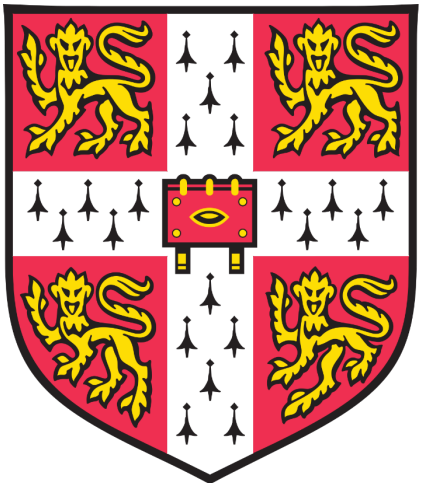
Mechanisms of CD4$^+$ Regulatory T Cell Heterogeneity in Health and Disease



By Francis Matthew Grant

Darwin College and the Babraham Institute

University of Cambridge

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee. The research in this thesis was carried out under the supervision of Dr Rahul Roychoudhuri at The Babraham Institute, Department of Lymphocyte Signalling and Development, UK, between October 2015 and July 2019.

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Summary

Regulatory T (Treg) cells are central to the maintenance of immune homeostasis and their dysfunction underlies the pathology of numerous diseases. Treg cell populations are phenotypically heterogeneous, comprising functionally quiescent resting Treg (rTreg) cells, which upon antigen stimulation, differentiate into functionally activated Treg (aTreg) cells. The purpose of rTreg cell populations and how their naïve-like phenotype is maintained despite chronic exposure to cognate self- and foreign antigens remains to be understood. The transcription factor BACH2 is critical for early Treg cell lineage specification, however, its function following Treg lineage-commitment is unresolved. The studies detailed herein demonstrate that *Bach2* is highly expressed during Treg cell development in the thymus. High levels of *Bach2* are maintained in post-thymic, lineage-committed rTreg cells but is downregulated in aTreg cells, and upon inflammation. Functionally, BACH2 acts to restrain T cell receptor (TCR)-driven activation in rTreg cells and constrain their differentiation into aTreg cells. Cell-autonomous expression of BACH2 is required following Treg cell lineage-commitment for functional quiescence and long-term maintenance of Treg cell populations. This is necessary for the restraint of excessive memory differentiation and IFN-γ production by $CD8^+$ T cells. Therefore, in lineage-committed Treg cells, BACH2-mediated restraint of aTreg cell differentiation is required for the maintenance of immune homeostasis. These findings deepen our understanding of Treg cell biology and extend our knowledge of the function of the transcription factor BACH2 in lymphocytes.

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- Babraham Institute Flow Cytometry Facility: flow cytometry and FACS.
- Babraham Institute Bioinformatics Department: bioinformatics analyses.

2. Data obtained from technical service providers

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- Babraham Institute Flow Cytometry Facility: FACS.
- Babraham Institute Bioinformatics Department: computational biology analyses.
- Babraham Institute BSU Transgenics: breeding and care of mouse strains.
- Babraham Institute BSU Experimental and Import unit: injections, irradiations and drawing of blood samples.
- Cancer Research UK Cambridge Institute Genomics Core: single-cell RNA-Sequencing data.

3. Data produced or analysed jointly

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List of Publications

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Nasrallah, R. and **Grant, F.M.**, *et al*. The 11q13.5 immune disease risk locus contains a distal enhancer required for Treg-mediated suppression of gut inflammation. *Under review at Nature*.

Gyori, D., Lim, E.L., and **Grant, F.M.**, *et al*. Compensation between $CSF1R^+$ macrophages and $Foxp3^+$ Treg cells drives resistance to tumor immunotherapy. JCI Insight. 2018 Jun 7;3(11).

Roychoudhuri, R., *et al*. The transcription factor BACH2 promotes tumor immunosuppression. J Clin Invest. 2016 Feb;126(2):599-604.

Abbreviations

APC	Antigen presenting cell
B6	Black 6
BAT	Brown adipose tissue
BCR	B cell receptor
BFA	Brefeldin A
BM	Bone marrow
BRIDA	BACH2-related immunodeficiency and autoimmunity
CKO	Conditional knockout
DMEM	Dulbecco's Modified Eagle's Medium
DP	Double-positive
FAP	Fibro/adipogenic progenitor
GFP	Green fluorescent protein
IFN	Interferon
IL	Interleukin
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
LN	Lymph node
MHC	Major histocompatibility complex
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-time polymerase chain reaction

SNP	Single nucleotide polymorphism
TCR	T cell receptor
tdRFP	Tandem red fluorescent protein
TF	Transcription factor
TGF-β	Transforming growth factor beta
TNF	Tumour necrosis factor
UMI	Unique molecular identifier
WAT	White adipose tissue
WT	Wild type

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1 Introduction

1.1 Adaptive immune responses and T cell immunity

The mammalian immune system comprises a repertoire of molecules, cells, barriers and organs that defend the host against diverse threats. All cellular organisms have innate mechanisms of self-defence. However, the evolution of adaptive immune mechanisms in jawed fish (the gnathostomes), around 500 million years ago, marked the beginning of acquired protection with specificity for a given threat (reviewed in Cooper & Alder, 2006, and Flajnik & Kasahara, 2010). Antigen specificity is a hallmark of adaptive immunity, which results from the construction of cellular receptors by rearranging parts of the genome. The two major lineages of adaptive immune cells are named according to the tissue where these genetic rearrangements occur: B lymphocytes or B cells develop in the bone marrow (or avian bursa of Fabricius), whereas T lymphocytes or T cells develop in the thymus (Cooper, Peterson, & Good, 1965). In order to construct a functional T cell receptor (TCR), T cells must undergo a selection process in the thymus. Indeed, of all haematopoietic progenitor cells that migrate to the thymus for development, 95–97% will cease to undergo further maturation and instead undergo death by neglect (Surh and Sprent 1994). The remaining 3–5% of mature thymocytes that join the peripheral T cell pool are those tasked with defence of the host.

The enormity of threats in nature is matched by an equally enormous diversity in TCR specificities. This is achieved by the combinatorial diversity of the V and J gene segments at the TCR α locus; the V, D and J gene segments at the TCR β locus; the variable nucleotide sequence changes at gene segment junctions that accompany the rearrangement process; and the combinatorial diversity introduced by pairing different α and β TCR chains. Theoretical models calculate that the human genome is capable of generating over a quadrillion (10^{15})

different TCRs (reviewed in Nikolich-Žugich, Slifka, & Messaoudi, 2004). The actual observed diversity however, is estimated at approximately 100 million (10^8) (Arstila 1999). Even between genetically redundant inbred mouse strains, over 75% of TCR-β sequences can be unique (Bousso et al. 1998). This astounding capacity for receptor heterogeneity enables T cells to recognize an astoundingly large number of peptide sequences not encoded within the host genome. Furthermore, the aforementioned estimates do not consider other T cell subgroups, such as those that construct their TCR from the γ and δ loci. The eradication of exogenous threats however, relies not just upon recognition. T cells also must have a means to overcome the multitudinous defence mechanisms presented by the pathogen itself.

After construction of the α:β TCR within the thymus, T cells begin their development toward functionally divergent lineages. Here, expression of either the CD4 or CD8 co-receptors are a phenotypic marker of these divergent lineages. $CD4^+$ T 'helper' cells generally orchestrate adaptive immunity via juxtacrine and cytokine signalling. $CD8^+$ 'cytotoxic' T cells are classically described by their capacity destroy targeted cells through direct cellular cytotoxicity, however, much of their function can also be attributed to cytokine release (Kelso et al. 1991). During thymic development, selection to either lineage begins with expression of both CD4 and CD8 co-receptors simultaneously. These double-positive (DP) thymocytes eventually downregulate one of the two co-receptors permanently. DP thymocytes with TCRs that recognize peptide complexed with major histocompatibility complex (MHC) class I on the surface of antigen-presenting cells (APC) develop toward CD8 single-positive (SP) thymocytes, whereas those recognizing peptide complexed with MHC class II develop toward CD4 SP thymocytes. $CD4^+$ thymocytes then undergo further development, either toward functionally pro- or anti-inflammatory subgroups. Here, a small population of CD4 SP thymocytes bearing TCRs specific for host-encoded molecules

upregulate the transcription factor Foxp3. These so-called regulatory T (Treg) cells fulfil immunosuppressive functions and balance immune responses in peripheral tissues (Sakaguchi *et al.* 1995; reviewed in Hsieh, Lee and Lio 2012; Josefowicz, Lu and Rudensky 2012). Mature $CD4^+$ or $CD8^+$ T cells thus emerge from the thymus to join the peripheral lymphocyte pools. However, selection to either the $CD4^+$ T helper cell, $CD4^+$ Treg cell or $CD8^+$ cytotoxic T cell lineage is not enough functional diversity for T cells to adequately combat pathogens confronted by the host. Yet more functional divergence arises post-thymically, in relation to the threat encountered.

