Multiple environmental signaling pathways control the differentiation of RORγt-expressing regulatory T cells

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Running Title:

Role of microbiota and cytokines in the regulation of RORγt expression by Tregs

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Abstract

RORγt-expressing Tregs form a specialized subset of intestinal $CD4^+$ $Foxp3^+$ cells which is essential to maintain gut homeostasis and tolerance to commensal microbiota. Recently, c-Maf emerged as a critical factor in the regulation of RORγt expression in Tregs. However, save c-Maf, the signaling pathways involved in the differentiation of $ROR\gamma t^+$ Tregs and their possible interplay with c-Maf in this process are largely unknown. We show that $ROR\gamma t^+$ Treg development strongly relies on signals derived from a complex microbiota, as well as IL-6/STAT3- and TGF-β-derived signals, although deficiency in either signaling pathway barely affects c-Maf expression. Ectopic expression of c-Maf did not rescue RORγt expression in STAT3-deficient Tregs, indicating additional effectors downstream of STAT3. Moreover, we show that an inflammatory IFN-γ/STAT1 signaling pathway opposes $ROR\gamma t^+$ Treg differentiation in a c-Maf independent fashion.

These data thus argue for a complex integrative signaling network that finely tunes RORγt expression in Tregs. The finding that type 1 inflammation impedes $ROR\gamma t^+$ Treg development even in the presence of an active IL-6/STAT3 pathway further suggests a dominant negative effect of STAT1 over STAT3 in this process.

Introduction

CD4 T cells expressing transcription factor forkhead box P3 (Foxp3) constitute a regulatory lineage of T cells (Treg) which maintains immune tolerance against self-antigens and prevents tissue destruction consequent to excessive immune responses. Tregs can be generated in the thymus from developing $CD4^+$ thymocytes (tTregs) or can result from the differentiation of mature T cells in the periphery (pTregs) (Chen et al., 2003; Panduro et al., 2016; Whibley et al., 2019). Recent findings indicate that, similarly to conventional helper T cells, Tregs are phenotypically and functionally heterogeneous. Distinct Treg populations adopt specialized phenotypes through the co-expression of Foxp3 and lineage-defining transcription factors such as PPARγ, BCL6, or RORγt in response to tissue- or inflammatory-driven signals (Panduro et al., 2016).

In particular, $ROR\gamma t^+$ Tregs are found in the intestinal tissue of naïve mice. Signals deriving from a complex microbiota as well as STAT3 signaling are necessary to their presence in the intestinal compartment. This subset of Tregs has been shown to protect efficiently from intestinal immunopathology in different colitis models (Lochner, 2011; Ohnmacht et al., 2015; Sefik et al., 2015; Yang et al., 2015) and to mediate immunological tolerance to the gut pathobiont *Helicobacter hepaticus* (Xu et al., 2018). A recent study showed that thymic-derived Tregs also expressed RORγt in lymph nodes following immunization and were able to protect mice from Th17 cell-mediated CNS inflammation (Kim et al., 2017). Although RORγt expression can be acquired by ex-Tregs during pathogenic Th17 conversion (Komatsu, 2014), RORγt-expressing Tregs mostly represent a Treg lineage participating in the immunological tolerance of barrier tissues and protecting from autoimmunity (Kim et al., 2017; Lochner, 2011; Ohnmacht et al., 2015; Sefik et al., 2015; Xu et al., 2018; Yang et al., 2015). $ROR\gamma t^+$ Tregs can also develop in the tumor microenvironment where they hinder anti-tumor

immunity, thus revealing a double-edged function of this Treg subset in immune homeostasis (Downs-Canner et al., 2017). However, despite their importance in physiological and pathological immune responses, the factors driving RORγt$^+$ Treg differentiation are still incompletely defined.

The transcription factor c-Maf, belonging to the AP-1 family of basic region/leucine zipper transcription factors, is expressed by distinct CD4$^+$ T cell subsets, including Th17, Th2, Tfh and Tr1 cells, and is thought to regulate the expression of IL-10, IL-4, and IL-21 through the transactivation of their promoters, downstream of Batf, ICOS, and STAT3 signaling (Andris et al., 2017; Apetoh et al., 2010; Bauquet et al., 2009; Hiramatsu et al., 2010; Sahoo et al., 2015). Transcriptomic studies conducted on Tregs found in different tissues revealed a strong enrichment for c-Maf in the intestinal compartment, and recent studies reported that c-Maf promotes the differentiation of RORγt$^+$ Tregs in this organ (Imbratta et al., 2019; Wheaton et al., 2017; Xu et al., 2018). Thus, and as previously described for Th17 cells (Tanaka et al., 2014a), expression of RORγt by Tregs is c-Maf-dependent. However, although RORγt$^+$ Tregs are restricted to gut-associated tissues in naïve mice, c-Maf is expressed in a wider proportion of Tregs in distinct organs. Of note, a subset of splenic CD44$^+$ CD62L$^-$ effector Tregs driven by ICOS signaling expresses high levels of c-Maf (Wheaton et al., 2017). The partial overlapping of RORγt and c-Maf expression along with the presence of a substantial population of c-Maf$^+$ RORγt$^-$ Tregs in lymphoid organs therefore suggests that high levels of c-Maf are not sufficient *per se* to drive RORγt$^+$ Treg cell differentiation.

Herein, extensive analysis of lymphoid organs and tissues of genetically invalidated or microbiota-altered mice revealed that, well beyond the c-Maf/RORγt interplay, multiple signaling pathways cooperate to exert a tight control over RORγt expression in Tregs.

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Results

c-Maf is highly expressed in intestinal Tregs and is required for RORγt expression

We first investigated the expression of c-Maf and RORγt in Tregs from distinct organs. In accordance with previous data (Ohnmacht et al., 2015; Sefik et al., 2015), we observed that RORγt^{+} Tregs were found mainly in the small intestine and colon lamina propria and, to a lesser extent, in mesenteric lymph nodes (Figure 1A, C). Of note, a large proportion of Tregs (ranging from 20% to 85%) expressed c-Maf in all the examined organs, with a notable exception for the thymus (Figure 1A, B). In the intestine, both RORγt^{+} and RORγt^{-} Treg subsets expressed c-Maf, although expression levels were higher in the RORγt^{+} compartment (Figure 1A, D, E). Treg-specific ablation of c-Maf resulted in increased Treg proportions in the intestine and lymphoid organs (Figure 1F, H and Figure S1A in Supplementary Material). Despite the wide distribution of c-Maf-expressing Tregs in distinct organs, Treg-conditional ablation of c-Maf did not result in systemic autoimmune disease, nor did it disturb conventional and regulatory T cell homeostasis in lymphoid organs. Moreover, c-Maf-deficient Tregs retained their *in vitro* suppressive capacity (Figure S1B and data not shown). In agreement with previous reports (Imbratta et al., 2019; Neumann et al., 2019; Wheaton et al., 2017; Xu et al., 2018), c-Maf$^{Treg\ KO}$ mice showed a near complete loss of RORγt expression in Tregs and spontaneously developed intestinal inflammation (Figure 1G, I and Figure S2). c-Maf deficiency did not affect the percentage of intestinal GATA3^{+} Tregs (Figure 1G, J), further indicating that c-Maf is specifically required for Tregs to acquire RORγt expression.

Strikingly, FACS analysis also revealed that a large fraction of c-Maf^{+} Tregs do not express RORγt. This was observed in all the examined organs and was most evident in the small intestine lamina propria, where two-thirds of the c-Maf^{+} Tregs lacked RORγt expression

(Figure 1A). c-Maf expression was mainly found among effector Tregs, which express high levels of ICOS and CD44 (Figure S3). In contrast to RORγt^{+} Tregs, which were mostly of peripheral origin, c-Maf^{+} Tregs were found both in Nrp1^{+} and Nrp1^{-} Tregs, suggesting that c-Maf^{+} Tregs can be of thymic or peripheral origin (Figure 2A, B). Thymic c-Maf^{+} Tregs were enriched in the spleen whereas their peripheral counterparts were enriched in mesenteric lymph nodes and formed the majority of intestinal Tregs (Figure 2C). Altogether, these data indicate that c-Maf^{+} Tregs can be divided into distinct compartments and that only a fraction of c-Maf^{+} Tregs co-express RORγt.

Complex microbiota, STAT3 and TGF-β signals control RORγt but are dispensable for c-Maf expression in Tregs

The presence of c-Maf^{+} RORγt^{-} Tregs in different organs and their distinct origins prompted us to further analyze the specific environmental signals responsible for the induction of c-Maf and RORγt expression by Tregs. IL-6/STAT3 signaling has been proposed to drive RORγt^{+} Treg generation and to promote c-Maf expression in Th cells (Ohnmacht et al., 2015; Veldhoen et al., 2006). However, mice genetically invalidated for IL-6 (IL-6$^{-/-}$), STAT3 (Stat3$^{flox/flox}$-CD4^{CRE}) or TGF-β (Tgfbr2$^{flox/flox}$-Foxp3^{CRE}) signaling showed normal to even slightly increased frequencies of c-Maf^{+} Tregs (Figure 3B, D, F; see also Figure S4 for representative FACS plots). Contrary to c-Maf, RORγt expression in Tregs was considerably decreased in all the aforementioned conditions (Figure 3A, C, E and S4). Thus, despite being necessary for the expression of RORγt by Tregs, c-Maf is not sufficient, and most likely cooperates with other signaling pathways to induce RORγt expression in Tregs.

