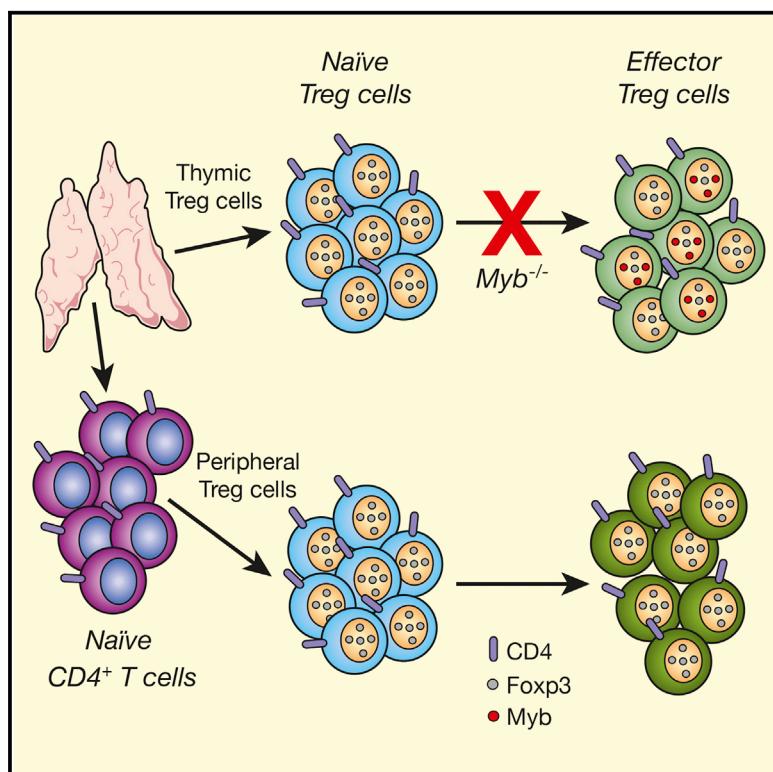


Effector Regulatory T Cell Differentiation and Immune Homeostasis Depend on the Transcription Factor Myb

Graphical Abstract



Authors

Sheila Dias, Angela D'Amico,
Erika Cretney, ..., Gordon K. Smyth,
Wei Shi, Stephen L. Nutt

Correspondence

nutt@wehi.edu.au

In Brief

Treg cells can derive from either thymic or peripheral sources and can undergo further differentiation into an effector state in response to environmental cues. Dias and colleagues demonstrate that Myb is specifically required for the differentiation of thymus-derived effector Treg cells that play a non-redundant role in controlling immune homeostasis.

Highlights

- Myb is specifically expressed in ICOS⁺ effector Treg cells
- Mice lacking Myb in Treg cells develop severe inflammatory disease
- Myb is essential for effector Treg cell differentiation of thymus-derived Treg cells
- Myb controls effector Treg cell proliferation

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Effector Regulatory T Cell Differentiation and Immune Homeostasis Depend on the Transcription Factor Myb

Sheila Dias,^{1,2} Angela D'Amico,¹ Erika Cretney,^{1,2} Yang Liao,^{1,2} Julie Tellier,^{1,2} Christine Bruggeman,^{1,5} Francisca F. Almeida,^{1,2} Jamie Leahy,¹ Gabrielle T. Belz,^{1,2} Gordon K. Smyth,^{1,3} Wei Shi,^{1,4} and Stephen L. Nutt^{1,2,6,*}

¹Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, VIC 3052, Australia

²Department of Medical Biology, University of Melbourne, Parkville, VIC 3010, Australia

³Department of Mathematics and Statistics, University of Melbourne, Parkville, VIC 3010, Australia

⁴Department of Computing and Information Systems, University of Melbourne, Parkville, VIC 3010, Australia

⁵Present address: Department of Blood Cell Research, Sanquin Research, 1006 AN Amsterdam, the Netherlands

⁶Lead Contact

*Correspondence: nutt@wehi.edu.au

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SUMMARY

FoxP3-expressing regulatory T (Treg) cells are essential for maintaining immune homeostasis. Activated Treg cells undergo further differentiation into an effector state that highly expresses genes critical for Treg cell function, although how this process is coordinated on a transcriptional level is poorly understood. Here, we demonstrate that mice lacking the transcription factor Myb in Treg cells succumbed to a multi-organ inflammatory disease. Myb was specifically expressed in, and required for the differentiation of, thymus-derived effector Treg cells. The combination of transcriptome and genomic footprint analyses revealed that Myb directly regulated a large proportion of the gene expression specific to effector Treg cells, identifying Myb as a critical component of the gene regulatory network controlling effector Treg cell differentiation and function.

INTRODUCTION

Regulatory T (Treg) cells are vital to the preservation of immune tolerance and prevention of exacerbated immune responses to foreign antigens. Treg cells also promote therapeutic tolerance to transplanted organs and can influence the ability of tumor cells or certain pathogens to escape immune surveillance by dampening the immune response (Josefowicz et al., 2012a). The transcription factor FoxP3 is required for the generation, identity, and suppressive function of CD4⁺ Treg cells, and loss-of-function mutations in its encoding gene are sufficient to trigger fatal systemic autoimmunity in both mice and humans (Rudensky, 2011). Referred to as thymus-derived Treg (tTreg) cells, the majority of Treg cells are generated in the thymus and are exported to the periphery as FoxP3-expressing cells with suppressive capacity. FoxP3 expression can also be induced in peripheral CD4⁺ T cells, and these peripheral Treg (pTreg) cells are, at least in ho-

meostatic conditions, distinguished from tTreg cells by the lack of surface neuropilin-1 (Nrp1) (Weiss et al., 2012) and the transcription factor Helios (Thornton et al., 2010). tTreg cells and pTreg cells are thought to play complementary roles in the regulation of the immune system (Haribhai et al., 2011; Josefowicz et al., 2012b). Naive Treg cells can undergo further differentiation in response to antigen (Levine et al., 2014; Vahl et al., 2014) and a variety of environmental cues, resulting in acquisition of distinct phenotypes and effector molecules (Cretney et al., 2013).

The proto-oncogene myeloblastosis (Myb) is a transcription factor implicated in the differentiation of multiple cell types (Greig et al., 2008). Myb acts at several stages during thymopoiesis to promote the proliferation and/or survival of specific T cell subsets (Allen et al., 1999; Bender et al., 2004; Lieu et al., 2004). Myb is required for natural killer T (NKT) cell generation and promotes the development of CD4⁺ T cells in the thymus through a GATA3-dependent mechanism (Hu et al., 2010; Maurice et al., 2007). Although Myb expression is downregulated in all mature T cells, it is transiently upregulated upon T cell receptor activation in vitro (Bich-Thuy et al., 1987). MYB is required for the proper polarization of human CD4⁺ T cells under T helper 2 (Th2) cell differentiation conditions in vitro (Nakata et al., 2010), and its expression is altered in autoimmune diseases (Gustafsson et al., 2015). Although it has been unclear whether Myb is also required for Treg cell differentiation, we reasoned that this is a likely scenario given that Myb-deficient CD4⁺CD8⁺ double-positive (DP) precursors overexpress CD25 (Lieu et al., 2004), a specific marker of Treg cells. Furthermore, in silico approaches have identified Myb binding sites as the most significantly enriched DNA sequences in the proximity of Treg-cell-specific regulatory regions (Morikawa et al., 2014), and MYB has also been identified in a screen for transcription factor binding sites that co-localize with FoxP3 binding sites in human Treg cells (Sadlon et al., 2010).

Here, we show that Myb is dispensable for Treg cell differentiation in the thymus but required for tTreg cell homeostasis in the periphery. In contrast, pTreg cells neither expressed nor required Myb for their differentiation. Myb was induced in tTreg cells upon activation, and the deletion of Myb in Treg cells resulted in the spontaneous development of a fatal multi-organ inflammatory disease. Myb bound to the regulatory regions of

