

1 **Multiple environmental signaling pathways control the differentiation of**
2 **ROR γ t-expressing regulatory T cells**

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12 *Running Title:*

13 Role of microbiota and cytokines in the regulation of ROR γ t expression by Tregs

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20

1 **Abstract**

2 ROR γ t-expressing Tregs form a specialized subset of intestinal CD4⁺ Foxp3⁺ cells which is
3 essential to maintain gut homeostasis and tolerance to commensal microbiota. Recently, c-
4 Maf emerged as a critical factor in the regulation of ROR γ t expression in Tregs. However,
5 aside from c-Maf signaling, the signaling pathways involved in the differentiation of ROR γ t⁺
6 Tregs and their possible interplay with c-Maf in this process are largely unknown. We show
7 that ROR γ t⁺ Treg development is controlled by positive as well as negative signals. Along
8 with c-Maf signaling, signals derived from a complex microbiota, as well as IL-6/STAT3-
9 and TGF- β -derived signals act in favor of ROR γ t⁺ Treg development. Ectopic expression of
10 c-Maf did not rescue ROR γ t expression in STAT3-deficient Tregs, indicating the presence of
11 additional effectors downstream of STAT3. Moreover, we show that an inflammatory IFN-
12 γ /STAT1 signaling pathway acts as a negative regulator of ROR γ t⁺ Treg differentiation in a c-
13 Maf independent fashion.

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

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19

1 **Introduction**

2

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

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

1 microenvironment where they hinder anti-tumor immunity, thus revealing a double-edged
2 function of this Treg subset in immune homeostasis (Downs-Canner et al., 2017). However,
3 despite their importance in physiological and pathological immune responses, the factors
4 driving ROR γ ⁺ Treg differentiation are still incompletely defined.

5 Recent studies reported that transcription factor c-Maf promotes the differentiation of
6 intestinal ROR γ ⁺ Tregs (Imbratta et al., 2019; Wheaton et al., 2017; Xu et al., 2018).

7 Coincidentally, transcriptomic studies conducted on Tregs originating from different tissues
8 revealed a strong enrichment for c-Maf in the intestinal compartment (Sefik et al., 2015).

9 Transcription factor c-Maf, a member of the AP-1 family of basic region/leucine zipper
10 transcription factors, is expressed by distinct CD4⁺ T cell subsets, including Th17, Th2, Tfh
11 and Tr1 cells, and is thought to regulate the expression of IL-10, IL-4, and IL-21 through the

12 transactivation of their promoters, downstream of Batf, ICOS, and STAT3 signaling (Andris
13 et al., 2017; Apetoh et al., 2010; Bauquet et al., 2009; Hiramatsu et al., 2010; Sahoo et al.,
14 2015). Thus, and similarly to what has been previously described for Th17 cells (Tanaka et

15 al., 2014a), expression of ROR γ in Tregs is c-Maf-dependent. However, unlike ROR γ
16 expression, which is restricted to gut-associated Tregs in naïve mice, c-Maf is expressed by a
17 wider proportion of Tregs found in distinct organs. Of note, high levels of c-Maf are found in

18 a subset of splenic CD44⁺ CD62L⁻ effector Tregs driven by ICOS signaling (Wheaton et al.,
19 2017). The partial overlapping of ROR γ and c-Maf expression along with the presence of a
20 substantial population of c-Maf⁺ ROR γ ⁻ Tregs in lymphoid organs therefore suggests that c-

21 Maf is not sufficient *per se* to drive ROR γ ⁺ Treg cell differentiation and supports the
22 existence of complementary signaling pathways.

23 Herein, extensive analysis of the lymphoid organs and tissues of genetically invalidated mice
24 or mice harboring an altered microbiota revealed that, well beyond the c-Maf/ROR γ
25 interplay, multiple signaling pathways cooperate to exert a tight control over ROR γ

1 expression in Tregs.

1 **Results**

2

3 **c-Maf is highly expressed in intestinal Tregs and is required for ROR γ t expression**

4 We first investigated the expression of c-Maf and ROR γ t in Tregs found in distinct organs. In
5 accordance with previous data (Ohnmacht et al., 2015; Sefik et al., 2015), we observed that
6 ROR γ t⁺ Tregs were mainly present in the small intestine and colon lamina propria and, to a
7 lesser extent, in mesenteric lymph nodes (Figure 1A, B). Of note, a large proportion of Tregs
8 (ranging from 20% to 85%) expressed c-Maf in all the examined organs, with a notable
9 exception for the thymus (Figure 1A, C). In the intestine, both ROR γ t⁺ and ROR γ t⁻ Treg
10 subsets expressed c-Maf, although expression levels were higher in the ROR γ t⁺ compartment
11 (Figure 1A, D, E). Strikingly, FACS analysis also revealed that a large fraction of c-Maf⁺
12 Tregs do not express ROR γ t. This was observed in all the examined organs and was most
13 evident in the small intestine lamina propria, where two-thirds of the c-Maf⁺ Tregs lacked
14 ROR γ t expression (Figure 1A).

15 In lymphoid organs, c-Maf expression was mainly found among effector Tregs, characterized
16 by the expression of high levels of ICOS and CD44 (Figure S1 in Supplementary Material). In
17 contrast to ROR γ t⁺ Tregs, which were mostly of peripheral origin, c-Maf⁺ Tregs were found
18 both in Nr ρ 1⁺ and Nr ρ 1⁻ Tregs, suggesting that c-Maf⁺ Tregs can be of thymic or peripheral
19 origin (Figure 2A, B). Thymic c-Maf⁺ Tregs were enriched in the spleen whereas their
20 peripheral counterparts were enriched in mesenteric lymph nodes and formed the majority of
21 intestinal Tregs (Figure 2C).

22 Treg-specific ablation of c-Maf resulted in increased Treg proportions in the intestine and
23 lymphoid organs (Figure 1F, H and Figure S2A). Despite the wide distribution of c-Maf-
24 expressing Tregs in distinct organs, Treg-conditional ablation of c-Maf did not result in
25 systemic autoimmune disease, nor did it disturb conventional and regulatory T cell

1 homeostasis in lymphoid organs. c-Maf-deficient Tregs also retained their *in vitro* suppressive
2 capacity (Figure S2B and data not shown). In agreement with previous reports (Wheaton et
3 al., 2017; Xu et al., 2018), c-Maf^{Treg KO} mice spontaneously developed intestinal inflammation
4 and showed a near complete loss of ROR γ t expression in Tregs (Figure 1G, I and Figure S3).
5 They nevertheless expressed normal percentages of intestinal GATA3⁺ Tregs (Figure 1G, J).
6 Altogether, these data indicate that c-Maf is required for the differentiation of ROR γ t⁺ Tregs
7 and that, contrary to ROR γ t, c-Maf expression is also found in a large proportion of effector
8 thymic Tregs, located in distinct organs.

