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The role of transcription factors in shaping regulatory T cell identity

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Abstract

Forkhead box protein 3 expressing ($FOXP3^+$) regulatory T cells (T_{reg} cells) suppress conventional T cells and are essential for immunological tolerance. FOXP3, the master transcription factor (TF) of T_{reg} cells, controls the expression of multiples genes to guide T_{reg} cell differentiation and function. However, only a small fraction (<10%) of T_{reg} cell-associated genes are directly bound by FOXP3 and FOXP3 alone is insufficient to fully specify the T_{reg} cell programme, indicating a role for other accessory TFs operating upstream, downstream and/or concurrently with FOXP3 to direct T_{reg} cell specification and specialized functions. Indeed, the heterogeneity of T_{reg} cells can be at least partially attributed to differential expression of TFs that fine-tune their trafficking, survival and functional properties, some of which are niche specific. In this Review, we discuss the emerging roles of accessory TFs in controlling T_{reg} cell identity. We specifically focus on members of the basic helix-loop-helix family (AHR), basic leucine zipper family (BACH2, NFIL3 and BATF), CUT homeobox family (SATB1), zinc finger domain family (BLIMP1, Ikaros and BCL-11B) and interferon regulatory factor family (IRF4), as well as lineage-defining TFs (T-bet, GATA3, RORγt and BCL-6). Understanding the imprinting of T_{reg} cell identity and specialized function will be key to unravelling basic mechanisms of autoimmunity and identifying novel targets for drug development.

Introduction

$CD4^+$ regulatory T cells (T_{reg} cells), characterized by expression of CD25 (IL-2 receptor α-chain) and forkhead box p3 (FOXP3), represent one of the key cellular mechanisms for peripheral tolerance induction in mammals[1-3]. The transcription factor (TF) FOXP3, a member of the forkhead-winged-helix family, is constitutively expressed in T_{reg} cells and is essential both for their specification and function[2-4]. The critical roles of FOXP3 and T_{reg} cells themselves are illustrated by mammalian T_{reg} cell-deficiency diseases that

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manifest as fatal multiorgan autoimmune inflammation. These include the human syndrome X-linked immunodysregulation polyendocrinopathy and enteropathy (IPEX) and the Scurfy mouse model, in which mutations in the forkhead domain of FOXP3, which is responsible for nuclear import and DNA binding, result in T_{reg} cell deficiency and failure to restrict self-reactive conventional T cells[5-7]. Despite its importance, FOXP3 is insufficient by itself to specify the complete T_{reg} cell transcriptome[8,9], exemplified by the lack of suppressive capability of FOXP3-expressing activated human conventional T cells. Indeed, chromatin immunoprecipitation with high-throughput sequencing (CHIP-seq) studies that pinpoint genome-wide FOXP3 binding in T_{reg} cells indicate that only a small proportion of genes that are dependent on intact FOXP3 expression are bound by FOXP3[10,11]. This suggests that a substantial part of the T_{reg} cell transcriptional programme is regulated by other TFs, either alone or in combination with FOXP3.

Recent advances in single cell transcriptomics and emerging concepts of mammalian T helper cell polarization suggest that it is common for “master” lineage-specifying TFs to be co-expressed. Thus, T cell behaviour and stability might best be understood when considering transcriptional outputs produced by interactions between gradients of competing TFs, non-coding RNAs and cellular epigenetics[12-15]. Conceptually, a modular model offers the most accurate framework for describing the T_{reg} cell lifecycle, with specific functions “added” to the essential FOXP3 programme by accessory TFs. In this Review, we focus on the emerging roles of some of the accessory TFs that control T_{reg} cell specification and/or maturation (Figure 1 and Tables 1-2). Although there are many such TFs, we restrict the discussion to members of the basic helix-loop-helix family (AHR), basic leucine zipper (bZIP) family (BACH2, NFIL3 and BATF), CUT homeobox family (SATB1), zinc finger domain family (BLIMP1, Ikaros and BCL-11B), interferon regulatory factor family (IRF4), as well as conventional T cell lineage-defining TFs (T-bet, GATA3, RORγt and BCL-6).

T_{reg} cell specification and maturation

The development of thymus-derived T_{reg} cells, referred to as ‘tT_{reg} cells’, takes place neonatally in the thymus. This is triggered by thymocytes that are activated through T cell receptors (TCRs) with an above-average affinity for self-antigens and by CD25 signalling, which lead to the expression of *FOXP3*[16-18]. In addition, T_{reg} cells also form postnatally in the periphery, known as ‘pT_{reg} cells’. Peripheral specification is driven by the sensing of environmental signals, including TCR and CD25 signalling. In both tT_{reg} cells and pT_{reg} cells, TFs downstream of TCR engagement bind to the promoter and conserved non-coding sequence (CNS) regions of the *FOXP3* gene locus, including a T_{reg} cell-specific demethylated region (TSDR)[16,17] (Figure 2). CpG dinucleotides at the TSDR are mostly demethylated in tT_{reg} cells, but partially or completely methylated in in vitro-induced T_{reg} cells (‘iT_{reg} cells’) and conventional T cells[19-23]. While there are many mechanisms by which T_{reg} cells repress immune cell activation[24], most are attributed to stable FOXP3 expression[24,25], which in turn is determined by specific epigenetic marks, such as those at the TSDR, and by the recruitment of multiple TFs driving *FOXP3* expression[26-28] (Figure 2). To imprint T_{reg} cell specification and function, cells require a network of accessory TFs, including nuclear factor of activated T cells (NFAT)[29], nuclear factor-κB (NF-κB)[30], signal transducer and activator of transcription 5 (STAT5)[27], runt-related transcription factor

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1 (RUNX1)[31], cAMP response element binding protein (CREB), activating transcription factor (ATF)[32] and SMAD proteins[33]. These TFs operate in concert to specify the mature T_{reg} cell programme (Table 2), which is characterized by expression of specific cell-surface molecules (such as CD25) and soluble factors and repression of genes associated with effector T cell function (such as *IL2*, *IFNG* and *IL4*)[4,17,24,29,30] (Table 2). The intricate nature of these networks could be exemplified by RUNX1, which activates transcription of *IL2* and *IFNG* when FOXP3 is absent[31]. Conversely, when FOXP3 is present, it interacts with RUNX1 and prevents induction of interleukin-2 (IL-2) and interferon-γ (IFNγ) and imprints T_{reg} cell-associated molecules and suppressive function[31]. Thus, deletion of *Runx1* in mouse naive $CD4^+$ T cells permits unhindered cellular activation and cytokine production, resulting in spontaneous, catastrophic autoimmunity[34]. As expected, single nucleotide variants in the RUNX1 binding site are associated with susceptibility to autoimmunity (psoriasis) in humans[35].

T_{reg} cell maturation in peripheral tissues is shaped by activating signals in the environment, which induce an effector phenotype (characteristically $CD62L^{low}CD44^{hi}CCR7^{low}$) and suppressive markers such as IL-10 and programmed cell death 1 (PD1)[22,36,37]. Recruitment of accessory TFs superimpose supplementary gene modules or ‘programmes’ that are hallmarks of effector T_{reg} cells and impart specialized function and specify trafficking to different tissues (Figure 1). Consistently, transcriptional profiling of $FOXP3^{hi}CD4^+$ subpopulations shows a small but reproducible (core) set of T_{reg} cell-specific genes (such as *Foxp3*, *Il2ra and Tnfrsf18* (which encodes GITR)) onto which additional programmes, sometimes with striking similarity to those observed in conventional T cells, are added[38,39]. Thus, there is substantial heterogeneity in T_{reg} cell programmes involving interplay and interactions between a number of transcriptional regulators[40].