1.2 T cell differentiation and effector function is controlled by transcription factors

T cell differentiation requires distinct gene modules to either be expressed or suppressed, thus much attention has been drawn toward the actions of transcription factors (TFs) in recent decades. TFs bind DNA and regulate gene expression, thus are fundamental in determining cellular fate and identity. During thymic development, the transition of DP thymocytes to $CD8^+$ SP thymocytes is primarily dependent upon the functions of the Runx family of TFs (Taniuchi et al. 2002), whereas transition toward $CD4^+$ SP thymocytes relies on the actions of Th-POK and GATA-3 (He et al. 2005; Pai et al. 2003). Post-thymic $CD4^+$ helper T cells undergo further differentiation into functionally distinct subsets. Defence against threats that mediate their pathogenicity intracellularly within the host, such as viruses, stimulate cells of the innate immune system to release the cytokines IFN-γ and IL-12. This in turn, leads to the expression of the TF T-bet within helper T cells and their differentiation into type 1 T helper (Th1) cells (Mosmann et al. 1986; Wenner et al. 1996; Szabo et al. 2000). Commitment toward Th1 cells endows $CD4^+$ T cells with the capacity to counter intracellular pathogens by releasing the cytokines IFN-γ, TNF-α and IL-2. In response, host cells increase antigen processing and production of MHC, B cells become activated and begin secreting IgG2a and

IgG3, and phagocytes become primed to endocytose pathogenic host cells (reviewed in Zhu, Yamane, & Paul, 2010). Similar paradigms exist during differentiation of other helper T cell subsets. Immune responses against extracellular parasites involves differentiation of type 2 T helper (Th2) cells, which requires expression of the TF GATA-3. Defence against extracellular bacteria and fungi at mucosal tissue sites requires type 17 T helper (Th17) cells, which express RORγt (Conti et al. 2009). Therefore, TFs such as T-bet, GATA-3 and RORγt, which are critical for differentiation and function of specific T cell subsets, are often termed 'lineage-specific' or 'lineage-defining' transcription factors (Vaquerizas et al. 2009; Rengarajan, Szabo, and Glimcher 2000).

1.3 Immune homeostasis is dependent upon Foxp3-expressing Treg cells

The lineage-defining TF for Treg cells is Foxp3. In contrast to inflammatory helper T cells, T cell populations bearing immunosuppressive function in mouse express high levels of Foxp3. Failure to express functional Foxp3—as seen in the Scurfy mouse strain and the human disease, IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome—manifests in severe multi-organ autoimmune pathology (Brunkow et al. 2001; Ochs et al. 2001). Naïve $CD4^+$ $CD25^-$ T cells retrovirally transduced with the *Foxp3* gene exhibit suppressor activity (Hori, Nomura, and Sakaguchi 2003; Fontenot, Gavin, and Rudensky 2003). Ectopic expression of *Foxp3* in cytotoxic CD8 T cells similarly induces suppressor function (Khattri et al. 2003). Attenuation of Foxp3 expression in Treg cells results in a reduced ability to constrain autoimmunity (Wan and Flavell 2007). Furthermore, conditional deletion of the *Foxp3* gene in Treg cells using the Cre-lox system results in their acquisition of a Th1-like phenotype, with the capacity to produce IL-2 (Williams and Rudensky 2007). These studies define the absolute requirement of Foxp3 for lineage commitment and immunosuppressive function of Treg cells.

1.3.1 Thymic and peripheral Foxp3 expression

As immunosuppressive Foxp3$^+$ Treg cells can originate either at the CD4 SP stage during thymic development, or in the periphery from naïve CD4$^+$ T cells, the terms thymic Treg (tTreg) cell and peripheral Treg (pTreg) cell are used for clarity (Figure 1.1). The term 'induced' Treg (iTreg) cell is used to describe Treg cells generated *in vitro* by the culture of naïve CD4$^+$ T cells with TGF-β (reviewed in Shevach and Thornton 2014).

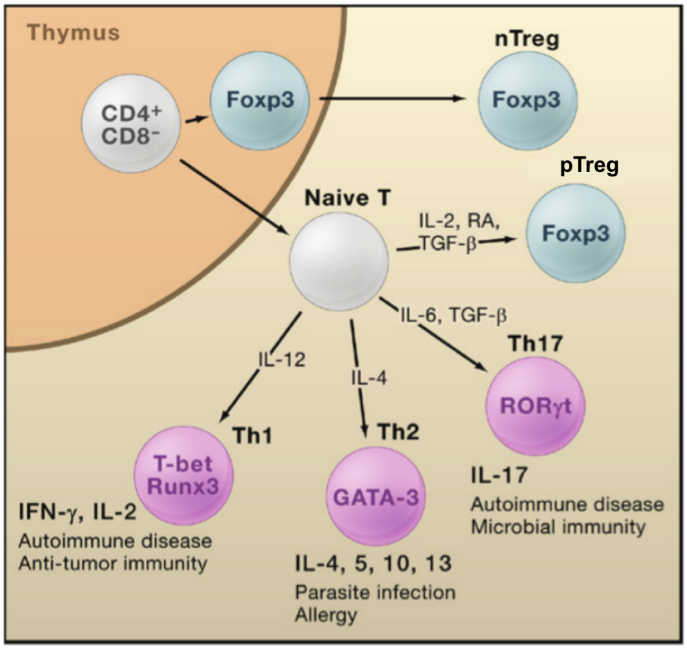

Figure 1.1: Transcription factors guide T cell differentiation toward functionally distinct subsets.
Figure is adapted from Sakaguchi *et al.* 2008.

Although the mechanisms that govern lineage-commitment and differentiation of tTreg cells are still not fully understood, a number of significant factors involved in this process are known. CD4 SP thymocytes with TCR bearing intermediate- to high-strength affinity for self-antigen, appear to be predisposed for tTreg cell differentiation. The first suggestion that TCR specificity played a role in instructing Treg cell fate came from the observation that

tTreg cells are absent from mice bearing the transgenic DO11.10 TCR, which recognizes chicken ovalbumin (OVA) (Itoh et al. 1999). A seminal publication from Jordon *et al*., then showed that TCR bearing high affinity self-reactivity was a critical determinant for tTreg cell fate specification (Jordan et al. 2001). One model proposes that the combined strength of interaction between the antigen receptors and multiple peptide-MHC complexes on the antigen-presenting cell (APC) drives CD25 expression. Thymocytes bearing upregulated CD25 thus become more sensitive to IL-2 signalling, which in turn facilitates induction of Foxp3 and instructs Treg cell fate commitment (Figure 1.2) (Burchill et al. 2008; Lio and Hsieh 2008). In addition, IL-7 and IL-15 signalling is known to be involved (Vang et al. 2008).