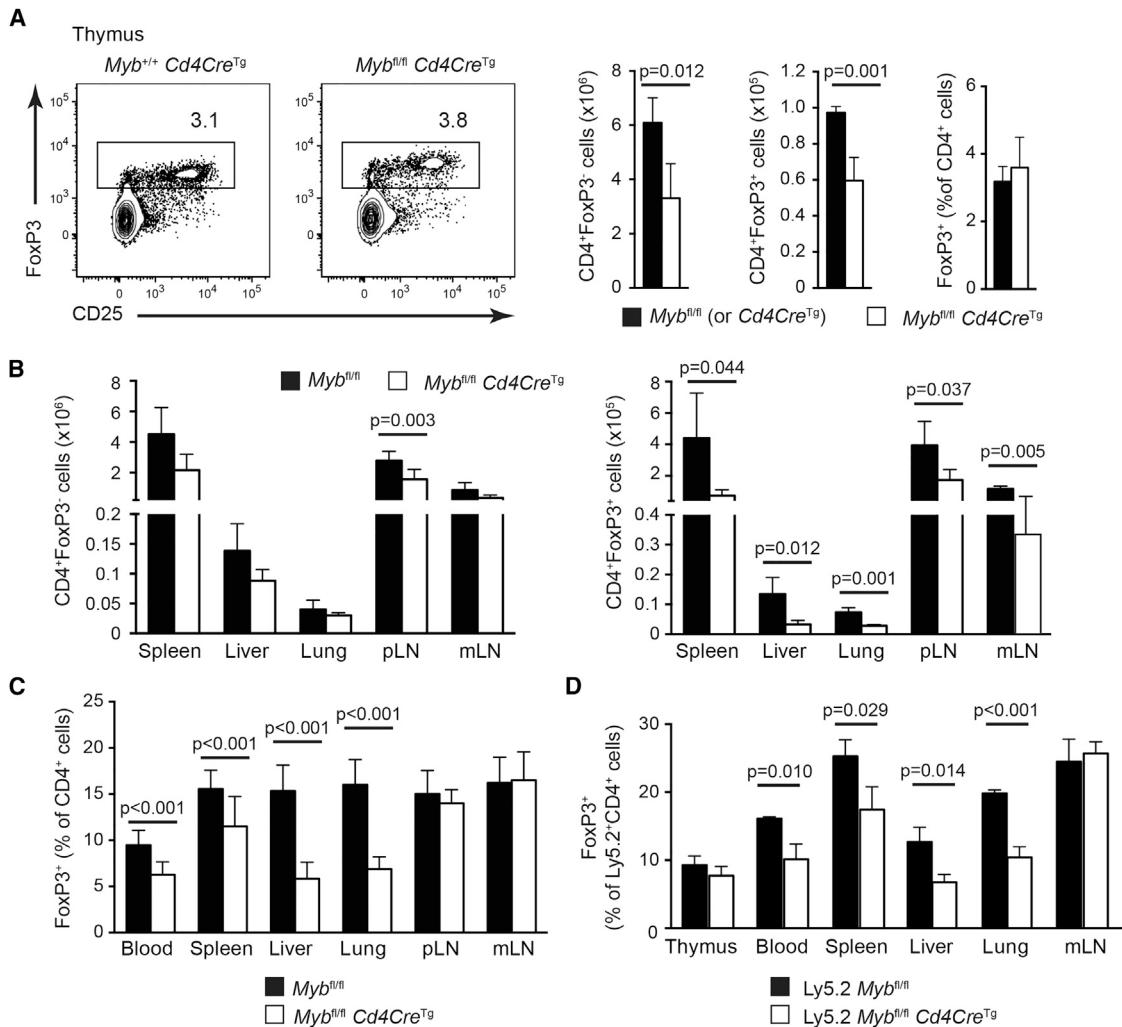


Figure 1. Reduced Proportion of Treg Cells in Peripheral Organs of *Myb^{fl/fl}Cd4cre^{Tg}* Mice

(A) Left: intracellular FoxP3 and cell-surface CD25 on CD4⁺CD8⁻ thymocytes from control *Myb^{+/+}Cd4cre^{Tg}* and *Myb^{fl/fl}Cd4cre^{Tg}* mice. Numbers indicate the proportion of gated Treg cells. Graphs show the number of CD4⁺FoxP3⁻ (left) and CD4⁺FoxP3⁺ (middle) thymocytes (data are from four littermate mice per genotype). Right: percentage of FoxP3⁺ cells among CD4⁺CD8⁻ thymocytes in control (*Myb^{fl/fl}* or *Myb^{+/+}Cd4cre^{Tg}*) and *Myb^{fl/fl}Cd4cre^{Tg}* mice (data are from eight mice per genotype and represent three individual experiments).

(B) Number of CD4⁺FoxP3⁻ (left) and CD4⁺FoxP3⁺ (right) T cells isolated from the indicated tissues. Data are from four littermate mice per genotype. pLN, peripheral lymph node; mLN, mesenteric lymph node.

(C) Percentage of FoxP3⁺ Treg cells among CD4⁺ T cells isolated from the indicated tissues. 5–20 mice were analyzed per organ per genotype and pooled from six individual experiments.

(D) Percentage of FoxP3⁺ Treg cells among Ly5.2⁺CD4⁺ T cells in 50:50 mixed BM chimeras of Ly5.1 and either Ly5.2 WT *Myb^{fl/fl}* or Ly5.2 *Myb^{fl/fl}Cd4cre^{Tg}* cells. Data are from three chimeras and represent three independent experiments.

Bar graphs depict the mean ± SD; p values compare the indicated samples.

many genes important in Treg cell biology and was required for optimal proliferation of effector Treg cells. Our findings demonstrate that Myb activity is deeply embedded in the transcriptional program of effector Treg cell differentiation.

RESULTS

Myb Controls the Size of the Treg Cell Compartment

To assess the importance of Myb in the biology of FoxP3⁺ Treg cells, we analyzed mice with a conditional *Myb* deletion (Eman-bokus et al., 2003) induced by the transgene *Cd4cre*, which

mediates deletion at the DP stage and before Treg cell lineage specification. In agreement with previous studies, we found reduced frequency of CD4⁺ T cells and ectopic expression of CD25 in DP thymocytes from *Myb^{fl/fl}Cd4cre^{Tg}* mice (Figure 1A and data not shown). *Myb* deletion did not alter the proportion of FoxP3⁺ Treg cells among thymus CD4⁺ T cells, given that Treg cell numbers were reduced proportionally to CD4⁺ thymocytes without Myb (Figure 1A). A different scenario emerged outside the thymus, where *Myb*-deficient Treg cells were under-represented both in absolute cell number and as a proportion of CD4⁺ T cells in the circulation, spleen, liver, and lung,

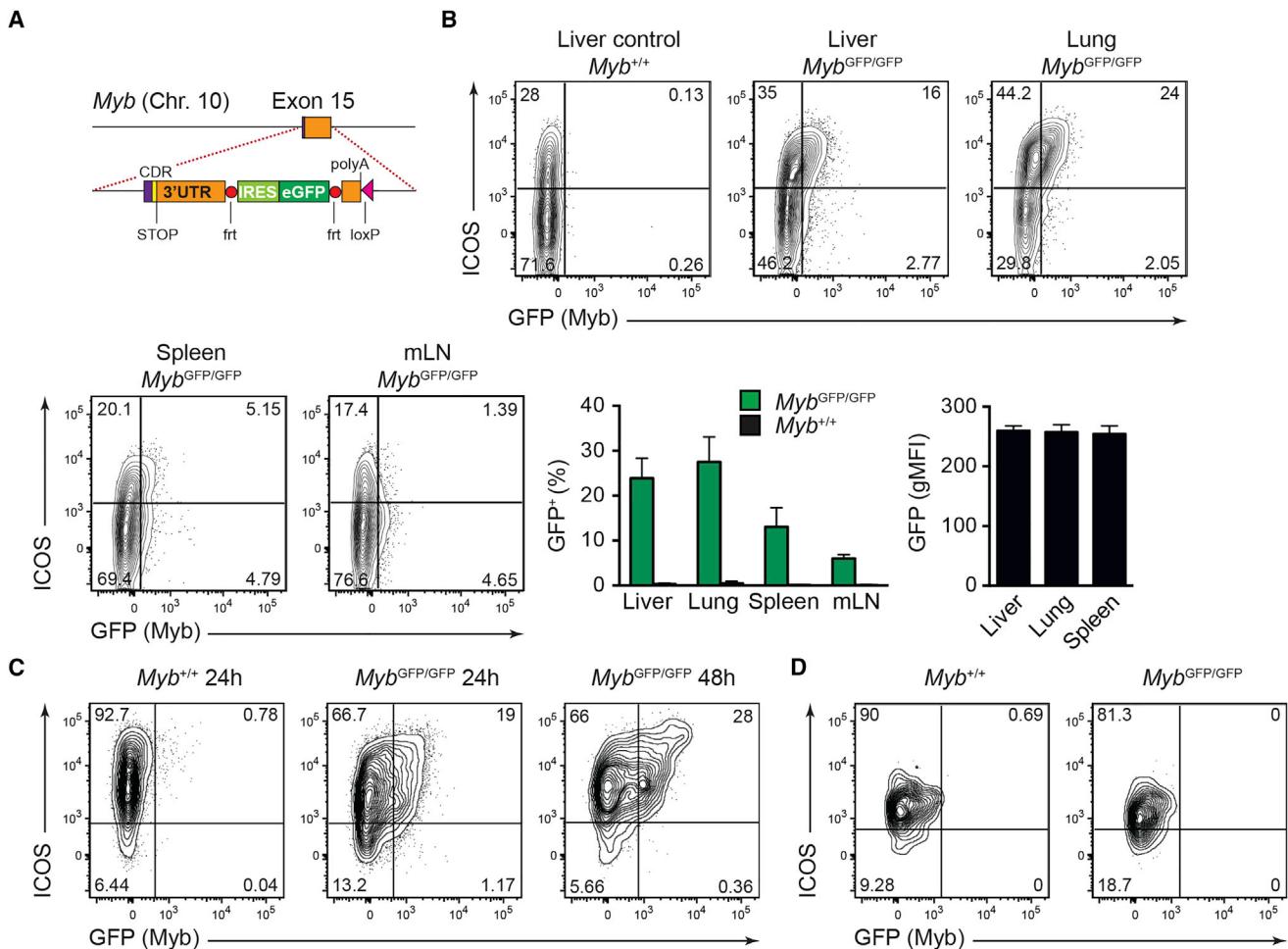


Figure 2. Myb Is Expressed in ICOS⁺ Treg Cells

(A) Schematic representation of the *Myb*^{GFP} reporter strain. An internal ribosome entry site (IRES)-eGFP cassette was introduced into the *Myb* 3' UTR by homologous recombination in embryonic stem cells, leaving the *Myb* coding region (CDR) and known microRNA binding sites intact.

(B) GFP and ICOS surface expression in CD4⁺RFP⁺ Treg cells isolated from the indicated organs of adult *Myb*^{GFP/GFP}*Foxp3*^{RFP/RFP} mice. Data represent eight mice analyzed in three independent experiments. Bar graphs summarize the data obtained in the experiment shown ($n = 3$ for each genotype). Lower middle: mean percentage of RFP⁺GFP⁺ cells \pm SD. Lower right: mean gMFI (geometric mean fluorescence intensity) of GFP \pm SD from RFP⁺GFP⁺ Treg cells. *Myb*^{+/+}*Foxp3*^{RFP/RFP} mice were analyzed as controls, and background fluorescence in the liver is shown for comparison. mLN, mesenteric lymph node.

(C) GFP and ICOS expression in CD4⁺RFP⁺ Treg cells isolated from the spleen and LNs of *Myb*^{+/+}*Foxp3*^{RFP/RFP} or *Myb*^{GFP/GFP}*Foxp3*^{RFP/RFP} mice and stimulated for 24 or 48 hr with anti-CD3 and anti-CD28 in the presence of IL-2. Plots represent three wells in each of two independent experiments.

(D) GFP and ICOS expression in RFP⁺ iTreg cells differentiated from naive CD4⁺RFP⁻ *Myb*^{+/+}*Foxp3*^{RFP/RFP} or *Myb*^{GFP/GFP}*Foxp3*^{RFP/RFP} T cells in vitro for 4 days. Plots represent two independent experiments.