Accessory transcription factors in T_{reg} cell specification

AHR

Aryl hydrocarbon receptor (AHR) is a member of the class I bHLH proteins, serving as sensors of diverse environmental factors, such as xenobiotics, oxygen tension and endogenous ligands generated from host cells, diet and the microbiota[41-43]. Initially recognized as a mediator of the toxic effects of dioxins, AHR is widely expressed in immune and non-immune cells[42,43]. AHR demonstrates promiscuity for endogenous and exogenous ligands with different structures and physiochemical characteristics, thus can produce opposing effects in different cells[44]. In immune cells, AHR is an immunoregulatory TF, influencing T cell differentiation and cytokine production[45]. For example, in parent-into-F1 acute graft versus host disease models, AHR signalling suppresses cytotoxic T cells[46] and generates $CD4^+CD25^+$ T cells with characteristics of suppressive T_{reg} cells expressing cytotoxic T lymphocyte antigen 4 (CTLA4) and glucocorticoid-induced TNFR-related protein (GITR)[47]. Consistently, *in vivo* injection of mice with the high affinity AHR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces T_{reg} cells, suppressing experimental autoimmune encephalomyelitis (EAE)[48] and experimental autoimmune uveoretinitis[49]. Approximately 40-50% of *Ahr*$^{-/-}$ mice die shortly after birth due to inflammatory infiltration in multiple organs[50]. These mice demonstrate ~30% reduction in

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T_{reg} cells, with the residual T_{reg} cells displaying decreased FOXP3 levels[51]. Interestingly, 6-formylindolo[3,2-b]carbazole (FICZ), a tryptophan-derived endogenous high affinity AHR ligand[52], does not induce FOXP3 expression[48]. Instead, FICZ synergizes with transforming growth factor-β (TGFβ), IL-6 and IL-23 to induce expression of retinoic acid receptor-related orphan receptor-γt (RORγt), expansion of T helper 17 (T_H17) cells[48,53] and increased severity of EAE[48].

The exact molecular mechanism or mechanisms of AHR in T_{reg} cell development and function is incompletely understood. Inactive AHR resides in a cytoplasmic complex containing heat shock protein 90, AHR-interacting protein, p23 chaperone and c-SRC protein kinase. These components prevent AHR from ubiquitylation and degradation, thus maintaining steady-state protein expression. AHR agonists induce conformational changes promoting its nuclear translocation to target genes containing the AHR binding DNA motif (the dioxin response element (DRE))[54,55] (Figure 2). The mouse *Foxp3* promoter contains an evolutionarily conserved AHR-binding site and three non-evolutionarily conserved AHR-binding sites[48]. AHR binds both sites in TCDD-treated naive $CD4^+$ T cells and transactivates *Foxp3* transcription[48]. The *Gpr15* locus also contains important AHR-binding sites[56] and encodes an orphan G protein-coupled chemoattractant receptor, the key factor for T cell homing to the large intestine[57]. AHR binds two open chromatin regions in the *Gpr15* locus to enhance its expression. FOXP3 also binds the same AHR-binding regions in T_{reg} cells and physically interacts with AHR[56]. By contrast, RORγt overexpression competitively antagonizes AHR binding at the *Gpr15* locus[56]. Interactions between AHR, FOXP3 and RORγt (identified by co-immunoprecipitation) indicate a T_{reg} cell-specific network that precisely regulates *Gpr15* expression[56] (Table 2).

TGFβ, a key cytokine influencing both *in vitro* and *in vivo* T_{reg} cell differentiation, also has synergic effects with AHR[58] (Figure 2). SMAD proteins, canonical signalling molecules of TGFβ, bind to the *FOXP3* CNS1 locus and to *IL2* promoter to induce and repress gene expression, respectively[33,59,60]. Thus, naive human $CD4^+$ T cells activated in the presence of TGFβ express FOXP3 without necessarily demonstrating suppressive activity[61]. However, if AHR is concurrently active, they do gain both the phenotypic characteristics of $FOXP3^+$ T_{reg} cells (such as high FOXP3 expression, but low *IFNA1*, *IL2* and *IL17* expression) and CD39-dependent suppressive function[58]. TGFβ-induced AHR^+ T_{reg} cells have high expression of SMAD1 and Aiolos (Ikaros family zinc finger 3), which are involved in repression of *IL2* transcription in T_{reg} cells[58]. Conversely, naive $CD4^+$ T cells stimulated through AHR alone develop a $FOXP3^-$ type 1 regulatory T cell phenotype, expressing the AHR-bound target *IL10*[58,62,63]. *IL10* is also bound by MAF at a MAF-recognition element, also known as MARE. AHR and MAF, individually and synergistically, transactivate *IL10* to control this key immunoregulatory cytokine[58,64]. Collectively, these data indicate that AHR is a FOXP3 agonist and that the AHR-regulated module aids development and enhances suppressive function of T_{reg} cells. Some of this is mediated directly and some through synergistic effects with TGFβ and recruitment of additional transcriptional regulators.

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BACH2

BTB domain and CNC homology 2 (BACH2) is a member of the BACH subfamily of bZIP TFs that function as transcriptional activators and repressors[65]. BACH proteins contain a cap'n'collar-type bZIP domain as well as an amino-terminal broad complex, tramtrack, bric-a-brac/poxvirus and zinc finger domain, which is typically a protein interaction motif[66]. BACH2 maintains the balance between networks of TFs that are critical to maturation and function of both T and B cells[65,67,68]. It modulates the differentiation and function of multiple immune cells, including T_{reg} cells, T_H1, T_H2 and T_H17 cells, $CD8^+$ T cells and natural killer (NK) cells, prevents terminal exhaustion, supports quiescence and long-term maintenance of T cell subsets and enforces stem-like transcriptional programmes[69-73]. Consistent with this, CHIP-seq shows BACH2-binding sites at key genes driving conventional T cell differentiation in mice, including *Jun*, *Prmd1*, *Gata3*, *Irf4*, *Nfil3* (see below), *Ahr* (see above) and *Gzmb*, which are targeted for repression[69,74] (Table 2). This repressive function is aided by competition for genome occupancy between BACH2 and other bZIP TFs, such as the AP-1 family member JUND[70,71,75].

T_{reg} cells are high expressors of BACH2 both in the thymus and, more heterogeneously, in the periphery[69,76]. BACH2 is critical for T_{reg} cell differentiation as demonstrated by BACH2 deficient mice that develop spontaneous lethal multi-organ (especially gut and lung) lymphocytic and macrophagic inflammation, together with antinuclear and anti-double-stranded DNA autoantibodies[69]. These mice are deficient in T_{reg} cells, their residual T_{reg} cells are low in FOXP3 and don't prevent transfer colitis[69]. *Bach2*$^{-/-}$ conventional T cells display spontaneous activation and produce elevated T_H1 and T_H2 cytokines[69], indicating de-repression of conventional T cell gene programmes. Moreover, *Bach2*$^{-/-}$ naive T cells differentiate poorly to T_{reg} cells in response to TGFβ *in vitro*[69]. All these predicates indicate that BACH2 is required for efficient generation of T_{reg} cells[77]. Indeed, nuclear BACH2 is an obligate homodimer[66], which in turn can form heterodimers through the bZIP domain with small MAF proteins, including MAFF, MAFG and MAFK, allowing binding to MAREs[66]. One such locus of BACH2 binding is in the *Foxp3* promoter in TGFβ-induced T_{reg} cells[78] (Figure 2).

BACH2 also has a functional role in fully differentiated mature $FOXP3^+$ T_{reg} cells, in which it is highly expressed[76]. T_{reg} cells from mice with genetic ablation of *Bach2* selectively in T_{reg} cells (*Bach2*$^{fl/fl}$*Foxp3*^{Cre}) demonstrate reduced capacity to regulate allergic inflammation in the lungs[79]. Consistent with the repressive function of BACH2, the actual level of BACH2 expression in mature T_{reg} cells may also be important. For example, BACH2 expression is downregulated in activated or effector T_{reg} cells, which explains why $BACH2^-FOXP3^+$ T_{reg} cells express chemokine receptors, co-stimulatory molecules, inhibitory molecules and proteins involved in T_{reg} cell function more highly than $BACH2^+FOXP3^+$ T_{reg} cells[76]. This is also seen in subpopulations of (human) T_{reg} cells with wound healing properties[80]. $CD161^+$ T_{reg} cells are highly suppressive retinoic acid-induced $FOXP3^+$ human T_{reg} cells that produce effector cytokines[80,81] and a wound healing gene programme[80]. These are recruited to the gastrointestinal tract and are particularly enriched in inflammatory bowel diseases[80,82]. In these cells, BACH2 downregulation works in concert with at least three other TFs, RORγt, FOSL2 and AP-1, to permit the expression of genes

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involved in wound healing[80]. Another example where BACH2 functions within networks of multiple immunoregulatory TFs is the contraction programme of human T_H1 cells. In this instance, BACH2 is recruited by signals initiated by vitamin D receptor (VDR) and cooperates with VDR, STAT3 and JUN to repress effector (T_H1) programmes and induce IL-10 production[83].