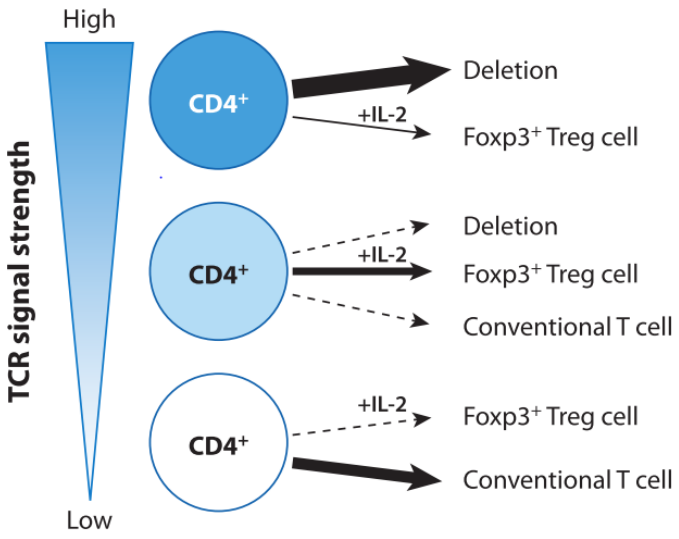

Figure 1.2: The differentiation of CD4 SP thymocytes toward Treg cell fate is guided by TCR signal strength.

Figure adapted from Josefowicz, Lu and Rudensky 2012.

Outside of the thymus, mature $CD4^+$ $Foxp3^-$ conventional T (Tconv) cells have the capacity to upregulate Foxp3 expression and differentiate into Treg cells in the presence of TGF-β (Chen et al. 2003; Apostolou and von Boehmer 2004). To distinguish their origin from Treg

cells produced in the thymus (tTreg), those that differentiate peripherally from $CD4^+$ $Foxp3^-$ Tconv cells are thus referred to as pTreg cells. By virtue of their self-antigen specific TCR, tTreg cells are essential for maintaining systemic tolerance for host antigen. However, to prevent unwarranted immune responses directed toward foreign antigens encountered, tolerance is also required for antigens that the host is exposed to in the environment. To this end, upon their discovery, pTreg were hypothesized to have evolved to fulfil this function as a regulator of immune responses toward non-self antigen. Indeed, results from numerous studies have since been found to support this theory. In an experimental model using hyper-IgE asthmatic mice lacking thymically-derived Treg cells, the feeding of oral OVA antigen was found to induce mucosal pTreg populations (Mucida et al. 2005). Subsequently, a reduction in the severity of asthmatic responses was observed in these mice. Verginis *et al*. were able to induce the differentiation of pTreg cells in female mice, specific for antigen from male mice, by exposure of these female mice to Y chromosome-encoded transplantation antigens (HY) (Verginis et al. 2008). Consequently, the female mice developed long-term tolerance to HY antigen and were consequently better able to sustain male-derived skin grafts. In the BALB/c *Foxp3*^{K276X} mouse strain, which are devoid of any Treg cells, systemic multi-organ inflammatory pathology was only prevented upon adoptive cell transfer of tTreg cells and Tconv cells (Haribhai et al. 2011). The donor Tconv cells were found to give rise to a population of pTreg cells upon transfer, and alternatively, when donor *Foxp3*$^{\Delta EGFP}$ Tconv cells—which express non-functional Foxp3—were transferred, the rescue of complete immune homeostasis was abrogated. In addition, upon comparison of the TCR sequences from tTreg and pTreg cells in the host mice, distinct TCR specificities were observed. These findings strongly suggest an important function for pTreg in immune homeostasis —non-redundant with that of tTreg—and particularly relevant in mucosal tissue, which is most frequently exposed to environmental antigen. Indeed, pTreg fulfil critical immunoregulatory

roles in the gut and other mucosal sites, which are discussed at greater depth later (section 1.5.4).

Given that Treg cells can arise from two distinct sources, studies were conducted to try and identify distinct molecular markers that could discriminate between tTreg and pTreg cells *in vivo*. The first attempts at this by Shevach and colleagues suggested that expression of the transcription factor, Helios, might be restricted to Treg cells of thymic origin (Thornton et al. 2010). They observed that all Foxp3^{+} CD4SP thymocytes were positive for Helios, and upon their development and emigration to the periphery, approximately 70% of tTreg cells retain Helios expression. In addition, Foxp3^{-} Tconv cells stimulated *in vitro* to differentiate into iTreg cells—using anti-CD3 and anti-CD28 antibodies, in the presence of TGF-β—maintained low expression of Helios. However, subsequent studies found that use of antigen-loaded APCs in iTreg differentiation assays, as opposed to anti-CD3 and anti-CD28 antibodies, results in the induction of Helios in cultured cells (Gottschalk, Corse, and Allison 2012). Furthermore, *in vivo* pTreg cell induction via intraperitoneal administration of antigen similarly resulted in Helios expression. Therefore, expression of Helios by Treg cells appears to be context-dependant and not indicative of their developmental origin (reviewed in (Shevach and Thornton 2014). Another marker suggested to demarcate tTreg cells, is the non-tyrosine kinase receptor, Neuropilin-1 (Nrp1). Foxp3^{+} CD4SP thymocytes were found to express Nrp1 at high levels, whereas the induction of pTreg *in vivo* under certain conditions did not result in Nrp1 expression (Weiss et al. 2012; Yadav et al. 2012). In models of *in vivo* pTreg induction, including proliferation in lymphopenic mice, microbiota-induced differentiation and antigen administration, Nrp1 expression remained low. However, models that induce inflammation, such as experimental autoimmune encephalomyelitis (EAE) and chronic asthma, did cause Nrp1 upregulation in peripherally-induced Treg cells. As paracrine

TGF-β signalling is involved in pTreg cell generation, and the expression of Nrp1 itself is regulated by TGF-β, many are reluctant to use Nrp1 as a tTreg marker. The authors of these studies thus concluded that under non-inflammatory conditions, Nrp1 can be used to identify tTreg cells.

1.3.2 Foxp3 expression in humans

Though Foxp3 expression is considered to be the definitive marker of Treg cells in mice, this does not appear to be true for humans. Although Foxp3 expression is required for the Treg cell phenotype and their appropriate restraint of inflammation—as evidenced in the disease, IPEX—the expression of Foxp3 alone is not sufficient to categorize a T cell as being suppressive. TCR stimulation of human $CD4^+$ $Foxp3^-$ Tconv cells was found to produce populations of T cells expressing Foxp3 (Walker et al. 2003), suggesting that Foxp3 can be induced following activation. Importantly, this Foxp3 upregulation did not confer suppressive functions (Wang et al. 2007; Allan et al. 2007). These studies identified that, in contrast to mice, human T cells have the propensity to upregulate Foxp3 transiently upon activation. Furthermore, the relative expression of Foxp3 also cannot account for the Treg cell phenotype, as human $Foxp3^+$ T cells with high levels of Foxp3 expression still have the capacity to secrete inflammatory cytokines (Tran, Ramsey, and Shevach 2007). Therefore, Foxp3 cannot be considered an exclusive marker of Treg cells in human. Further studies will shed light on the additional molecular mechanisms that determine human Treg cell identity. Some of these likely factors are discussed below.

1.3.3 The role of Foxp3 in thymic Treg cell development

Though Foxp3 expression is absolutely required for Treg cell function, its role in tTreg cell lineage commitment is reliant upon other factors. Studies utilizing a transgenic mouse strain

with non-functional *Foxp3*$^{\Delta EGFP}$ allele, found that the characteristic Treg cell transcriptional signature and cell surface phenotype is mostly preserved in the absence of Foxp3 (Lin et al. 2007). Here, gene expression microarrays from male *Foxp3*$^{\Delta EGFP}$ (non-functional Foxp3) and *Foxp3*^{EGFP} (functional Foxp3) revealed that EGFP^{+} cells from both genotypes were able to express signature Treg genes, such as: *Il2ra, Ctla4, Itgae, Cd83, Gpr83, Gzmb, Ikzf2, Icos, Klrg1, S100a4, S100a6 and Tnfrsf9*. This work was expanded upon by Samstein, *et al.*, who showed that chromatin at signature Treg cell loci was largely accessible prior to Foxp3 expression. Instead of directly shaping the chromatin landscape, Foxp3 was found to bind pre-established enhancers, which were created by TCR signalling events (Samstein, Arvey, et al. 2012). This led to the hypothesis that co-ordinated expression or repression of a network of TFs is required for Treg cell lineage-commitment. A number of important factors, which act in a combinatorial manner, have since been identified. These include: Foxo1, Helios, Xbp1, Eos, IRF4, Satb1, Lef1 and GATA-1 (Ouyang et al. 2012; Samstein, Arvey, et al. 2012; Fu et al. 2012). These results suggest that several factors act collectively as pioneer factors to determine Treg cell fate in developing thymocytes, then late-acting TFs like Foxp3 define identity and fully establish functionality by interacting with pre-formed enhancers.