RORγt^{+} Tregs are absolutely dependent on microbiota for their development (Ohnmacht et al., 2015; Sefik et al., 2015). Analysis of germ-free and antibiotics-treated mice revealed that, in contrast to RORγt expression, which was lost in both cases, c-Maf expression by Tregs was

only marginally affected in microbiota-deficient mice (Figure 4A-D).

Short chain fatty acids (SCFA) are gut microbiota-derived bacterial fermentation products, which include acetate, propionate, and butyrate, that regulate the size and function of the colonic Treg pool (Arpaia et al., 2013). Treg cells were induced *in vitro* with TGF-β in the absence or presence of acetate, propionate, or butyrate. In this experimental setting, SCFA did not affect or slightly decreased the differentiation of Foxp3$^+$ Treg cells (Figure S5). Addition of SCFA to the culture medium induced a 4 to 5-fold upregulation of RORγt expression, while minimally affecting c-Maf expression (Figure 4E, F, left panels). Of note, SCFA still promoted RORγt expression in c-Maf KO induced Tregs, although to a lower extent (Figure 4F, right panel). Overall, these observations suggest that microbiota-derived products regulate RORγt expression in Tregs through both c-Maf-dependent and independent pathways.

STAT3 and c-Maf control RORγt expression in iTregs through partly overlapping pathways

In presence of TGF-β and IL-2, about 75% of *in vitro* activated naïve CD4 T cells differentiated into Tregs, as assessed by their Foxp3 expression. Addition of IL-6, a pro-Th17 cytokine, to the TGF-β/IL-2 cytokine cocktail decreased the frequency of induced Tregs but led to the differentiation of a population of double positive c-Maf$^+$ RORγt$^+$ induced Tregs (iTreg17 cells; Figure 5A, lower panels and B, C). In agreement with *in vivo* observations (Figure 1G, I), ablation of c-Maf expression led to a significant reduction of *in vitro* generated RORγt$^+$ Tregs (Figure 5D, E, H). In the absence of STAT3, the expression of c-Maf and RORγt in iTreg17 was heavily decreased (Figure 5F-I). Despite showing residual c-Maf expression, STAT3 KO iTregs displayed a more severe down-regulation of RORγt than their c-Maf KO counterparts (90% versus 50 %; Figure 5J), suggesting a prominent role of STAT3 in RORγt expression in this context. Analysis of STAT3 and c-Maf genome mapping from

public ChIPseq databases (Ciofani et al., 2012) revealed that both transcription factors bind to the *Rorc* locus, albeit at distinct preferential sites (Figure 5K).

To determine whether the role of STAT3 in promoting RORγt induction solely relies on c-Maf, we restored c-Maf expression in STAT3-deficient iTreg17 cells. WT and c-Maf KO CD4 T cells were infected with a control-GFP or a c-Maf-GFP encoding retrovirus. As shown in Figure 6A, infection with the c-Maf-GFP retrovirus restored optimal levels of c-Maf in both c-Maf KO and STAT3 KO Tregs. Although the c-Maf encoded RV did restore RORγt expression in c-Maf-deficient Tregs, it was unable to do so in the STAT3 KO Tregs (Figure 6B). Collectively, these data indicate that in Tregs, an additional STAT3-driven but cMaf-independent pathway is required to promote optimal RORγt.

A pro-Th1 inflammatory environment dampens RORγt expression in Tregs

While IL-6 promoted RORγt expression in wild type iTregs, it surprising led to a marked reduction of this transcription factor in STAT3-deficient iTregs (Figure 5, compare panels F and G), thus suggesting the presence of an inhibitory pathway downstream of the IL-6 receptor. Studies by Costa-Pereira et al have shown that in STAT3 KO fibroblasts, IL-6 signals through STAT1 and has IFNγ-like effects (Costa-Pereira et al., 2002). We observed that in contrast to their wild type counterparts, STAT3 KO $CD4^+$ T cells cultured in the presence of IL-6 expressed higher levels of phospho-STAT1 (Figure S6). This prompted us to investigate the consequences of a STAT1/Th1 inflammatory pathway on the differentiation of $ROR\gamma t^+$ Tregs.

Infection with *Toxoplasma gondii* results in a highly Th1-polarized microenvironment leading to altered Treg cell homeostasis (Oldenhove, 2009). In particular, the strong Th1 environment triggered by *T. gondii* infection has been shown to induce T-bet and IFN-γ expression in Treg cells (Hall et al., 2012; Oldenhove et al., 2009). Analysis of Tregs from the

lamina propria of *T. gondii* infected C57BL/6 mice confirmed the emergence of a T-bet$^+$ Treg subset (Figure 7A and B, upper panels). Interestingly, *T. gondii* infection resulted in reduced numbers of tissue-associated RORγt$^+$ Tregs, with minimal effect on Maf expression (Figure 7A and B, middle and lower panels). Th17 cell differentiation was also suppressed during *T. gondii* infection (Figure S7), consistent with the observation that Th17 cell differentiation and Th1 cell differentiation are mutually suppressive (Harrington et al., 2005; Park et al., 2005). This reciprocal exclusion was also reflected among Tregs, as T-bet and RORγt were expressed in distinct Treg cell subsets in the infected mice (Figure 7C).

We next wished to evaluate the effect of Th1-promoting signals on RORγt expression by Tregs, Addition of IFN-γ to the iTreg17 differentiation media led to the selective accumulation of the phosphorylated form of STAT1, without affecting phospho-STAT3 accumulation (Figure S8). IFN-γ increased the expression of Tbet, with a concomitant reduction in RORγt$^+$ expression (Figure 7D, upper panels). On the contrary, c-Maf was barely affected by addition of IFN-γ to the iTreg17 culture medium (Figure 7D, upper middle panel). STAT1 KO Tregs were insensitive to the IFN-γ-driven inhibition of RORγt expression (Figure 7D, lower panels). Altogether these results strongly suggest that the IFN-γ/STAT1 signaling pathway negatively regulates RORγt expression even in the presence of an active IL-6/STAT3-driven pathway.

Discussion

RORγt$^+$ Tregs form a distinct population of regulatory T cells that is crucial to maintain gastrointestinal homeostasis and prevent colitis (Ohnmacht et al., 2015; Sefik et al., 2015; Yang et al., 2015). Whereas RORγt$^+$ Treg function has been amply documented, the factors driving RORγt$^+$ Treg differentiation remain ill-defined. We herein show that multiple signaling pathways cooperate to exert both positive and negative control over RORγt$^+$ expression by Tregs.

We confirmed and extended previous data showing that the transcription factor c-Maf plays a major role for the acquisition of RORγt expression by Tregs (Neumann et al., 2019; Wheaton et al., 2017; Xu et al., 2018). However, contrary to RORγt, which is confined to a subset of intestinal Tregs, c-Maf expression is found in a wider proportion of Tregs, located in distinct organs. This observation suggests that c-Maf cooperates with others pathways to induce optimal RORγt expression by these regulatory cells.

Extensive analysis of microbiota deficient and genetically manipulated mice revealed that microbiota, and IL-6-, STAT3- and TGF-β-signaling pathways are required for RORγt$^+$ Treg cell differentiation in a non-redundant manner. On the contrary, blocking any one of these pathways only had minimal effect on c-Maf expression *in vivo*, suggesting that several redundant pathways cooperate to induce c-Maf expression in Tregs.

Neumann et al recently showed that c-Maf expression in Tregs is involved in establishing and controlling host-microbiota homeostasis, thus identifying c-Maf$^+$ Tregs as a key regulator of intestinal function (Neumann et al., 2019). They also reported that, in addition to the loss of RORγt expression in Tregs, which we also observed, germ-free mice exhibited a near complete loss of c-Maf expression in intestinal Tregs. While we could not reproduce this observation, we observed that Tregs found in germ-free mice expressed reduced levels of c-

Maf. Reminding that the RORγt$^+$ population expresses the highest levels of c-Maf among intestinal Tregs, we can therefore speculate that RORγt expression in Tregs requires a high c-Maf threshold and that even a minimal decrease in c-Maf expression could hinder RORγt expression in microbiota-driven intestinal Tregs.

Short chain fatty acids (SCFA) produced by gut commensal microbes induce functional colonic Tregs and protect against T cell-dependent experimental colitis (Arpaia et al., 2013; Furusawa et al., 2013). Depending on the cytokine environment and immunological context, butyrate, acetate, and propionate, the most available SCFA in the gut, can also support IL-10 expression in Th1 and Th17 effector cells, thereby inhibiting colitis caused by pathogenic T cells (Park et al., 2015; Sun et al., 2018). We show herein that TGF-β-induced Tregs acquire both c-Maf and RORγt expression when cultured in the presence of butyrate, acetate, or propionate. In absence of c-Maf, SCFA still promote RORγt expression in Tregs, but to a lesser extent, thus suggesting that SCFA rely on c-Maf-dependent and independent pathways to promote RORγt expression in Tregs. Further studies could help clarify whether SCFA regulate RORγt expression through GPR41 or GPR43-dependent signaling, or through HDAC inhibitor activity and subsequent enhancement of mTOR–S6K activity, as previously shown in Th17 cells (Park et al., 2015).

Rather surprisingly, while STAT3 and TGF-β signaling drive c-Maf expression in Tfh and Th17 cells (Ciofani et al., 2012; Mari et al., 2013; Rutz et al., 2011), we found that mice deficient for STAT3 or TGF-β in the T cell compartment harbored normal to even slightly increased numbers of c-Maf$^+$ Tregs. The proportion of RORγt$^+$ Tregs was nonetheless severely reduced in these mice, indicating that, while STAT3 and TFG-β seem dispensable for the induction of c-Maf expression, they are both essential to achieve optimal differentiation of RORγt$^+$ Tregs in the intestine.