Numbers in (B)–(D) indicate the proportion of cells in each quadrant. See also Figure S1.

but not in the lymph nodes (LN; Figures 1B and 1C). These results indicate that Myb activity was necessary for maintaining the normal frequency of Treg cells. We then generated mixed bone marrow (BM) chimeras of Ly5.1 wild-type (WT) and either Ly5.2 WT (*Myb*^{fl/fl}) or Ly5.2 *Myb*^{fl/fl}*Cd4cre*^{Tg} cells and found the same pattern of under-representation among Ly5.2⁺ Myb-deficient Treg cells (Figure 1D), showing that Myb maintained the normal frequency of Treg cells in a cell-intrinsic manner.

Myb Is Upregulated in ICOS-Expressing Treg Cells

To understand how Myb regulates Treg cell homeostasis, we analyzed the pattern of *Myb* expression in vivo. We engineered

the *Myb* locus to synthesize GFP under the control of the endogenous *Myb* regulatory elements (*Myb*^{GFP}), allowing for the quantitative analysis of *Myb* transcription in single cells (Figure 2A). Importantly, the previously described miR150 binding sites in the 3' UTR were left intact, allowing for the regulation of *Myb* by this microRNA (Xiao et al., 2007). Homozygous *Myb*^{GFP} mice were viable and healthy and had normal frequencies of B and T lymphocytes (data not shown). As predicted from earlier studies, *Myb*^{GFP} was highly and uniformly expressed in populations enriched with hematopoietic stem cells and common lymphoid progenitors (Greig et al., 2008), as well as CD4⁺ thymocytes (Figures S1A and S1B). We then crossed our *Myb*^{GFP} line with mice carrying a *Foxp3*^{RFP} allele

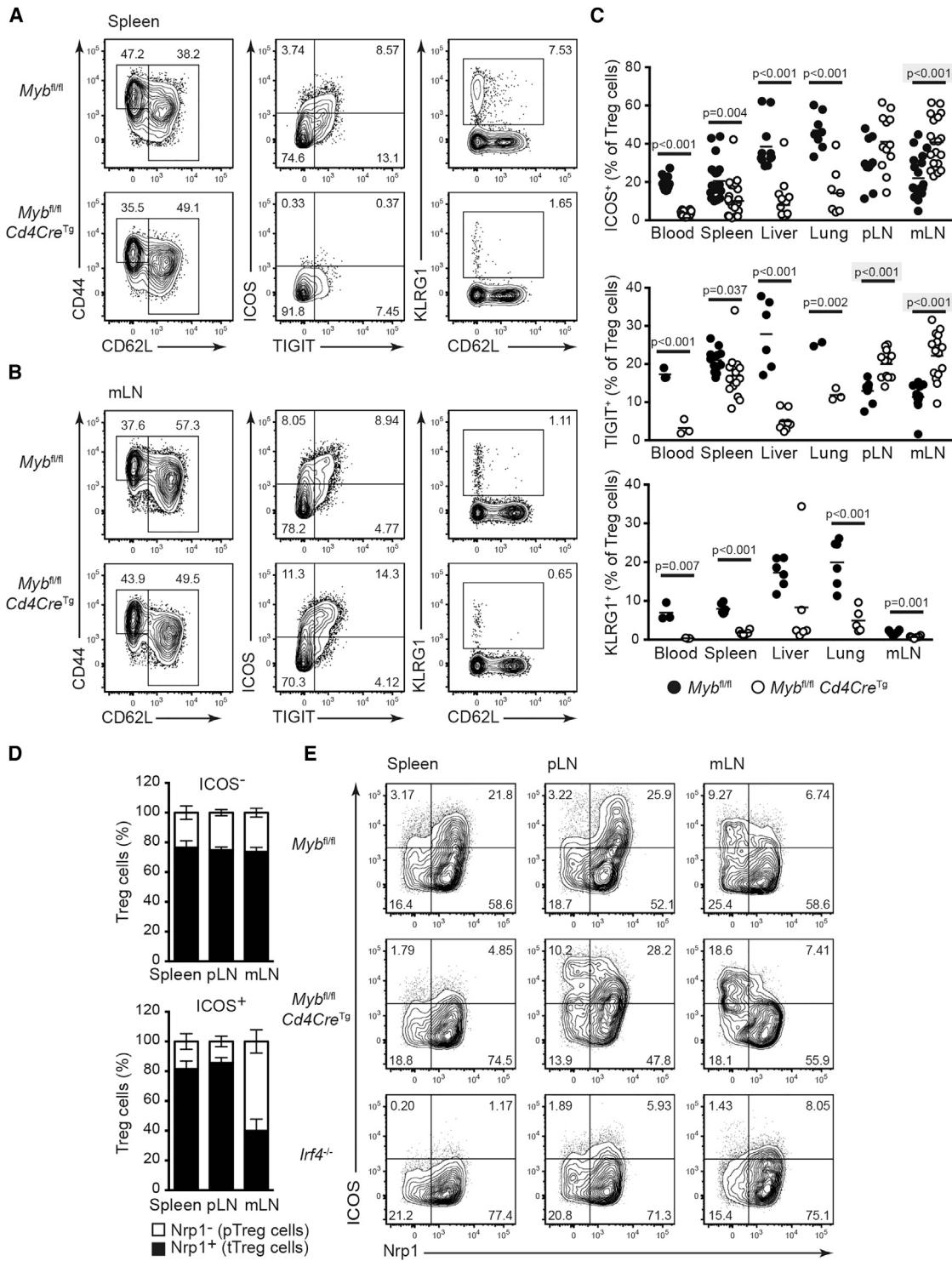


Figure 3. Myb-Deficient Treg Cells Lack Multiple Activation Markers and Effector Molecules

(A and B) Flow cytometric analysis of CD4⁺FoxP3⁺ Treg cells isolated from (A) spleen or (B) mesenteric lymph nodes (mLN) of *Myb*^{fl/fl} and *Myb*^{fl/fl}*Cd4cre*^{Tg} mice shows the expression of CD44, CD62L, ICOS, TIGIT, and KLRG1. Plots represent 3–13 mice analyzed over four independent experiments.

(C) Percentage of ICOS⁺ (top), TIGIT⁺ (middle), or KLRG1⁺ (bottom) cells among CD4⁺FoxP3⁺ Treg cells from adult *Myb*^{fl/fl} and *Myb*^{fl/fl}*Cd4cre*^{Tg} mice. Each dot represents an individual mouse pooled from four to six individual experiments; horizontal bars show the mean. p values compare the indicated samples.

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that allows the identification of Foxp3⁺ Treg cells and found that *Myb* was highly expressed in newly generated thymic FoxP3⁺Nrp1⁻ Treg cells and then downregulated in FoxP3⁺ Nrp1⁺ Treg cells in the thymus and periphery (Figures 2B and S1B–S1D). *Myb*^{GFP} expression was subsequently induced in Treg cells with high expression of ICOS, a marker of effector Treg cells (Herman et al., 2004; Ito et al., 2008), most prominently in the liver and lung (Figures 2B and S2D). Furthermore, Treg cells stimulated in vitro with anti-CD3 and anti-CD28 in the presence of interleukin-2 (IL-2) upregulated ICOS and *Myb*^{GFP} expression (Figure 2C). In contrast, *Myb*^{GFP} was not expressed in in-vitro-polarized induced Treg (iTreg) cells (Figure 2D). Thus, *Myb*^{GFP} is specifically expressed in ICOS⁺ effector Treg cells generated in vivo.

Myb-Deficient Treg Cells Lack Multiple Effector Molecules

The highly restricted expression of *Myb* to ICOS⁺ effector Treg cells raises the possibility that *Myb* activity is specifically required for the differentiation of these cells. Indeed, we found a reduced proportion of ICOS⁺ Treg cells in the absence of *Myb* in a variety of organs, with the notable exception of the LNs (Figures 3A–3C and S2A–S2C). The loss of ICOS expression in Treg cells in the absence of *Myb* could be due to the requirement for *Myb* for the transcription of the *Icos* locus or reflect the reduced representation of the effector Treg cell subset. To distinguish between these possibilities, we analyzed the expression of additional effector molecules. We found that *Myb*-deficient Treg cells were able to downregulate CD62L and upregulate CD44 and therefore achieve an activated state (Figures 3A, 3B, S2A, and S2B). Surface expression of the immune receptor TIGIT in Treg cells correlates with their effector functions (Joller et al., 2014), and KLRG1-expressing Treg cells are a subset of short-lived terminally differentiated cells with an effector signature (Cheng et al., 2012). We found that the proportion of both TIGIT⁺ and KLRG1⁺ Treg cells was greatly reduced in the absence of *Myb* in a pattern similar to that of ICOS (Figures 3A–3C, S2A, and S2B), suggesting that *Myb* is required for the appearance of effector Treg cells.

Myb Is Required for Thymic, but Not Peripheral, Treg Cells

To ascertain why *Myb* inactivation affected Treg cells differently between the LNs and all other peripheral locations analyzed, we sought to identify any distinguishing features of Treg cells from these locations. We found that in WT mice, most ICOS⁺ Treg cells in the spleen, peripheral LNs (pLNs), liver, and lung expressed Nrp1 (Figures 3D–3E and S2C). By contrast, only ~40% of ICOS⁺ Treg cells in the mesenteric LNs (mLNs) expressed Nrp1 (Figures 3D and 3E). These results suggest that, under steady-state conditions, most ICOS⁺ Treg cells in the spleen and pLNs are iTreg cells, whereas

in the mLNs they are predominantly pTreg cells (Weiss et al., 2012).