9

10 **Complex microbiota, STAT3 and TGF- β signals control ROR γ t but are dispensable for** 11 **c-Maf expression in Tregs**

12 The presence of c-Maf⁺ ROR γ t⁻ Tregs in different organs and their distinct origins prompted
13 us to further analyze the specific environmental signals responsible for the induction of c-Maf
14 and ROR γ t expression by Tregs. IL-6/STAT3 and TGF- β signaling has been shown to drive
15 ROR γ t⁺ and c-Maf expression in a variety of T cells (Ohnmacht et al., 2015; Veldhoen et al.,
16 2006). Mice genetically invalidated for IL-6 (IL-6^{-/-}), STAT3 (Stat3^{flox/flox}-CD4^{CRE}) or TGF- β
17 (Tgfbr2^{flox/flox}-Foxp3^{CRE}) signaling showed normal to even slightly increased frequencies of c-
18 Maf⁺ Tregs, although ROR γ t expression was considerably decreased in all the aforementioned
19 conditions (Figure 3; see also Figure S4 for representative FACS plots). Thus, despite being
20 necessary for the expression of ROR γ t by Tregs, c-Maf is not sufficient, and most likely
21 cooperates with other signaling pathways to induce ROR γ t expression in Tregs.

22 ROR γ t⁺ Tregs are absolutely dependent on microbiota for their development (Ohnmacht et
23 al., 2015; Sefik et al., 2015). Analysis of germ-free and antibiotics-treated mice revealed that,
24 in contrast to ROR γ t expression, which was lost in both cases, c-Maf expression by Tregs was
25 only marginally affected in microbiota-deficient mice (Figure 4A-D).

1 Short chain fatty acids (SCFA) are gut microbiota-derived bacterial fermentation products,
2 which include acetate, propionate, and butyrate, that regulate the size and function of the
3 colonic Treg pool (Arpaia et al., 2013). Treg cells were induced *in vitro* with TGF- β in the
4 absence or presence of acetate, propionate, or butyrate. In this experimental setting, SCFA did
5 not affect or slightly decreased the differentiation of Foxp3⁺ Treg cells (Figure S5). Addition
6 of SCFA to the culture medium induced a 4 to 5-fold upregulation of ROR γ t expression,
7 while minimally affecting c-Maf expression (Figure 4E, F, left panels). In absence of c-Maf,
8 intermediate levels of ROR γ t were induced in Tregs treated with SCFA (Figure 4F, right
9 panel). Overall, these observations suggest that microbiota-derived products regulate ROR γ t
10 expression in Tregs through both c-Maf-dependent and independent pathways.

11

12 **STAT3 and c-Maf control ROR γ t expression in iTregs through partly overlapping** 13 **pathways**

14 In presence of TGF- β and IL-2, about 75% of *in vitro* activated naïve CD4 T cells
15 differentiated into Tregs, as assessed by their Foxp3 expression. Addition of IL-6, a pro-Th17
16 cytokine, to the TGF- β /IL-2 cytokine cocktail decreased the frequency of induced Tregs but
17 led to the differentiation of a population of double positive c-Maf⁺ ROR γ t⁺ induced Tregs
18 (iTreg17 cells; Figure 5A, lower panels and B, C). In agreement with *in vivo* observations
19 (Figure 1G, I), ablation of c-Maf expression led to a significant reduction of *in vitro* generated
20 ROR γ t⁺ Tregs (Figure 5D, E, H). In the absence of STAT3, the expression of c-Maf and
21 ROR γ t in iTreg17 was heavily decreased (Figure 5F-I). Despite showing residual c-Maf
22 expression, STAT3 KO iTregs displayed a more severe down-regulation of ROR γ t than their
23 c-Maf KO counterparts (90% versus 50 %; Figure 5J), suggesting a prominent role of STAT3
24 in ROR γ t expression in this context. Analysis of STAT3 and c-Maf genome mapping from
25 public ChIPseq databases (Ciofani et al., 2012) revealed that both transcription factors bind to

1 the *Rorc* locus, albeit at distinct preferential sites (Figure 5K).
2 To determine whether the role of STAT3 in promoting ROR γ t induction solely relies on c-
3 Maf, we restored c-Maf expression in STAT3-deficient iTreg17 cells. WT and c-Maf KO
4 CD4 T cells were infected with a control-GFP or a c-Maf-GFP encoding retrovirus. Although
5 the c-Maf encoded retrovirus did restore ROR γ t expression in c-Maf-deficient Tregs and
6 induced optimal levels of c-Maf in STAT3 KO Tregs, it was unable to restore ROR γ t
7 expression in the latter cells (Figure 6). Collectively, these data indicate that in Tregs, an
8 additional STAT3-driven but cMaf-independent pathway is required to promote optimal
9 ROR γ t.

10

11 **A pro-Th1 inflammatory environment dampens ROR γ t expression in Tregs**

12 While IL-6 promoted ROR γ t expression in wild type iTregs, it surprisingly led to a marked
13 reduction of this transcription factor in STAT3-deficient iTregs (Figure 5F-I), thus revealing
14 the presence of an inhibitory pathway regulating ROR γ t expression. Studies by Costa-Pereira
15 et al have shown that in STAT3 KO fibroblasts, IL-6 signals through STAT1 and has IFN γ -
16 like effects (Costa-Pereira et al., 2002). We observed that in contrast to their wild type
17 counterparts, STAT3 KO CD4⁺ T cells cultured in the presence of IL-6 expressed higher
18 levels of phospho-STAT1 (Figure S6). This prompted us to investigate the consequences of a
19 STAT1/Th1 inflammatory pathway on the differentiation of ROR γ t⁺ Tregs.

