cycle threshold value for ubiquitin and the gene of interests for each sample, the equation 1.8 e (Ct ubiquitin minus Ct gene of interest) x 104 was used to obtain the normalized values.)

Chromatin immunoprecipitation and massive parallel sequencing (ChIP-seq):

Naïve cells from IL-17-EGFP reporter mice were activated with anti-CD3 (2.5μg/ml) and anti-CD28 (1μg/ml) and polarized in the presence of TGF-β (10ng/ml), IL-6 (100ng/ml), anti-IFN-γ (10μg/ml) and anti-IL-4 (10μg/ml) to generate Th17 cells. After stimulation for 3 days, cells were allowed to rest for next 3 days, followed by reactivation for 3 days with anti-CD3 (1μg/ml) and anti-CD28 (1μg/ml) in the presence of IL-23 (20ng/ml). On day 9, IL-17-$EGFP^+$ cells were sorted, stimulated with the phorbol ester PMA and ionomycin for 1.5 h and cross-linked for ChIP with Blimp1 or p300 antibodies. To generate $Foxp3^+$ Treg cells, naïve cells from Foxp3-GDL reporter mice were activated with anti-CD3 (5μg/ml)

and anti-CD28 (5μg/ml) and polarized in the presence of TGF-β (1ng/ml), IL-2 (100U/ml) and all-trans retinoic acid (2.5nM). After stimulation for 3 days, Foxp3-EGFP^{+} cells were sorted, stimulated with the phorbol ester PMA and ionomycin for 1.5 h and cross-linked for ChIP with Blimp1 or p300 antibodies. For STAT3 ChIP, naïve cells from WT B6 mice were cultured for 3 – 4 days in serum-free medium (X-VIVO 20; Lonza) with plate bound anti-CD3 and anti-CD28 (10 μg/ml each), IL-6 (20 ng/ml), IL-1β (20ng/ml), IL-23 (50 ng/ml) and anti-IFN-γ (10 μg/ml). For ChIP, fragmented chromatin lysate were prepared from 10 million cells per IP and target protein of interest was precipitated with anti-Blimp1 (Cell Signaling 9115), anti- STAT3 (eBioscience 14–6727) or anti-p300 (Santa Cruz sc-58). Recovered DNA fragments of approximately 10 to 20 ng were made into libraries with illumine adaptors and sequenced for single read 50 cycles using HiSeq 2000 (Illumina, San Diego, CA). Only uniquely matching reads are retained and mapped to mouse mm9 reference genome into 50 bps windows using bowtie-0.12.8. To find significant peaks for Blimp1 and p300, “macs14” with p-value=1e-8 was utilized and the input whole cell extract data was used as the negative control. To find the list of genes bound by Blimp1, RefSeq was used as the reference genome and target genes were identified if a peak was detected within −10 kbp of transcription start site and $^{+}$10 kbp of transcription end site.

STAT-3KO and STAT-1KO assay:

Mice and media.—*Stat3*$^{fl/fl}$ mice were from D. Levy (Lee et al., 2002) and bred with *Cd4*- Cre^{+} Tg mice. *Stat1*$^{-/-}$ mice were also from D. Levy (Durbin et al., 1996). All animal studies were performed according to the NIH guidelines for the use and care of live animals and were approved by the Institutional Animal Care and Use Committee of NIAMS. Cells were cultured in RPMI medium with 10% (vol/vol) FCS, 2mM glutamine, 100IU ml-1 of penicillin, 0.1 mg ml-1 of streptomycin and 20mM HEPES buffer, pH 7.2–7.5 (all from Invitrogen) and 2mM β–mercaptoethanol (Sigma-Aldrich).

Cell isolation and differentiation.—CD4^{+} T cells from spleens and lymph nodes of 6–8 week-old mice were purified by negative selection and magnetic separation (Miltenyi Biotec) followed by sorting of naïve CD4^{+}CD62L^{+}CD44^{-}CD25^{-} population using FACSAria II (BD). Naïve CD4^{+} T cells were activated by plate-bound anti-CD3 (10 μg ml^{-1}; eBioscience) and anti-CD28 (10 μg ml^{-1}; eBioscience) in media for 3 days with IL-1β (20 ng ml^{-1}, R&D Systems), IL-6 (20 ng ml^{-1}, R&D Systems), IL-23 (50 ng ml^{-1}, R&D Systems), plus anti-TGF-β1, TGF-β2 neutralizing antibodies (10 μg ml^{-1}, R&D Systems), anti-IFN-γ neutralizing antibodies (10 μg ml^{-1}, BDPharmingen) and anti-IL-4 neutralizing antibodies (10 μg ml^{-1}, BioXCell) for Th17 cell-polarizing condition.