1.4 Mechanisms of regulatory T cell-mediated suppression of inflammatory responses

The specific mechanisms of Treg cell immunosuppression have been a matter of intense exploration over the last two decades. The perplexing array of immunosuppressive mechanisms exerted by Treg cells *in vitro* and *in vivo* has resulted in considerable discussion as to how, exactly, their immunodominance is achieved. Such findings do, however, collectively suggest that Treg cells have a plethora of immunoregulatory mechanisms, which are employed depending on the particular tissue and inflammatory setting (reviewed in Vignali, Collison and Workman 2008). Treg cells were initially distinguished phenotypically

by their high expression of the IL-2 receptor alpha chain (IL-Rα), or CD25 (Sakaguchi et al. 1995). IL-2 represents a prominent lymphocyte growth factor, which when competitively depleted from the extracellular environment by Treg cells expressing high affinity IL-2 receptors, acts non-specifically to prevent paracrine signalling and counter effector lymphocyte expansion (Fontenot et al. 2005; Pandiyan et al. 2007). Constitutive high expression of cell surface cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, or CD152) is also a notable feature of Treg cells (Read, Malmström, and Powrie 2000). CTLA-4 binds to CD80 and CD86 on antigen presenting cells (APCs) with higher affinity than CD28. This competitive sequestering of an important T cell co-stimulatory ligand acts to reduce the capacity of APCs to activate T cells (Wing et al. 2008). Furthermore, binding of CTLA-4 to CD80 or CD86 is also known to initiate their trans-endocytosis to the cytosol of Treg cells, where they are subsequently degraded (Qureshi et al. 2011). Pro-inflammatory effector T (Teff) cell function can be suppressed via the adenosine receptor 2A ($A2_AR$). Treg cells directly contribute to production of pericellular adenosine by expressing the ectoenzymes CD39 and CD73, which catabolize ATP to ADP and AMP (Kobie et al. 2006). In addition, $A2_AR$ signalling inhibits IL-6 secretion by T cells, whilst promoting TGF-β production, thus establishing an environment supportive of pTreg cell differentiation and antagonistic to Th17 cell development (Zarek et al. 2008). Treg cells are prominent producers of the anti-inflammatory cytokine IL-10, which not only acts to suppress proliferation of Th1 and Th2 T cell subsets, but also acts in an autocrine manner to support Treg cell proliferation and function (Del Prete et al. 1993; Rubtsov et al. 2008). Treg cells also secrete TGF-β, which in the context of immunosuppression, directly restrains proliferation of B and T cells (Kehrl, Roberts, et al. 1986; Kehrl, Wakefield, et al. 1986). Though classically considered to be a property of NK and $CD8^+$ T cells alone, cytotoxic mechanisms are also employed by Treg cells, where expression of molecules such as perforin and granzyme B are utilized to directly

induce cell death in Teff cells (Cao et al. 2007). Thus conceptually, the immunosuppressive mechanisms exhibited by Treg cells can be broadly categorised as those that attenuate inflammation via targeting of other T cell subsets (e.g, environmental IL-2 depletion, cytotoxicity, and the suppressor cytokines IL-10 and TGF-β) or those that target antigen-presenting cells (CTLA-4, CD39 and CD73). The described mechanisms here only represent a brief summary of the Treg immunosuppressive repertoire of functions, which are further explored with relevance to health and disease (section 1.5) and in the context of Treg cell phenotypic heterogeneity (section 1.6).

1.5 Immune homeostasis in health and disease

As Treg cells fulfil a dominant role in balancing immune responses across the entire body, it is perhaps unsurprising that they are essential for maintaining homeostasis within numerous tissue types. Indeed, their dysfunction associates with a diversity of diseases. Here, their influence lies upon a dichotomous pathological spectrum. At one end, excessive restraint of effector immune cells results in a failure of the immune system to launch a robust defence. Conversely, defective restraint of effector immune cells results in exacerbated responses of the immune system toward antigen.

1.5.1 Autoimmune and inflammatory disease

The observation that mutations rendering Foxp3 non-functional lead to severe multi-organ autoimmune disease in humans and mice (described in section 1.3) led to an intense exploration into the association between Treg cell dysfunction and common autoimmune and inflammatory diseases. Such studies have been confounded in part by the complexities of Treg cells and by autoimmune disease. With regard to Treg cells: standard markers for lineage-committed Treg cells are less reliable in humans (described in section 1.3); the

proportion or absolute number of Treg cells—a parameter measured in many studies—is not strictly proportional to the capacity of Treg cells to restrain inflammation; Treg cells possess numerous mechanisms of immunosuppression (described in section 1.4), which can be difficult to ascertain from *ex vivo* studies; different subsets exist within the Treg cell lineage and these each contribute functionally in different contexts (described in section 1.6). With regard to autoimmune disease: various environmental triggers and genetic factors contribute to disease susceptibility; tissue biopsies of pathological sites used for analysis can be invasive, or practically challenging, which has led mostly to patient blood (a non-pathological tissue site) being used for analysis; and mechanisms underlying the pathology of certain diseases may vary among patients. Despite these complexities, functional defects in Treg cells have been observed in autoimmune diseases, these include: multiple sclerosis (Viglietta et al. 2004), polyglandular syndrome type II (Kriegel et al. 2004), rheumatoid arthritis (Ehrenstein et al. 2004), type I diabetes (Lindley et al. 2005), psoriasis (Sugiyama et al. 2005) and myasthenia gravis (Balandina et al. 2005). In addition to the aforementioned autoimmune diseases, defective Treg function has been observed in atopic humans. Indeed, circulating Treg cells from atopic individuals were found defective in their ability to suppress Th2-type immune responses against pollen allergens (Ling et al. 2004; Grindebacke et al. 2004).

1.5.2 Cancer

Perhaps the most predominant association between Treg cells and disease—particularly within the non-specialist and non-scientific communities—involves neoplastic disease. This is especially true given the recent Nobel Prize in physiology or medicine award to immunologists Jim Allison and Tasuku Honjo, who jointly pioneered the cancer immunotherapy field by elucidating the mechanisms of negative immune regulation (or

immune checkpoint inhibition). The association between Treg cells and tumour immunity was first identified from the observation that mice treated with an anti-CD25 antibody, which causes systemic Treg cell depletion, are more resistant to tumour growth in the B16 melanoma model (Shimizu, Yamazaki, and Sakaguchi 1999). This antibody-mediated resistance was further shown in models of leukaemia, myeloma and sarcoma (Onizuka et al. 1999). Accompanying these studies were the seminal contributions from the laboratories of Jim Allison, who published on the successful rejection of B16 melanoma in mice via antagonism of CTLA-4 juxtacrine signalling (van Elsas, Hurwitz, and Allison 1999), and Tasuku Honjo, who published on the involvement of the PD-1 immuno-inhibitory receptor in the negative regulation of lymphocyte activation (Freeman et al. 2000). These studies began a radical leap in cancer therapeutics, by acknowledging that tumour growth is supported by mechanisms—in part mediated by Treg cells—that negatively regulate effector T cells and impair their capacity to exert robust anti-tumour responses.