Besides c-Maf, many genes involved in Th17 differentiation, such as Irf4, Batf, Rora, Ahr,

Sox5t and HIF-1α, are expressed in response to STAT3 activation (Brüstle et al., 2007; Dang et al., 2011; Durant et al., 2010; Schraml et al., 2009; Tanaka et al., 2014b; Yang et al., 2008). Sox5t is a T cell isoform of Sox5 which induces RORγt expression in Th17 cells via physical interaction with c-Maf (Tanaka et al., 2014b). As enforced expression of c-Maf was not sufficient to induce Foxp3$^+$ RORγt$^+$ T cell differentiation in absence of STAT3, we speculate that Sox5, or other molecules downstream of STAT3, act together with c-Maf to achieve RORγt expression in Tregs. Interestingly, ChIP-seq data revealed that STAT3 and c-Maf bind with different intensities to distinct sites of the *Rorc* locus. The inability of c-Maf to compensate STAT3-deficiency in RORγt$^+$ Treg differentiation could thus also be explained by a direct effect of STAT3 on RORγt expression. This is in agreement with previous data showing that STAT3 binds to intron 1 of *Rorc* gene and induces chromatin remodeling of the locus (Durant et al., 2010). Overall, it would seem that STAT3 controls RORγt$^+$ Treg cell fate both through direct activation of the *Rorc* locus and by regulating the expression of a set of genes, including c-Maf, that is essential for RORγt$^+$ Treg differentiation (Figure S9).

In T cells, IL-6 predominantly signals via STAT3 and to a lesser extent via STAT1. It has been proposed that the accessibility of different STATs within the cell influences the outcome of cytokine signaling (Regis et al., 2008), as illustrated by the observation that IL-6 acquired the ability to induce the expression of STAT1-dependent genes in STAT3-deficient cells (Costa-Pereira et al., 2002; Schiavone et al., 2011). However, Hirahara et al showed an asymmetric action of STAT3 and STAT1 at the genomic level where much of STAT1 chromatin binding was STAT3-dependent. This challenged the classical view that, in the absence of its major STAT module, a cytokine would acquire an alternative STAT-signaling profile (Hirahara et al., 2015). Yet, in the absence of STAT3, and despite a global reduction in STAT1 chromatin binding, some preferential STAT1 binding sites were conserved in a group

of IL-6 downregulated genes. With this in mind we hypothesized a negative influence of STAT1 on RORγt expression by Tregs. Indeed, STAT3-deficient T cells showed increased STAT1 phosphorylation in response to IL-6, compatible with a switch from STAT3 to STAT1 signaling in these cells. We further showed that IFN-γ-driven activation of STAT1 opposed RORγt expression in Tregs, without affecting Foxp3 and c-Maf expression or STAT3 phosphorylation. Naïve STAT1 KO mice did not show altered proportions of intestinal RORγt^{+} Tregs (data not shown), but that could be explained by the lack of inflammatory Th1 components at steady state. Indeed, in wild type mice infected with the Th1-prototypic *Toxoplasma gondii* intestinal parasite, RORγt expression was decreased in intestinal Tregs, confirming the antagonistic role of inflammatory Th1 responses on RORγt expression in Tregs *in vivo*.

Different integrative pathways have been proposed to explain the functional outcome of multiple STAT signaling in distinct T cell subsets (Lin and Leonard, 2019). Meyer zu Horste et al recently reported that death receptor Fas promotes Th17 cell differentiation and inhibits Th1 cell development by preventing STAT1 activation. In this model, Fas regulated the STAT1 versus STAT3 balance by binding and sequestering STAT1 (Meyer zu Horste et al., 2018). Although not formerly excluded, sequestration of STAT3 is unlikely in RORγt^{+} Tregs as addition of IFN-γ to the iTreg17 culture media did not affect the phosphorylation status of STAT3. Our data rather suggest that Treg cell fate results from the balance of STAT1 and STAT3 driven signals. Gene expression could also conceivably be fine-tuned by the formation of STAT1/STAT3 heterodimers, as proposed for the IL-21 signaling (Wan et al., 2015). Of interest, patients with STAT3 mutations or with gain-of-function STAT1 mutations show similar susceptibility to fungal infections (Casanova et al., 2012; O'Shea et al., 2013). In the latter group, overactive STAT1 appears to limit STAT3-driven antifungal responses (Casanova et al., 2012).

Further work is required to decipher whether STAT1 interacts with STAT3 or exerts an independent negative role on RORγt$^+$ Treg cell fate. As T-bet is induced in Tregs that develop during *Toxoplasma* infection or in response to IFN-γ, we can also envision that STAT1 signaling inhibits RORγt expression through T-bet blocking of Runx1-mediated transactivation of *Rorc*, as previously reported for the Th1/Th17 lineage specification (Lazarevic et al., 2011). Regardless of the molecular mechanism, the observation that IFN-γ/STAT1 signaling pathway negatively regulates RORγt, even in the presence of an active IL-6/STAT3 pathway, suggests a dominant negative effect of STAT1 over STAT3 in these experimental conditions.

The antagonism between STAT1 and STAT3 seems to be cell type-specific or specific to a certain gene locus, as a cooperation between STAT1 and STAT3 downstream of IL-6 has been described for optimal Bcl6 induction and Tfh differentiation in response to viral infections (Choi et al., 2013).

Collectively, our data reveal that, beyond the previously established c-Maf/RORγt interplay, multiple signaling pathways cooperate to exert a tight control over RORγt expression in Tregs.

Material and Methods

Mice. C57BL/6 mice were purchased from Envigo (Horst, The Netherlands). c-Maf-flox mice (C. Birchmeier, Max Delbrück Center for Molecular Medicine, Berlin, Germany) were crossed with CD4-CRE mice (G. Van Loo, Ghent University, Ghent, Belgium) or FOXP3-CRE-YFP mice which were developed by A. Rudensky (Rubtsov et al., 2008) and kindly provided by A. Liston (KU Leuven, Leuven, Belgium). IL-6$^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). STAT3-flox mice were kindly provided by S. Akira (Osaka University, Osaka, Japan); STAT1$^{-/-}$ mice by D.E. Levy (New York University School of Medicine, NYC, USA). Germ-free mice were obtained from the Ghent Germfree and Gnotobiotic mouse facility (Ghent University, Ghent, Belgium) and were compared to SPF control mice. c-Maf-flox, CD4-CRE, FOXP3-CRE-YFP, IL-6$^{-/-}$, STAT3-flox, STAT1$^{-/-}$ and germ-free mice were bred on a C57BL/6 background. Tgfbr2-flox mice (Chytil et al., 2002) on a NOD background crossed with Foxp3-Cre mice (JAX 008694) were kindly provided by Q. Tang (University of California San Francisco, SF, USA) and were housed and bred at the UCSF Animal Barrier Facility.

All mice were used between 6 and 12 weeks of age. The experiments were carried out in compliance with the relevant laws and institutional guidelines and were approved by the Université Libre de Bruxelles Institutional Animal Care and Use Committee (protocol number CEBEA-4).

Antibodies, intracellular staining and flow cytometry. The following monoclonal antibodies were purchased from eBioscience: CD278 (ICOS)-biotin, CD304 (Nrp1)-PerCP eF710, c-Maf-EF660, Foxp3-FITC, RORγt-PE, T-bet-PE; or from BD Biosciences: CD25-BB515, CD44-PECy7, CD4-A700, CD4-PB, CD62L-A700, GATA3-PE, RORγt-PECF594, STAT1 (pY701)-A488, STAT3 (pY705)-A647, streptavidin-PECy7.

Live/dead fixable near-IR stain (ThermoFisher) was used to exclude dead cells. For transcription factor staining, cells were stained for surface markers, followed by fixation and permeabilization before nuclear factor staining according to the manufacturer's protocol (FOXP3 staining buffer set from eBioscience). For phosphorylation staining, cells were fixed with formaldehyde and permeabilized with methanol before staining. Flow cytometric analysis was performed on a Canto II (BD Biosciences) or CytoFLEX (Beckman Coulter) and analyzed using FlowJo software (Tree Star).

Isolation of lymphocytes. After removal of Peyer's patches and mesenteric fat, intestinal tissues were washed in HBSS 3% FCS and PBS, cut in small sections and incubated in HBSS 3% FCS containing 2,5mM EDTA and 72,5 µg/mL DTT for 30 min at 37°C with agitation to remove epithelial cells, and then minced and dissociated in RPMI containing liberase (20 µg/ml, Roche) and DNase (400 µg/ml, Roche) at 37 °C for 30 min (small intestine) or 45 min (colon). Leukocytes were collected after a 30% Percoll gradient (GE Healthcare). Lymph nodes, thymus and spleens were mechanically disrupted in culture medium.

After anesthesia, mice were perfused with PBS. Liver and lung samples were digested with collagenase (200U, Worthington) and DNase I (40µg/mL, Roche) at 37°C and mechanically disrupted. Leukocytes were collected at the interphase of a 40%/70% Percoll gradient.

Antibiotics treatment. Wide spectrum antibiotics (ampicillin 1g/L and neomycin 1g/L, Sigma-Aldrich; vancomycin 0,5g/L and metronidazole 1 g/L, Duchefa) were added to the sweetened drinking water of mice treated with antibiotics for three to four weeks. Control mice were given sweetened drinking water in parallel.

Toxoplasma infection. ME-49 type II *T. gondii* was kindly provided by Dr De Craeye (Institut Scientifique de Santé Publique, Belgium) and was used for the production of tissue cysts in C57BL/6 mice, which were inoculated 1–3 months previously with three cysts by gavage. Animals were killed, and the brains were removed. Tissue cysts were counted and

mice were infected by intragastric gavage with 10 cysts. Mice were sacrificed at day 8 after infection.