Quantitative real-time PCR.—Total RNA was isolated with the use of *mir*Vana miRNA kit (Ambion); cDNA was synthesized with Taqman reverse transcription Kit according to the manufacturer’s instructions (Applied Biosystems). Quantitative PCR was performed with an ABI PRISM 7700 sequence detection system with site-specific primers and probes (Applied Biosystems). The comparative threshold cycle method and an internal control (β-actin) were used to normalize the expression of the target genes. The primers and probes are from Applied Biosystems: mouse ACTB 4352341E; Prdm1, Mm00476128_m1; Il23r, Mm00519942_m1.

Statistics:

Normal distribution was assumed for all samples. Unless otherwise indicated, an unpaired parametric student's t-test was used for comparison of data sets. Where shown, error bars indicate standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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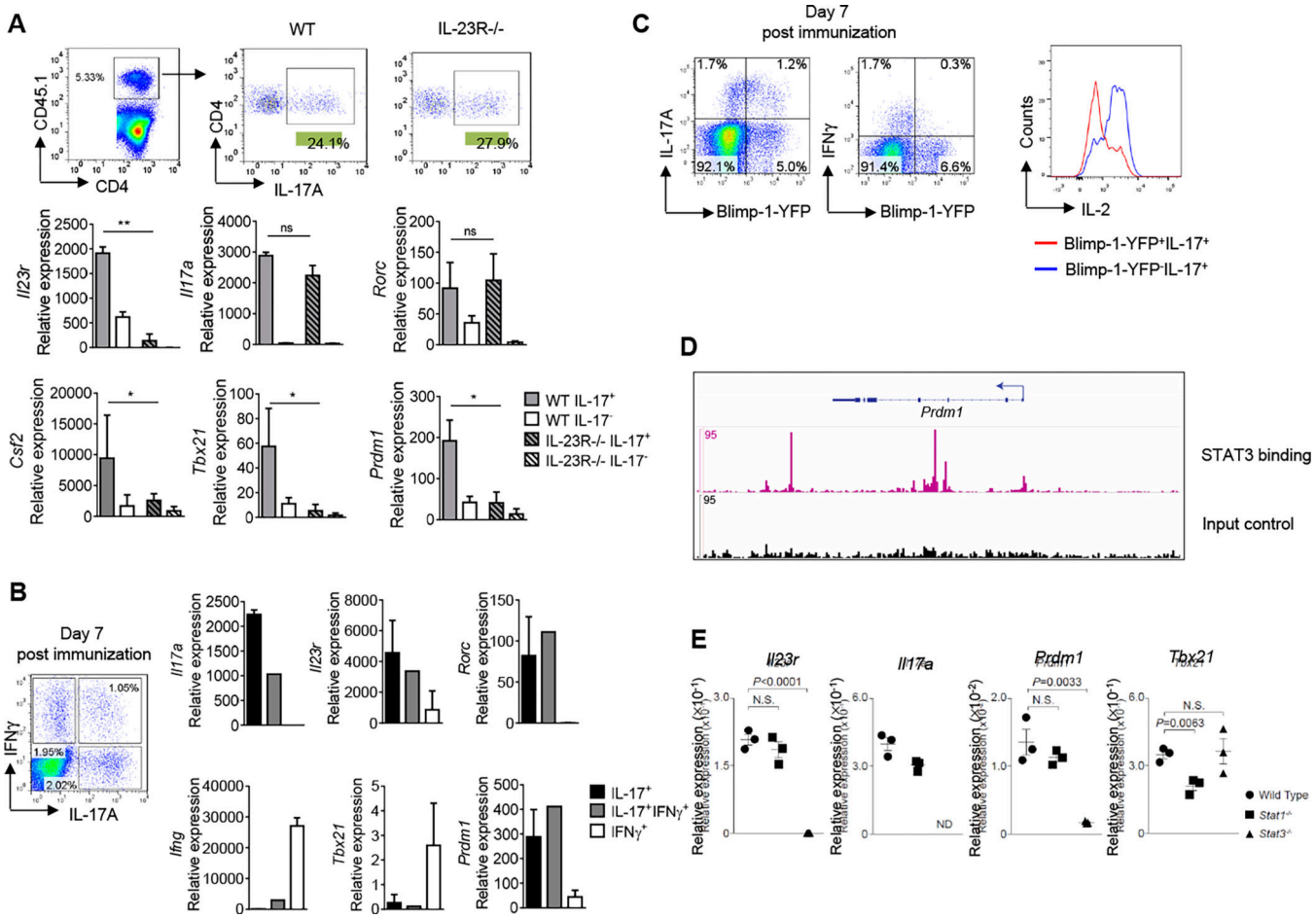

Figure 1. IL-23 signaling induces *Prdm1* expression in Th17 cells.