On a population level, BACH2 is associated with both polygenic and Mendelian diseases, indicating its critical immunoregulatory role. Genetic variations in the human *BACH2* locus are associated with susceptibility to several autoimmune diseases, including rheumatoid arthritis[84], Crohn disease[85], multiple sclerosis[86], type 1 diabetes[87] and asthma[88]. The *BACH2* gene is tightly regulated by an extensive regulatory region composed of multiple enhancers, collectively termed a super enhancer. Super enhancers ensure appropriate and finely tuned expression of critically important genes and polymorphisms within these loci associate with multiple autoimmune diseases[65]. BACH2 itself is recruited to other super enhancer loci within T cells to act as a 'guardian' TF preventing autoimmunity[65]. In turn, single nucleotide variants in *BACH2* identified in genome-wide association studies commonly occur within its associated super enhancer region, some of which (such as rs72928038) functionally impair *BACH2* expression[89]. Homozygous deficiency of *BACH2* in human populations have not been described because this gene has low tolerance to loss of function. Nevertheless, rare patients with *BACH2* haploinsufficiency do exist and present with monogenic BACH2-related immunodeficiency and autoimmunity syndrome, characterized by decreased FOXP3 expression in T_{reg} cells, increased expression of T-bet and gut homing receptors (CCR9 and integrin-β7) on $CD4^+$ T cells, and clinical colitis together with B cell immunodeficiency[68].

BACH2 shuttles between the nucleus and cytoplasm guided by a carboxy-terminal nuclear localization signal[90]. Serine residues in BACH2 can also be phosphorylated by phosphoinositide 3-kinase–AKT signalling and regulate nuclear trafficking[91], as can sumoylation or desumoylation events at lysine residues[92]. Sumoylation, the addition of small ubiquitin-like modifiers (SUMO) to proteins is a reversible post-translational modification regulating trafficking, stability and biology of TFs[93]. Desumoylation of BACH2 catalysed by SUMO-specific protease 3 (SENP3) prevents nuclear export, resulting in nuclear accumulation and stabilization of T_{reg} cell-associated gene loci[92]. SENP3 deficiency, therefore, causes spontaneous autoimmunity and enhanced antitumour immunity[92] from T_{reg} cell dysregulation. Reactive oxygen species (ROS) induce SENP3 expression[94], thus ROS-rich environments, such as in cancer, drive T_{reg} cell-mediated tumour immunosuppression, whereas ROS-low states impair T_{reg} cell function[95] (Figure 2). Indeed, this mechanism represents a plausible explanation for the link between low ROS levels and increased susceptibility to autoimmunity[96]. Collectively, the BACH2 module enhances FOXP3 expression and suppresses pro-inflammatory genes, aiding the development, function and steady-state maintenance of T_{reg} cells.

BCL-11B

BCL-11B, a C2H2 zinc finger TF, has essential roles in T cell specification. It is expressed in thymocytes at the double negative 2 stage, promotes T cell lineage commitment and

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represses alternative lineage specification, particularly NK cells[97,98]. BCL-11B suppresses T_H2 cell programmes in mature lymphocytes to restrict T_H17 cell plasticity[99]. Likewise, by enhancing group 2 innate lymphoid cell (ILC2) programmes (and repressing group 3 innate lymphoid cell programmes) BCL-11B maintains peripheral ILC2 populations[100]. BCL-11B has both transcriptional repressor and activator functions in association with the nucleosome remodeling and deacetylase (NuRD) complex (a key transcriptional corepressor) and histone acetyltransferases (HATs, such as p300), respectively[101,102]. Deletion of *Bcl11b* in T cells (*Bcl11b*$^{fl/fl}$*Cd4*^{Cre}) causes colitis and wasting disease, which is preventable by transferring wild-type T_{reg} cells[103]. T_{reg} cell-specific *Bcl11b* deletion (*Bcl11b*$^{fl/fl}$*Foxp3*^{Cre}) results in lethal multi-organ autoimmunity similar to mice lacking T_{reg} cells[104,105]. T_{reg} cells of *Bcl11b*$^{fl/fl}$*Cd4*^{Cre} mice and *Bcl11b*$^{fl/fl}$*Foxp3*^{Cre} mice demonstrate impaired[103] and almost complete loss, of suppressive function[104,105], respectively. T_{reg} cells lacking BCL-11B have lower fitness than wild-type counterparts and significant loss of characteristic T_{reg} cell-associated genes[104,105], including *Il10* and *Foxp3* expression[103-105]. There is, in fact, considerable inter-dependence between the gene regulatory programmes of BCL-11B and FOXP3. Since BCL-11B directly binds the *Il10* promoter, *Foxp3* promoter and *Foxp3* CNS0–CNS2 regions and is required for their efficient transcription[103,105] (Figure 2 and Table 2), there is significant overlap between BCL-11B-bound and FOXP3-bound genes[104,105]. Mechanistically, FOXP3 is required for optimal recruitment of BCL-11B to its targets and BCL-11B is, in turn, required for optimal recruitment of FOXP3 to its target loci[104,105]. Thus, FOXP3 is misdirected to alternative loci when BCL-11B is absent and expression of characteristic T_{reg} cell genes is lost. Collectively, BCL-11B is an essential TF supporting the T_{reg} cell programme and restraining alternative lineages. The BCL-11B-regulated module enhances FOXP3 expression and T_{reg} cell-associated genes and works cooperatively with FOXP3 protein.

Ikaros

Members of the Ikaros zinc finger TF family, especially Helios and possibly EOS, have been associated with T_{reg} cell biology and extensively discussed elsewhere[106-112]. More recently, Ikaros, a repressor of pro-inflammatory genes encoded by *IKZF1*, has been suggested as an essential TF in the induction of iT_{reg} cells. Ikaros-deficient $CD4^+$ T cells (*Ikaros*$^{fl/fl}$*Lck*^{Cre}) can differentiate to T_H1, T_H2 and T_H17 cell lineages *in vitro* but fail to generate iT_{reg} cells[113,114]. In fact, Ikaros-deficient $CD4^+$ T cells cultured under iT_{reg} cell-polarizing conditions demonstrate aberrant production of IL-17 and IL-22 upon TCR activation[113] and their fully differentiated T_H17 cells take on the phenotype of pathogenic cells ($ROR\gamma t^+$T-bet^+IL-17^+)[114]. In the absence of Ikaros, expression of another TF, FOXO1[115], and IL-7RA decreases in $CD4^+$ T cells[116]. FOXO1 promotes iT_{reg} cell differentiation by binding to the promoter and CNS regions of *Foxp3*[117] (Figure 2 and Table 2). T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), a receptor on T_{reg} cells[118], further regulates FOXO1 activity by increasing its availability in the nucleus via inhibition of AKT[119]. Ikaros is, in fact, required for development of all lymphoid lineages, thus mice that are deficient for all Ikaros isoforms are lymphopenic and fail to thrive from 1-3 weeks of life due to opportunistic infections[120]. Thus, mutations of *IKZF1* strongly correlate with poor outcomes in high-risk acute lymphoblastic leukaemia[121], single nucleotide variants in *IKZF1* are associated with autoimmunity (such as systemic lupus erythematosis and

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Sjogren syndrome)[122,123], and immune dysregulation characterized by abnormal T and B cell differentiation is a feature of Mendelian diseases mediated by haploinsufficient, dominant negative or gain-of-function mutations in *IKZF1*[124,125]. Although some of these patients exhibit reduced T_{reg} cells, the broad immune dysregulation makes it currently unclear to what extent these clinical phenotypes are related to T_{reg} cells, as opposed to other immune cells[126]. Collectively, Ikaros represses pro-inflammatory genes and provides an essential module for the induction of iT_{reg} cells.

SATB1

DNA binding protein SATB1 controls transcriptional and epigenetic changes by forming long-range chromatin loops, bringing distal regions together and recruiting epigenetic modifying enzymes and transcriptional machineries to target gene loci[127,128]. SATB1 is highly expressed by thymocytes and is essential for controlling genes participating in T cell development and activation[129,130]. It is upregulated at the single-positive and double-positive stages of thymocytes development, where it binds to CNS0 and, with less affinity, to CNS3 of *FOXP3*, before being downregulated again in mature T_{reg} cells[131] (Figure 2). Mature (especially human) T_{reg} cells express low levels of SATB1 compared with conventional T cells even after TCR and IL-2 stimulations[132], because FOXP3 negatively regulates *SATB1* expression. FOXP3 binds *SATB1* in T_{reg} cells and represses its expression whereas FOXP3 knockdown increases *SATB1* in T_{reg} cells[132].