Whether or not Treg cells contribute to cancer pathology relates to the cancer and tissue type. Heavy infiltration and/or expansion of Treg cells is observed in melanoma, non-small cell lung, colorectal and ovarian cancers (reviewed in Sakaguchi *et al.* 2010; Nishikawa and Sakaguchi 2014). A low ratio of Treg to Teff cells associates with higher survival rates in the aforementioned tumour types, along with breast cancer (Bates et al. 2006), hepatocellular carcinoma (Gao et al. 2007), renal cell carcinoma (Griffiths et al. 2007), pancreatic ductal adenocarcinoma (Hiraoka et al. 2006) and cervical cancer (Jordanova et al. 2008). The current repertoire of clinically approved cancer immunotherapies, designed to obstruct the negative signalling mechanisms elucidated by Allison and Honjo, show greater clinical efficacy in tumour types that present high Treg cell infiltration and/or high Treg:Teff cell ratios. Ipilimumab (Yervoy)—a fully human IgG1 monoclonal antibody, which binds to

CTLA-4—is currently approved for the treatment of malignant melanoma and renal cell carcinoma. Nivolumab (Opdivo)—a fully human IgG4 monoclonal antibody, which binds to PD-1—is approved for the same cancer types as ipilimumab and generally used as a combination therapy alongside ipilimumab. In addition, nivolumab has approval for treatment of non-small cell lung cancer, small cell lung cancer, renal cell carcinoma, Hodgkin lymphoma, squamous cell carcinoma of the head and neck, urothelial carcinoma, colorectal cancer and hepatocellular carcinoma. Prior to the advent of these immunotherapeutic agents, for patients with metastatic melanoma: there was no accepted standard of care; no therapy had been shown in a phase 3 clinical trial to improve overall survival in patients; and the median survival of patients presenting distant metastases was less than one year. In a seminal study published in the New England Journal of Medicine, a phase 3 clinical trial was conducted on patients with metastatic melanoma, where ipilimumab administered with or without the cancer vaccine glycoprotein 100 (gp100) was compared with gp100 alone. The median overall survival in the gp100-alone group was 6.4 months, compared to 10.1 months in the ipilimumab-alone group and 10.0 months in the ipilimumab-plus-gp100 group (Hodi et al. 2010). This initial positive result led to a rush of clinical trials to understand the full clinical potential of newly emerging immune checkpoint inhibitors. The observation that combinatorial blockade of CTLA-4 and PD-1 signalling was synergistic in treatment of advanced melanoma lead to a further leap in patient treatment (Curran et al. 2010; Wolchok et al. 2013). The most recent clinical data demonstrated that the median overall survival for patients with advanced melanoma was: 19.9 months for those treated with ipilimumab; 37.6 months for those treated with nivolumab; and beyond 36 months (the median overall survival had not yet been reached at time of publication of the study) for those treated with nivolumab-plus-ipilimumab (Wolchok et al. 2017). Thus in our current age, where therapeutic agents exist with the capacity to relieve immune checkpoint inhibition, a greater

proportion of patients afflicted with cancer can experience a partially durable tumour regression.

1.5.3 Infection

Though academic and pharmaceutical research has chiefly been directed toward the implications of Treg cell function in autoimmune and oncogenic disease, much has also been uncovered regarding their function in infectious disease. One of the earliest suggestions that Treg cells played a role during chronic infection, came from the observation that persistent *Leishmania major* infection was dependent upon continual IL-10 release (Y Belkaid et al. 2001). Subsequent studies found that Treg cells, specific for *L. major*, accumulate in the dermis during chronic infection, where they impair effector T cell responses via both IL-10-dependant and IL-10-independent mechanisms (Yasmine Belkaid et al. 2002; Suffia et al. 2006). Indeed, studies have since shown that Treg cell populations proliferate and accumulate at sites of infection in several human diseases. For example, this phenomenon was observed in: viral infections, such as hepatitis B (D. Xu et al. 2006); fungal infections, such as *Paracoccidioides brasiliensis* (Cavassani et al. 2006); and bacterial infection, such as *Helicobacter pylori* (Kandulski et al. 2008). Compellingly, Treg cell-mediated immunosuppression was shown to be implicated in the progression of malaria. Mice infected with a lethal strain of *Plasmodium yoelii* are protected from death upon systemic depletion of Treg cells, using an anti-CD25 antibody (Hisaeda et al. 2004). Furthermore, a longitudinal study of malaria sporozoite infection in humans observed the expansion of Treg cells following blood-stage infections, which associated with upregulation of TGF-β, decreased proinflammatory cytokine secretion and a reduction in antigen-specific immune responses (Walther et al. 2005). In contrast to chronic infection, studies involving acute infection are far

less frequent and data from these studies have often shown contradictory functions of Treg cells during the pathology (reviewed in Sanchez and Yang 2011; Stephen-Victor *et al.* 2017).

1.5.4 Gastrointestinal inflammation

The mammalian digestive tract performs the task of food digestion and absorption, whilst acting as the interface between organism and environment. The immunology of the gut is thus dynamic and unique, responsible for protecting the host against approximately 4 x 10^{13} bacteria in the human colon, as well as facilitating the entry of nutrients to sustain the host (Sender, Fuchs, and Milo 2016). The intestinal lamina in the colon harbours 25–35% Treg cells, with the small intestine resident to 10–15% Treg cells of total $CD4^+$ T cells (Sefik et al. 2015; K. S. Kim et al. 2016). In mice raised in completely sterile environments, or germ-free conditions, where mice are devoid of any microbiota, the proportions of colonic Treg cells are severely reduced (Geuking et al. 2011). These observations suggest a complex interplay between tissues of the host gastrointestinal tract, environment and cells of the immune system. Cell transfer studies by Powrie *et al*. highlighted how distinct T cell subsets could impart profound gastrointestinal phenotypes in donor mice (F Powrie et al. 1993). This seminal work resulted in researchers adopting a mouse model of colitis, where adoptive transfer of purified $CD4^+$ $CD45RB^{high}$ T cells into donor mice of the SCID mouse strain resulted in severe colonic inflammation. Subsequent studies revealed that this inflammatory phenotype could be ameliorated by co-transfer of purified $CD4^+$ $CD25^+$ $CD45RB^{low}$ Treg cells, which was dependant on IL-10 and TGF-β (F Powrie et al. 1994, 1996). These data directly implicate T cell dynamics in the pathogenesis of colitis and suggest that Treg cells may perform a central role in its prevention. Importantly, as transfer of $CD4^+$ $CD45RB^{high}$ Teff cells alone into donor mice housed in germ-free conditions failed to induce colitis (Fiona

Powrie et al. 2003), we again gained additional insight into the complex interactions between host immunity, microbiota and pathology.

The direct involvement of Treg cells in the maintenance of immune homeostasis at mucosal tissue sites was further elaborated by Rubtsov *et al*., who used the *Foxp3*$^{\text{YFP-Cre}}$ *Il10*$^{\text{flox}}$ transgenic mouse strain to abrogate IL-10 secretion specifically within Treg cells (Rubtsov et al. 2008). Mice bearing Treg cells incapable of IL-10 release suffered spontaneous colitis, increased lung inflammation during OVA sensitisation, and increased skin hypersensitivity during dinitrofluorobenzene sensitisation. Interestingly, colonic Treg cell function requires the expression of RORγt, the archetypal Th17 lineage-defining transcription factor (Sefik et al. 2015). A topic of which, is discussed further in section 1.6. Therefore, maintenance of excessive inflammation in the gut and other mucosal sites is dependent on the action of Treg cells, where IL-10 release is the principal mechanism of immunosuppression.

1.5.5 Non-immune physiology

Treg cells have increasingly become associated with processes outside of classical immunological roles. Burzyn, *et al.* demonstrated that Treg cells bearing a unique repertoire of TCR sequences accumulate and actively expand at injured skeletal muscle tissue in mice (Burzyn et al. 2013). Furthermore, this muscle Treg cell population was required for effective repair of damaged tissue. Indeed, in the *Foxp3*$^{\text{DTR}}$ mouse strain—where the gene encoding the Diphtheria toxin (DTx) receptor is knocked into the *Foxp3* locus, such that expression of Foxp3 is not disrupted, but all Foxp3^{+} cells are sensitive to DTx—transient ablation of Treg cells resulted in: altered histological features of the regenerated muscle tissue, such as greater collagen deposition and a reduction in regenerative centrally nucleated fibres; a reduction in the myogenic capacity of muscle progenitor satellite cells; and a prolonged gene expression

pattern in muscle tissue, consistent with an ineffective and drawn-out repair process. Muscle repair processes are impaired with age and Treg cell accumulation was found to be diminished at injured skeletal muscle tissue in aged mice (Kuswanto et al. 2016). The observed defects in Treg cell recruitment, proliferation and retention associated with the impaired release of IL-33 by fibro/adipogenic progenitor (FAP) cells upon muscle wounding. Furthermore, exogenous supplementation of IL-33 in aged mice could restore Treg cell populations in injured muscle and promote tissue regeneration.