(A) Differential gene expression analysis of intracellularly stained IL-17$^+$ and IL-17$^-$ wild-type or *Il23r*–/– CD45.1$^+$CD4$^+$ OT-II cells from draining lymph nodes of CD45.2$^+$ mice at d7 post immunization with OVA(323–339) in CFA. **(B)** Differential gene expression analysis of intracellularly stained IL-17$^+$ and IFNγ$^+$ cells from draining lymph nodes of C57BL/6J mice at d7 post immunization with $MOG_{35–55}$ in CFA. **(C)** Intracellular staining for IL-17, IFNγ and IL-2 expression by CD4$^+$Blimp-1-YFP$^+$ cells from draining lymph nodes of $MOG_{35–55}$ immunized Blimp-1-YFP reporter mice (n=5 per group). Data are representative of 3 independent experiments. Error bars in all graphs are SEM. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test). **(D)** STAT binding sites on *Prdm1* locus as assessed by chromatin immunoprecipitation and massive parallel sequencing of Th17 cells differentiated in the presence of TGFβ, IL-6 and IL-23. **(E)** Naïve CD4$^+$ T cells from STAT-3 CKO (*Stat3*$^{fl/fl}$*Cd4*^{cre}), STAT-1 KO (*Stat1*$^{-/-}$) or wild type control mice were cultured under Th17 condition (plate bound anti-CD3, anti-CD28, IL-1β, IL-6, IL-23, anti-TGF-β1, anti-TGF-β2, anti-IFNγ and anti-IL-4) for 3 days. *Prdm1* and *Il23r* mRNA expression were assessed by quantitative RT-PCR (NS, not significant). See also Suppl. Fig. 1