A different picture emerged when we analyzed Nrp1 expression in *Myb*^{f/f}*Cd4cre*^{Tg} mice, where we found a prominent contribution of Nrp1⁻ cells to the ICOS⁺ pool in the LNs, but not in the spleen, liver, or lung (Figures 3E and S2C). Expression of Helios, an independent marker of tTreg cells (Thornton et al., 2010), followed the same trend (Figures S2D–S2F). Thus, we conclude that the proportion of ICOS⁺ Treg cells is maintained in the pLNs (and even increased in the mLNs) of *Myb*^{f/f}*Cd4cre*^{Tg} mice because of an increased contribution of pTreg cells. In contrast to *Myb* deficiency, the lack of IRF4 compromised the development of both Nrp1⁻ and Nrp1⁺ ICOS⁺ Treg cells (Figure 3E). These results indicate that *Myb* is required for the emergence of ICOS⁺ tTreg cells, but not ICOS⁺ pTreg cells. In keeping with this conclusion, *Myb* was dispensable for the in vitro generation and proliferation of iTreg cells and the in vivo generation of pTreg cells in adoptive-transfer assays (Figures S2G and S2H). Taken together, our results indicate that *Myb* activity is essential for the emergence of tTreg cells expressing high amounts of the effector molecules ICOS, TIGIT, and KLRG1 but is dispensable for pTreg cell homeostasis.

Myb Deletion in Treg Cells Results in Fatal Immune Pathology

Despite the low numbers of effector tTreg cells, *Myb*^{f/f}*Cd4cre*^{Tg} mice showed no signs of clinical disease. Because previous work has suggested that *Myb* also plays a role in mature conventional T cells (Lieu et al., 2004; Nakata et al., 2010) and that *Myb* deficiency ablates the NKT cell population (Hu et al., 2010), it is possible that the pathological manifestations of Treg-cell-intrinsic defects in *Myb*^{f/f}*Cd4cre*^{Tg} mice were being masked by defects in other cell types. To ascertain the physiological relevance of *Myb* activity in Treg cells, we generated *Myb*^{f/f}*Foxp3*^{YFP-cre} mice, where *Myb* was specifically deleted in Treg cells, simultaneously traceable by YFP. These mice spontaneously developed a multi-organ inflammatory disease with early onset and lethality by ~6 months of age (Figure 4A). *Myb*^{f/f}*Foxp3*^{YFP-cre} mice failed to gain weight and showed an enlarged spleen, neutrophilia, and chronic anemia (Figures 4B, 4C, and S3A–S3C). We confirmed *Myb* deletion in all Treg cells, including ICOS⁺ (Nrp1⁻ enriched) Treg cells isolated from mLNs (Figure S3D). In the absence of *Myb* in Treg cells, a higher proportion of FoxP3⁻CD4⁺ and CD8⁺ T cells produced interferon-γ (IFN-γ) (Figure 4D), but not tumor necrosis factor (TNF) or IL-17a (data not shown), whereas FoxP3⁺ Treg cells characteristically produced very little of any of these cytokines (Figure S3E). Although Foxp3⁺ Treg cells were readily detected in *Myb*^{f/f}*Foxp3*^{YFP-cre} mice (data not shown), inflammation and leukocyte infiltration were evident in several non-lymphoid organs, including but not limited to the liver, lung, and gut (Figures 4E and S3A). All together, these

(D) Proportion of ICOS⁻ (top) or ICOS⁺ (bottom) CD4⁺FoxP3⁺ Treg cells expressing (black bar) or lacking (white bar) Nrp1 in adult WT mice. Seven to ten mice were analyzed and pooled from at least three individual experiments. Bar graphs depict the mean ± SD.

(E) Flow cytometric analysis of CD4⁺FoxP3⁺ Treg cells isolated from the spleen, pLNs, or mLNs of adult *Myb*^{f/f}, *Myb*^{f/f}*Cd4cre*^{Tg}, or *Irif4*^{-/-} mice shows differential expression of Nrp1 and ICOS. Plots represent two *Irif4*^{-/-} and 11–18 *Myb* mice analyzed over eight independent experiments.

Numbers in (A), (B), and (E) indicate the proportion of cells in each box or quadrant. See also Figure S2.

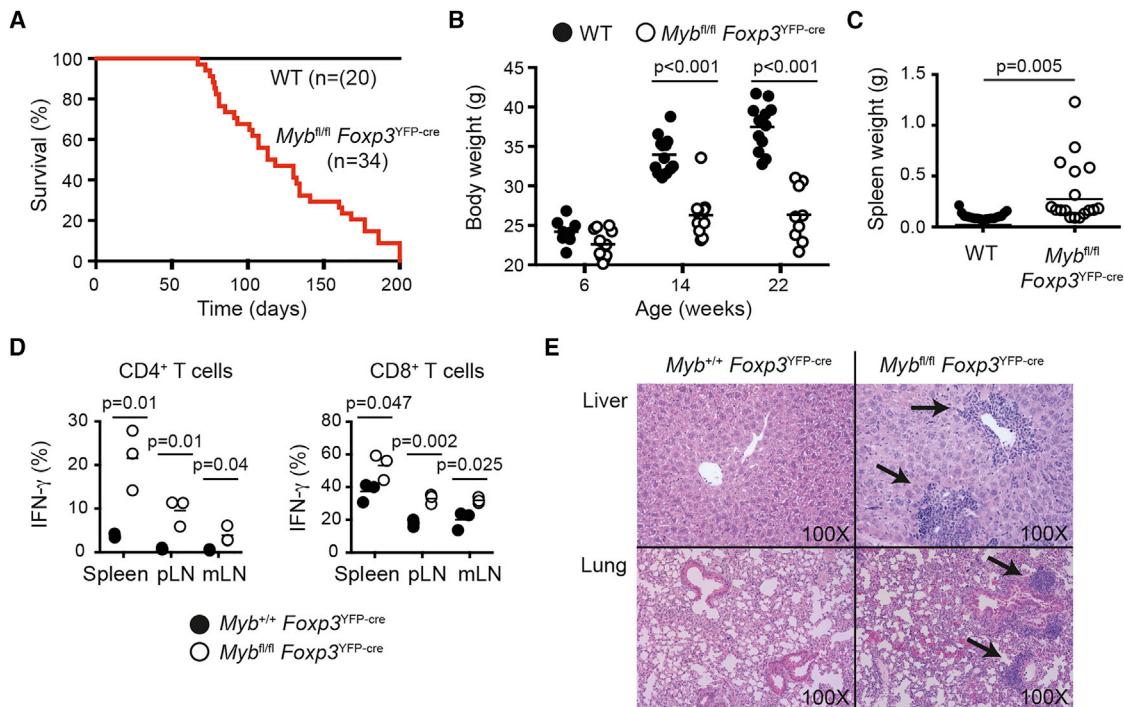


Figure 4. Fatal Autoimmune Pathology in Mice Lacking Myb in Treg Cells

(A) Survival curve of *Myb^{fl/fl} Foxp3^{YFP-cre}* mice (n = 34, red line) and WT control mice (n = 20, black line); p < 0.0001.

(B) Body weight of *Myb^{fl/fl} Foxp3^{YFP-cre}* and WT control males at the indicated ages.

(C) Splenic weight of *Myb^{fl/fl} Foxp3^{YFP-cre}* mice (n = 20) and WT control mice (n = 19).

(D) Percentage of CD4⁺ (top) and CD8⁺ (bottom) T cells isolated from *Myb^{+/+} Foxp3^{YFP-cre}* or clinically ill *Myb^{fl/fl} Foxp3^{YFP-cre}* mice that expressed IFN- γ after in vitro stimulation with PMA and ionomycin for 4 hr.

(E) H&E staining of tissue sections from the liver and lung of *Myb^{+/+} Foxp3^{YFP-cre}* and *Myb^{fl/fl} Foxp3^{YFP-cre}* mice (magnification 100 \times). Arrows indicate examples of immune infiltrates in the liver and lung of *Myb^{fl/fl} Foxp3^{YFP-cre}* mice.

In (B)–(D), each dot represents an individual mouse, and horizontal bars show the mean. p values compare the indicated samples. See also Figure S3.

observations demonstrate that Myb is essential for Treg cell function in vivo.

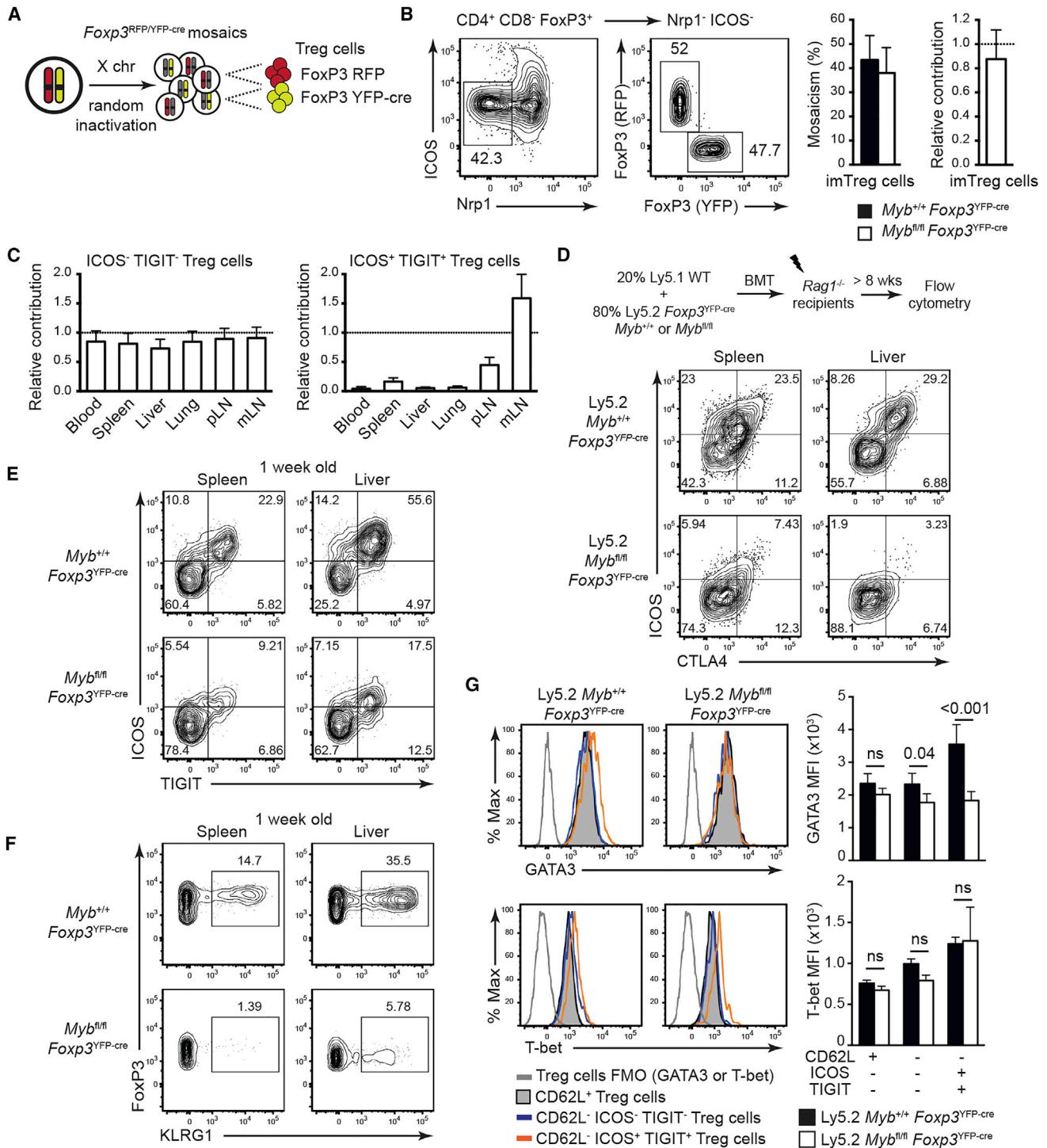
Myb Is Required for the Generation of Effector tTreg cells