Evidence also shows an intriguing association between Treg cells and metabolic function. Adipose tissue forms a natural caloric reserve in mammals. Whereas visceral white adipose tissue (WAT) is responsible for the storage of nutrients during over-nutrition and its release under conditions of energy deficit, brown adipose tissue (BAT) generates body heat by UCP1-mediated non-shivering thermogenesis. An integrated unit is formed by the associations between adipose tissue, the nervous system and the immune system. Mediated in part by adipose tissue cytokines, or adipokines, this interconnected system functions as an endocrine organ and exerts profound effects on metabolic homeostasis (reviewed in Kershaw and Flier 2004). Pro-inflammatory cytokines originating from adipose tissue, such as type 1 IFNs, TNF-α and IL-6, have been increasingly been shown to be causative of metabolic syndrome—a medical term for a combination of diabetes, high blood pressure and obesity—and insulin resistance (Hotamisligil, Shargill, and Spiegelman 1993; H. Xu et al. 2003; Ganguly 2018). Therefore, functionally immunosuppressive Treg cells could understandably be involved in the regulation of metabolism, exerted by adipose tissue. Indeed, a substantially higher percentage of $CD4^+$ T cells expressing Foxp3 can be found in murine adipose tissue (50% of $CD4^+$ T cells, versus 10–15% in peripheral lymphoid compartments) (Feuerer et al. 2009). In a seminal study by Feuerer *et al*., Treg cell populations were found to be reduced in

abdominal fat, but not in the spleens, of mice from three different models of obesity (the leptin-deficiency model, heterozygous yellow spontaneous mutation model and chronic high-fat diet model). Using non-obese diabetic (NOD) mice possessing the aforementioned *Foxp3*^{DTR} transgene, depletion of Treg cells resulted in a significant increase in expression of inflammatory genes (e.g., genes encoding TNF-a, IL-6, RANTES) in adipose tissue, but not in the spleen and lung, as well as an increase in insulin, which indicated insulin resistance. Furthermore, the restoration of abdominal fat Treg cell populations in mice fed a high-fat diet via administration of the IL-2 / anti-IL-2 antibody complex—which is known to selectively expand Treg cells (Boyman et al. 2006)—led to a reduction of blood glucose. Subsequent studies from the same research group identified a selectively expressed master transcriptional regulator of adipose tissue Treg cells, PPAR-γ (Cipolletta et al. 2012). Whereas the synthetic ligand of PPAR-γ, pioglitazone—which modulates lipid metabolism—was able to restore insulin sensitivity in wild-type mice, it failed to have the same affect in mice bearing PPAR-γ-deficient Treg cells. In humans, obese patients have similarly been found to be deficient in Treg cell populations, and this has been statistically correlated with increased body weight and body mass index measures (Wagner et al. 2013). Therefore, these studies highlight how Treg cells are essential in adipose tissue for regulating local inflammation, and so impact non-immune physiology, such as during metabolic dysfunction and insulin resistance.

During mammalian gestation, immune responses directed toward non-self, paternal-derived antigen developing in the fetus must be sequestered in the maternal immune system. $CD4^+$ $CD25^+$ T cell populations expand in the circulation and lymphatics progressively during murine pregnancy, and their presence is required for successful breeding of different mouse strains (Aluvihare, Kallikourdis, and Betz 2004). Early human pregnancy decidua—a modified mucosal lining, which forms the maternal part of the placenta—similarly comprises

an abundant population of $CD4^+$ $CD25^+$ T cells, expressing high levels of CTLA-4. However, their abundance was significantly lower in samples taken from females who experienced spontaneous abortion, than from those who underwent induced abortion (Sasaki et al. 2004). In addition, women experiencing recurrent spontaneous abortions show low numbers of $Foxp3^+$ Treg cells at both the follicular and luteal phases of the menstruation cycle (Arruvito et al. 2007). By using transgenic mice in which the CNS1 region of the *Foxp3* locus was knocked out, Samstein *et al*. demonstrated that pTreg cells are essential for maintaining maternal tolerance toward fetal development. Indeed, increased fetal resorption was observed in CNS1-deficient females, with concomitant increased immune cell infiltration (Samstein, Josefowicz, et al. 2012). Interestingly, studies on pregnancy have provided striking evidence for the importance of 'memory' Treg cell populations. Rowe *et al.* found that antigen-specific Treg cells—with antigen specificity toward a transgenic, paternally-derived antigen—not only rapidly expand during gestation, but also remain elevated postnatally, then undergo repeated expansion during subsequent pregnancy (Rowe et al. 2012). This Treg cell population, which accumulated during secondary pregnancy, was derived from those originating from prior pregnancy, and their function was required to prevent fetal resorption. These studies collectively demonstrate that Treg cells enforce maternal-fetal tolerance and are thus essential for healthy development of the fetus.

1.5.6 Contribution of other suppressive lymphocytes to immune homeostasis

Though the literature regarding immune homeostasis is predominately focused upon the regulatory capacity of the T cell lineage, studies suggest that the B cell lineage also presents the capacity to form regulatory cell types. These so-called regulatory B (Breg) cells have been shown to produce IL-10 and suppress inflammatory responses in EAE, collagen-induced arthritis, and colitis models (Mizoguchi et al. 2002; Fillatreau et al. 2002; Mauri et

al. 2003). Interestingly, Bregs have been shown, in part, to function by promoting regulatory phenotypes in T cells in mouse models and human studies (Carter et al. 2011; Flores-Borja et al. 2013). This is thought to be mediated by both direct juxtacrine signalling (Yoshizaki et al. 2012; Mann et al. 2007) and by indirect effects upon dendritic cells (Matsumoto et al. 2014; Sun et al. 2005). In addition, Breg cells have the capacity to produce TGF-β, which was shown to exert anergic effects on $CD8^+$ T cells (Parekh et al. 2003). Unlike Treg cells, no defining transcription factor has been identified, which confers the Breg cell phenotype. This has led some to question whether Breg cells indeed represent a distinct B cell subtype, or instead, arise reactively in response to environmental cues. Furthermore, future studies should clarify whether Breg cells are important in normal human immune homeostasis and whether Breg cell dysfunction has any involvement in disease pathology.

1.6 Regulatory T cells are a phenotypically heterogeneous cell population

Treg cells comprise a cell population with varied tissue specificities and functions. Not only do they exert immunosuppressive roles across different tissue types, using a variety of different mechanisms, but also engage in non-immune functions during unexpected physiological conditions. We have already discussed how Foxp3 expression, and thus Treg cell lineage commitment, can occur either within developing T cells in the thymus or within peripherally circulating $Foxp3^-$ Tconv cells (section 1.3.1). Their heterogeneous origin is, therefore, one factor that contributes to a diversity of Treg cell phenotypes within the body. After genesis, their subsequent residency within different tissue sites appears to be driven by intrinsic heterogeneity. Although Treg cells found at different tissues show a certain degree of phenotypic overlap, distinct Treg cell phenotypes can be observed in relation to which tissue they reside in. For example, Burzyn *et al*. found that the muscle resident Treg cells transcriptome differed more from their spleen and lymph node (LN) counter parts to a greater

degree than the latter did from each other (Burzyn et al. 2013). The greatest phenotypic overlap observed from the tissues chosen for study was between muscle Treg cells and adipose tissue resident Treg cells. Whereas genes encoding IL-10, CCR1, PDGF and AREG were notably upregulated in muscle Treg cells—with additional upregulation of genes encoding KLRG-1, CCR2 and ST2 upon muscle injury—those notably downregulated in muscle Treg cells were genes encoding CXCR5, CCR7, TCF7, LEF1 and SATB1. In addition, the muscle Treg cell populations that expanded upon injury were clonally derived and displayed unique TCR repertoires. In comparison to spleen and LN Treg cells, adipose tissue resident Treg cells are enriched for hallmark Treg genes, such as those encoding CD25, GITR, CTLA-4, OX40, KLRG-1 and Foxp3 itself (Feuerer et al. 2009). Many genes involved in migration and extravasation were similarly found enriched, these included the genes for CCR1, CCR2, CCR9, CCL6, CXCL2 and CXCL10. Conversely, other migratory genes were downregulated, such as those encoding CCL5 and CXCR3. Cipolletta *et al*. demonstrated that the adipose tissue resident Treg cell phenotype is driven by PPAR-γ, which is now considered to be a signature transcription factor for this Treg cell population (Cipolletta et al. 2012). Indeed, in the absence of functional PPAR-γ adipose tissue resident Treg cell populations were diminished by approximately four-fold. Therefore, we see that Treg cells present distinct arrays of transcription factors, functional molecules and migratory receptors in relation to where they reside within the body.