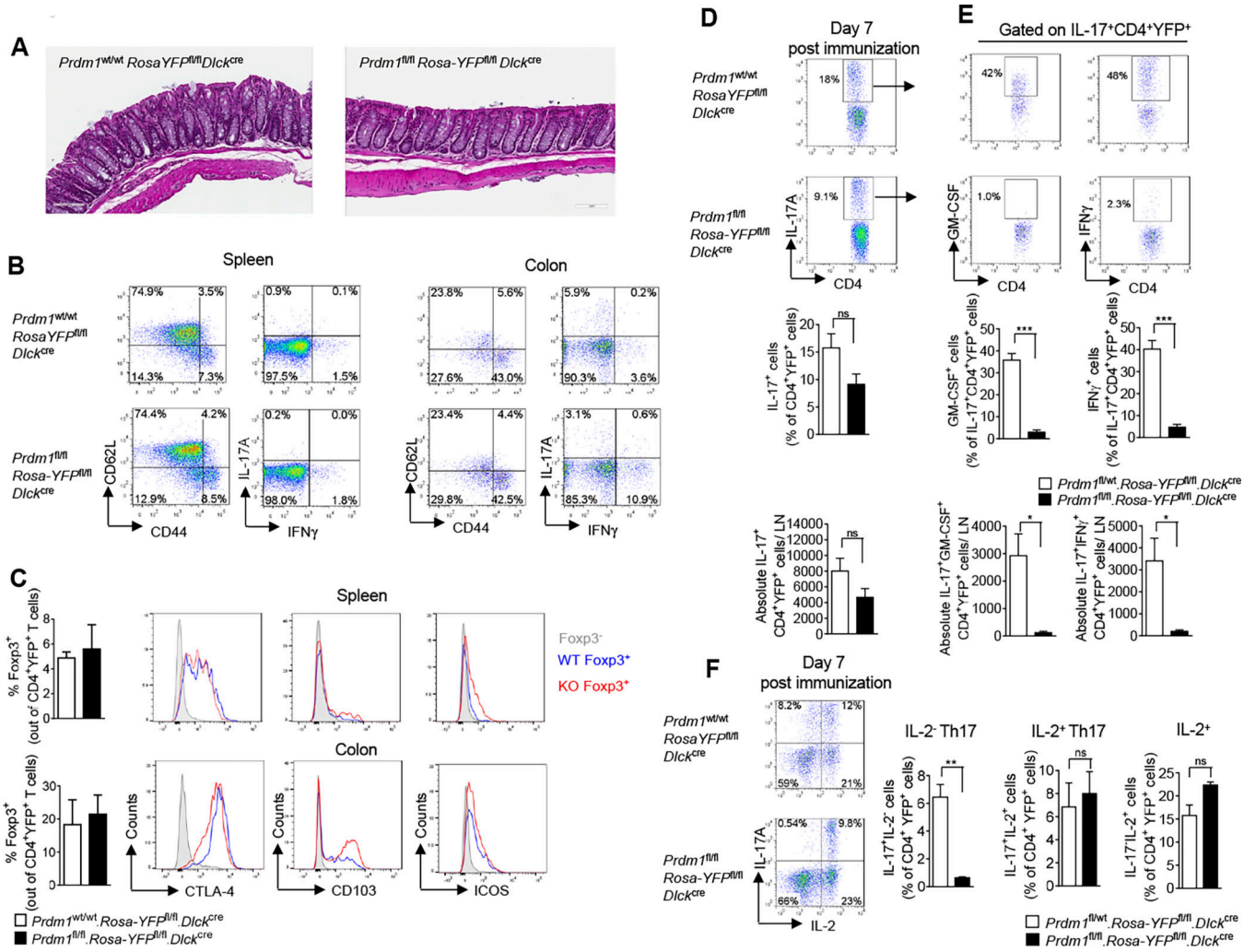

Figure 2. Blimp-1 deficiency blocks development of inflammatory Th17 cells co- expressing IL-17, GM-CSF and IFNγ.

(A) Haematoxylin and eosin staining (original magnification, ×200) of the colon (G) from 18wk old Blimp-1 CKO mice. **(B)** Peripheral $CD4^+$ T cell development in Blimp-1 CKO mice. Activation (CD44 and CD62L) and functional (intracellular IL-17 and IFNγ) status of $CD4^+$ T cells from spleen and colonic lamina propria of WT and CKO mice. **(C)** Frequency and functional status (CTLA-4, CD103, ICOS) of $CD4^+YFP^+Foxp3^+$ Treg cells from colonic lamina propria of Blimp-1 CKO mice (n=4 per group). (D) Frequency and absolute number of IL-17-producing $CD4^+$**YFP^+** T cells from d7 draining lymph nodes of MOG35–55 immunized Blimp-1 CKO (*$Prdm1^{fl/fl}Rosa\text{-}YFP^{fl}Dlck^{cre}$*) and heterozygous (*$Prdm1^{fl/wt}Rosa\text{-}YFP^{fl}Dlck^{cre}$*) mice (n=5 per group). **(E)** Frequency and absolute number of GM-CSF and IFNγ producing $IL\text{-}17^+$ cells from (D). **(F)** Blimp-1 represses IL-2 expression by Th17 cells. Intracellular staining of IL-2 and IL-17 by $CD4^+YFP^+$ T cells from (F). Data are representative of 3 independent experiments. Error bars in all graphs are SEM. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test). See also Suppl. Fig. 2