20 Infection with *Toxoplasma gondii* results in a highly Th1-polarized microenvironment leading
21 to altered Treg cell homeostasis (Oldenhove, 2009). In particular, the strong Th1
22 environment triggered by *T. gondii* infection has been shown to induce T-bet and IFN- γ
23 expression in Treg cells (Hall et al., 2012; Oldenhove et al., 2009). Analysis of Tregs from the
24 lamina propria of *T. gondii* infected C57BL/6 mice confirmed the emergence of a T-bet⁺ Treg
25 subset (Figure 7A and B, upper panels). Interestingly, *T. gondii* infection resulted in a reduced

1 frequency of tissue associated ROR γ t⁺ Tregs, despite having minimal effect on Maf
2 expression (Figure 7A and B, middle and lower panels). Th17 cell differentiation was also
3 suppressed during *T. gondii* infection (Figure S7), consistent with the observation that Th17
4 cell differentiation and Th1 cell differentiation are mutually suppressive (Harrington et al.,
5 2005; Park et al., 2005). This reciprocal exclusion was also reflected among Tregs, as T-bet
6 and ROR γ t were expressed in distinct Treg cell subsets in the infected mice (Figure 7C).
7 We next wished to evaluate the effect of Th1-promoting signals on ROR γ t expression by
8 Tregs, Addition of IFN- γ to the iTreg17 differentiation media led to the selective
9 accumulation of the phosphorylated form of STAT1, without affecting neither phospho-
10 STAT3 accumulation nor c-Maf expression by iTregs (Figure S8). Of note, IFN- γ led to the
11 accumulation of Tbet-expressing Tregs, with a concomitant reduction in the number of
12 ROR γ t⁺ Tregs (Figure 7D, upper panels). STAT1 KO Tregs were insensitive to the IFN- γ -
13 driven inhibition of ROR γ t expression (Figure 7D, lower panels). Altogether these results
14 strongly suggest that the IFN- γ /STAT1 signaling pathway negatively regulates ROR γ t
15 expression even in the presence of an active IL-6/STAT3-driven pathway.

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17

1 **Discussion**

2

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

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

15 We showed that microbial signals, as well as IL-6-, STAT3-, and TGF- β -signaling pathways
16 promote ROR γ ⁺ Treg cell differentiation in a non-redundant manner. Constrastingly,
17 blocking any one of these pathways only had minimal effect on c-Maf expression *in vivo*,
18 suggesting that several redundant pathways cooperate to induce c-Maf expression in Tregs.

19 Neumann et al reported that, in addition to the loss of ROR γ expression in Tregs, which we
20 also observed, germ-free mice exhibited a near complete loss of c-Maf expression in intestinal
21 Tregs. While we could not reproduce this observation, we observed that Tregs found in germ-
22 free mice expressed slightly reduced levels of c-Maf. Reminding that the ROR γ ⁺ population
23 expresses the highest levels of c-Maf among intestinal Tregs, we therefore speculate that
24 ROR γ expression in Tregs requires a high c-Maf threshold and that even a minimal decrease

1 in c-Maf expression could hinder ROR γ t expression in microbiota-driven intestinal Tregs.
2 Short chain fatty acids (SCFA) produced by gut commensal microbes induce functional
3 colonic Tregs and protect against T cell-dependent experimental colitis (Arpaia et al., 2013;
4 Furusawa et al., 2013). Depending on the cytokine environment and immunological context,
5 butyrate, acetate, and propionate, the most available SCFA in the gut, can also support IL-10
6 expression in Th1 and Th17 effector cells, thereby inhibiting colitis caused by pathogenic T
7 cells (Park et al., 2015; Sun et al., 2018). We show herein that SCFA induce ROR γ t
8 expression in *in vitro* differentiated Tregs in a c-Maf-dependent and independent manner.
9 Further studies could help clarify whether SCFA regulate ROR γ t expression through GPR41
10 or GPR43-dependent signaling, or through HDAC inhibitor activity and subsequent
11 enhancement of mTOR–S6K activity, as previously shown in Th17 cells (Park et al., 2015).
12 The proportion of ROR γ t⁺ Tregs was severely reduced in mice deficient for STAT3 or TGF- β
13 signaling. Rather surprisingly, while STAT3 and TGF- β signaling drive c-Maf expression in
14 Tfh and Th17 cells (Ciofani et al., 2012; Mari et al., 2013; Rutz et al., 2011), we found that
15 mice deficient for STAT3 or TGF- β in the T cell compartment harbored normal to even
16 slightly increased numbers of c-Maf⁺ Tregs, indicating that, while STAT3 and TGF- β seem
17 dispensable for the induction of c-Maf expression, they are both essential to achieve optimal
18 differentiation of ROR γ t⁺ Tregs in the intestine.
19 Besides c-Maf, many genes involved in Th17 differentiation, such as Irf4, Batf, Rora, Ahr,
20 Sox5t and HIF-1 α , are expressed in response to STAT3 activation (Brüstle et al., 2007; Dang
21 et al., 2011; Durant et al., 2010; Schraml et al., 2009; Tanaka et al., 2014b; Yang et al., 2008).
22 Sox5t is a T cell isoform of Sox5 which induces ROR γ t expression in Th17 cells via physical
23 interaction with c-Maf (Tanaka et al., 2014b). As enforced expression of c-Maf was not
24 sufficient to induce Foxp3⁺ ROR γ t⁺ T cell differentiation in absence of STAT3, we speculate
25 that Sox5, or other molecules downstream of STAT3, act together with c-Maf to achieve

1 ROR γ t expression in Tregs. Interestingly, ChIP-seq data revealed that STAT3 and c-Maf bind
2 with different intensities to distinct sites of the *Rorc* locus. The inability of c-Maf to
3 compensate STAT3-deficiency in ROR γ t⁺ Treg differentiation could thus also be explained
4 by a direct effect of STAT3 on ROR γ t expression. This is in agreement with previous data
5 showing that STAT3 binds to intron 1 of *Rorc* gene and induces chromatin remodeling of the
6 locus (Durant et al., 2010). Overall, it would seem that STAT3 controls ROR γ t⁺ Treg cell fate
7 both through direct activation of the *Rorc* locus and by regulating the expression of a set of
8 genes, including c-Maf, that is essential for ROR γ t⁺ Treg differentiation (Figure S9).

9

10 In T cells, IL-6 predominantly signals via STAT3 and to a lesser extent via STAT1. It has
11 been proposed that the accessibility of different STATs within the cell influences the outcome
12 of cytokine signaling (Regis et al., 2008), as illustrated by the observation that IL-6 acquired
13 the ability to induce the expression of STAT1-dependent genes in STAT3-deficient cells
14 (Costa-Pereira et al., 2002; Schiavone et al., 2011). However, Hirahara et al showed an
15 asymmetric action of STAT3 and STAT1 at the genomic level where much of STAT1
16 chromatin binding was STAT3-dependent. This challenged the classical view that, in the
17 absence of its major STAT module, a cytokine would acquire an alternative STAT-signaling
18 profile (Hirahara et al., 2015). Yet, in the absence of STAT3, and despite a global reduction in
19 STAT1 chromatin binding, some preferential STAT1 binding sites were conserved in a group
20 of IL-6 downregulated genes. With this in mind we hypothesized a negative influence of
21 STAT1 on ROR γ t expression by Tregs. Indeed, STAT3-deficient T cells showed increased
22 STAT1 phosphorylation in response to IL-6, compatible with a switch from STAT3 to
23 STAT1 signaling in these cells. We further showed that IFN- γ -driven activation of STAT1
24 opposed ROR γ t expression in Tregs, without affecting Foxp3 and c-Maf expression or
25 STAT3 phosphorylation. Naïve STAT1 KO mice did not show altered proportions of

1 intestinal ROR γ ⁺ Tregs (data not shown), which could be explained by the lack of
2 inflammatory Th1 components at steady state. Indeed, in wild type mice infected with the
3 Th1-prototypic *Toxoplasma gondii* intestinal parasite, ROR γ t expression was decreased in
4 intestinal Tregs, confirming the antagonistic role of inflammatory Th1 responses on ROR γ t
5 expression in Tregs *in vivo*.