The canonical lineage specifying transcription factors that guide differentiation of $CD4^+$ $Foxp3^-$ Tconv toward helper subsets are also expressed by Treg cells for appropriate restraint of the corresponding type of inflammatory response. For example, whereas T-bet expression is required in Tconv cells for their differentiation toward the Th1 cell subset, its expression is similarly upregulated by Treg cells that counter the Th1 cell inflammatory response. T-bet

expression in Treg cells was found to promote expression of CXCR3, which facilitated their migration to sites of Th1-type inflammation. Critically, loss of T-bet function in Treg cells resulted in their inability to restrain the expansion of Th1 cells releasing IFN-γ (Koch et al. 2009). Similarly, the expression of the transcription factor IRF4—which is involved in Th2 cell differentiation and the control of IL-4 production—was necessary for Treg cell-mediated constraint of Th2-type inflammation. Accordingly, a lymphoproliferative disease—characterized by the selective expansion of $CD4^+$ T cells secreting IL-4 and IL-5—was observed in mice bearing a conditional knockout (CKO) of *Irf4* within Treg cells (Zheng et al. 2009). As previously mentioned, a distinct population of $ROR\gamma t^+$ Treg cells maintain homeostasis in the gut (section 1.5.4). The transcription factor STAT3 acts in conjunction with RORγt to establish Th17 cell identity, and its deletion in Treg cells resulted in the spontaneous fatal intestinal inflammation (Chaudhry et al. 2009). Importantly, this pathology was characterized by excessive production of IL-17, with no differences in secretion of Th1- or Th2-type inflammatory cytokines, which indicated the specific dysregulation of gut resident Th17 cell responses. Thus Treg cells demonstrate a striking degree of functional heterogeneity, where distinct Treg cell populations differentiate into specific subsets alongside inflammatory helper T cells, to counter specific types of inflammation that occur during the immune response (Figure 1.3).

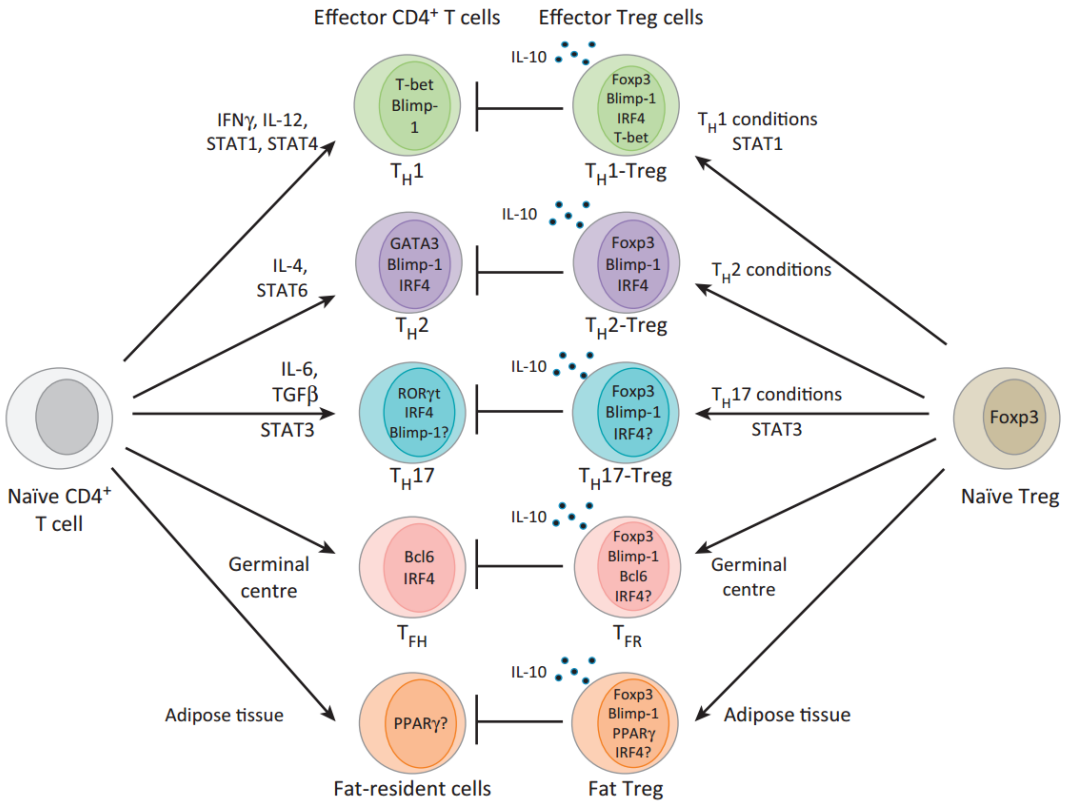

Figure 1.3: Hallmark T helper subset transcription factors are reciprocally expressed by Treg cells for appropriate restraint of the corresponding type of inflammatory response

Figure adapted from Cretney, Kallies and Nutt 2013.

Although the molecular mechanisms that underlie the reliance of Treg cells on these hallmark T helper cell transcription factors for their functional heterogeneity remain to be elucidated, a number of important observations have been made. These transcription factors may regulate the localisation of Treg cell subsets to the appropriate tissue. Indeed, Treg cells lacking functional T-bet, IRF4 or STAT3 demonstrated impaired expression of the chemokine receptors CXCR3, CCR8 and CCR6, respectively, which are required for guiding their tissue localisation during inflammatory responses. The mechanisms by which Treg cells exert immunosuppression may also be influenced. For example, deficiency in these transcription factors all led to a reduction in IL-10 expression. In addition, reduced expression of *Icos*, *Fgl2*, *Ebi3*, *Prf1* and *Gzmb* were observed in IRF4-deficient or STAT3-deficient Treg cells.

A hallmark of T cell biology is their responsiveness to antigen, clonal expansion, differentiation toward short-lived effector subsets, the acquisition of greater functional capacity, and eventual death. As previously discussed, tTreg cells and pTreg cells respond to diverse self- and foreign antigens, respectively, and clonally expanded Treg cells are observed in tissues, such as the muscle. Different populations of Treg cells also express functional molecules to varying degrees. Therefore, this has led researchers to question whether Treg cells follow a similar resting to activated phenotypic trajectory, that is so well characterised in their effector counterparts. Evidence does indeed support the existence of a subset of resting Treg (rTreg) cells—exhibiting a quiescent or naïve phenotype and are functionally less mature—and activated Treg (aTreg) cells, exhibiting an effector phenotype, which have encountered cognate antigen and present a greater capacity for immunosuppression. In a similar paradigm to $CD4^+$ $Foxp3^-$ Tconv and $CD8^+$ Teff cells, resting Treg cell populations principally reside within secondary lymphoid structures, express high levels of CCR7 and CD62L, but low levels of CD44. However, upon antigenic stimulation, activated Treg cells undergo a phenotypic switch to downregulate CCR7 and CD62L, but upregulate CD44, and migrate away from secondary lymphoid sites to the tissue (Huehn et al. 2004; Lee, Kang, and Kim 2007). Cheng, *et al.* observed that KLRG1 (Killer cell lectin-like receptor subfamily G member 1)—a marker canonically associated with terminally differentiated Teff cells—is likewise, highly expressed on a subset of Treg cells, located in non-lymphoid tissue, which present high expression of other activation molecules, such as CD69, CD103, CD25 and Blimp-1 (Cheng et al. 2012). These $KLRG1^+$ Treg cells expressed higher levels of functional molecules, such as CTLA-4, CD39, CD73, and demonstrated a greater capacity for restraint of Teff cell proliferation in a Treg cell suppression assay. By using adoptive transfer of $KLRG1^-$ and $KLRG1^+$ Treg cells into