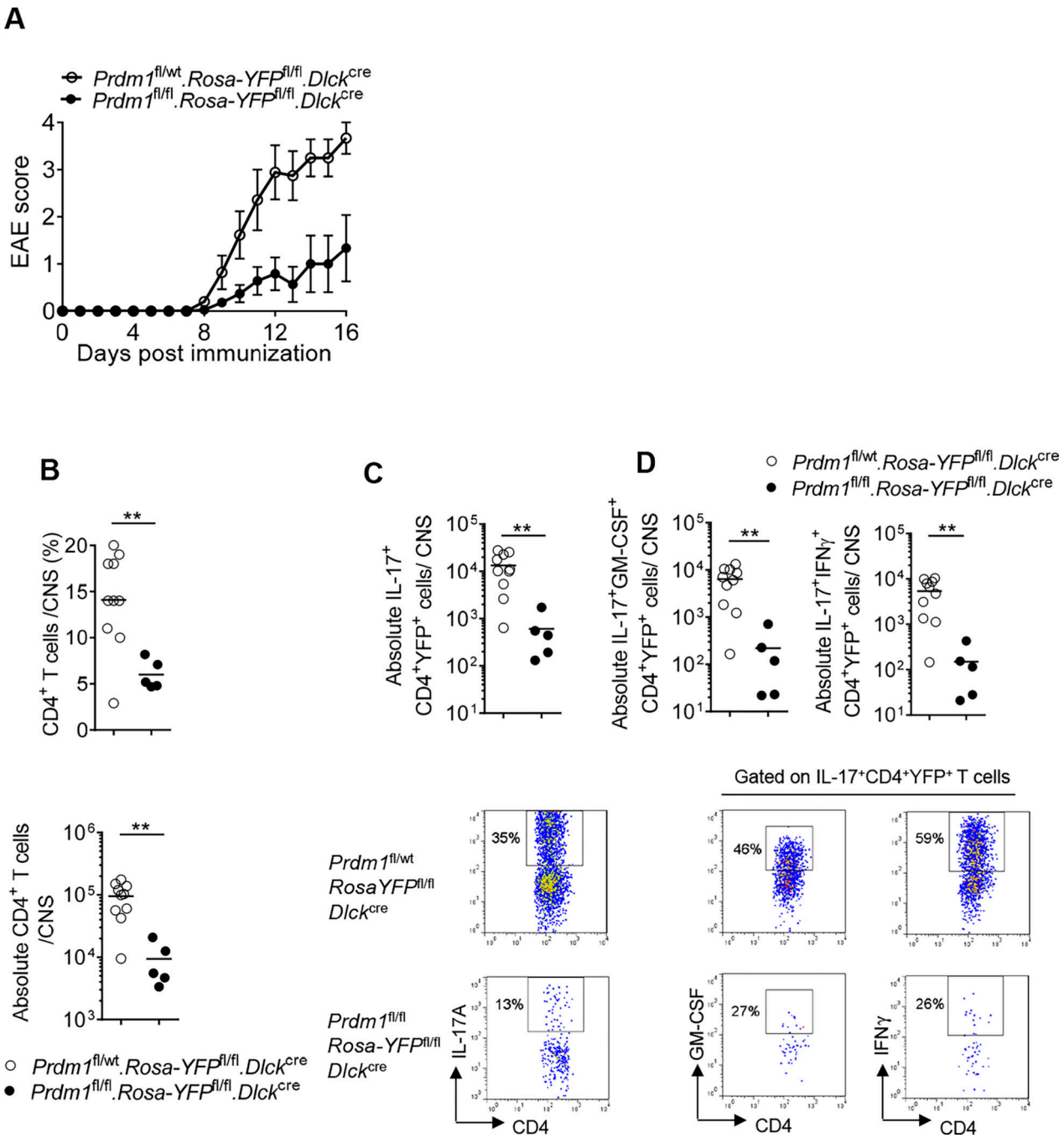

Figure 3. Blimp-1 deficiency reduces EAE incidence and severity.

(A) Clinical scores of Blimp-1 CKO mice after EAE induction. (n=9 per group). **(B)** Frequency and absolute number of $CD4^+$ T cells in the CNS of *Prdm1* conditional knockout mice 16 d post immunization (n=10 for het, n=5 for ko). **(C)** Frequency and absolute number of IL-17-producing YFP^+CD4^+ T cells from the CNS of mice in (B). **(D)** Frequency and absolute number of GM-CSF and IFNγ-producing $IL\text{-}17^+YFP^+CD4^+$ T cells from (B). Data are representative of 3 independent experiments. Error bars in all graphs are SEM. ns, not significant; $^{**}P < 0.01$ (Student's t-test). See also Suppl. Fig. 3