To better characterize the cell-intrinsic defects caused by Myb deletion specifically in Treg cells, we generated female Treg cell mosaics by crossing *Myb^{fl/fl} Foxp3^{YFP-cre}* males with *Myb^{fl/fl} Foxp3^{RFP/RFP}* females. As a result of random inactivation of the X chromosome, all F1 females (*Myb^{fl/fl} Foxp3^{YFP-cre/RFP}*) deleted Myb in their YFP-cre-expressing Treg cells and were protected from disease by the presence of Myb-sufficient RFP⁺ Treg cells (Figure 5A). To ensure that all defects identified were due to the lack of Myb rather than deleterious effects of cre activity, we compared *Myb^{fl/fl} Foxp3^{YFP-cre/RFP}* with *Myb^{+/+} Foxp3^{YFP-cre/RFP}* females. We calculated the relative contribution of YFP⁺ Treg cells and WT RFP⁺ Treg cells. In the thymus, Myb-deficient and sufficient immature (Nrp⁻) Treg cells were equally represented (Figure 5B). Similarly, we found equal contributions of both genotypes among Treg cells lacking ICOS and TIGIT in all peripheral organs analyzed (Figures 5C and S4A). In contrast, Myb-deficient Treg cells were barely detectable among ICOS⁺ TIGIT⁺ and KLRG1⁺ Treg cells (Figures 5C and S4A). Also consistent with our previous analysis was the differential effect in the

LNs, compatible with an increased contribution of Nrp1⁻ pTreg cells in these organs (Figure 5C).

To confirm that Myb loss impaired the generation of effector Treg cells, we analyzed the expression of additional surface markers that have a differential pattern in these cells. CD103 and CXCR3 were enriched among ICOS⁺ TIGIT⁺ WT Treg cells (Figure S4C). However, their pattern of expression in the Myb-deficient Treg cells mirrored that of the ICOS⁻ TIGIT⁻ WT subset, confirming the absence of the effector Treg cell population. Furthermore, whereas completely Myb-deficient Treg cells showed no impaired capacity to suppress the proliferation of CD4⁺ T cells in vitro (Figure S4B), analysis of mixed BM chimeras (20% Ly5.1⁺ WT; 80% *Myb^{fl/fl} Foxp3^{YFP-cre}* or *Myb^{+/+} Foxp3^{YFP-cre}* [both Ly5.2⁺]) showed that Myb-deficient Treg cells were deficient in CTLA4 (Figure 5D), known to be essential for the suppressive capacity of Treg cells in vivo (Wing et al., 2008).

It was recently shown that the initial wave of Treg cells, generated within the first 10 days after birth, have functional properties distinct from those of adult Treg cells (Yang et al., 2015). We found that 1-week-old *Myb^{fl/fl} Foxp3^{YFP-cre}* mice also had markedly impaired generation of ICOS⁺ TIGIT⁺ and KLRG1⁺ Treg cells (Figures 5E, 5F, and S4D), suggesting that Myb is essential for the establishment of a tolerogenic environment in neonates. All together, these results support the conclusion

**Figure 5. Myb Is Required for the Generation of Effector tTreg Cells**(A) Schematic representation of FoxP3-reporter mosaicism in *Foxp3^{YFP-cre/RFP}* females.(B) Contour plots indicate the gating of ICOS and Nrp1 surface expression in thymic Treg cells and the percentage of RFP⁺ and YFP⁺ Treg cells among the most immature (im) Nrp1⁻ ICOS⁻ subset. The graph shows the mean percentage \pm SD of YFP⁺ cells among Nrp1⁻ ICOS⁻ thymic Treg cells in *Myb^{+/+} Foxp3^{YFP-cre/RFP}* ($n = 13$) or *Myb^{fl/fl} Foxp3^{YFP-cre/RFP}* ($n = 7$) females. The relative contribution was calculated as the ratio of mosaicism in *Myb^{fl/fl} Foxp3^{YFP-cre/RFP}* females to that in *Myb^{+/+} Foxp3^{YFP-cre/RFP}* females (set at 1, dotted line).(C) Mean relative contribution \pm SD of Myb-deficient cells, calculated as in (B), to the ICOS⁻ TIGIT⁻ or ICOS⁺ TIGIT⁺ subsets of Treg cells in different organs. Data derive from 7 *Myb^{+/+} Foxp3^{YFP-cre/RFP}* and 13 *Myb^{fl/fl} Foxp3^{YFP-cre/RFP}* mice. pLN, peripheral lymph node; mLN, mesenteric lymph node.

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that Myb is required for the efficient generation of effector tTreg cells throughout life.

The requirement for Myb in the generation of effector Treg cells was not paralleled in other T cells. For example, Myb was dispensable for the generation of antigen-specific (NP⁺) CD8⁺ T cells in response to influenza infection, and a proportion of these cells expressed KLRG1, consistent with an effector phenotype (Figures S5A and S5B). Follicular T helper (Tfh) cells were also detected in the mediastinal LNs of both WT and Myb-deficient influenza-infected mice (Figures S5C and S5D). In vitro polarization and proliferation of CD4⁺ T cells in Th1, Th2, and Th17 cell differentiation conditions were also unaffected by Myb loss (Figure S5E). These results indicate that Myb is dispensable for the generation of effector CD8⁺ and CD4⁺ T cell subsets, at least in response to acute viral infection, although whether these effector populations are fully functional remains to be experimentally determined.

GATA3 Induction in Effector Treg Cells Depends on Myb

GATA3 is induced when Treg cells become activated and is required for Treg cell suppressor function, although possibly only under specific physiological circumstances (Wang et al., 2011; Wohlfert et al., 2011; Yu et al., 2015). The transcription factor T-bet is also expressed in a subset of Treg cells with an activated phenotype and is important for the maintenance of Treg cell homeostasis (Koch et al., 2009). We confirmed that GATA3 was upregulated in ICOS⁺TIGIT⁺ Myb^{+/+}Foxp3^{YFP-cre} effector Treg cells, but the few ICOS⁺TIGIT⁺ Myb-deficient Treg cells in the chimeric mice failed to modulate GATA3 expression (Figure 5G). In contrast, Myb-deficient ICOS⁺TIGIT⁺ Treg cells and their WT ICOS⁺TIGIT⁺ counterparts had similar amounts of T-bet (Figure 5G). Our results show that, unlike T-bet expression, increased expression of GATA3 in effector Treg cells is dependent on Myb.

Genome-wide Analysis of Myb-Deficient Treg Cells

To uncover Myb-dependent genes relevant in Treg cells, we performed a global analysis of gene expression by RNA sequencing (RNA-seq). We compared the transcriptome of CD62L⁺ICOS⁻TIGIT⁻ (“naive” phenotype), CD62L⁻ICOS⁻TIGIT⁻ (“activated” phenotype, defined by downregulation of CD62L but a lack of surface effector molecules), and CD62L⁻ICOS⁺TIGIT⁺ (“effector” phenotype) Treg cells isolated from healthy female Myb^{+/+}Foxp3^{YFP-Cre/YFP-cre} WT or Myb^{f/f} Foxp3^{YFP-cre/+} mice; Myb-deficient CD62L⁻ICOS⁺TIGIT⁺ Treg cells were excluded because of their paucity (Figures S6A and S6B). Exons 3–6 of Myb were efficiently removed in the naive and activated populations derived from Myb^{f/f} Foxp3^{YFP-cre/+} mice (Figure S6C). Pairwise comparisons among all three WT subpopulations revealed 4,107 differentially expressed (DE) genes, the vast majority (94%) of which were expressed at an

intermediate rate in activated Treg cells (Figure 6A). It was also clear that more genes differed, and to a higher degree, between effector and naive Treg cells than between activated and naive Treg cells or between activated and effector subpopulations, suggesting a hierarchical relationship among naive, activated, and effector Treg cells (Figure 6B).

To investigate the extent to which Myb directly regulates gene expression in Treg cells, we applied a procedure termed digital genomic footprinting, developed by the ENCODE Consortium (Neph et al., 2012) and recently used to map MYB binding sites in human blood cell types (Bengtzen et al., 2015). We utilized two existing genome-wide DNase I hypersensitivity (DHS) datasets encompassing total splenic Treg cells (termed steady-state Treg cells) and in-vivo-activated Treg cells that result from the inflammatory environment elicited by the transient depletion of Treg cells (Arvey et al., 2014; ENCODE Project Consortium, 2012). The DNase I genomic footprints were downloaded from ENCODE and used for de novo discovery of Myb-binding motifs. Myb digital genomic footprints (DGFs) were assigned to genes if they occurred within 20 kb upstream of the transcriptional start site and 5 kb downstream of the 3' UTR. Within these criteria, multiple gene assignments for individual Myb DGPs were allowed. On the basis of our analysis of the Myb-deficient phenotype, we expected that Myb would bind considerably more genes in the activated dataset than in the steady-state situation, where only a small proportion of Treg cells express Myb (Figure 2B). Indeed, we found 2.6-fold more genes with Myb DGPs in activated Treg cells (6,966 genes) than in steady-state Treg cells (2,707 genes; Figure 6C). Genome-wide analysis of the average DNase I cleavage sites in Treg cells showed a footprint centered on the Myb motif (Figure 6D), and 66% of Myb DGPs in steady-state Treg cells were also found in the in-vivo-activated Treg cell dataset (Figure 6E).