T cell culture. Naive $CD4^+$ T cells were purified from spleen of mice with indicated genotypes. $CD4^+$ T cells were positively selected from organ cell suspensions by magnetic-activated cell sorting using CD4 beads (MACS, Miltenyi) according to the product protocol, and then isolated as $CD4^+$ $CD44^{lo}CD62L^{hi}CD25^-$ or $CD4^+$ $CD44^{lo}$ $CD62L^{hi}$ YFP^- by FACS. T cells were cultured at 37°C in RPMI supplemented with 5% heat-inactivated FBS (Sigma-Aldrich), 1% non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamin (Invitrogen), 500 U/mL penicillin/500 µg/ml streptomycin (Invitrogen), and 50 µM β-mercaptoethanol (Sigma-Aldrich).

To generate iTreg cells, cells were cultured 72h in 24 or 96 well plates coated with 5µg/mL anti-CD3 (BioXcell, 145-2c11) at 37°C for 72h. The culture was supplemented with anti-CD28 (1 µg/mL, BioXcell, 37.51) and TGF-β alone (3 ng/ml, eBioscience), TGF-β and IL-2 (10 ng/mL, Peprotech), or TGF-β, IL-2 and IL-6 (10 ng/mL, eBioscience) for optimal iTreg cell polarization. Acetate (C2, 10 mM), propionate (C3, 0,5 mM), butyrate (C4, 0,125 mM), all from Sigma-Aldrich, and IFN-γ (10 and 100 ng/mL, Peprotech) were also used and added to the culture for the whole duration of the experiment.

Retroviral infection. Platinum-E retroviral packaging cells (T. Kitamura, University of Tokyo, Tokyo, Japan) were transfected with a c-Maf encoding retroviral plasmid (pMIEG-c-Maf-IRES-GFP) or a control retroviral plasmid (pMIEG-IRES-GFP) to produce retrovirus-containing supernatants. 24 hours after activation, naïve CD4 T cells polarized in presence of TGF-β, IL-2 and IL-6, as described above, were infected during a 90-minute centrifugation with 1 mL retrovirus-containing supernatant and polybrene. 48 hours later, infected cells were FACS-sorted based on GFP expression and were stimulated with anti-CD3 for 24 hours (5µg/mL, coated) before flow cytometry staining.

Treg cell *in vitro* suppression assay. Naïve T cells with the phenotype $CD4^+$ $CD44^{lo}$ $CD62L^{hi}$ YFP^- were isolated from the spleen of FOXP3-CRE-YFP mice by FACS after positive enrichment for $CD4^+$ cells using MACS LS columns (Miltenyi) and labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, ThermoFisher). Treg cells with the phenotype $CD4^+$ FOXP3-CRE-YFP^+ were isolated from the mesenteric lymph nodes of Foxp3-CRE-YFP or c-$Maf^{Treg\ KO}$ mice by FACS. Splenocytes from wild-type B6 mice were depleted in T cells (anti-CD90.2 beads) using MACS LS columns (Miltenyi). 4×10^4 CFSE-labelled naive T cells were cultured for 72 h with APCs (1×10^5) and soluble anti-CD3 (0,5 μg/mL) in the presence or absence of various numbers of Treg cells as indicated.

RT-qPCR. RNA was extracted using the TRIzol method (Invitrogen) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using the SYBR Green Master mix kit (ThermoFisher). Primer sequences were as follow: RPL32 (F) ACATCGGTTATGGGAGCAAC; RPL32 (R) TCCAGCTCCTTGACATTGT; IL-10 (F) CCTGGGTGAGAAGCTGAAGA; IL-10 (R) GCTCCACTGCCTTGCTCTTA; IL-17A (F) ATCCCTCAAAGCTCAGCGTGTC; IL-17A (R) GGGTCTTCATTGCGGTGGAGAG; IL-22 (F) CAGCAGCCATACATCGTCAA; IL-22 (R) GCCGGACATCTGTGTTGTTA; TNF-α (F) GCCTCCCTCTCATCAGTTCTA; TNF-α (R) GCTACGACGTGGGCTACAG.

ChIP-seq data. Publicly available ChIP-seq data (GSE40918) for c-Maf and Stat3 was downloaded and mapped to the mm9 genome using Bowtie2 with sensitive-local predefined parameters. Resulted BAM files were converted to bigwig files and visualized by IGV genome browser.

Statistical analysis. For unpaired data, statistical difference between groups was determined by an unpaired t test when the sample size was sufficient and both groups passed the normality test, and by a Mann-Whitney test for two-tailed data otherwise. Mutant and control

groups did not always have similar standard deviations and therefore an unpaired two-sided Welch's t-test was used. For paired data, a paired t test was used. Error bars represent mean ± SD. No samples were excluded from the analysis.

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Conflict of Interest Disclosure

The authors declare no conflict of interest

References

Andris F, Denanglaire S, Anciaux M, Hercor M, Hussein H, Leo O. 2017. The transcription factor c-Maf promotes the differentiation of follicular helper T cells. *Front Immunol* **8**. doi:10.3389/fimmu.2017.00480

Apetoh L, Quintana FJ, Pot C, Joller N, Xiao S, Kumar D, Burns EJ, Sherr DH, Weiner HL, Kuchroo VK. 2010. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol* **11**:854–861. doi:10.1038/ni.1912

Arpaia N, Campbell C, Fan X, Dikiy S, Van Der Veeken J, Deroos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**:451–455. doi:10.1038/nature12726

Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho I-C, Sharpe AH, Kuchroo VK. 2009. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* **10**:167–75. doi:10.1038/ni.1690

Brüstle A, Heink S, Huber M, Rosenplänter C, Stadelmann C, Yu P, Arpaia E, Mak TW, Kamradt T, Lohoff M. 2007. The development of inflammatory TH-17 cells requires interferon-regulatory factor 4. *Nat Immunol* **8**:958.

Casanova J-L, Holland SM, Notarangelo LD. 2012. Inborn Errors of Human JAKs and STATs. *Immunity* **36**:515–528. doi:10.1016/j.immuni.2012.03.016

Chen W, Jin W, Hardegen N, Lei K-J, Li L, Marinos N, McGrady G, Wahl SM. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* **198**:1875–1886.

doi:10.1084/jem.20030152

Choi YS, Eto D, Yang JA, Crotty S. 2013. Cutting Edge: STAT1 Is Required for IL-6 − Mediated Bcl6 Induction for Early Follicular Helper Cell Differentiation. *J Immunol* **190**:3049–3053. doi:10.4049/jimmunol.1203032

Chytil A, Magnuson MA, Wright CVE, Moses HL. 2002. Conditional inactivation of the TGF-β type II receptor using Cre:Lox. *genesis* **32**:73–75. doi:10.1002/gene.10046

Ciofani M, Madar A, Galan C, Sellars M, MacE K, Pauli F, Agarwal A, Huang W, Parkurst CN, Muratet M, Newberry KM, Meadows S, Greenfield A, Yang Y, Jain P, Kirigin FK, Birchmeier C, Wagner EF, Murphy KM, Myers RM, Bonneau R, Littman DR. 2012. A validated regulatory network for Th17 cell specification. *Cell* **151**:289–303. doi:10.1016/j.cell.2012.09.016

Costa-Pereira AP, Tininini S, Strobl B, Alonzi T, Schlaak JF, Is'harc H, Gesualdo I, Newman SJ, Kerr IM, Poli V. 2002. Mutational switch of an IL-6 response to an interferon-γ-like response. *Proc Natl Acad Sci* **99**:8043 LP – 8047. doi:10.1073/pnas.122236099

Dang E V, Barbi J, Yang H-Y, Jinasena D, Yu H, Zheng Y, Bordman Z, Fu J, Kim Y, Yen H-R, Luo W, Zeller K, Shimoda L, Topalian SL, Semenza GL, Dang C V, Pardoll DM, Pan F. 2011. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell* **146**:772–84. doi:10.1016/j.cell.2011.07.033

Downs-Canner S, Berkey S, Delgoffe GM, Edwards RP, Curiel T, Odunsi K, Bartlett DL, Obermajer N. 2017. Suppressive IL-17A(+)Foxp3(+) and ex-Th17 IL-17A(neg)Foxp3(+) T(reg) cells are a source of tumour-associated T(reg) cells. *Nat Commun* **8**:14649. doi:10.1038/ncomms14649

Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, Takahashi H, Sun H-W, Kanno Y, Powrie F, O'Shea JJ. 2010. Diverse Targets of the Transcription Factor

STAT3 Contribute to T Cell Pathogenicity and Homeostasis. *Immunity* **32**:605–615. doi:https://doi.org/10.1016/j.immuni.2010.05.003

Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* **504**:446–450. doi:10.1038/nature12721

Hall AO, Beiting DP, Tato C, John B, Oldenhove G, Lombana CG, Pritchard GH, Silver JS, Bouladoux N, Stumhofer JS, Harris TH, Grainger J, Wojno EDT, Wagage S, Roos DS, Scott P, Turka LA, Cherry S, Reiner SL, Cua D, Belkaid Y, Elloso MM, Hunter CA. 2012. The cytokines interleukin 27 and interferon-γ promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity* **37**:511–523. doi:10.1016/j.immuni.2012.06.014

Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. 2005. Interleukin 17-producing CD4+effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* **6**:1123–1132. doi:10.1038/ni1254