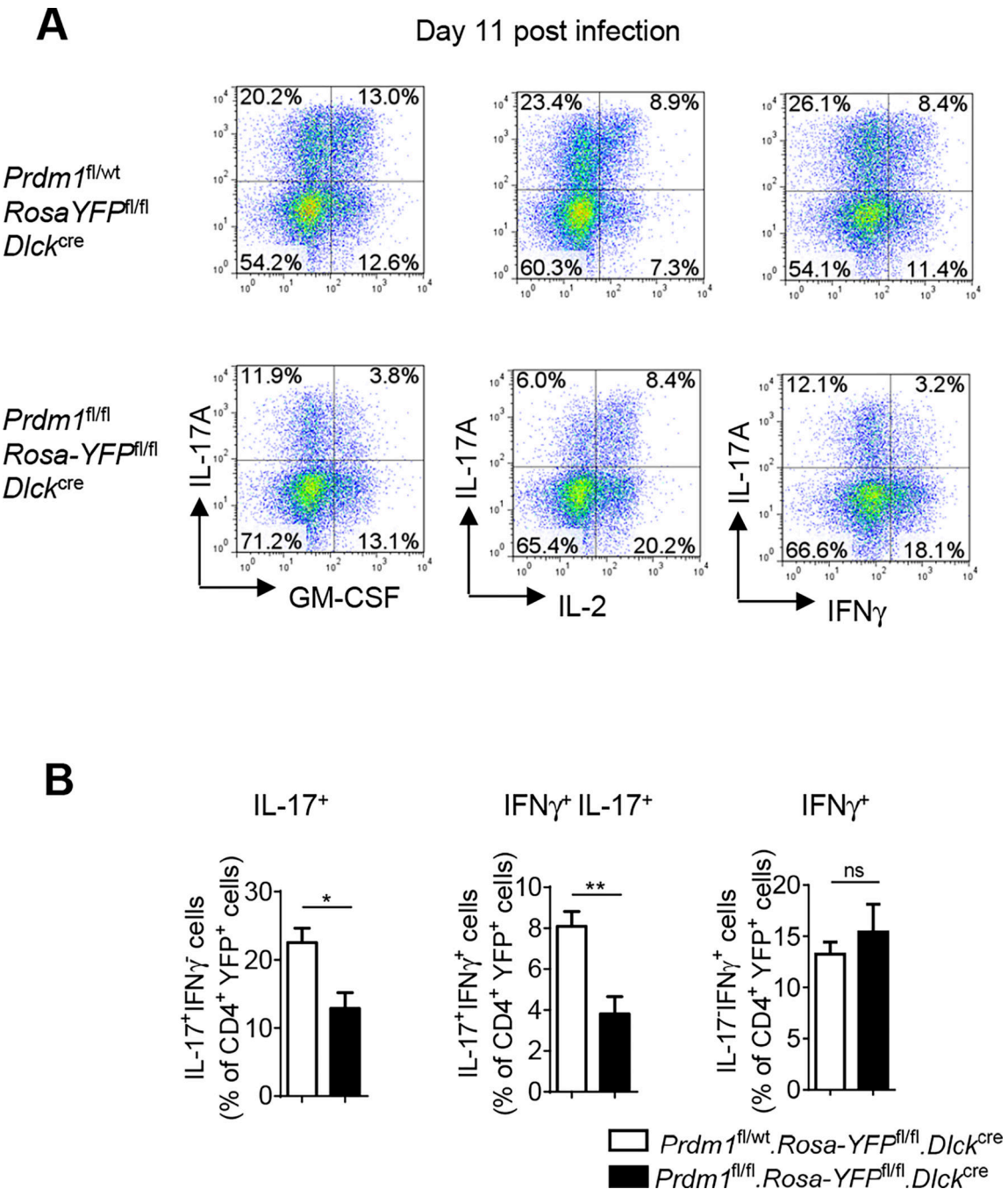

Figure 4. Blimp-1 regulates inflammatory Th17 cells in gut.

(A) Intracellular expression of IL-17, GM-CSF, IL-2 and IFNγ by $CD4^+YFP^+$ T cells from the colonic lamina propria cells of mice at d11 post infection with *Citrobacter rodentium*. **(B)** Frequency of $IL\text{-}17^+$, $IFN\gamma^+$ and $IFN\gamma^+IL\text{-}17^+$ cells from (A)(n=5 per group). Data are representative of 3 independent experiments. Error bars in all graphs are SEM. ns, not significant; *P < 0.05 and **P < 0.01 (Student's t-test).