6 Different integrative pathways have been proposed to explain the functional outcome of
7 multiple STAT signaling in distinct T cell subsets (Lin and Leonard, 2019). Meyer zu Horste
8 et al recently reported that death receptor Fas promotes Th17 cell differentiation and inhibits
9 Th1 cell development by preventing STAT1 activation. In this model, Fas regulated the
10 STAT1 versus STAT3 balance by binding and sequestering STAT1 (Meyer zu Horste et al.,
11 2018). Although not formerly excluded, sequestration of STAT3 is unlikely in ROR γ ⁺ Tregs
12 as addition of IFN- γ to the iTreg17 culture media did not affect the phosphorylation status of
13 STAT3. Our data rather suggest that Treg cell fate results from the balance of STAT1 and
14 STAT3 driven signals. Gene expression could also conceivably be fine-tuned by the
15 formation of STAT1/STAT3 heterodimers, as proposed for the IL-21 signaling (Wan et al.,
16 2015). Of interest, patients with STAT3 mutations or with gain-of-function STAT1 mutations
17 show similar susceptibility to fungal infections (Casanova et al., 2012; O’Shea et al., 2013).
18 In the latter group, overactive STAT1 appears to limit STAT3-driven antifungal responses
19 (Casanova et al., 2012).

20 Further work is required to decipher whether STAT1 interacts with STAT3 or exerts an
21 independent negative role on ROR γ ⁺ Treg cell fate. As T-bet is induced in Tregs that develop
22 during *Toxoplasma* infection or in response to IFN- γ , we can also envision that STAT1
23 signaling inhibits ROR γ t expression through T-bet blocking of Runx1-mediated
24 transactivation of *Rorc*, as previously reported for the Th1/Th17 lineage specification
25 (Lazarevic et al., 2011). Regardless of the molecular mechanism, the observation that IFN-

1 γ /STAT1 signaling pathway negatively regulates ROR γ t, even in the presence of an active IL-
2 6/STAT3 pathway, suggests a dominant negative effect of STAT1 over STAT3 in these
3 experimental conditions.

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

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

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1 **Material and Methods**

2

3 **Mice.** C57BL/6 mice were purchased from Envigo (Horst, The Netherlands). c-Maf-flox mice
4 (C. Birchmeier, Max Delbrück Center for Molecular Medicine, Berlin, Germany) were
5 crossed with CD4-CRE mice (G. Van Loo, Ghent University, Ghent, Belgium) or FOXP3-
6 CRE-YFP mice which were developed by A. Rudensky (Rubtsov et al., 2008) and kindly
7 provided by A. Liston (KU Leuven, Leuven, Belgium). IL-6^{-/-} mice were obtained from The
8 Jackson Laboratory (Bar Harbor, ME, USA). STAT3-flox mice were kindly provided by S.
9 Akira (Osaka University, Osaka, Japan); STAT1^{-/-} mice by D.E. Levy (New York University
10 School of Medicine, NYC, USA). Germ-free mice were obtained from the Ghent Germfree
11 and Gnotobiotic mouse facility (Ghent University, Ghent, Belgium) and were compared to
12 SPF control mice. c-Maf-flox, CD4-CRE, FOXP3-CRE-YFP, IL-6^{-/-}, STAT3-flox, STAT1^{-/-}
13 and germ-free mice were bred on a C57BL/6 background. Tgfbr2-flox mice (Chytil et al.,
14 2002) on a NOD background crossed with Foxp3-Cre mice (JAX 008694) were kindly
15 provided by Q. Tang (University of California San Francisco, SF, USA) and were housed and
16 bred at the UCSF Animal Barrier Facility.

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

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

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

8 **Isolation of lymphocytes.** After removal of Peyer's patches and mesenteric fat, intestinal
9 tissues were washed in HBSS 3% FCS and PBS, cut in small sections and incubated in HBSS
10 3% FCS containing 2,5mM EDTA and 72,5 µg/mL DTT for 30 min at 37°C with agitation to
11 remove epithelial cells, and then minced and dissociated in RPMI containing liberase (20
12 µg/ml, Roche) and DNase (400 µg/ml, Roche) at 37 °C for 30 min (small intestine) or 45 min
13 (colon). Leukocytes were collected after a 30% Percoll gradient (GE Healthcare). Lymph
14 nodes, thymus and spleens were mechanically disrupted in culture medium.

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

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

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

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

3 **T cell culture.** Naive CD4⁺ T cells were purified from spleen of mice with indicated
4 genotypes. CD4⁺ T cells were positively selected from organ cell suspensions by magnetic-
5 activated cell sorting using CD4 beads (MACS, Miltenyi) according to the product protocol,
6 and then isolated as CD4⁺ CD44^{lo}CD62L^{hi}CD25⁻ or CD4⁺ CD44^{lo} CD62L^{hi} YFP⁻ by FACS. T
7 cells were cultured at 37°C in RPMI supplemented with 5% heat-inactivated FBS (Sigma-
8 Aldrich), 1% non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 2
9 mM L-glutamin (Invitrogen), 500 U/mL penicillin/500 µg/ml streptomycin (Invitrogen), and
10 50 µM β-mercaptoethanol (Sigma-Aldrich).

11 To generate iTreg cells, cells were cultured 72h in 24 or 96 well plates coated with 5µg/mL
12 anti-CD3 (BioXcell, 145-2c11) at 37°C for 72h. The culture was supplemented with anti-
13 CD28 (1 µg/mL, BioXcell, 37.51) and TGF-β alone (3 ng/ml, eBioscience), TGF-β and IL-2
14 (10 ng/mL, Peprotech), or TGF-β, IL-2 and IL-6 (10 ng/mL, eBioscience) for optimal iTreg
15 cell polarization. Acetate (C2, 10 mM), propionate (C3, 0,5 mM), butyrate (C4, 0,125 mM),
16 all from Sigma-Aldrich, and IFN-γ (10 and 100 ng/mL, Peprotech) were also used and added
17 to the culture for the whole duration of the experiment.