Hirahara K, Onodera A, Villarino AV, Bonelli M, Sciumè G, Laurence A, Sun H-W, Brooks SR, Vahedi G, Shih H-Y, Gutierrez-Cruz G, Iwata S, Suzuki R, Mikami Y, Okamoto Y, Nakayama T, Holland SM, Hunter CA, Kanno Y, O'Shea JJ. 2015. Asymmetric Action of STAT Transcription Factors Drives Transcriptional Outputs and Cytokine Specificity. *Immunity* **42**:877–889. doi:10.1016/j.immuni.2015.04.014

Hiramatsu Y, Suto A, Kashiwakuma D, Kanari H, Kagami S, Ikeda K, Hirose K, Watanabe N, Grusby MJ, Iwamoto I, Nakajima H. 2010. c-Maf activates the promoter and enhancer of the IL-21 gene, and TGF-beta inhibits c-Maf-induced IL-21 production in

CD4+ T cells. *J Leukoc Biol* **87**:703–12. doi:10.1189/jlb.0909639

Imbratta C, Leblond MM, Bouzourène H, Speiser DE, Velin D, Verdeil G. 2019. Maf deficiency in T cells dysregulates T(reg) - T(H)17 balance leading to spontaneous colitis. *Sci Rep* **9**:6135. doi:10.1038/s41598-019-42486-2

Kim B-S, Lu H, Ichiyama K, Chen X, Zhang Y-B, Mistry NA, Tanaka K, Lee Y-H, Nurieva R, Zhang L, Yang X, Chung Y, Jin W, Chang SH, Dong C. 2017. Generation of RORγt(+) Antigen-Specific T Regulatory 17 Cells from Foxp3(+) Precursors in Autoimmunity. *Cell Rep* **21**:195–207. doi:10.1016/j.celrep.2017.09.021

Komatsu N. 2014. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat Med* **20**:62–68.

Lazarevic V, Chen X, Shim J-H, Hwang E-S, Jang E, Bolm AN, Oukka M, Kuchroo VK, Glimcher LH. 2011. T-bet represses TH17 differentiation by preventing Runx1-mediated activation of the gene encoding RORγt. *Nat Immunol* **12**:96–104. doi:10.1038/ni.1969

Lin J-X, Leonard WJ. 2019. Fine-Tuning Cytokine Signals. *Annu Rev Immunol* **37**:295–324. doi:10.1146/annurev-immunol-042718-041447

Lochner M. 2011. Microbiota-induced tertiary lymphoid tissues aggravate inflammatory disease in the absence of RORgamma t and LTi cells. *J Exp Med* **208**:125–134.

Mari N, Hercor M, Denanglaire S, Leo O, Andris F. 2013. The capacity of Th2 lymphocytes to deliver B-cell help requires expression of the transcription factor STAT3. *Eur J Immunol* **43**:1489–98. doi:10.1002/eji.201242938

Meyer zu Horste G, Przybylski D, Schramm MA, Wang C, Schnell A, Lee Y, Sobel R, Regev A, Kuchroo VK. 2018. Fas Promotes T Helper 17 Cell Differentiation and Inhibits T Helper 1 Cell Development by Binding and Sequestering Transcription Factor STAT1. *Immunity* **48**:556-569.e7. doi:10.1016/j.immuni.2018.03.008

Neumann C, Blume J, Roy U, Teh PP, Vasanthakumar A, Beller A, Liao Y, Heinrich F,

Arenzana TL, Hackney JA, Eidenschenk C, Gálvez EJC, Stehle C, Heinz GA, Maschmeyer P, Sidwell T, Hu Y, Amsen D, Romagnani C, Chang H-D, Kruglov A, Mashreghi M-F, Shi W, Strowig T, Rutz S, Kallies A, Scheffold A. 2019. c-Maf-dependent Treg cell control of intestinal TH17 cells and IgA establishes host–microbiota homeostasis. *Nat Immunol*. doi:10.1038/s41590-019-0316-2

O'Shea JJ, Holland SM, Staudt LM. 2013. JAKs and STATs in Immunity, Immunodeficiency, and Cancer. *N Engl J Med* **368**:161–170. doi:10.1056/NEJMra1202117

Ohnmacht C, Park J-H, Cording S, Wing JB, Atarashi K, Obata Y, Gaboriau-Routhiau V, Marques R, Dulauroy S, Fedoseeva M, Busslinger M, Cerf-Bensussan N, Boneca IG, Voehringer D, Hase K, Honda K, Sakaguchi S, Eberl G. 2015. MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORγt^+ T cells. *Science* **349**:989–93. doi:10.1126/science.aac4263

Oldenhove G. 2009. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity* **31**:772–786.

Oldenhove G, Bouladoux N, Wohlfert EA, Hall JA, Chou D, Dos Santos L, O'Brien S, Blank R, Lamb E, Natarajan S, Kastenmayer R, Hunter C, Grigg ME, Belkaid Y. 2009. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity* **31**:772–86. doi:10.1016/j.immuni.2009.10.001

Panduro M, Benoist C, Mathis D. 2016. Tissue Tregs. *Annu Rev Immunol* **34**:609–633. doi:10.1146/annurev-immunol-032712-095948

Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* **6**:1133–1141. doi:10.1038/ni1261

Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, Kim CH. 2015. Short-chain

fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. *Mucosal Immunol* **8**:80–93. doi:10.1038/mi.2014.44

Regis G, Pensa S, Boselli D, Novelli F, Poli V. 2008. Ups and downs: The STAT1:STAT3 seesaw of Interferon and gp130 receptor signalling. *Semin Cell Dev Biol* **19**:351–359. doi:https://doi.org/10.1016/j.semcdb.2008.06.004

Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR, Muller W, Rudensky AY. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**:546–58. doi:10.1016/j.immuni.2008.02.017

Rutz S, Noubade R, Eidenschenk C, Ota N, Zeng W, Zheng Y, Hackney J, Ding J, Singh H, Ouyang W. 2011. Transcription factor c-Maf mediates the TGF-[beta]-dependent suppression of IL-22 production in TH17 cells. *Nat Immunol* **12**:1238–1245.

Sahoo A, Alekseev A, Tanaka K, Obertas L, Lerman B, Haymaker C, Clise-Dwyer K, McMurray JS, Nurieva R. 2015. Batf is important for IL-4 expression in T follicular helper cells. *Nat Commun* **6**:7997. doi:10.1038/ncomms8997

Schiavone D, Avalle L, Dewilde S, Poli V. 2011. The immediate early genes Fos and Egr1 become STAT1 transcriptional targets in the absence of STAT3. *FEBS Lett* **585**:2455–2460. doi:10.1016/j.febslet.2011.06.020

Schraml BU, Hildner K, Ise W, Lee W-L, Smith WA-E, Solomon B, Sahota G, Sim J, Mukasa R, Cemerski S, Hatton RD, Stormo GD, Weaver CT, Russell JH, Murphy TL, Murphy KM. 2009. The AP-1 transcription factor Batf controls TH17 differentiation. *Nature* **460**:405.

Sefik E, Geva-Zatorsky N, Oh S, Konnikova L, Zemmour D, McGuire AM, Burzyn D, Ortiz-Lopez A, Lobera M, Yang J, Ghosh S, Earl A, Snapper SB, Jupp R, Kasper D, Mathis D,

Benoist C. 2015. MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of RORγ^{+} regulatory T cells. *Science* **349**:993–7. doi:10.1126/science.aaa9420

Sun M, Wu W, Chen L, Yang W, Huang X, Ma Caiyun, Chen F, Xiao Y, Zhao Y, Ma Chunyan, Yao S, Carpio VH, Dann SM, Zhao Q, Liu Z, Cong Y. 2018. Microbiota-derived short-chain fatty acids promote Th1 cell IL-10 production to maintain intestinal homeostasis. *Nat Commun* **9**:3555. doi:10.1038/s41467-018-05901-2

Tanaka S, Suto A, Iwamoto T, Kashiwakuma D, Kagami S, Suzuki K, Takatori H, Tamachi T, Hirose K, Onodera A, Suzuki J, Ohara O, Yamashita M, Nakayama T, Nakajima H. 2014a. Sox5 and c-Maf cooperatively induce Th17 cell differentiation via RORγt induction as downstream targets of Stat3. *J Exp Med* **211**:1857–1874. doi:10.1084/jem.20130791

Tanaka S, Suto A, Iwamoto T, Kashiwakuma D, Kagami S, Suzuki K, Takatori H, Tamachi T, Hirose K, Onodera A, Suzuki J, Ohara O, Yamashita M, Nakayama T, Nakajima H. 2014b. Sox5 and c-Maf cooperatively induce Th17 cell differentiation via RORγt induction as downstream targets of Stat3. *J Exp Med* **211**:1857–1874. doi:10.1084/jem.20130791

Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. 2006. TGFβ in the Context of an Inflammatory Cytokine Milieu Supports De Novo Differentiation of IL-17-Producing T Cells. *Immunity* **24**:179–189. doi:https://doi.org/10.1016/j.immuni.2006.01.001

Wan C-K, Andraski AB, Spolski R, Li P, Kazemian M, Oh J, Samsel L, Swanson PA, McGavern DB, Sampaio EP, Freeman AF, Milner JD, Holland SM, Leonard WJ. 2015. Opposing roles of STAT1 and STAT3 in IL-21 function in CD4⁺ T cells. *Proc Natl Acad Sci* **112**:9394 LP – 9399.

doi:10.1073/pnas.1511711112

Wheaton JD, Yeh C-H, Ciofani M. 2017. Cutting Edge: c-Maf Is Required for Regulatory T Cells To Adopt RORγt $^{+}$ and Follicular Phenotypes. *J Immunol* ji1701134. doi:10.4049/jimmunol.1701134

Whibley N, Tucci A, Powrie F. 2019. Regulatory T cell adaptation in the intestine and skin. *Nat Immunol* **20**:386–396. doi:10.1038/s41590-019-0351-z

Xu M, Pokrovskii M, Ding Y, Yi R, Au C, Harrison OJ, Galan C, Belkaid Y, Bonneau R, Littman DR. 2018. C-MAF-dependent regulatory T cells mediate immunological tolerance to a gut pathobiont. *Nature* **554**:373–377. doi:10.1038/nature25500

Yang B-H, Hagemann S, Mamareli P, Lauer U, Hoffmann U, Beckstette M, Föhse L, Prinz I, Pezoldt J, Suerbaum S, Sparwasser T, Hamann a, Floess S, Huehn J, Lochner M, Peduto L, Cherrier M, Sawa S, Langa F, Varona R, Riethmacher D, Si-Tahar M, Di Santo JP, Eberl G. 2015. Foxp3+ T cells expressing RORγt represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunol* **205**:1381–1393.

Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS, Watowich SS, Tian Q, Jetten AM, Dong C. 2008. T Helper 17 Lineage Differentiation Is Programmed by Orphan Nuclear Receptors RORα and RORγ. *Immunity* **28**:29–39. doi:10.1016/j.immuni.2007.11.016

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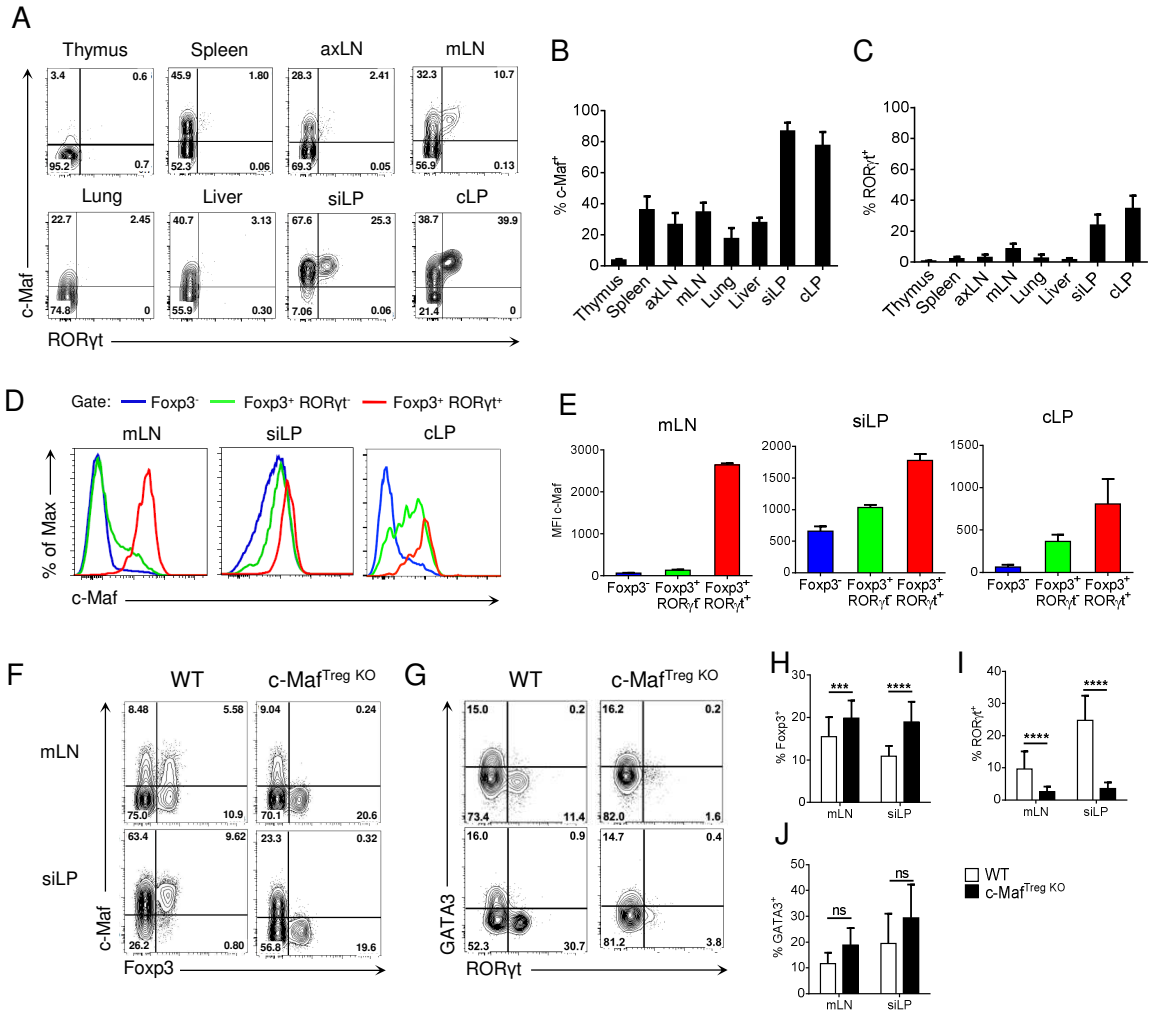

Figure 1. **Transcription factor c-Maf is required for the differentiation of RORγt⁺ Tregs.** (A) Representative flow cytometry expression profiles of c-Maf versus RORγt of Treg cells in the indicated organs of naïve C57BL/6 mice (gate CD4⁺ Foxp3⁺). (B, C) Histograms showing the frequency of c-Maf⁺ (B) or RORγt⁺ (C) Treg cells in the indicated organs. (D, E) Expression profile (D) and median of fluorescence intensity (E) of c-Maf among Foxp3⁻, Foxp3⁺ RORγt⁻ and Foxp3⁺ RORγt⁺ CD4 T cells in the indicated organs. (F, G) Representative flow cytometry expression profiles of c-Maf versus Foxp3 (F, gate CD4⁺) and GATA3 versus RORγt (G, gate CD4⁺ Foxp3⁺) from mLN and siLP of WT and c-Maf$^{Treg\ KO}$ mice. (H-J) Histograms showing the frequency of total Tregs (H), RORγt⁺ Tregs (I) and GATA3⁺ Tregs (J) in mLN and siLP of WT and c-Maf$^{Treg\ KO}$ mice. Results are representative of at least three independent experiments; histograms in (B, C, E, H-J) represent the mean ± SD of at least five individual mice. Difference between groups is determined by an unpaired t test (H, I) or a Mann–Whitney test for two-tailed data (J). ***$p < 0.001$; ****$p < 0.0001$. (axLN: axillary lymph nodes, mLN: mesenteric lymph nodes, siLP: small intestine lamina propria, cLP: colon lamina propria)

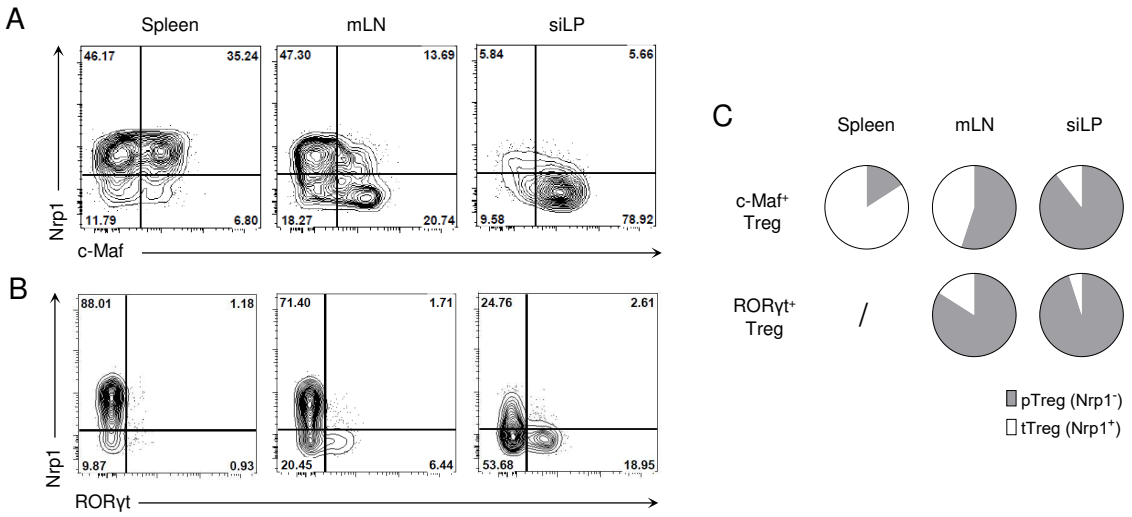

Figure 2. **c-Maf-expressing Tregs can be of thymic or peripheral origin**. (A, B) Representative flow cytometry expression profiles of Nrp1 versus c-Maf (A) or RORγt (B) of Treg cells in the indicated organs of naïve C57BL/6 mice (gate CD4$^+$ Foxp3$^+$ cells). (C) Pie charts show the relative frequencies of thymic (tTreg) and peripheral (pTreg) Treg cells among c-Maf$^+$ and RORγt$^+$ Treg subsets in the spleen, mLN and siLP. Results are representative of at least three independent experiments.