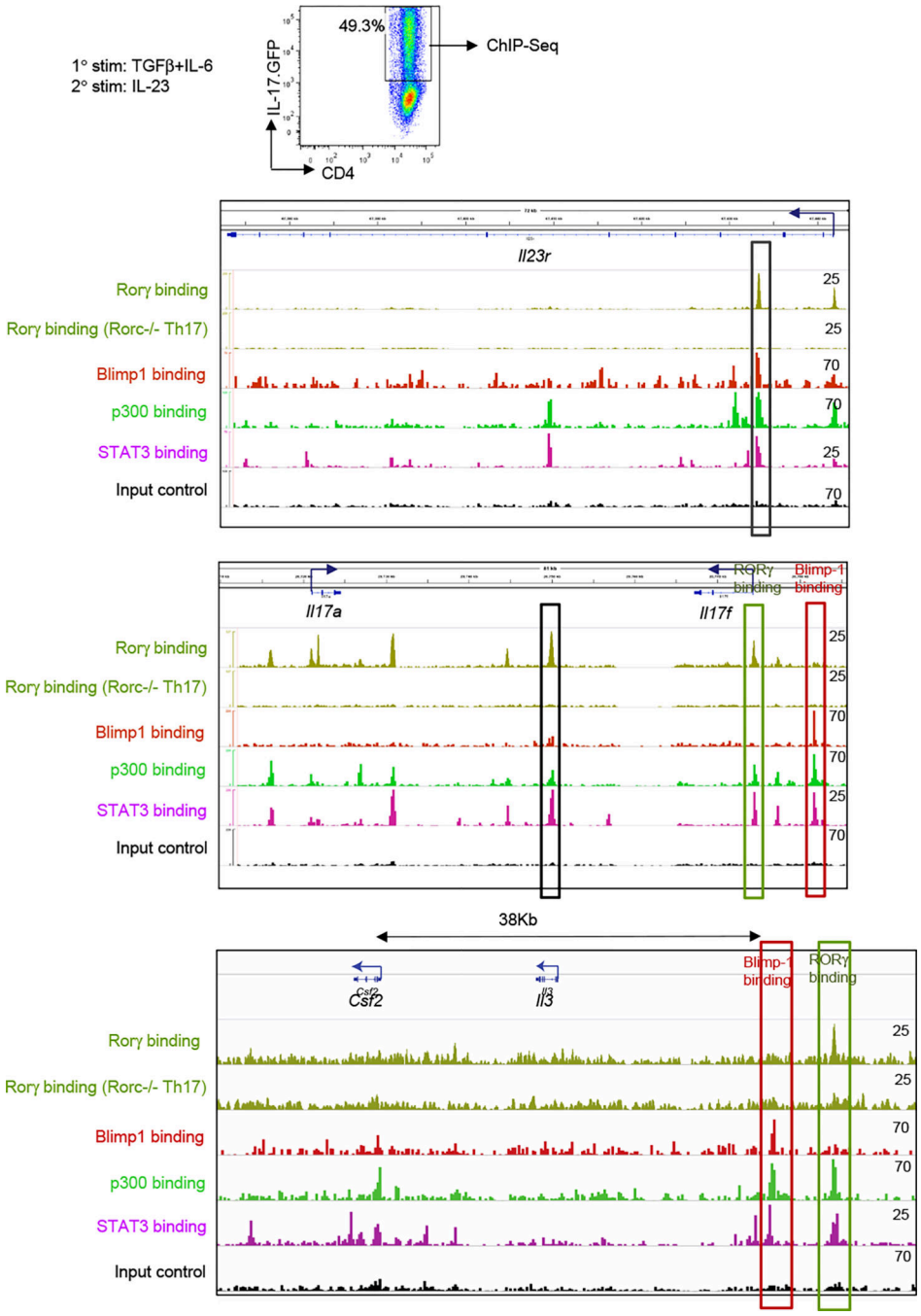

Figure 5. Blimp-1 directly binds Th17 signature genes.

Blimp-1, STAT-3, co-activator p300 and RORγt binding on *Il23r*, *Csf2* and *Il17f* loci as assessed by chromatin immunoprecipitation and massive parallel sequencing of purified IL-17.EGFP^{+} cells differentiated in the presence of TGFβ, IL-6 and IL-23. The RORγt data is extracted from Ciofani et al. *Cell* 151:289, 2012 (GEO# GSM1004855 WT Rorg ChIP; GSM1004854 RORγKO RORγ ChIP). See also Suppl. Fig. 4 & 5