18 **Retroviral infection.** Platinum-E retroviral packaging cells (T. Kitamura, University of
19 Tokyo, Tokyo, Japan) were transfected with a c-Maf encoding retroviral plasmid (pMIEG-c-
20 Maf-IRES-GFP) or a control retroviral plasmid (pMIEG-IRES-GFP) to produce retrovirus-
21 containing supernatants. 24 hours after activation, naïve CD4 T cells polarized in presence of
22 TGF-β, IL-2 and IL-6, as described above, were infected during a 90-minute centrifugation
23 with 1 mL retrovirus-containing supernatant and polybrene. 48 hours later, infected cells were
24 FACS-sorted based on GFP expression and were stimulated with anti-CD3 for 24 hours
25 (5µg/mL, coated) before flow cytometry staining.

1 **Treg cell *in vitro* suppression assay.** Naïve T cells with the phenotype CD4⁺ CD44^{lo}
2 CD62L^{hi} YFP⁻ were isolated from the spleen of FOXP3-CRE-YFP mice by FACS after
3 positive enrichment for CD4⁺ cells using MACS LS columns (Miltenyi) and labelled with
4 carboxyfluorescein diacetate succinimidyl ester (CFSE, ThermoFisher). Treg cells with the
5 phenotype CD4⁺ FOXP3-CRE-YFP⁺ were isolated from the mesenteric lymph nodes of
6 Foxp3-CRE-YFP or c-Maf^{Treg KO} mice by FACS. Splenocytes from wild-type B6 mice were
7 depleted in T cells (anti-CD90.2 beads) using MACS LS columns (Miltenyi). 4 × 10⁴ CFSE-
8 labelled naive T cells were cultured for 72 h with APCs (1 × 10⁵) and soluble anti-CD3 (0,5
9 µg/mL) in the presence or absence of various numbers of Treg cells as indicated.

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

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

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

- 1 groups did not always have similar standard deviations and therefore an unpaired two-sided
- 2 Welch's t-test was used. For paired data, a paired t test was used. Error bars represent mean \pm
- 3 SD. No samples were excluded from the analysis.
- 4

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10

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12 **Conflict of Interest Disclosure**

13 The authors declare no conflict of interest

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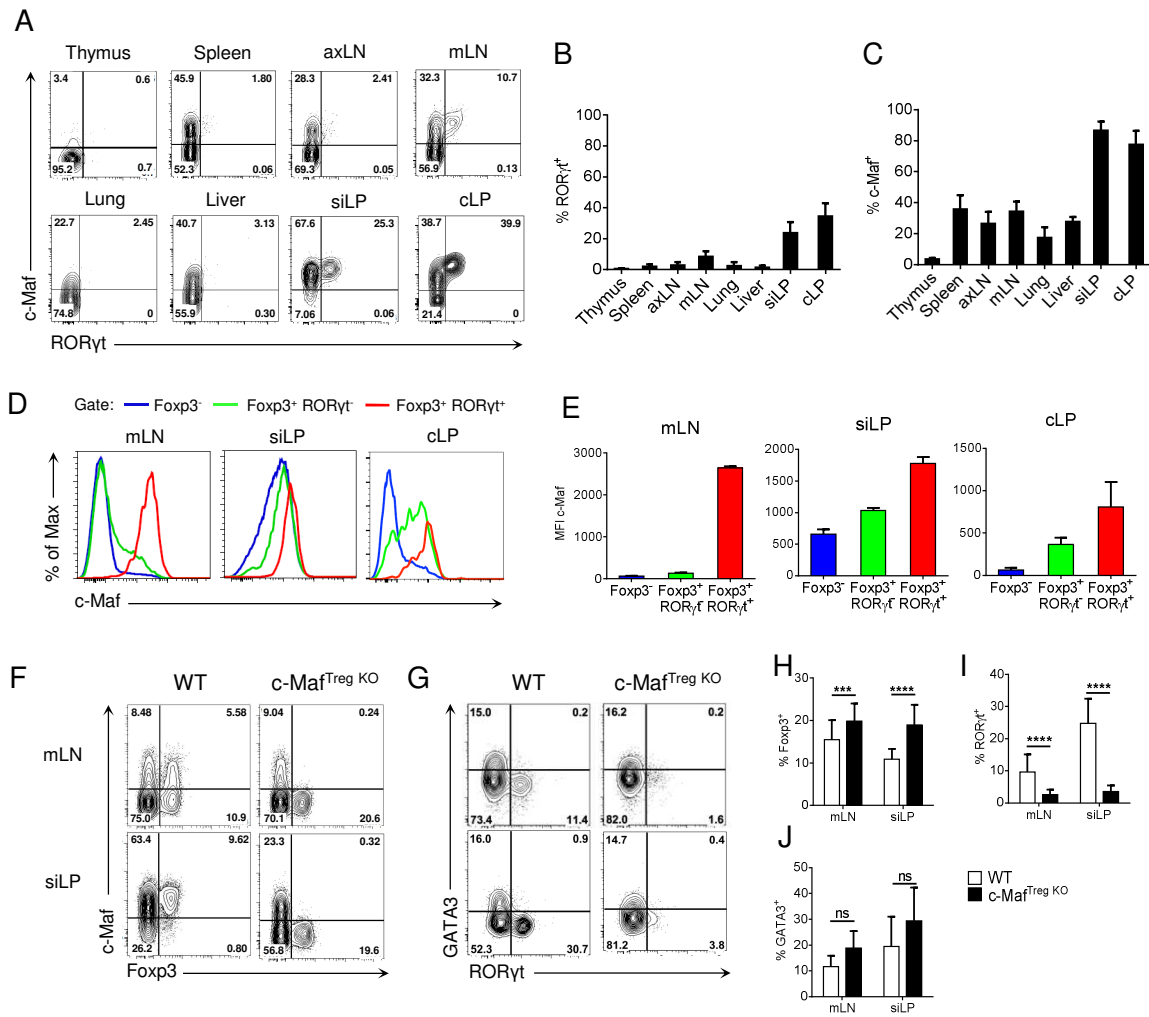


Figure 1. Transcription factor c-Maf is required for the differentiation of ROR γ t⁺ Tregs. (A) Representative flow cytometry expression profiles of c-Maf versus ROR γ t of Treg cells in the indicated organs of naïve C57BL/6 mice (gate CD4⁺ Foxp3⁺). (B, C) Histograms showing the frequency of ROR γ t⁺ (B) or c-Maf⁺ (C) Treg cells in the indicated organs. (D, E) Expression profile (D) and median of fluorescence intensity (E) of c-Maf among Foxp3⁺, Foxp3⁺ ROR γ t⁺ and Foxp3⁺ ROR γ t⁺ 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 ROR γ t (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), ROR γ t⁺ 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 \pm 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)