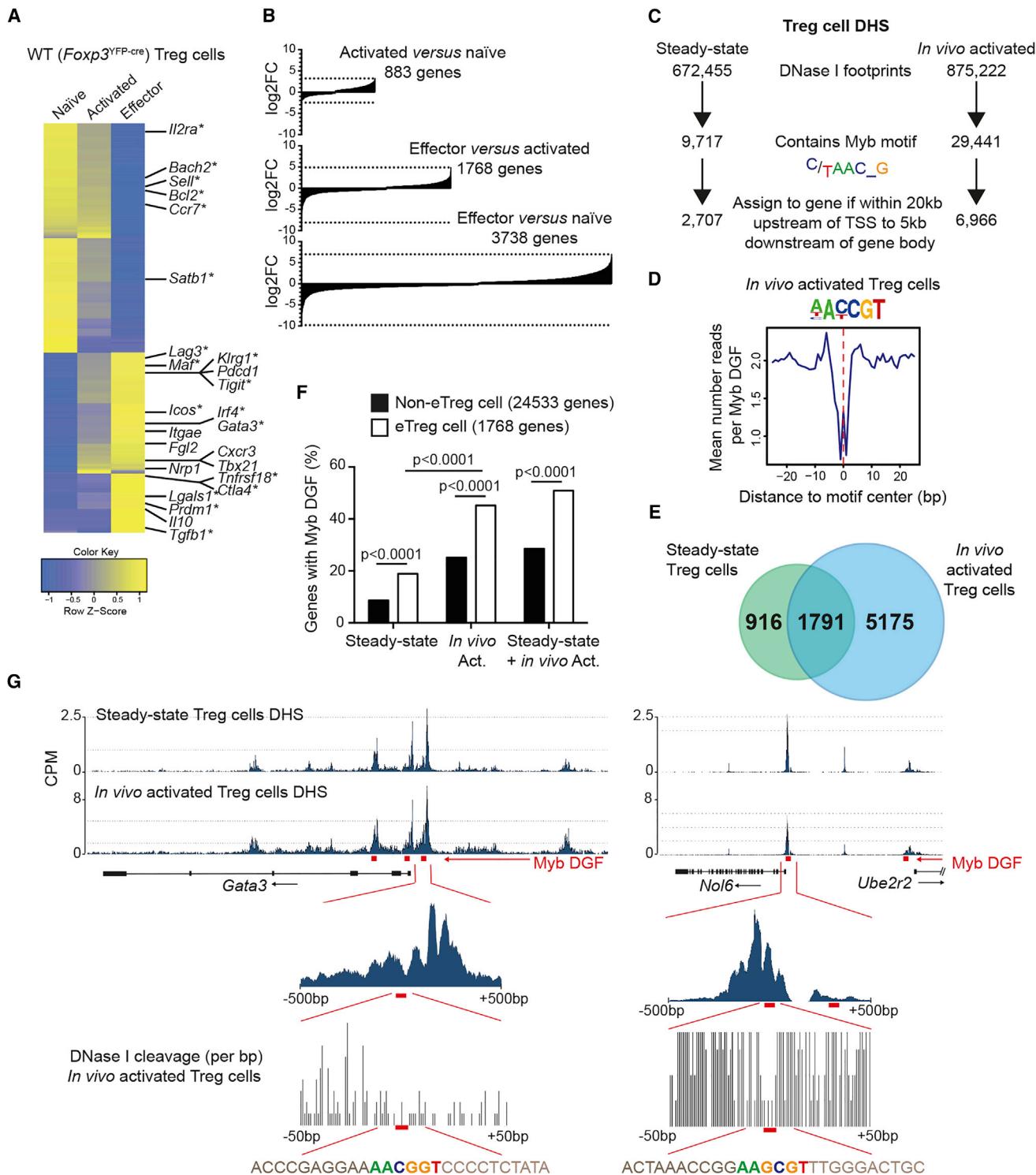
Analysis of the 1,768 genes that were DE between effector and activated Treg cells, herein referred to the effector Treg cell profile, showed that these genes were much more enriched in Myb DGPs than in the rest of the genome (Figure 6F). More than 50% of the effector Treg cell genes had Myb DGPs (Figures 6F, 6G, and S7F and Table S1). This list contained many genes with known functions in effector Treg cells, including the transcriptional regulators Gata3, Bach2, Satb1, Irf4, Maf, and Prdm1 and the effector molecules Ctl4a, Klr1, Icos, Lag3, and Lgals1 (Figure 6A and Table S1). Moreover, for a large proportion of these effector Treg cell genes, chromatin immunoprecipitation (ChIP)-identified Myb binding sites in human T-ALL cell lines correlate with the active chromatin modification H3K27Ac and DHS regions from human Treg cells (examples in Figure S6D; ENCODE Project Consortium, 2012; Mansour et al., 2014), suggesting a broad and evolutionary conserved role for Myb in Treg cell differentiation.

(D) ICOS and intracellular CTLA4 in Ly5.2⁺CD4⁺FoxP3⁺ Treg cells isolated from the spleen and liver of 20:80 BM chimeras of Ly5.1 WT and Ly5.2 Myb^{f/f} Foxp3^{YFP-Cre} or Ly5.2 Myb^{+/+}Foxp3^{YFP-cre} control cells. Plots represent four mice per group.

(E and F) Expression of (E) ICOS and TIGIT and (F) KLRG1 on Treg cells from 1-week-old Myb^{+/+}Foxp3^{YFP-Cre} or Myb^{f/f} Foxp3^{YFP-cre} mice. Plots represent (E) ten or (F) six mice.

(G) Flow cytometric analysis of GATA3 and T-bet in CD62L⁺ICOS⁻TIGIT⁻, CD62L⁻ICOS⁻TIGIT⁻, or CD62L⁻ICOS⁺TIGIT⁺ Ly5.2⁺CD4⁺FoxP3⁺ splenic Treg cells from 20:80 BM chimeras as in (D). Histograms represent four mice per group. Bar graphs depict the mean fluorescence intensity (MFI) ± SD for each genotype; p values compare the indicated groups (ns, non-significant). FMO, fluorescence minus one staining.

Numbers in (B) and (D)–(F) indicate the proportion of cells in each box or quadrant. See also Figures S4 and S5.

**Figure 6. Transcriptional Profiling of Treg Cell Subpopulations**

(A) Heatmap showing all differentially expressed (DE) genes among naïve (CD62L⁺ICOS⁻TIGIT⁻), activated (CD62L⁻ICOS⁻TIGIT⁻), and effector (CD62L⁻ICOS⁺TIGIT⁺) Treg cells isolated from the spleen and pLNs of WT (*Myb*^{+/+}*Foxp3*^{YFP-Cre}) mice (described in Figures S6A and S6B). The relative expression (Z scores) of genes is shown and is color coded according to the legend. Rows are scaled to have a mean of 0 and an SD of 1, and the position of genes of interest is highlighted. Asterisks indicate genes with Myb digital genomic footprints (DGFs). Data are from two experiments.

(B) Log₂ fold change (FC) of the DE genes (false-discovery rate < 0.05; reads per kilobase of transcript per million mapped reads > 2 in at least one sample) between activated and naïve (top), effector and activated (middle), or effector and naïve (bottom) Treg cells; each bar represents one gene, and the dotted lines mark the limit FC values. Data are from two experiments.

(legend continued on next page)

The complete loss of Myb-deficient effector Treg cells precluded a direct determination of the proportion of effector Treg cell genes regulated by Myb. To overcome this limitation, we identified Myb-dependent genes in activated Treg cells, whose transcriptional trajectory appeared to be in transition between the naive and effector states (Figure 7A). The comparison between WT and Myb-deficient activated Treg cells identified 330 Myb-dependent (DE) genes, 43.3% of which belonged to the effector Treg cell profile, whereas 47% had Myb DGFs (Figures S7A–S7D and Table S1). We found a strong enrichment of Myb-dependent genes among the most regulated genes in the transition from an activated to an effector Treg cell (Figures S7A and S7B). *Lgals1*, which encodes the immune suppressor galectin-1, is shown as an example of a Myb-dependent gene that has Myb DGFs in mouse Treg cells and binding in human T-lineage cells within the effector Treg cell profile (Mansour et al., 2014; Figures S7A, S7C, and S7E). Collectively these two approaches demonstrate that Myb directly regulates the expression of a substantial fraction of the effector Treg cell program.

Myb Is Required for the Optimal Proliferation of Effector Treg Cells

We used the PANTHER classification tool of the Gene Ontology (GO) database to perform pathway analysis of the genes that contributed to the effector Treg cell profile and also contained Myb DGFs, which revealed highly significant enrichment for many genes associated with a variety of signaling, protein processing, metabolic, and protein translation pathways, reflective of the changes in metabolic activity associated with the transition between activated and effector Treg cells (Figure 7A). This analysis revealed pronounced changes in the subset of genes associated with the cell cycle and apoptosis (Figures 7A–7C), two processes that Myb has previously been implicated in (Greig et al., 2008; Lieu et al., 2004).

Myb DGFs were identified in 38 genes that were associated with the “apoptotic process” GO term and DE in effector Treg cells, including downregulation of the anti-apoptotic genes *Bcl2* and *Mcl1* and upregulation of the pro-death molecules *Bak1* and *Casp7* (Figure 7B). Although these changes suggest an increased apoptotic rate in effector Treg cells, they were balanced by a decrease in *Bcl2l11* (*Bim*) and an increase in *Bcl2l1* (*Bcl-xL*). To assess the impact of Myb deficiency on cell survival, we cultured Treg cells and conventional CD4⁺ T cells for 2 days in conditions that induce Myb expression (Figure 2C) and measured active caspase-3 as a readout for the apoptotic

rate. Myb-deficient Treg cells showed a small and statistically significant increase in apoptosis rate, although it is noteworthy that Treg cells of both genotypes were markedly less prone to apoptosis than conventional CD4⁺ T cells (Figures 7D and 7E).