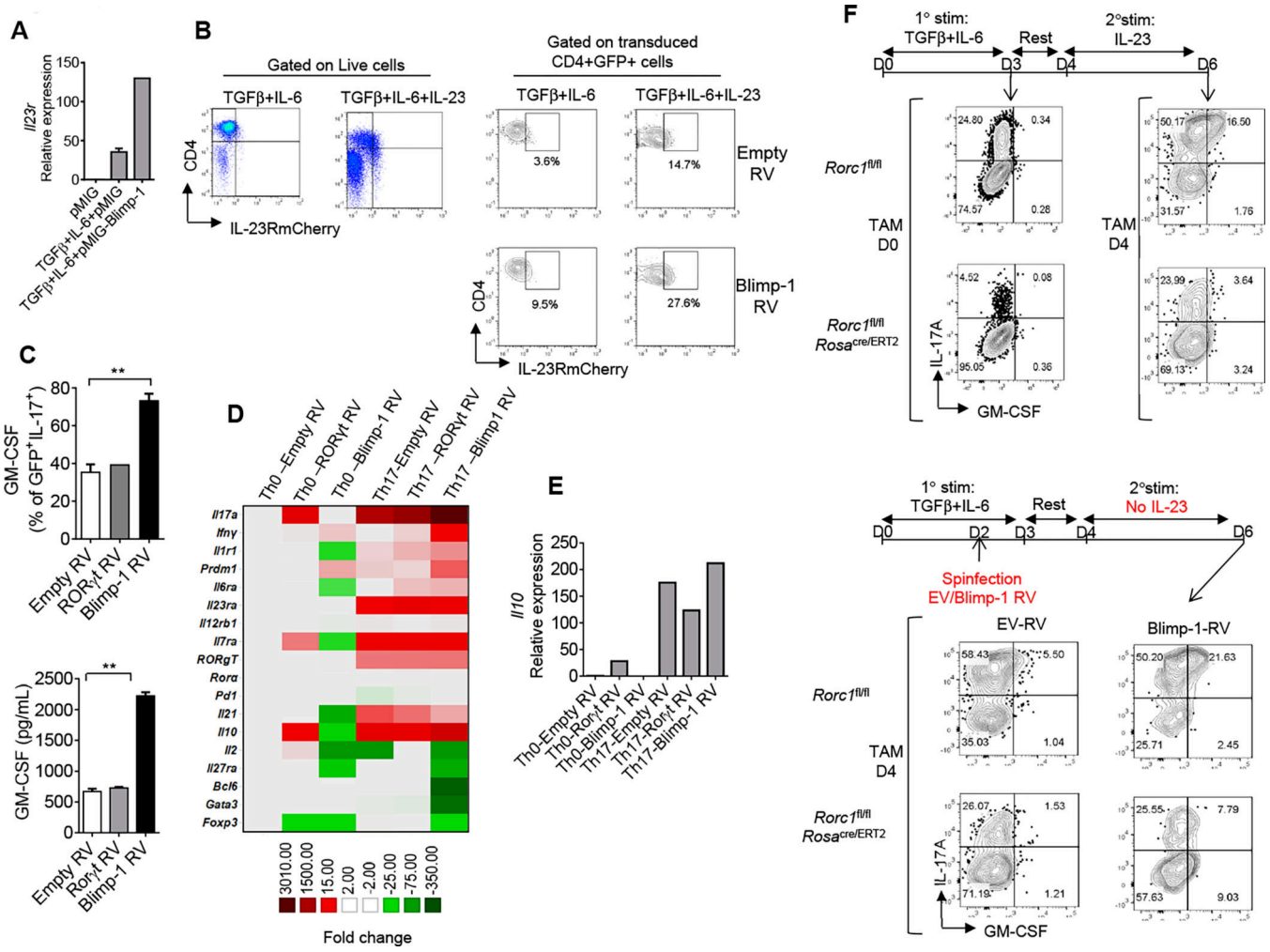

Figure 6. Ectopic Blimp-1 expression upregulates Th17 signature genes.

(A) Naïve OT-II cells were activated under Th0 or Th17 conditions, retrovirally transduced on day 2 with pMIG (Empty) or pMIG-Blimp-1 RV, rested and reactivated for 72h followed by gene expression analysis of sorted GFP$^+$ cells. (B) IL-23R-mCherry cells were activated under Th17 conditions, retrovirally transduced on day 2 with pMIG (Empty) or pMIG-Blimp-1 RV, rested and reactivated for 72h followed by surface analysis of IL-23R-mCherry on bulk live cells (top) or transduced GFP$^+$CD4$^+$ cells (bottom). (C) Naïve OT-II cells were activated under Th17 conditions, retrovirally transduced on day 2 with pMIG (Empty), pMIG-RORγt or pMIG-Blimp-1 RV, rested and reactivated for 72h followed by GM-CSF analysis by ELISA of transduced culture supernatants (D) Differential gene expression analysis of retrovirally transduced and sorted GFP$^+$ Th0 or Th17 cells 72h post-secondary stimulation. Heat map represents fold differences compared to the control Th0-pMIG-GFP. The absolute values were normalized to ubiquitin and are shown in Suppl.Fig.5. Error bars in all graphs are SEM. **P < 0.01 (Student's t-test). (E) IL-10 expression analysis of retrovirally transduced and sorted GFP$^+$ cells from (D). (f) Intracellular cytokine analysis in RORγ CKO (*Rorc*$^{fl/fl}$*Rosa*^{creERT2}) cells activated under Th17 conditions, transduced with

MIG or Blimp-1 RV on day 2, rested and reactivated for 72 h in the presence or absence of IL-23 and Tamoxifen to inhibit *Rorc* transcription. See also Suppl. Table 1

Table 1.

Induction of EAE in Blimp-1 deficient mice.

Genotype	Disease incidence (%)	Max clinical score (Mean)	Day of onset (Mean)
Prdm1$_{fl/wt}$*Rosa-YFP*^{fl} *Dlck*^{cre}	7/7 (100%)	3,4,2,3,3,4,4,4,3 (3.3)	9,9,13,10,12,9,10,8,9 (9.8)
Prdm1$^{fl/fl}$*Rosa-YFP*^{fl} *Dlck*^{cre}	3/7 (42%)	0,2,0,0,0,2,1,1,0 (0.6)	10,10,14,11 (11.2)

EAE in control (*Prdm1*$^{fl/wt}$*Rosa-YFP*^{fl}*Dlck*^{cre}) and Blimp-1 CKO mice (*Prdm1*$^{fl/fl}$*Rosa-YFP*^{fl}*Dlck*^{cre}). Disease incidence is presented as mice affected/total mice (with percentage in parentheses). Mean day of onset (mean ± s.e.m.) was calculated for mice that developed clinical signs of EAE. Data is representative of 3 independent experiments.