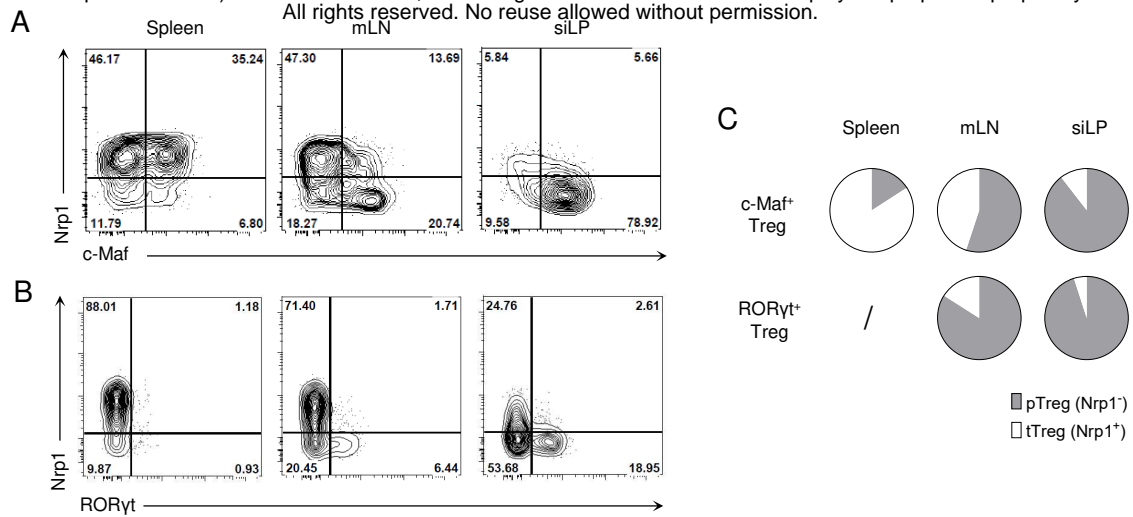


Figure 2. **c-Maf-expressing Tregs can be of thymic or peripheral origin.** (A, B) Representative flow cytometry expression profiles of Nrp1 versus c-Maf (A) or RORyt (B) of Treg cells in the indicated organs of naïve C57BL/6 mice (gate CD4⁺ Foxp3⁺ cells). (C) Pie charts show the relative frequencies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

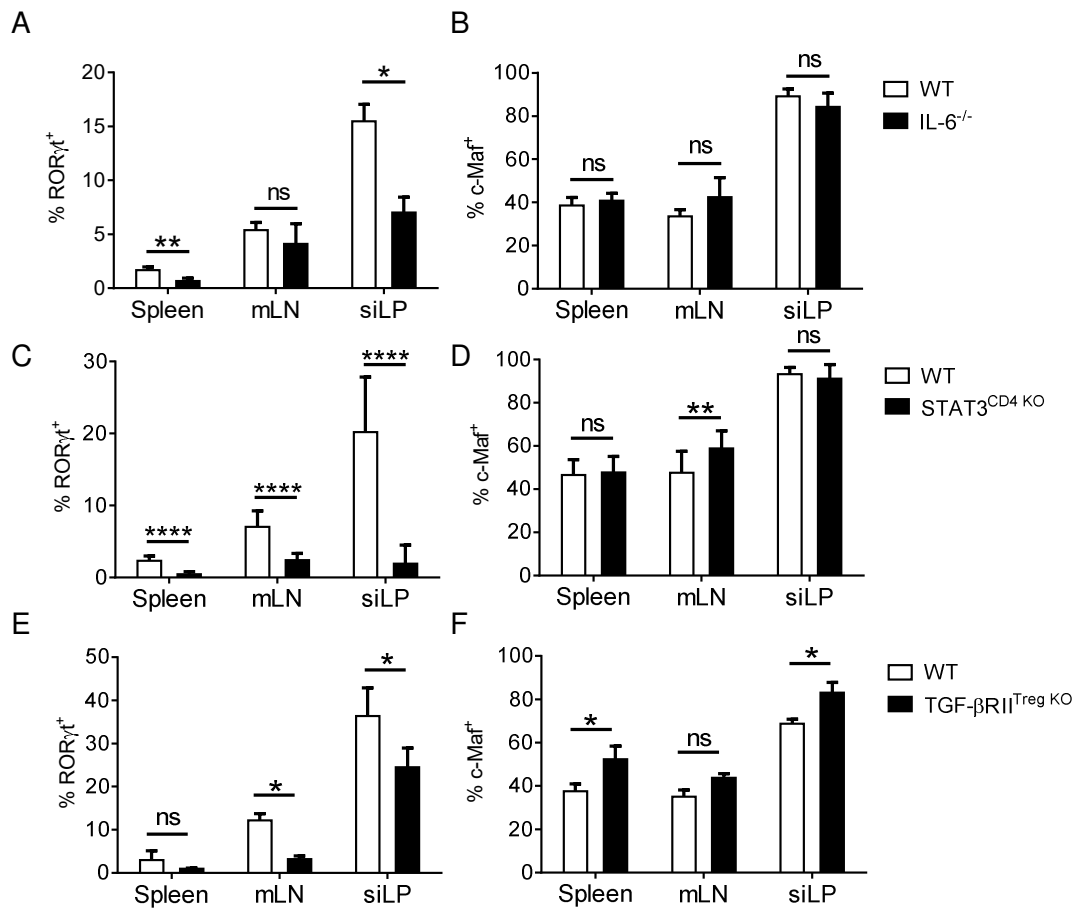


Figure 3. **IL-6/STAT3 and TGF- β signaling promote ROR γ t expression in Tregs independently of c-Maf.** (A-F) Histograms show the frequency of ROR γ t⁺ (A, C, E) or c-Maf⁺ (B, D, F) cells among Treg cells (gate CD4⁺ Foxp3⁺) in indicated organs of WT and IL-6^{-/-} (A, B), STAT3^{CD4 KO} (C, D) and TGF- β RII^{Treg KO} (E, F) mice. Results are representative of at least three independent experiments; histograms represent the mean \pm 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.