Satb1$^{-/-}$ mice are viable, but small, with small immune organs, and die at ~3 weeks of age, possibly from apoptosis of non-immune cells[129]. Mice with haematopoietic cell-specific *Satb1* knockout (*Stat1b*$^{fl/fl}$ *Vav*^{Cre}) produce autoantibodies and develop spontaneous autoimmunity affecting multiple organs[133]. They have activated conventional T cells and reduced T_{reg} cell number and function[133]. Selective deletion of *Satb1* in $CD4^+$ cells (*Satb1*$^{fl/fl}$*Cd4*^{Cre}) results in a significant reduction of CD4 single-positive thymocytes, but an almost complete absence of tT_{reg} cells[131]. This is because a T_{reg} cell-specific super enhancer is activated in T_{reg} cell precursors in a SATB1-dependent manner and required for expression of signature genes attributed to T_{reg} cells[131]. Thus, SATB1 is required for tT_{reg} cell lineage specification and its deletion impairs tT_{reg} cell development before FOXP3 expression. In mature T_{reg} cells, FOXP3 represses *Satb1* expression to prevent conventional T cell polarization and maintain stable T_{reg} cell identity. Thus, overexpressing SATB1 in mature T_{reg} cells results in loss of suppressive function and gain of conventional T cell programmes producing IFNγ, IL-4 and IL-17A[132]. SATB1 binds the promoter of *Bhlhe40*, which encodes a TF that drives granulocyte-macrophage colony-stimulating factor (GM-CSF) production, which is an essential T_H17 cell pathogenic chemokine[134]. Thus, SATB1 in conventional T cells regulates pathogenic T_H17 cells and its ablation in T_H17 cells protects mice from EAE, due to marked reduction of GM-CSF[134]. Notably, *SATB1* single nucleotide variants (rs11719975) associate with multiple sclerosis[135]. Likewise, high SATB1 expression and lineage instability is a feature of tumour-infiltrating T_{reg} cells[136]. Conversely, T_{reg} cells in chronic hepatitis B virus infection characteristically have low levels of SATB1[137], which may contribute to impaired viral clearance[138]. Overall, SATB1 is critical for tT_{reg} cell development prior to FOXP3 expression by controlling transcriptional and

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epigenetic changes. The SATB1 module in mature T_{reg} cells is antagonistic to the T_{reg} cell phenotype and fosters conventional T cell programmes that associate with autoimmunity.

Accessory transcription factor programmes in T_{reg} cell maturation

IRF4

Interferon regulatory factor 4 (IRF4) is a TF involved in the expression of interferon-induced genes[139] and found in a variety of cells, including B and T cells, macrophages and dendritic cells[140-147]. The protein is rapidly induced in T cells following TCR stimulation and subsequently regulates the commitment of T cells to T_H2 and T_H17 fates[142,143,148]. IRF4 expression in thymic epithelial cells in response to RANK signalling is crucial for inducing tT_{reg} cells[149] and IRF4-deficient thymic epithelial cells (*Irf4*$^{fl/fl}$*FoxN1*^{Cre}) poorly stimulate tT_{reg} cell differentiation, without affecting the pT_{reg} cell pool[149]. Both thymic and peripheral mouse FOXP3^{+} T_{reg} cells have high IRF4 levels because FOXP3 directly induces *Irf4* expression after binding the *Irf4* promoter, hence *Foxp3* deletion markedly reduces *Irf4* mRNA levels in T_{reg} cells[150]. Deleting *Irf4* in differentiated T_{reg} cells (*Irf4*$^{fl/fl}$*Foxp3*^{Cre}) causes spontaneous and lethal autoimmunity from 6-8 weeks of age[150]. In fact, *Irf4* deficiency in T_{reg} cells compromises maturation into effector T_{reg} cells, illustrated by low expression of characteristic markers, such as inducible T cell costimulator (ICOS), CTLA4 and IL-10, as well as suppressive function[36]. Because the TF *Pparg* is directly induced by IRF4 and is essential for development of adipose-tissue T_{reg} cells[37], which are involved in preserving insulin sensitivity and glucose tolerance[151], complete IRF4-deficient mice have a near total absence of this T_{reg} cell subset[37]. These mice do not, however, develop overwhelming autoimmunity similar to *Irf4*$^{fl/fl}$*Foxp3*^{Cre} strains, confirming that IRF4 also has key roles in conventional effector T cell programmes. Human IRF4 deficiency also leads to reduced frequency of T_{reg} cells[152]. A subset of intratumoral human effector T_{reg} cells that have high expression of IRF4 and potent suppressive function are associated with poor cancer prognosis[136,153]. This observation is corroborated by mice in which inducible deletion of *Irf4* in T_{reg} cells (*Irf4*$^{fl/fl}$*Foxp3*$^{EGFPCre\text{-}ERT2}$ mice fed tamoxifen) accelerates tumour clearance[153].

IRF4 interacts with FOXP3 in T_{reg} cells (they co-immunoprecipitate from nuclear lysates[150]) and has a role in gene transcription, but its biology in T_{reg} cells is not fully understood. IRF4 is in the nucleoplasm and makes homomeric and heteromeric interactions with other TFs, especially other members of the IRF and AP-1 families, through its C-terminal region[139]. These interactions are important to enhance its weak DNA binding and transactivation. The N-terminal DNA binding domain of IRF4 binds DNA consensus motifs akin to classical interferon-stimulated response elements[139,154]. One such locus is *IL10*, where it works cooperatively with B lymphocyte-induced maturation protein 1 (BLIMP1), which is encoded by *PRDM1* (see below), or PU.1 to remodel active chromatin and transactivate gene transcription[36,155] (Tables 1-2). It worth noting that IRF4 also binds the *PRDM1* locus[36] and induces BLIMP1 expression and that BLIMP1 is absent in IRF4-deficient T_{reg} cells[36]. In summary, the IRF4-regulated module is essential for T_{reg} cell maturation in the periphery into effector T_{reg} cells and for synergism with FOXP3 and BLIMP1 to transactivate *IL10*.

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BLIMP1

BLIMP1 is a transcriptional repressor interacting with other TFs, such as IRF4, through its proline-rich N-terminal domain[156]. It is well characterized as a master regulator orchestrating plasma cell development and immunoglobulin secretion[157,158]. It also has a role in T cells[159,160], as seen in the association between *PRDM1* polymorphisms and multiple autoimmune diseases, including inflammatory bowel diseases[161-164] and development of colitis in mice with T cell ablation of *Prdm1*[165]. However, this TF is not essential for T_{reg} cell differentiation as T_{reg} cell numbers are generally unaltered in BLIMP1 deficiency. Rather, BLIMP1 is required for optimal function of activated and effector T_{reg} cells. In fact, only 10-20% of mature T_{reg} cells express BLIMP1 in lymphoid organs, whereas most T_{reg} cells in tissues (such as the gut, lungs and central nervous system) do so, suggesting that its expression is likely to be key in specialized tissue-resident or effector T_{reg} cells[36,166]. This is intuitive because BLIMP1 expression is induced by inflammatory signals, which are more likely to be present in tissues. These signals include factors such as IFNγ-induced STAT1 and IRF4, which directly bind to the promoter of *Prdm1*[36,166]. In these T_{reg} cells, BLIMP1 helps to maintain regulatory function by stabilizing T_{reg} cell-associated genes and repressing conventional T cell-associated genes. For example, BLIMP1 in $FOXP3^{+}ROR\gamma t^{+}$ T_{reg} cells is crucial for suppressing the production of T_H17 cytokines and maintaining regulatory function (see below) by binding to CNS regions at the *Il17a* gene[167]. Consistently, EAE is exacerbated by T_{reg} cell-selective deficiency of BLIMP1 (*Prdm1*$^{fl/fl}$*Foxp3*^{Cre}) due to increased inflammatory features, such as IL-17 and IFNγ production, and loss of expression of classical T_{reg} cell genes including *Foxp3*, *Gzmb* and *Il10*[166].

As discussed previously, BLIMP1 works with IRF4 to activate *IL10* transcription[36] (Tables 1-2). IL-10 production is downregulated in T_{reg} cells from BLIMP1-deficient mice and BLIMP1 overexpression rescues their suppressive function[36,159]. As expected, BLIMP1 deficiency leads to similar consequences as IL-10 insufficiency. For example, IL-10Rα signalling in adipocytes causes insulin resistance and glucose intolerance in mice by altering chromatin accessibility and repressing transcription of thermogenic genes[168], but *Prdm1*$^{fl/fl}$*Foxp3*^{Cre} mice are protected[169]. In contrast, MOG35–55-induced EAE and autoimmune diabetes in non-obese diabetic mice are more severe due to lack of IL-10 and expansion of T_H1 and T_H17 cells, respectively[170,171]. As anticipated, $BLIMP1^{+}$ T_{reg} cells in tissues have enhanced suppressive function, which can be both advantageous (as an important component of graft-infiltrating $FOXP3^{+}$ T_{reg} cells that maintain spontaneously induced kidney allograft tolerance[172]) or detrimental (as a mechanism for tumour immune evasion[173]). Furthermore, expression of BLIMP1 is a cardinal feature of a subset of T_{reg} cells found in germinal centres, known as follicular regulatory T (T_{FR}) cells, which are discussed below. In summary, the BLIMP1-programme is required for optimal function of activated and effector T_{reg} cells and synergism with FOXP3 and IRF4 to transactivate *Il10* and *GzmB* and suppress IL-17 production.