54 genes associated with the “cell cycle” GO term also contained Myb DGFs and were DE in effector Treg cells, suggesting that deregulation of the cell cycle might also be a consequence of the absence of Myb (Figure 7A). *Cdk6*, *Cdc14a*, *Cdkn1a*, *E2f3*, *E2f4*, and *Myc* are examples of loci identified by this approach (Figure 7C and Table S1). On the basis of these findings, we hypothesized that Myb might be required for the proliferation of effector Treg cells. Indeed, we found that Myb-deficient Treg cells had reduced proliferation capacity in vitro (Figure 7F). Moreover, using the 20:80 BM chimeras described above, we found that only the effector Treg subset was actively proliferating, as shown by the proportion of Ki67-expressing cells (Figure 7G). Analysis of Myb-deficient ICOS⁺TIGIT⁺ Treg cells in the blood, spleen, and liver revealed a significant reduction in the proportion of proliferating cells among Myb-deficient ICOS⁺TIGIT⁺ Treg cells (Figure 7G), further supporting the conclusion that Myb activity contributes to the full proliferation potential of ICOS⁺TIGIT⁺ effector Treg cells. Collectively, these data suggest a broad function for Myb in regulating genes required for effector Treg cell differentiation, function, survival, and proliferation.

DISCUSSION

Although the importance of Treg cells for immune homeostasis has been known for two decades, it is only more recently that the diversity of differentiation options of Treg cells has become apparent. Here, we show that the conditional inactivation of the transcription factor Myb in mouse Treg cells results in multi-organ immune pathology and early lethality. Myb plays a stage-dependent role in Treg cells, whereby it is dispensable for their development in the thymus and for their maturation to a “naive” phenotype in peripheral tTreg cells but essential for their further differentiation to effector tTreg cells. In contrast, the differentiation of pTreg cells appears to be Myb independent. Genome-wide DNase I footprinting revealed that Myb bound to the regulatory regions of many effector-Treg-cell-specific genes and was required for their expression, demonstrating that Myb is a core component of the tTreg cell differentiation program.

Myb is recognized as a critical regulator of hematopoietic stem cell formation and lymphopoiesis; however, its activity in controlling the function of mature blood cells has remained poorly understood (Greig et al., 2008). Myb is relatively highly expressed

(C) Workflow for the identification of Myb DGFs. Genome-wide DHS mapping and DNase I footprints were from GEO: GSM1014200 and 1014148. Myb motifs within footprints were identified de novo and mapped to genes if they occurred within 20 kb of the transcriptional start site and 5 kb downstream of the 3' UTR. Only those Myb DGFs with >3 CPM (counts per million mapped reads) were retained.

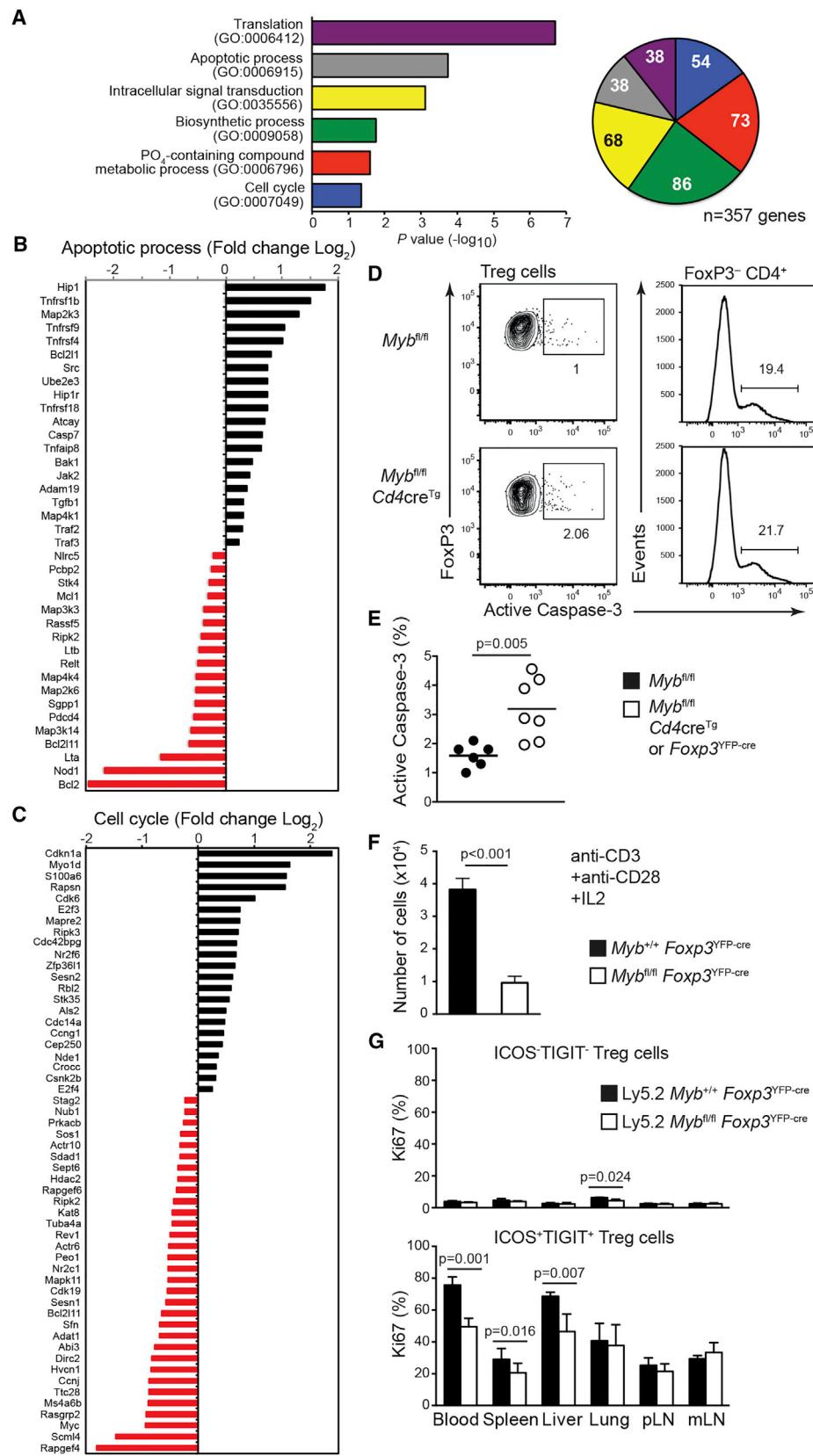
(D) Plot of the average DNase I cleavage pattern centered on the identified Myb DGF in in-vivo-activated Treg cells. The consensus Myb-binding motif identified in the dataset is shown. Base position 0 on the horizontal axis corresponds to the third base in the motif (from the left). The vertical axis shows the total number of reads for which the 5' end maps to each base position (in relation to the Myb motif center) divided by the total number of Myb DGFs.

(E) Venn diagram showing the overlap in the Myb DGFs in steady-state and in-vivo-activated Treg cells.

(F) Graph showing the proportion of the effector Treg cell profile genes and remaining non-DE genes that have Myb DGFs in either steady-state or in-vivo-activated Treg cells and both combined. p values from Chi-square tests compare the indicated samples.

(G) Representative tracks showing DHS in steady-state and in-vivo-activated Treg cells for the indicated loci. Red bars indicate Myb DGFs. Upper plots show DHS across the entire loci. Middle plots show a close up of a single DHS region containing a Myb DGF. Lower plots show the DNase I cleavage per nucleotide and the Myb consensus sequence (in color) underlying the DGF.

Please see also Figures S6 and S7 and Table S1.



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in most BM and thymic progenitors before it is downregulated in mature lymphocytes. Several studies have subsequently demonstrated that *Myb* is at least transiently re-expressed in activated T and B cells (Gustafsson et al., 2015; Nakata et al., 2010; Sadlon et al., 2010; Xiao et al., 2007); however, the importance of this re-expression has not been thoroughly addressed. The *Myb*^{GFP} reporter allele we generated confirmed the re-expression of Myb in effector Treg cells in vivo and after the stimulation of naive Treg cells in vitro. RNA-seq analysis revealed little modulation of *Myb* abundance during Treg cell differentiation, suggesting that the regulation of Myb (and GFP) abundance occurs predominantly post-transcriptionally. miR150, a well-characterized regulator of Myb abundance, is highly expressed in naive lymphocytes and repressed upon differentiation (Xiao et al., 2007) and has been shown to repress Myb, but not mRNA abundance, in colonic epithelial cells (Bian et al., 2011). Thus, we propose that activation of naive Treg cells induces proliferation and the downregulation of miR150, whose loss allows the derepression of Myb in effector Treg cells.

Our analysis of Myb DGFs from mouse Treg cells, along with RNA-seq data, revealed a large number of genes whose expression was modulated by this differentiation process and directly bound by Myb. We observed Myb binding in genes involved in effector Treg cell differentiation (*Prdm1*, *Bach2*, *Batf*, and *Gata3*), localization (*Itgae* and *Ccr7*), and function (*Icos*, *Lgals1*, and *Ctla4*) in mice, and this was also evident in human T cell lines. *Gata3*, a previously characterized target in thymocytes (Maurice et al., 2007), was also directly regulated by Myb in effector Treg cells. Analysis of mice lacking GATA3 in Treg cells has yielded contradictory findings. Whereas earlier studies reported a function for GATA3 in Treg cell function and for the maintenance of FoxP3 expression (Wang et al., 2011; Wohlfert et al., 2011), a more recent report has suggested that GATA3 on its own is dispensable for Treg cell function but that mice doubly deficient for GATA3 and T-bet in Treg cells develop severe autoimmunity (Yu et al., 2015). Although Myb-deficient effector Treg cells failed to upregulate GATA3, they showed normal expression of T-bet, suggesting that the deficiency in GATA3, although likely to contribute, on its own does not fully explain the autoimmune pathology observed.