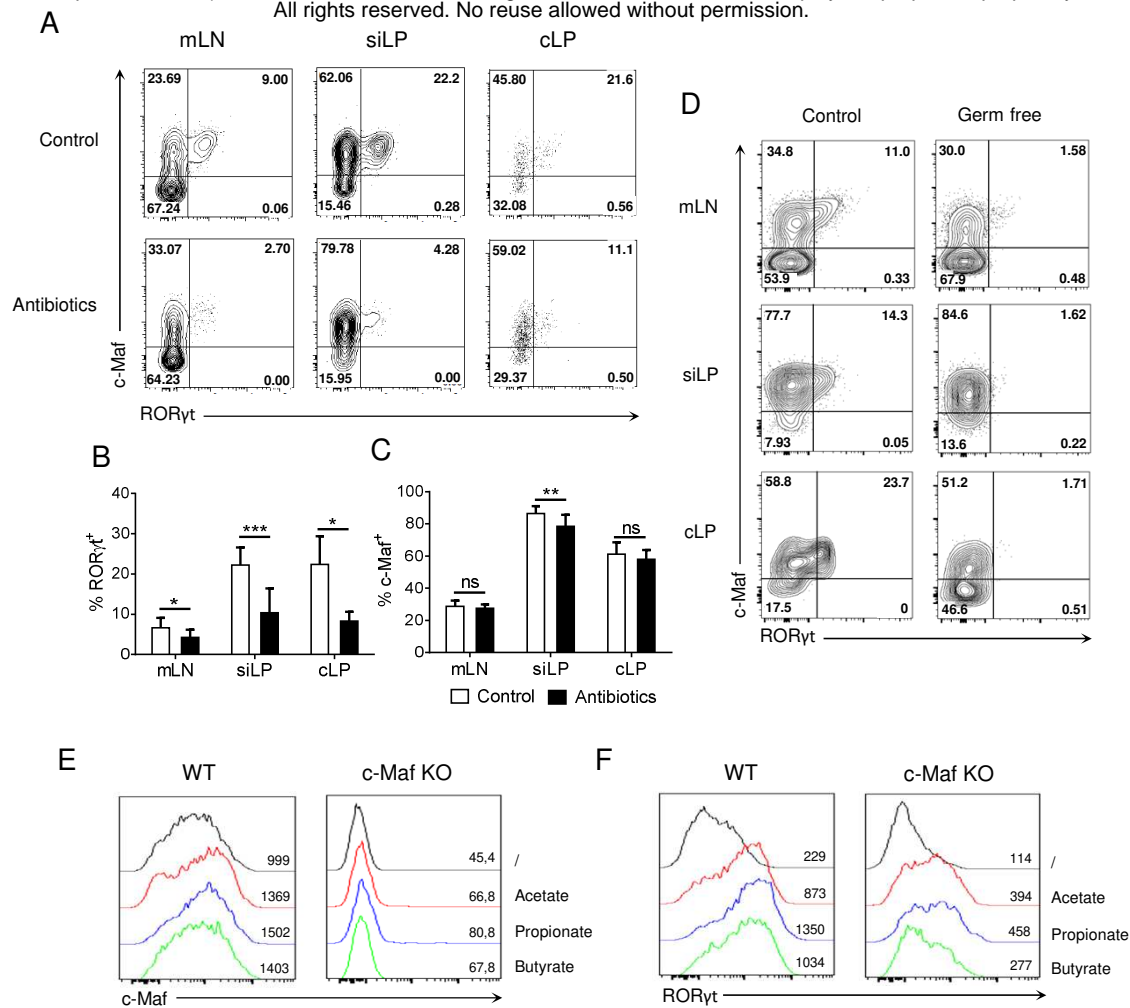


Figure 4. Microbial signals induce ROR γ t expression in Tregs but marginally affect c-Maf expression. (A-C) WT mice were treated with wide-spectrum antibiotics or a control solution for 4 weeks. Representative flow cytometry expression profiles of c-Maf versus ROR γ t in Treg cells (A) and histograms showing the frequency of ROR γ t⁺ (B) and c-Maf⁺ (C) Tregs in the indicated organs (gate CD4⁺ Foxp3⁺). (D) Representative flow cytometry expression profiles of c-Maf versus ROR γ t 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 (E) and ROR γ t (F) among Treg cells. Results are representative of at least three independent experiments; histograms in (B, C) represent the mean \pm 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