BATF

Basic leucine zipper ATF-like transcriptional factor (BATF), an AP-1 subfamily bZIP TF, is a regulator of T cells, most notably in the differentiation of T follicular helper

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(T_{FH}), T_H2 and T_H17 cells[174-176]. BATF is particularly prominent in precursors of T_{reg} cells that share transcriptional programmes with T_H2 cells (see below) and are found within tissues[177]. Some of these tissue-resident T_{reg} cells express CC-chemokine receptor 8 (CCR8) and have tissue repair capacity[178]. *Batf*$^{-/-}$ mice have significantly reduced tissue-infiltrating T_{reg} cells but no spontaneous autoimmunity[37,177,179]. T_{reg} cells of these mice do not express ST2, the IL-33 receptor, required for development and maintenance of adipose-tissue T_{reg} cells[37]. BATF and IRF4 (see above) both bind *Il1rl1* (the gene encoding ST2) and induce ST2 expression[37]. *Batf*$^{-/-}$ mice lack autoimmunity most likely due to the important role it has in specifying inflammatory conventional T cell lineages, because T_{reg} cell-specific *Batf* ablation (*Batf*$^{fl/fl}$*Foxp3*^{Cre}) causes spontaneous T_H2 cell-dominant multi-organ inflammatory disorder[180]. T_{reg} cells from these mice selectively fail to suppress T_H2 cell inflammation, but function normally with respect to suppression of T_H1 cells and conventional T cell proliferation[180]. This may in part be explained by excess production of T_H2 cytokines by *Batf*$^{fl/fl}$*Foxp3*^{Cre} T_{reg} cells themselves[180]. In humans with IPEX syndrome, an interesting *FOXP3* mutation, *FOXP3*^{A384T}, causes tissue-restricted autoimmunity, attributable to abnormally low BATF expression. This mutation has a gain-of-function effect, broadening DNA-binding specificity of FOXP3 and enhancing interaction with the *BATF* promoter[181]. The importance of BATF to tissue-infiltrating T_{reg} cells is highlighted by comprehensive mapping through CHIP-seq and assay for transposase-accessible chromatin with sequencing (ATAC-seq) of human T_{reg} cells in tumour microenvironments. These corroborate overlapping functions of BATF and IRF4 and indicate that BATF binds at key genetic loci, such as *IL10*, *CTLA4*, *TIGIT* and *TNFRSF4*, to enhance T_{reg} cell fitness in the tumour microenvironment[182] (Table 2). As anticipated, high and low BATF expression in these T_{reg} cells correlate with poor and favourable prognoses, respectively[182]. To summarize, the BATF-driven module is necessary for the development of non-lymphoid tissue T_{reg} cell precursors, as well as for the development and sustainability of T_{reg} cells that reside in tissues.

NFIL3

Nuclear factor interleukin-3 (NFIL3) is a member of the bZIP family[183] that was initially identified by its ability to bind and repress an E4 promoter sequence containing an ATF consensus site[184] and later characterized as binding and activating transcription of *IL3*[185]. NFIL3 regulates transcription in different immune cells. In B cells it regulates IgE class switching[186], in NK cells and dendritic cells it promotes development and function[187-189] and in T cells it regulates cytokine production[190,191]. NFIL3 expression is induced in T_{reg} cells during chronic infections[192] and impairs T_{reg} cell function by downregulating *FOXP3* expression[193]. It has also been proposed as an early marker gene for non-lymphoid tissue T_{reg} cells[177]. Expressions of NFIL3 and FOXP3 are reciprocally linked, as TGFβ, which drives iT_{reg} cell differentiation[33,59,60], represses *NFIL3*, whereas overexpression of *NFIL3* in T_{reg} cells represses *FOXP3* expression and impairs suppressive function *in vitro* and *in vivo*[193]. NFIL3 directly binds the gene promoter and CNS elements of *FOXP3* (including the TSDR), and physically interacts with FOXP3 protein itself[193] (Figure 2 and Table 2). Moreover, NFIL3 binds the *IL10* gene; thus, multiple immune cell subsets, including T_{reg} cells, have defective IL-10 production in the NFIL3-deficient state[194]. In other immune lineages, including ILCs and dendritic cells, NFIL3 functions upstream of a transcriptional

circuit involving other TFs, including DNA-binding protein inhibitor ID2 and zinc finger E-box-binding homeobox 2 (ZEB2), that imprint specification in precursors[195]. These TFs are also active in T_{reg} cells. Notably, ID2 is required for differentiation of $GATA3^+$ adipose tissue-associated T_{reg} cells (see below) [196] and, together with ID3, for maintenance of T_{reg} cells in general[197]. There are suggestions that ID2 expression may play a role in T_{reg} cell plasticity (see below) and also negatively regulate *IL10* transcription indirectly[198,199]. ZEB2 is a negative regulator of T_{reg} cell function. Thus, iT_{reg} cells differentiated from *Zeb2*$^{fl/fl}$ER^{Cre} precursors treated with tamoxifen or mature T_{reg} cells with short hairpin RNA-mediated *Zeb2* knockdown exhibit enhanced suppressive function[200]. However, it remains unclear whether NFIL3 regulates either of these TFs in T_{reg} cells. Humans with homozygous mutations in *NFIL3* develop autoimmunity, notably juvenile idiopathic arthritis and autoimmune thyroiditis. *Nfil3*$^{-/-}$ mice phenocopy the arthritis susceptibility, and the disease mechanism in both humans and mice is attributable to myeloid cell dysregulation and IL-1β hyperproduction[201]. Numbers and function of T_{reg} cells have not been reported in such patients. Although the mutation in *NFIL3*-deficient kindreds (c.G510A, p.M170I) reduces NFIL3 protein levels (~50%), these data suggest that immunomodulatory NFIL3 biology is more complex and intertwined with FOXP3. In summary, the NFIL3-driven module is a feature of chronic inflammatory states and impairs FOXP3 expression and antagonizes T_{reg} cell function.

Lineage-specifying transcription factors

As discussed, T_{reg} cells can take on effector phenotypes and co-opt additional transcriptional programmes that are typically restricted to other cell lineages, licensing them to enter specific tissues or endowing specialized function. Depending on the environment and tissue, T_{reg} cells can express lineage-specifying TFs from alternative lineages, such as T_H1, T_H2, T_H17 and T_{FH} cells (see below) and produce cytokines traditionally considered to be restricted to conventional T cells, such as IFNγ and IL-17A. This raises the question of T_{reg} cell "stability" and its role in autoimmune diseases and cell therapies[202]. As discussed above, stable FOXP3 expression is maintained by key TFs and epigenetic modifications including those at the *FOXP3* CNS regions[19-22]. As expected, mouse iT_{reg} cells, with higher TSDR methylation, lose *Foxp3* expression more readily than tT_{reg} cells[20,203]. Naive and memory human T_{reg} cells cultured with IL-1β together with IL-2 or IL-6 downregulate FOXP3 expression and suppressive function and express characteristic T_H17 cell-associated genes including IL-17 and CCR6[204,205]. Likewise, repeated stimulation of human T_{reg} cells through the TCR alone leads to loss of FOXP3 expression[206]. Mouse T_{reg} cells exposed to IL-6, with or without IL-1β, also produce IL-17[60,207] as do T_{reg} cells adoptively transferred into lymphopenic recipient mice[208]. In fact, fate mapping mice can identify ex-$FOXP3^+$ T_{reg} cells *in vivo* and some of these can be diabetogenic[209]. Similarly, excess IL-4 signalling can render mouse T_{reg} cells allergenic[210]. There remains a debate on the degree of T_{reg} cell plasticity and its contribution to inflammation in humans, particularly as other fate-mapping studies show T_{reg} cells to be remarkably stable *in vivo*[211]. The arguments for and against the plasticity model have been rehearsed elsewhere[202,212-214] but the full extent of T_{reg} cell instability and its impact on human diseases is yet to be understood.