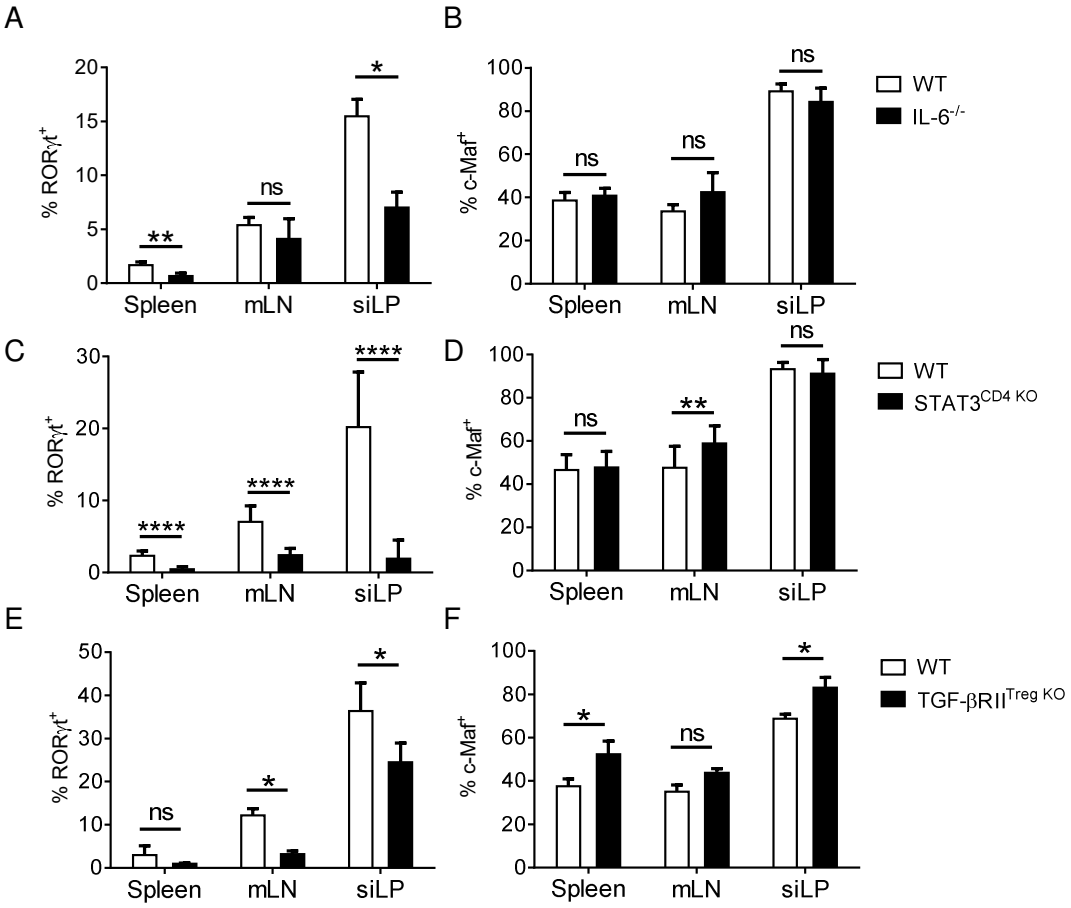

Figure 3. **IL-6/STAT3 and TGF-β signaling promote RORγt expression in Tregs independently of c-Maf**. (A-F) Histograms show the frequency of RORγt+ (A, C, E) or c-Maf+ (B, D, F) cells among Treg cells (gate CD4+ Foxp3+) in indicated organs of WT and IL-6-/- (A, B), STAT3CD4 KO (C, D) and TGF-βRIITreg KO (E, F) mice. Results are representative of at least three independent experiments; histograms represent the mean ± SD of at least four individual mice. Difference between groups is determined by an unpaired t test or a Mann–Whitney test for two-tailed data (A, B, C siLP, D mLN, E, F). See figure S4 for representative dot plots. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

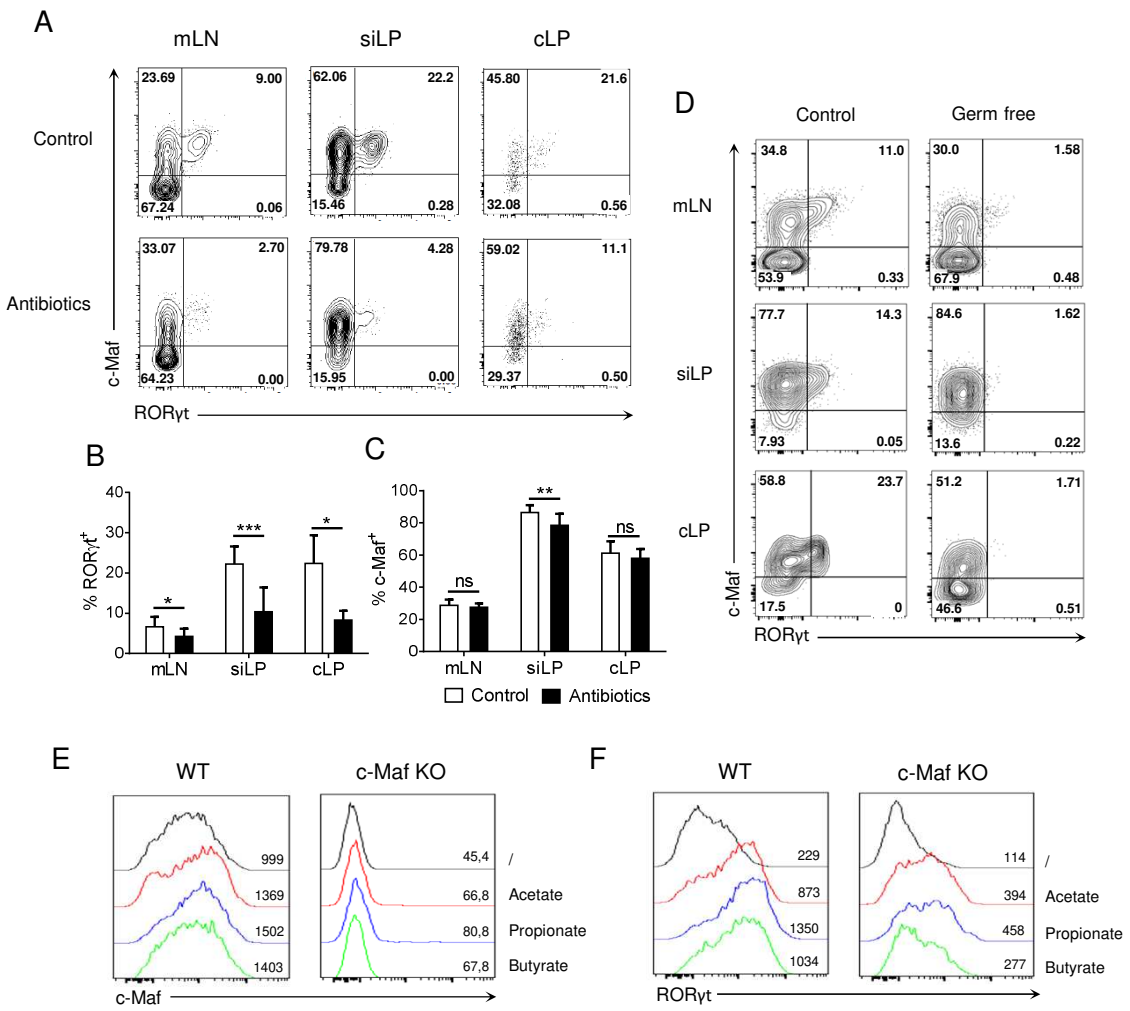

Figure 4. **Microbial signals induce RORγt expression in Tregs but marginally affect c-Maf expression.** (A-C) WT mice were treated with wide-spectrum antibiotics or a control solution for 4 weeks. Representative flow cytometry expression profiles of c-Maf versus RORγt in Treg cells (A) and histograms showing the frequency of RORγt$^+$ (B) and c-Maf$^+$ (C) Tregs in the indicated organs (gate CD4$^+$ Foxp3$^+$). (D) Representative flow cytometry expression profiles of c-Maf versus RORγt by Treg cells in the indicated organs of germ-free and control mice. (E-F) Naïve WT or c-Maf-deficient CD4 T cells were activated *in vitro* for 72h in presence of TGF-β and small chain fatty acids. Histograms show the expression profiles of c-Maf (F) and RORγt (G) among Treg cells. Results are representative of at least three independent experiments; histograms in (B, C) represent the mean ± SD of at least four individual mice. Difference between groups is determined by an unpaired t test (mLN, siLP) or a Mann–Whitney test for two-tailed data (cLP). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