Prior studies have revealed functions for Myb in regulating cell proliferation and survival. In thymocytes, Myb regulates *Bcl2l1* expression (Yuan et al., 2010), whereas *Bcl2* is a Myb target in multiple cell types (Greig et al., 2008). GO analysis revealed that genes involved in the “apoptotic process” were enriched with Myb DGFs and were DE in effector Treg cells. This included

Bcl2, *Bcl2l1*, *Bcl2l11*, *Bak1*, *Mcl1*, and several members of the TNFR superfamily. Cultured Myb-deficient Treg cells showed a 2-fold increase in their apoptotic rate, which most likely contributes to the loss of effector Treg cells in vivo. Our data also supports a strong role for Myb in promoting the cell cycle. Myb-deficient Treg cells showed a lower proliferative response in vitro and reduced proportion of Ki67⁺ effector Treg cells in vivo. Pathway analysis revealed that genes involved in the cell cycle and signal transduction were strongly enriched with direct Myb-target genes, including the cycle promoter *Cdk6* and the cell-cycle inhibitor *Cdkn1a*. Given that the ICOS⁺TIGIT⁺ effector population harbors most proliferating Treg cells, it is likely that the impaired proliferation and deregulated expression of cell-cycle genes is a major functional consequence of Myb loss in Treg cells. It is also noteworthy that *Myc*, another major regulator of cell proliferation, was downregulated at the transition between activated and effector Treg cells (Figure 7C), potentially heightening the effector Treg cell reliance on Myb to promote cell proliferation.

The expression of *Myb* in ICOS⁺ Treg cells and the disease induced by the selective loss of this population in mice suggest that effector Treg cells are essential for the immune homeostatic function of Treg cells. It was initially surprising that this deficiency occurred in the presence of a normal number of effector Treg cells in the LNs; however, further analysis revealed that these effector Treg cells lacked Nrp1 and Helios expression and were most likely pTreg cells (Thornton et al., 2010; Weiss et al., 2012) that did not express *Myb*. This finding clearly shows that effector tTreg cells play a non-redundant role that cannot fully be compensated by increased numbers of pTreg cells. A similar lethality and immune pathology was observed with mice lacking IRF4 (Zheng et al., 2009) in Treg cells; however, those mice lacked virtually all CD62L⁻ activated and effector Treg cells (Cretney et al., 2011), whereas a selective block was observed in effector tTreg cells without Myb. This distinction is important given that IRF4 deficiency in Treg cells results in Th2-type autoimmunity, whereas mice lacking Myb in Treg cells show a Th1-mediated autoimmune pathology.

Recently, it has become apparent that, in addition to the diversity of effector Treg cells observed in adult mice, a functionally distinct compartment of Treg cells is produced early in life (Yang et al., 2015). Neonatal Treg cells persist into adult life and are important for establishing and maintaining self-tolerance against autoimmune attack in peripheral tissues. In keeping with this model, we observed a very high proportion of either ICOS⁺TIGIT⁺ or KLRG1⁺ effector Treg cells in the blood and tissues such as the

Figure 7. Myb Controls Effector Treg Cell Survival and Proliferation

(A–C) GO analysis of the effector Treg cell profile genes that contain Myb DGFs. (A) Left: enriched GO categories ($p < 0.05$). Right: pie chart showing the number of genes in each GO category. (B and C) Differential expression of the genes in the (B) “apoptotic process” and (C) “cell cycle” GO terms. Genes whose expression increased in effector Treg cells are shown in black, and those that decreased are in red. All genes have Myb DGFs. (D and E) Detection of active caspase-3 in splenic Treg cells and FoxP3⁺CD4⁺ T cells cultured for 2 days in anti-CD3, anti-CD28, and IL-2. (D) Representative flow cytometry plots. Numbers indicate the percentage of cells in the indicated gate. (E) Graph shows the proportion of active caspase-3⁺ Treg cells. Each dot represents a cell derived from an independent mouse; the horizontal line shows the mean. Data are pooled from two experiments. (F) Impaired in vitro proliferation of Myb-deficient Treg cells. The graph shows the mean number \pm SD of cells recovered after 4 days in culture (as in B). Data represent three independent experiments. (G) Mean percentage of Ki67-expressing cells \pm SD among ICOS⁺TIGIT⁺Ly5.2⁺CD4⁺FoxP3⁺ or ICOS⁺TIGIT⁺Ly5.2⁺CD4⁺FoxP3⁺ Treg cells from the indicated tissue of 20:80 BM chimeras of Ly5.1 WT and Ly5.2 *Myb*^{+/+}*Foxp3*^{YFP-cre} or Ly5.2 *Myb*^{f/f}*Foxp3*^{YFP-cre} cells. Three to nine chimeras were analyzed per genotype in two individual experiments. (E–G) Significant p values compare the indicated groups.

liver and lung of 1-week-old WT mice. Because the presence of these neonatal effector Treg cells was strongly Myb dependent, it will be important in the future to determine the extent to which the lack of this unique population of protective cells contributes to the autoimmunity observed in *Myb^{f/f}Foxp3^{YFP-cre}* mice.

EXPERIMENTAL PROCEDURES

Mice

All mice were maintained on a C57BL/6 background in specific-pathogen-free conditions. Animal experiments were approved by the Walter and Eliza Hall Institute animal ethics committee. The *Myb^{f/f}Cd4cre^{Tg}* phenotype was analyzed in both the absence and presence of the *Foxp3^{RFP}* reporter, which was incorporated to facilitate the phenotypic characterization of their Treg cells. Adult mice were analyzed between 8 and 12 weeks of age.

Monitoring of *Myb^{f/f}Foxp3^{YFP-cre}* Mice

Mice were monitored weekly, in a blinded fashion, for the development of clinical manifestations of disease and were euthanized according to guidelines of the Walter and Eliza Hall Institute animal ethics committee. Blood was analyzed on the ADVIA2120i Hematology System (Siemens). For histology, organs were fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained with H&E.

Cell Isolation and Flow Cytometry

Cell preparation and flow cytometry was performed as described in the [Supplemental Experimental Procedures](#).

In Vitro Activation and Cytokine Assays

For analysis of *Myb^{GFP}* expression upon in vitro activation, CD4⁺RFP⁺ cells were sorted from the spleen and LNs of *Myb^{GFP/GFP}Foxp3^{RFP/RFP}* or control *Myb^{+/+}Foxp3^{RFP/RFP}* mice and cultured in wells coated overnight with anti-CD3 (2C11, generated in house) in the presence of 2 µg/mL anti-CD28 (37.51, generated in house) and 200 U/mL rhIL-2 (Peprtech). At 24 or 48 hr, cells were analyzed by flow cytometry. For cytokine assays, cells were isolated from the spleen and LNs of healthy *Myb^{+/+}Foxp3^{YFP-cre}* or sick *Myb^{f/f}Foxp3^{YFP-cre}* mice and bulk cultured in the presence of 50 ng/mL PMA (Sigma-Aldrich), 500 ng/mL ionomycin (Sigma-Aldrich), and GolgiStop (BD Biosciences) for 4 hr at 37°C. Cells were stained with fixable viability dye eFluor 506 (eBioscience) and fluorochrome-labeled antibodies against cell-surface CD4 and CD8 and intracellular IFN-γ (AN18, generated in house) with the BD Biosciences Cytofix/Cytoperm Fixation/Permeabilization Solution Kit.

RNA-Seq

RNA-seq data were generated and analyzed as described in the [Supplemental Experimental Procedures](#).

Digital Genomic Footprint Analysis

DHS sequencing data for steady-state and in-vivo-activated Treg cell samples were downloaded from the Gene Expression Omnibus (GEO: GSM1014200 and 1014148). Sequence reads were mapped to mouse genome mm9 with the Subread aligner. Footprints called for Treg cell samples were downloaded from ENCODE (<http://www.encodede.org/proj/footprints/footprinting.html>). Footprint coordinates were provided to the Homer program for de novo discovery of the Myb-binding motif. The discovered motif was then used for identifying the footprints that included the Myb-binding motif. Each Myb-binding footprint was extended from its center by 100 bp from both sides, and the number of mapped reads was counted for each footprint with the featureCounts program. Myb-binding footprints with >3 CPM (counts per million mapped reads) were retained in the analysis. For calling Myb target genes, the filtered list of Myb-binding footprints was assigned to genes. The body of genes was extended 20 kb upstream and 5 kb downstream in the search for Myb-binding footprints. The 5' ends of mapped reads surrounding the footprints were used for revealing DNase I cleavage sites at Myb-binding loci.

GO Analysis

GO analysis was carried out with the PANTHER GO-slim classification tool of the GO Reference Genome Project ([Mi et al., 2016](#)).

Statistical Analysis

The statistical significance of non-RNA-seq data was assessed with Prism6 (GraphPad); t tests for all single comparisons were unpaired and assumed Gaussian distribution and equivalent standard deviation (SD); t tests for all multiple comparisons were performed with fewer assumptions (each set was analyzed individually, and consistent SD was not assumed) and corrected by the Holme-Sidak method ($\alpha = 0.05$). Survival curves were compared with the Long-rank (Mantel-Cox) test. Enrichment of Myb DGFs in datasets was determined with Chi-square tests. p values < 0.05 were considered significant. Bar graphs display the arithmetic mean ± SD unless otherwise stated.

ACCESSION NUMBERS

Raw sequence reads, read counts, and normalized expression values have been deposited into the Gene Expression Omnibus under accession number GEO: GSE72494.

SUPPLEMENTAL INFORMATION

Supplemental Information include Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.jimmuni.2016.12.017>.

AUTHOR CONTRIBUTIONS

S.D. designed and performed virtually all experiments. A.D. provided technical support. E.C., J.T., C.B., F.F.A., and J.L. assisted in some experiments. G.T.B. assisted in the virus infection models, Y. L., W.S., and G.K.S. analyzed the RNA-seq data and performed the footprinting analysis. S.L.N. supervised the experimental design. S.D. and S.L.N. interpreted the results and wrote the manuscript.

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