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T-bet

T-bet is a well-established lineage-defining TF for T_H1 cells[215]. It drives T_H1 cell differentiation while repressing other lineages, such as T_H2 cells[215,216] and T_H17 cells[217]. T-bet in T_H1-polarized cells binds and transactivates the *IFNG* locus[218] and binds *FOXP3* promoter and represses its activation[219] (Figure 2). However, many (~30-70%) effector T_{reg} cells exhibit T_H1 cell-like characteristics, defined by co-expression of T-bet and CXC-chemokine receptor 3 (CXCR3), which is regulated by T-bet, in lymphoid and non-lymphoid tissues. Similarly, intestinal T_{reg} cells exhibit prevalent co-expression of T-bet and RORγt[220]. Such T_H1-like T_{reg} cells are readily identifiable in patients with multiple sclerosis[221]. Surprisingly, IFNγ induces FOXP3 expression in conventional T cells[222] and chemically-induced colitis in mice is characterized by increased proportions of IFNγ-expressing T_H1-like T_{reg} cells in the lamina propria[223]. T-bet expression in T_{reg} cells is likely induced in a similar manner to conventional T cells, where T_H1 cell specification involves IFNγ signalling through STAT1 and IL-12 signalling through STAT4[224]. Specifically, $CXCR3^+T\text{-}bet^+$ T_{reg} cells are dramatically reduced in mice lacking either STAT1 or IFNγ receptor[225]. Full T_H1 cell specification in T_{reg} cells is usually not completed because repressive histone 3 lysine 27 trimethylation (H3K27me3) epigenetic marks at *Il12rb2* delay IL-12Rβ2 expression and prevent timely STAT4 signalling[203].

T-bet regulates T_{reg} cell function during T_H1 cell responses, although the mechanism is not clear. T-bet-deficient mice lack $CXCR3^+FOXP3^+$ T_{reg} cells[225] and T-bet ablation (such as *Tbx21*$^{-/-}$ and *Tbx21*$^{fl/fl}$*Foxp3*^{Cre} strains) causes spontaneous autoimmune disease[220,225,226] and increased severity of *Toxoplasma gondii* infection[227], similar to the exacerbated EAE seen in IFNγ-deficient mice[222]. T-bet-expressing T_{reg} cells have high levels of CXCR3, GITR, CTLA4 and CD103 expression and abundant *IL10* and *TGFB* mRNA[225]. Indeed, TGFβ expression increases $T\text{-}bet^+FOXP3^+$ T_{reg} cells under T_H1 cell-polarizing conditions[228]. CXCR3-expressing T_{reg} cells suppress T_H1 cell and $CD8^+$ T cell proliferation[219,225,229]; CXCR3 expression is likely to be key, as it licenses T_{reg} cells for access to sites of T_H1 cell-mediated disease where they suppress inflammation[225]. However, T-bet in some models, such as in experimental colitis, has also been proposed to act as a pathological mediator in T_{reg} cells[223]. In summary, the T-bet programme licenses T_{reg} cells to enter sites of T_H1 cell-associated inflammation where they are required for repressing T_H1 cells (and $CD8^+$ T cells). However, the role of T-bet as a potential pathogenic factor in T_{reg} cells needs further investigation.

GATA3

GATA3 is the master lineage-specifying TF of T_H2 cells[230,231]. T_{reg} cell and T_H2 cell biology shows overlap[232], including high expression of GATA3 in dermal and intestinal T_{reg} cells and its induction after TCR and IL-2 stimulation[233]. Some T_{reg} cells even express higher GATA3 levels than conventional T cells[234]. GATA3-expressing T_{reg} cells are marked by surface expression of ST2[235], a cytokine receptor transcriptionally controlled by GATA3[236]. GATA3 expression in T_{reg} cells is independent of T_H2-polarizing cytokines but can be opposed by T_H1-specifying or T_H17-specifying cytokines, notably IL-12 and IL-6[233], and is repressed by BCL-6[237]. This may explain why GATA3 binds and represses genes for lineage-specifying TFs of opposing conventional T cell lineages, notably *TBX21*

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and *RORC*[233]. GATA3 binds to the TSDR in T_{reg} cells (but not in conventional T cells) to transactivate *FOXP3* transcription and also cooperates with FOXP3 protein to maintain FOXP3 expression (Figure 2); thus, *FOXP3* mRNA is reduced in GATA3-deficient T_{reg} cells[233,234]. T_{reg} cell-specific GATA3 deficient (*Gata3*$^{fl/fl}$*Foxp3*$^{EGFP\text{-}Cre}$) mice develop lymphadenopathy, splenomegaly and lymphocytic infiltration of multiple organs and show enhanced production of IFNγ, IL-4 and IL-17A by 16 weeks[234]. GATA3-deficient T_{reg} cells can suppress conventional T cells but don't accumulate in tissues, thus can't prevent transfer colitis, and some even produce IL-17[233,234]. GATA3 expression is also important in T_{reg} cells that resolve inflammation in acute kidney injury[238]. Depletion of T_{reg} cells in the skin or T_{reg} cell-specific *Gata3* deletion (*Gata3*$^{fl/fl}$*Foxp3*^{CreERT2} mice fed tamoxifen), leads to enhanced T_H2 cytokine expression, T_H2 cell infiltration, fibroblast activation and production of pro-fibrotic genes[239]. In summary, the GATA3 programme stabilizes FOXP3 expression, enhances suppressive capacity and permits expression of tissue-specific homing receptors.

RORγt

RORγt is an isoform of RORγ which is encoded by *RORC* and found in immune cells during thymopoiesis and lymphopoiesis[240,241]. It is commonly expressed by (predominantly group 3) ILCs[242,243] and $CD4^+$ T cells including T_H17 cells[244] and T_{reg} cells[245-247]. T_H17 cell differentiation is orchestrated by RORγt, which induces expression of IL-17[244,248]. However, pathogenicity of T_H17 cells depends on microenvironmental availability of additional STAT3-activating cytokines, such as IL-23[249] or IL-1[250]. Mouse iT_{reg} cells and tT_{reg} cells have repressive H3K27me3 epigenetic modifications at *Il17a*, but permissive and bivalent histone 3 lysine 4 trimethylation (H3K4me3) modifications at *Rorc*, respectively[251], theoretically permitting FOXP3 and RORγt co-expression following appropriate stimulation[60,207]. Unsurprisingly, a significant proportion of $FOXP3^+$ T_{reg} cells, especially those in the gastrointestinal tract, express RORγt, along with markers such as IL-10 and ICOS[245,252]. There is, in fact, large overlaps in gene expression profiles between $FOXP3^+$RORγt^- T_{reg} cells, $FOXP3^+$RORγt^+ T_{reg} cells and RORγt^+ T cells, indicating high similarity between these populations[253].

T_{reg} and T_H17 cell differentiation is induced from naive precursors. TGFβ induces FOXP3 expression via activation of SMAD proteins[33,59], whereas IL-6-mediated activation of STAT3 is required for RORγt expression[254]. Interestingly, IL-6-deficient mice have fewer RORγt^+ T_{reg} cells[252]. The RORγt programme is peripherally induced, for example by the gut microbiota through short-chain fatty acids and retinoic acid, or via antigen presentation by RORγt-expressing antigen-presenting cells; thus, germ-free mice have fewer RORγt^+ T_{reg} cells[246,252,255-257]. Epigenetic studies of RORγt^+ T_{reg} cells indicate demethylation at T_{reg} cell-specific signature genes[253]. This suggests a stable regulatory, rather than inflammatory, phenotype and that they may occupy an important immunoregulatory niche in the gut, for example to prevent inflammation in response to the microbiota. Indeed, colonic conventional T cells in unchallenged mice harbouring specific deletion of *Rorc* in T_{reg} cells (*Rorc*$^{fl/fl}$*Foxp3*^{Cre}) have dysregulated T_H1 and T_H17 cells and develop significantly more severe colitis when challenged with trinitrobenzenesulfonic acid[246]. Likewise, selective deletion of *Stat3* in T_{reg} cells (*Stat3*$^{fl/fl}$*Foxp3*^{Cre}) causes spontaneous gastrointestinal inflammation through failure to regulate local T_H17 cell-mediated inflammation[258]. In

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humans, RORγt$^+$ T_{reg} cells are induced by retinoic acid, are enriched in the gastrointestinal tract, produce IL-17 in a STAT3-dependent manner[80,259] and can be identified by expression of CCR6[205] and the C-type lectin-like receptor CD161[80]. These cells retain suppressive function and express a wound healing programme that is regulated by a TF network that includes BACH2[80,259] (see above).