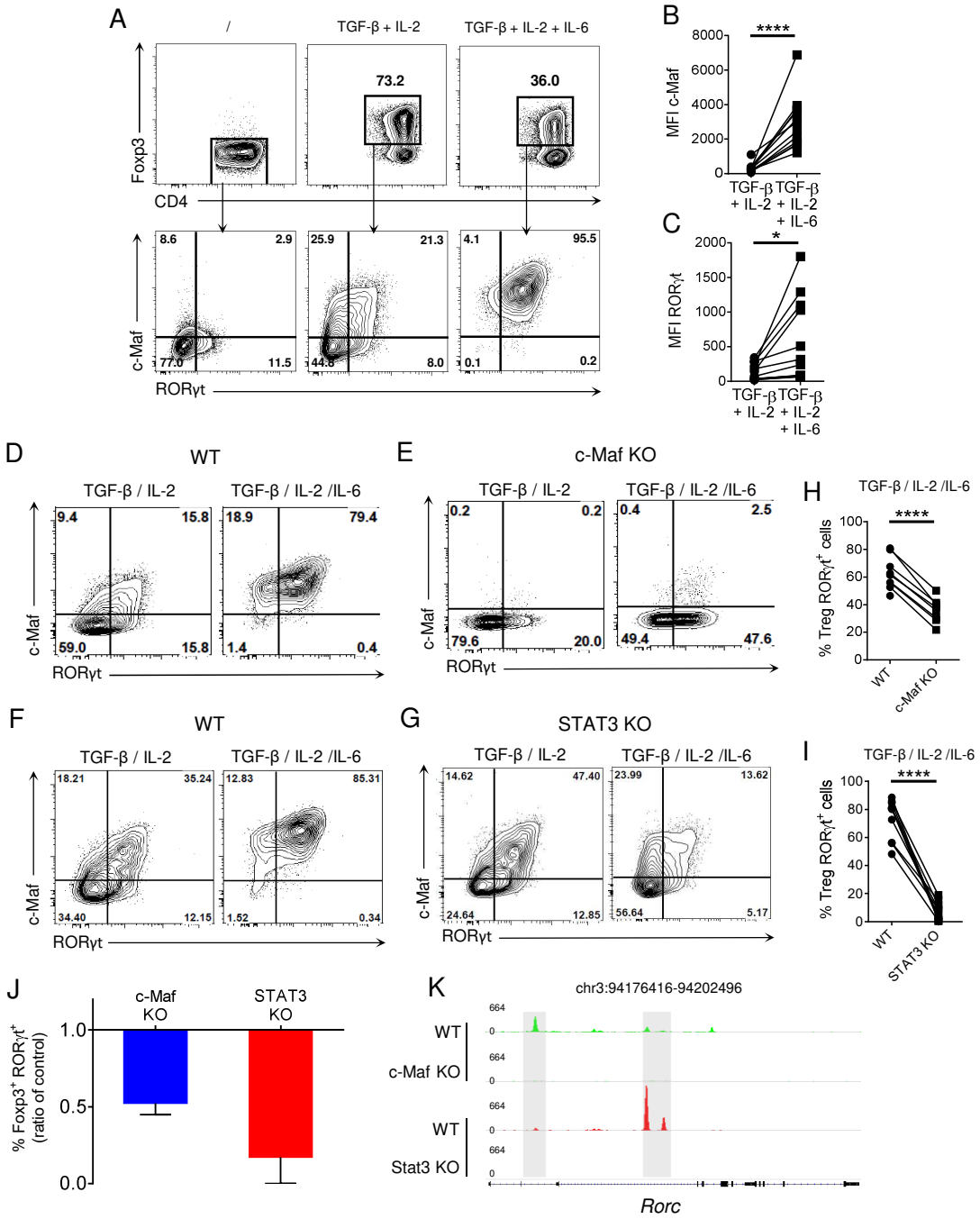

Figure 5. **c-Maf and STAT3 are required for optimal RORγt expression in *in vitro* polarized Tregs.** WT, c-Maf or STAT3-deficient naïve CD4 T cells were activated *in vitro* in presence of polarizing cytokines for 72h. Protein expression was then assessed by flow cytometry. (A) Representative flow cytometry expression profiles of Foxp3 versus CD4 and c-Maf versus RORγt in the indicated gate by WT Treg cells polarized *in vitro* in the indicated conditions. (B, C) Histograms show the MFI of c-Maf (B) or RORγt (C) in WT Treg cells *in vitro*. (D-G) Representative flow cytometry expression profiles of c-Maf versus RORγt by WT (D, F), c-Maf-deficient (E) and STAT3-deficient (G) Treg cells polarized in presence of TGF-β and IL-2 or TGF-β, IL-2 and IL-6 . (H, I) Histograms show the frequency of RORγt+ cells among WT, c-Maf (H) and STAT3-deficient (I) Tregs polarized in presence of TGF-β, IL-2 and IL-6. (J) Proportions of RORγt-expressing cells among CD4+ Foxp3+ Tregs is expressed as a ratio of control. Values from c-Maf or STAT3-KO Tregs were divided by the value from WT Tregs in each experimental data set. (K) Profiles generated from c-Maf and STAT3 ChIP-seq in WT, c-Maf- and STAT3-KO *in vitro* Th17 cells. Representative IGV tracks showing c-Maf (green), Stat3 (red) binding sites highlighted in grey at the Rorc locus among the indicated cell population. Gene location is indicated at the top of the panel. Y axis indicates the normalized read coverage for each track.
Results are representative of at least three independent experiments. Symbols in histograms represent individual mice. Difference between groups is determined by a paired t test. *$p < 0.05$; ****$p < 0.0001$.

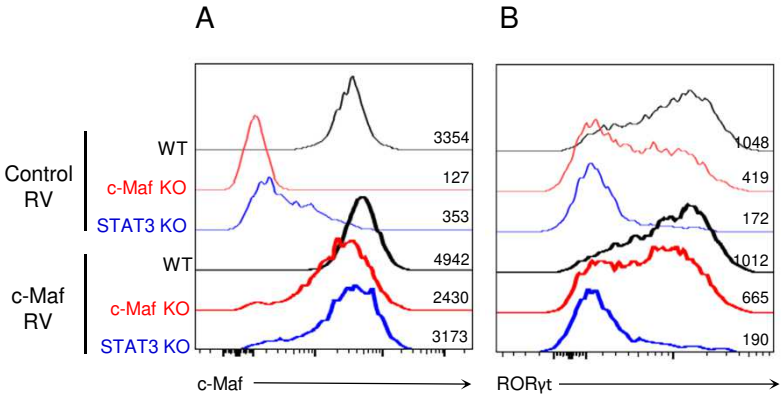

Figure 6. **c-Maf does not rescue RORγt expression in Tregs in the absence of STAT3.** WT, c-Maf or STAT3-deficient naïve CD4 T cells were activated *in vitro* in presence of TGF-β, IL-2 and IL-6 and infected with a control or c-Maf-expressing retrovirus. c-Maf (A) and RORγt (B) expression profiles and MFI as assessed by flow cytometry after 72h (gate $CD4^+$ $Foxp3^+$). Results are representative of three independent experiments

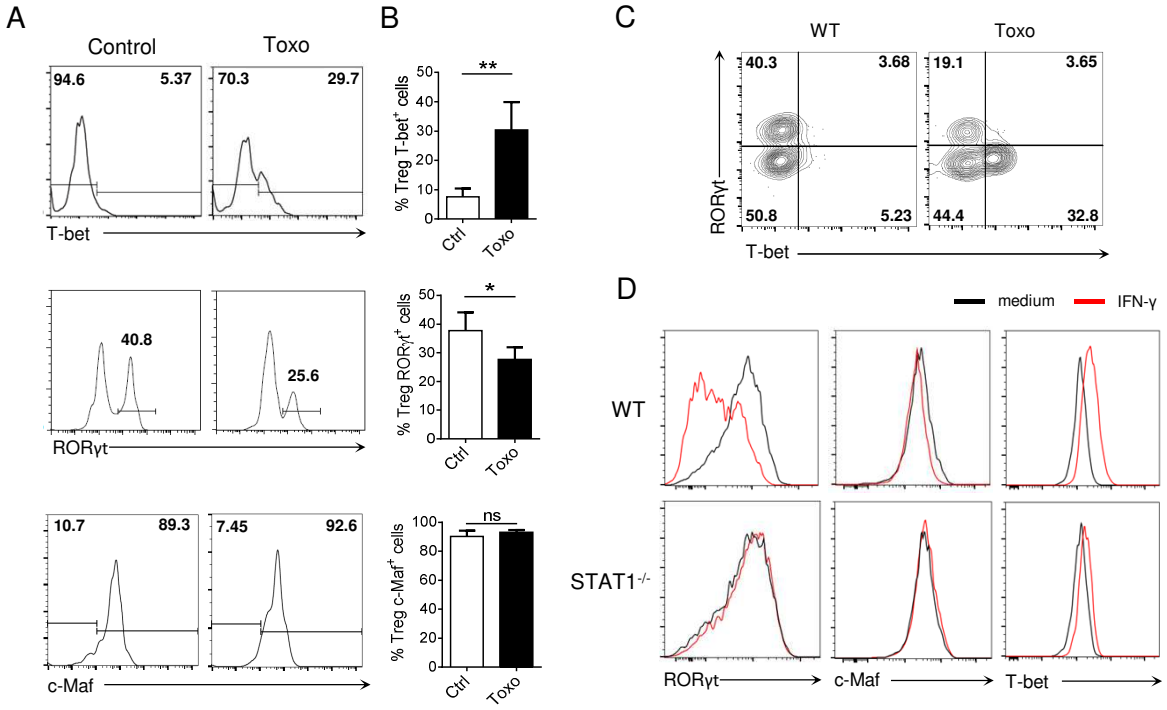

Figure 7. **Inflammatory Th1 responses opposes RORγt expression in Tregs**. (A) Histograms show RORγt, c-Maf and T-bet expression among Tregs in the siLP of WT mice 8 days after infection with *Toxoplasma gondii* or control mice (gate CD4⁺ Foxp3⁺). (B) Frequency of RORγt⁺, c-Maf⁺ and T-bet⁺ cells among Treg cells in the siLP of WT mice 8 days after infection with *Toxoplasma gondii* or control mice. (C) Representative flow cytometry expression profiles of RORγt versus T-bet among Treg cells isolated from mLN of WT mice infected or not with *Toxoplasma gondii* (gate CD4⁺ Foxp3⁺). (D) Histograms show RORγt, c-Maf and T-bet expression in WT and STAT1-deficient *in vitro* Treg17 cells stimulated with or without IFN-γ (100 ng/mL) for 72h. Results are representative of at least two (A-C) or three (D) independent experiments; histograms represent the mean ± SD of five individual mice. Difference between groups is determined by a Mann–Whitney test for two-tailed data. *$p < 0.05$