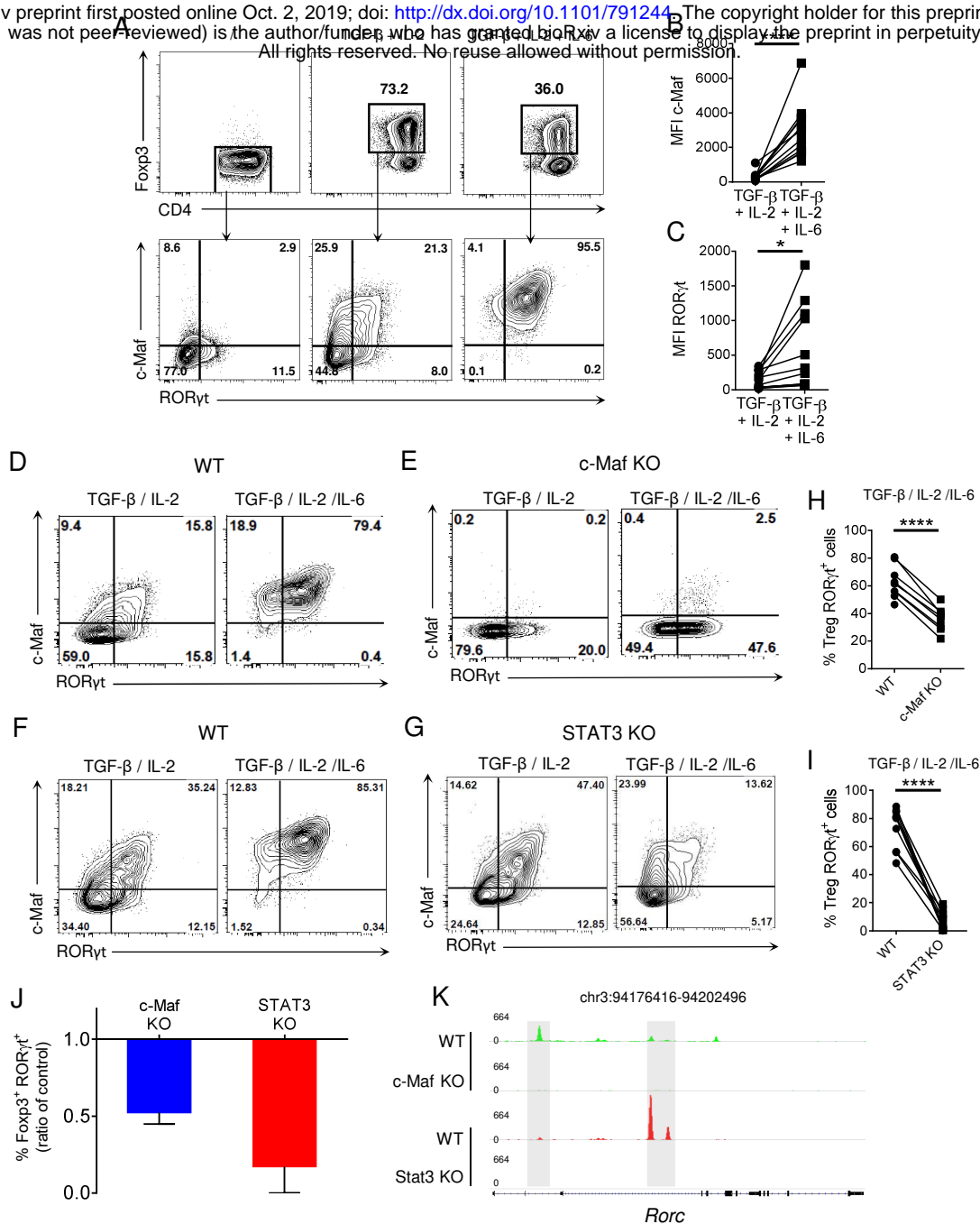


Figure 5. c-Maf and STAT3 are required for optimal RORyt expression in *in vitro* polarized Tregs. WT, c-Maf or STAT3-deficient naive CD4 T cells were activated *in vitro* in presence of polarizing cytokines for 72h. Protein expression was then assessed by flow cytometry. (A) Representative flow cytometry expression profiles of Foxp3 versus CD4 and c-Maf versus RORyt 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 RORyt (C) in WT Treg cells *in vitro*. (D-G) Representative flow cytometry expression profiles of c-Maf versus RORyt 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 RORyt⁺ 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 RORyt⁺-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.

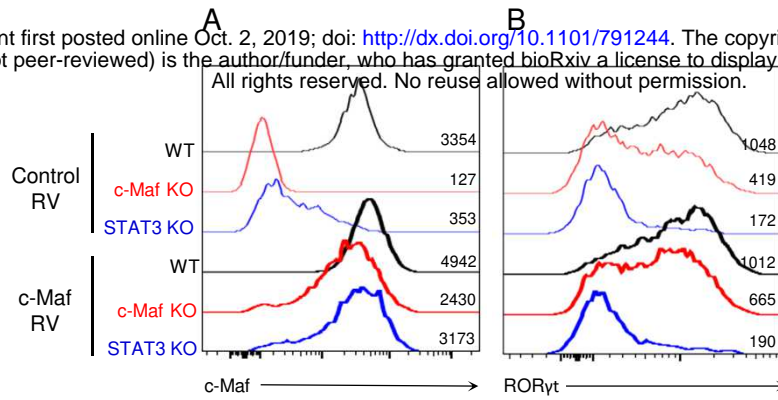


Figure 6. **c-Maf does not rescue RORγt expression in Tregs in the absence of STAT3.** WT, c-Maf or STAT3-deficient naïve CD4 T cells were activated *in vitro* in presence of TGF-β, IL-2 and IL-6 and infected with a control or c-Maf-expressing retrovirus. c-Maf (A) and RORγt (B) expression profiles and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

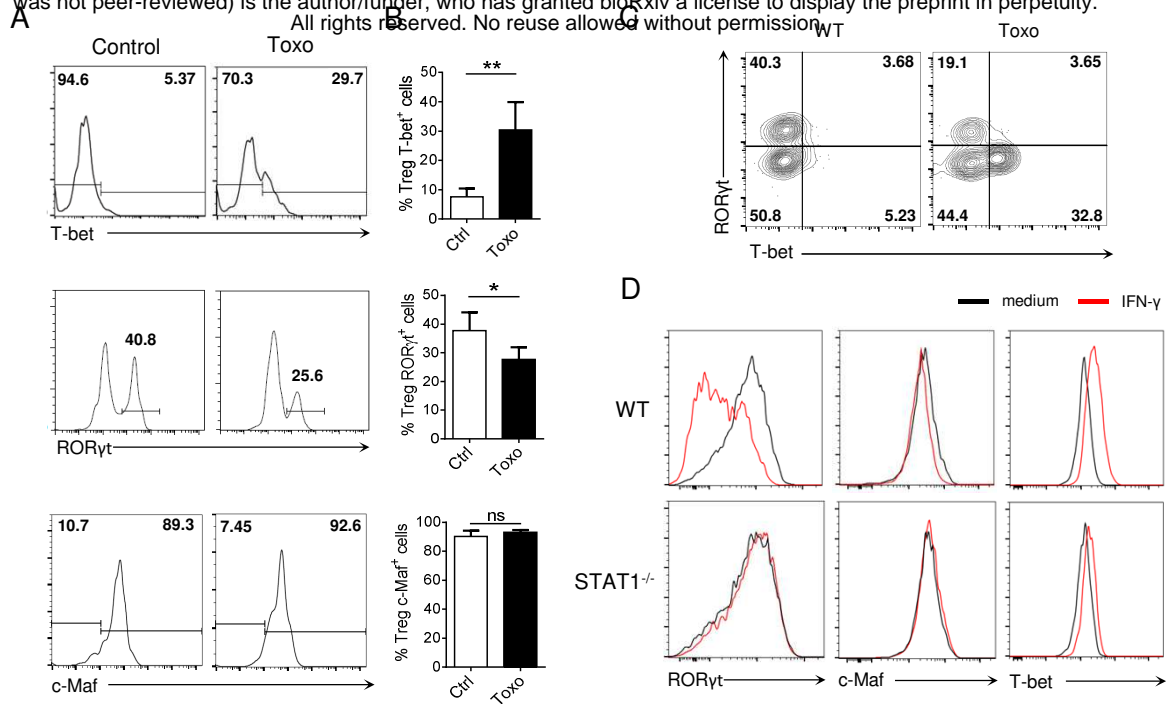


Figure 7. Inflammatory Th1 responses opposes RORyt expression in Tregs. (A) Histograms show RORyt, c-Maf and T-bet expression among Tregs in the siLP of WT mice 8 days after infection with *Toxoplasma gondii* or control mice (gate CD4⁺ Foxp3⁺). (B) Frequency of RORyt⁺, c-Maf⁺ and T-bet⁺ cells among Treg cells in the siLP of WT mice 8 days after infection with *Toxoplasma gondii* or control mice. (C) Representative flow cytometry expression profiles of RORyt versus T-bet among Treg cells isolated from mLN of WT mice infected or not with *Toxoplasma gondii* (gate CD4⁺ Foxp3⁺). (D) Histograms show RORyt, 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