The intracellular biology of RORγt in T_{reg} cells is not fully understood. FOXP3 interacts with RORγt in co-expression studies conducted in HEK293T cells[245] and, in fact, does so through the region encoded by *FOXP3* exon 2, which is necessary to suppress RORγt-mediated *IL17A* promoter activation[260] (Figure 2 and Table 2).

The RORγt programme in T_{reg} cells overlaps with the MAF programme[246,253,261,262], which induces IL-10 in multiple T helper cell subsets[64,263,264]. *Helicobacter hepaticus* is a pathobiont that induces multiple gut T cell lineages, including pT_{reg} cells, T_{FH} cells and pathogenic T_H17 cells[265 265] and drives enterocolitis in mice lacking immunoregulatory cytokines (such as *Il*10$^{-/-}$ strains)[266]. In *H. hepaticus*-challenged mice, T_{reg} cell-specific deletion of *Maf* (*Maf*$^{fl/fl}$*Foxp3*^{Cre}) leads to colitis due to lack of IL-10 in pT_{reg} cells, low levels of RORγt$^+$ T_{reg} cells and expansion of pathogenic T_H17 cells[265]. However, *Rorc*$^{fl/fl}$*Foxp3*^{Cre} mice are not susceptible to colitis, showing T_{reg} cell-expressed MAF is non-redundant for immune tolerance to gut pathobionts[265]. In fact, MAF represses *Il17a* transcription in RORγt$^+$ T_{reg} cells, which become the main source of T_{reg} cell-derived IL-17 in MAF-deficient T_{reg} cells[261]. Collectively, the RORγt programme enhances the suppressive capacity of T_{reg} cells and permits expression of tissue-specific homing receptors, for example to the gut, where they occupy an immunoregulatory niche to prevent or ameliorate inflammation in response to the microbiota or initiate wound healing.

BCL-6

BCL-6, a zinc finger TF, is essential for germinal centre formation and is critical both for germinal centre B cells and as the lineage-specifying TF of T_{FH} cells[267-270]. It is also expressed in a subset of T_{reg} cells found within germinal centres, known as T_{FR} cells, which share characteristics with T_{FH} cells, including expression of the chemokine receptor CXCR5. The entry of T_{FR} cells into B cell follicles is facilitated by CXCR5. As discussed above, expression of BLIMP1 is a cardinal feature of T_{FR} cells. Despite being normally repressed by BLIMP1, the presence of BCL-6, together with BLIMP1 and NFATc1–NFATαA complex allows for CXCR5 expression to occur in these cells[271] (Table 2). T_{FR} cells are absent in the thymus but induced in the periphery mostly from pre-existing T_{reg} cells (rather than naive conventional T cells)[272,273]. Under some circumstances, T_{FR} cells can also be induced from FOXP3$^-$ precursors[274]. Their function is to regulate germinal centre reactions to ensure dominance of antigen-specific B cell clones over self-reactive clones that may cause autoimmunity[272,273,275,276]. Indeed, mice with genetic ablation of *Bcl6* in T_{reg} cells (*Bcl6*$^{fl/fl}$*Foxp3*^{Cre}) clear viral infections more effectively but develop spontaneous antibody-mediated autoimmunity[276]. Likewise, abnormalities in T_{FR} cell proportions have been linked to antibody-mediated diseases in humans[277]. T_{FR} cells are induced through some of the same developmental cues as T_{FH} cells, including SLAM-associated protein-mediated signals[272], but not through the same cytokines that

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induce BCL-6 in T_{FH}– cells (IL-6 and IL-21) [273]. In humans, T_{FR} cells in the circulation are less mature and distinct from those in tissues, possibly representing spill-over from previous immunizations[275,278]. Thus, BCL-6 is a cardinal feature of T_{FR} cells, which enter germinal centres via BCL-6-encoded CXCR5 and ensure dominance of antigen-specific B cell clones over self-reactive clones and prevent autoimmunity.

Concluding remarks

FOXP3$^+$ T_{reg} cells play a crucial role in maintaining peripheral tolerance by suppressing other immune cells. FOXP3 is clearly the non-redundant and key regulator of T_{reg} cell development and function. However, only a proportion of the T_{reg} cell-specific transcriptome can be directly attributed to areas that are bound by FOXP3 itself. This aligns with a modular framework for describing the T_{reg} cell lifecycle in which specific functions are added to the essential FOXP3 programme by multiple accessory TFs to imprint and maintain the T_{reg} cell phenotype and tissue-specific functions. Many of these are non-redundant, thus result in T_{reg} cell deficiency when genetically ablated in mice. Some of these may represent normal responses to maintain tolerance to the microbiota and may potentially be abnormally appropriated to participate in the pathogenesis of pathogenic organisms or immunological evasion by cancers. It is anticipated that knowledge of T_{reg} cell biology will expand with the help of high-throughput methods and screening technologies that can assess multiple TFs simultaneously and ranging from the single cell to the tissue level. Likewise, future studies and robust markers categorically distinguishing tT_{reg} cells from pT_{reg} cells may enable closer examination of the role of transcriptional regulators in T_{reg} cells induced from precursors at different sites. A deeper understanding of T_{reg} cells will bring insights into the basic mechanisms of disease and pave the way for next-generation therapies for autoimmunity and transplant rejection.

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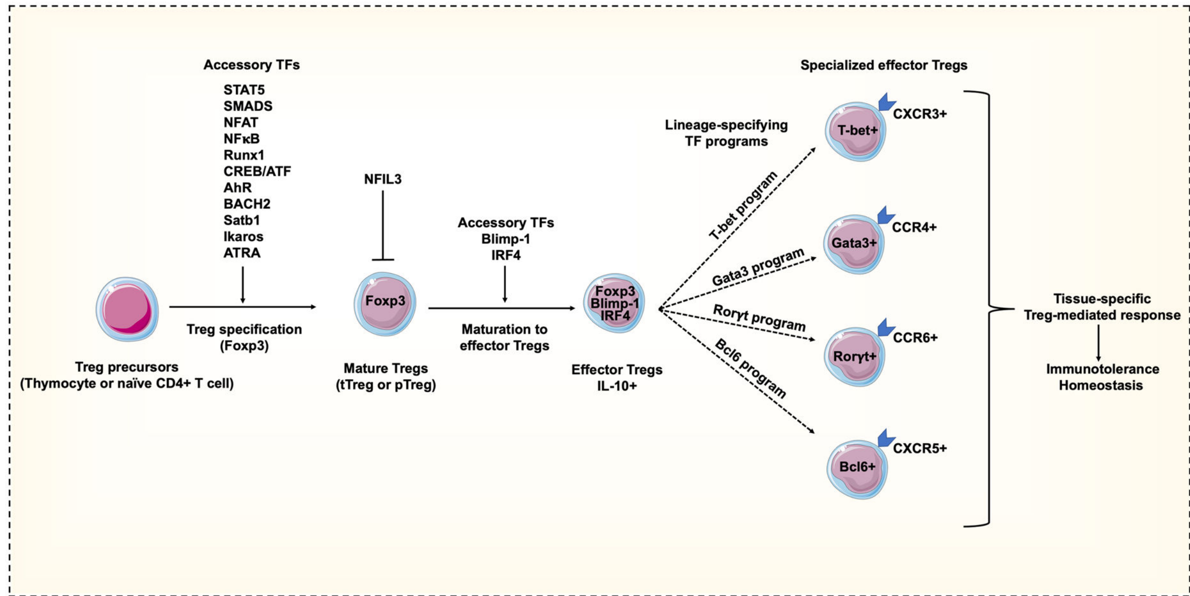

Figure 1. Accessory transcription factors in regulatory T cell specification and maturation.
Thymocytes or naive $CD4^+$ T cells differentiate into regulatory T (T_{reg}) cells following T cell receptor (TCR) engagement within microenvironments rich in T_{reg} cell-inducing soluble factors, such as interleukin-2 (IL-2) and transforming growth factor-β (TGFβ). The coordinated integration of multiple accessory transcription factors (TFs) drive T_{reg} cell specification and epigenetic changes (such as at the T_{reg} cell-specific demethylated region (TSDR)) that are indispensable for stable expression of forkhead box protein 3 (FOXP3). T_{reg} cell maturation to effector T_{reg} cells is driven by FOXP3-dependent and FOXP3-independent accessory TFs that induce FOXP3 expression and enhance production of effector (suppressive) cytokines. Some TFs, such as NFIL3, have the ability to repress FOXP3 expression. Mature effector T_{reg} cells can also be induced to express additional accessory programmes driven by TFs usually associated with lineage-specification in conventional T cells. These shape the unique features of specialized subpopulations of T_{reg} cells, such as tissue homing.

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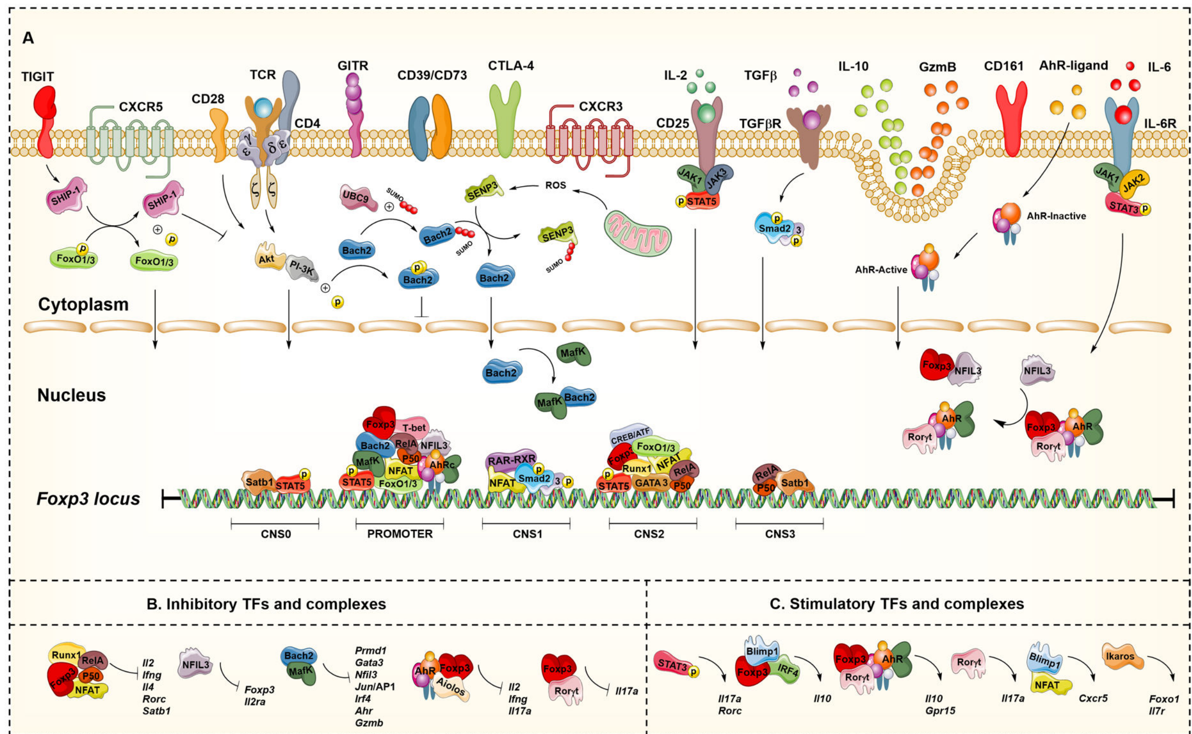

Figure 2. The coordinated network of accessory and lineage-specifying transcription factors regulating FOXP3 expression.

Engagement of T cell receptors (TCRs) by antigen-presenting cells (APCs) through the MHC class II-antigen complex, signalling of interleukin-2 (IL-2) via the CD25-STAT5 module, and activation of canonical transforming growth factor-β (TGFβ)-dependent SMAD pathways all work together to promote the differentiation of regulatory T cells (T_{reg} cells) and expression of forkhead box protein 3 (FOXP3). Other signals, such as cytokines and endogenous chemical compounds present in the environment, are detected by specific cell-surface receptors and transcription factors, such as AHR and RORγt. These signals are integrated along with additional transcriptional regulators (such as BACH2, MAF, Ikaros, Aiolos, RUNX1, FOXO1, SATB1 and NFIL3) at conserved noncoding sequence (CNS) regions of *FOXP3*. Together, these regulate expression of FOXP3 and the intracellular mechanisms required to activate T_{reg} cell suppressive functions through cell contact or soluble factors. These transcriptional regulators also form stimulatory and inhibitory complexes that regulate genes involved in the maintenance of hallmark and specialized genes expressed by T_{reg} cells, as summarized in Table 2.

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Table 1 |

Accessory and lineage-specifying transcription factors that shape regulatory T cell phenotype

Transcription factor	Transcription factor family	Function in T_{reg} cells	Location
FOXP3	FOX protein family	Development and function	Expressed in $CD4^{+}$ T_{reg} cells
AHR	Class I basic helix-loop-helix transcriptional regulator	FOXP3 agonist in development and enhanced suppressive function (IL-10, GZMB and homing receptors)	Organ specific (central nervous system, gut-associated lymphoid tissue)
BACH2	Basic leucine zipper transcriptional regulator	FOXP3 agonist in development, function, maintenance of steady state and suppressor of pro-inflammatory genes	Expressed in $CD4^{+}$ T_{reg} cells
SATB1	CUT homeobox factor	FOXP3 expression in early developmental stages but antagonist in mature T_{reg} cells	Expressed mostly in T_{reg} cell precursors
BCL-11B *	Zinc finger domain protein	FOXP3 agonist in T_{reg} cells; inter-dependent function with FOXP3 in T_{reg} cells	Expressed in $CD4^{+}$ T_{reg} cells
Ikaros	Zinc finger domain protein	Development and differentiation of in vitro-induced T_{reg} cells	Expressed in $CD4^{+}$ T_{reg} cells
IRF4	Interferon regulatory factor	Generation of effector T_{reg} cells; synergism with FOXP3 and BLIMP1 to transactivate *IL10*	Mucosa and visceral adipose tissue
BLIMP1	Zinc finger domain protein	Required for optimal function of effector T_{reg} cells; synergism with FOXP3 and IRF4 to transactivate *IL10*, *GZMB* and suppress IL-17 production	Organs (kidney, pancreas, lung, central nervous system) and gut-associated lymphoid tissue
BATF	Basic leucine zipper transcriptional regulator	Development of non-lymphoid T_{reg} cell precursors; growth and sustainability of tissue T_{reg} cells	Non-lymphoid T_{reg} cell precursors; tissue T_{reg} cells
NFIL3 *	Basic leucine zipper transcriptional regulator	FOXP3 antagonist; directly binds the *FOXP3* locus as well as FOXP3 protein	Inducible during chronic infections
T-bet	Nuclear receptor family	Enhances suppressive capacity and expression of tissue-specific homing receptors for sites of $T_{H}1$-type inflammation	Organs (central nervous system, pancreas) and gut-associated lymphoid tissue
GATA3	Nuclear receptor family	Enhanced suppressive capacity with expression of tissue-specific homing receptors	Organs (skin, kidney) and gut-associated lymphoid tissue
RORγt	Nuclear receptor family	Enhanced suppressive capacity and expression of tissue-specific homing receptors	Gut-associated lymphoid tissue
BCL-6	Zinc finger domain protein	Expression of homing receptors for germinal centres; regulation of germinal centre reactions	Germinal centre T_{reg} cells

* Transcription factors for which comparable human data are limited or not available. For all other transcription factors, there is functional evidence in both human and mouse T_{reg} cells.

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Table 2 |

Inhibitory and stimulatory transcription factors and complexes in regulatory T cells

TFs and TF complexes	Genes regulated
Inhibitory TFs and complexes	
AHR–FOXP3–Aiolos	*IL2, IFNG, IL17A*
BACH2–MAFK	*PRMD1, GATA3, NFIL3, JUN–AP-1, IRF4, AHR, GZMB*
FOXP3–RORγt	*IL17A*
FOXP3–RUNX1–RELA–p50–NFAT	*IL2, IFNG, IL4, RORC, SATB1*
NFIL3	*FOXP3, IL2RA*
Stimulatory TFs and complexes	
BATF	*IL10, CTLA4, TIGIT, TNFRSF4, TNFRSF9, IL1RL1*
BCL11B	*IL10, FOXP3*
BLIMP1–BCL-6–NFAT	*CXCR5*
BLIMP1–FOXP3–IRF4	*IL10*
Ikaros	*IL7R, FOXO1*
FOXP3	*BATF*
FOXP3–RORγt–AHR	*IL10, GPR15*
RORγt	*IL17A*
STAT3	*IL17A, RORC*

During regulatory T cell differentiation, transcriptional interactions result in the transient assembly of both inhibitory and stimulatory transcription factors (TFs) and complexes, which independently or in conjunction with FOXP3, repress or induce the expression of genes involved in the maintenance of hallmark regulatory T cell genes and specialized function within tissues.