lymphopenic mouse models, the authors observed that KLRG1^{+} Treg cells could originate only from KLRG1^{-} Treg cells (not *vice versa*), and that KLRG1^{+} had a reduced capacity for survival. These findings strongly suggest that Treg cells share an equivocal paradigm to Teff cells, consisting of a progressive differentiation toward functionally more capable, short-lived effector subsets following antigen stimulation. Other markers have been suggested to delineate memory-like or activated Treg cells from resting populations, such as CCR6 and TIGIT (Kleinewietfeld 2005; Joller et al. 2014). Such phenotypically and functionally distinct Treg cell populations have similarly been identified in humans, where rTreg cells are positive for CD45RA and express low levels of Foxp3, whereas aTreg cells are concomitantly CD45RA^{-} and high in Foxp3 expression (Miyara et al. 2009). Although the mechanisms that govern the transition of rTreg to aTreg cells still remain to be elucidated, TCR signalling appears to play a central role. In a mouse model where the TCRα chain was ablated in Foxp3-expressing cells upon administration of tamoxifen, which prevented a complete TCR being maintained on the cell surface of lineage-committed Treg cells, Levine *et al*. observed a failure of CD62L^{high} CD44^{low} rTreg cells to progress toward CD62L^{low} CD44^{high} aTreg cells during the steady-state (Levine et al. 2014). Despite normal percentages of Treg cells existing in spleen and lymph nodes, elevated numbers of CD4^{+} Tconv and CD8^{+} Teff cells, expressing increased amounts of inflammatory cytokines, were found in these tissues. Gene expression profiling revealed defective upregulation of key transcription factors and functional molecules within the TCR-deficient Treg cells, including the genes encoding NFATc1, c-Rel, Bcl6 and IRF4, CTLA-4 and IL-10. These results highlight the absolute dependence of Treg cells on TCR signalling to transition from a naïve phenotype, toward a functionally competent, activated state.

Recent advancements in technology have provided further evidence for the presence of distinct Treg cell populations and the necessity of TCR signalling in shaping Treg phenotypic heterogeneity. Single-cell RNA-Sequencing (scRNA-Seq) studies show how murine Treg cells residing in different tissue types harbour distinct transcriptional profiles (Miragaia et al. 2019). In addition, dimensionality reduction analysis of scRNA-Seq data from murine splenic Treg cells revealed that clusters expressing hallmark resting genes separated from those expressing hallmark activated genes (Zemmour et al. 2018). Clustering between the populations expressing hallmark rTreg and aTreg cell genes, were cell clusters enriched for gene set signatures associated with TCR-delivered signals. Analysis of Treg cells from *Nr4a1*^{GFP} reporter mice—where NR4A1 expression is proportional to TCR signalling intensity—revealed that, surprisingly, high TCR signals were not predictive of the Treg cell activation status. In contrast, across the entire spectrum of *Nr4a1*^{GFP} expression in Treg cells, all displayed equivalent levels of CD62L expression. Although no relationship between TCR signalling and aTreg status was found, the phenotypic divergence within the activated Treg clusters did indeed, associate with differences in the TCR signalling strength. The authors concluded that although the level of TCR signalling does not dictate the proportion of aTreg cells, it does dictate phenotypic heterogeneity amongst aTreg cell populations. Such results suggest unidentified factors, aside from TCR signalling strength, which fulfil critical roles in mediating the transition of lineage-committed rTreg cells toward aTreg cells.

1.7 The role of BACH2 in lymphoid cell lineage commitment, differentiation and function

Given the significance of the actions of transcription factors (TFs) in shaping lymphocyte cellular identity and thus, their importance in orchestrating effective immune responses,

much attention has been drawn toward understanding TF biology. Historically, focus was principally directed toward TFs that upregulate gene expression following TCR engagement, such as the NFAT, NF-κB and AP-1 families. Despite the necessity for these activating complexes—and those discussed in section 1.2—to drive the expression of keys genes involved in a particular phenotype for a given cell lineage, equally important are the actions of transcriptional repressors, which inhibit the expression of genes involved in the differentiation toward alternate lineages. Therefore, if cell differentiation is likened to a trajectory along a given path, transcriptional repressors prevent that path from diverging in alternate directions. BACH2 (BTB and CNC homolog 2) is a member of the basic region leucine zipper (bZIP) transcription factor family (Oyake et al. 1996) (Figure 1.4). The BACH2 bZIP domain enables formation of dimeric complexes with numerous other bZIP domain-containing TFs at DNA sequences containing TPA response elements (TREs) or cAMP response elements (CREs) (Reinke et al. 2013). Here, the palindromic TGA(G/C)TCA sequence serves as a template for the conserved bZip region of BACH2 to bind, where the leucine zipper then forms coiled-coil interactions with other bZIP TF monomers (Turner and Tjian 1989). In addition to the bZIP domain, BACH2 contains a BTB (bric-à-brac–tramtrack–broad complex) domain, which is also known as the POZ (pox virus and zinc finger) domain (Chaharbakhshi and Jemc 2016). The BACH2 BTB domain enables interaction with molecules in addition to bZIP TFs, and thus extends its functional capability (Tanaka et al. 2016).

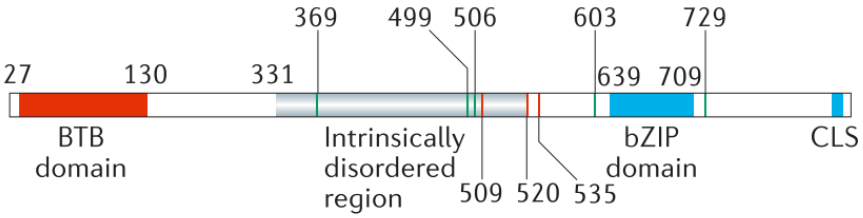

Figure 1.4: Schematic representation of the mouse BACH2 protein.

Cysteine–proline residues are highlighted by green lines, where the numbers indicate the position of the cysteine, and selected phospho-residues are highlighted by red lines. Figure adapted from Igarashi, Kurosaki and Roychoudhuri 2017.

1.7.1 BACH2 coordinates B cell maturation and directs memory B cell differentiation

BACH2 was originally described as a transcriptional repressor of gene regulatory networks required for plasma B cell differentiation (A. Muto 1998; Akihiko Muto et al. 2004). Following encounter with cognate antigen, activated B cells migrate to the germinal centre. Here, B cell clones, which produce mutated immunoglobulins through the process of somatic hypermutation (SHM), are positively selected by follicular T cells for survival according to their affinity for antigen. During this selection, class-switch recombination (CSR) occurs, generating B cell clones that express high-affinity and functionally diverse immunoglobulin. The entry of B cells into the germinal centre either results in short-lived, antibody-secreting plasma B cells being generated, or the production of long-lived, memory B cell populations. The TF BLIMP-1 (encoded by *Prdm1*) is essential for driving the terminal differentiation of B cells to plasma cells (Shaffer et al. 2002). Here, BACH2 acts in conjunction with BCL-6, to restrain *Prdm1* expression, and is thus plays a key role in SHR and CSR (Akihiko Muto et al. 2004; Huang et al. 2014). In addition, BACH2 expression in germinal centre B cells correlates with their propensity to form memory B cells, where higher BACH2 expression denotes B cells with lower affinity BCR and a high proclivity for memory cell differentiation. Indeed, memory B cell differentiation was diminished in *Bach2* haploinsufficient mice (Shinnakasu et al. 2016; Kometani et al. 2013). Because of its importance in B cell maturation and differentiation, BACH2 has attracted attention with regard to prognostic and mechanistic relationships to B cell pathologies (Sakane-Ishikawa et al. 2005; Ichikawa et al. 2014).

1.7.2 BACH2 regulates T cell differentiation

Though BACH2 was known to perform critical roles in B cells, *Bach2*^{KO} mice were found to display a striking phenotype, which could not be accounted for solely due to B cell dysfunction. Roychoudhuri *et al*. observed that *Bach2*^{KO} mice displayed a severe multi-organ autoimmune pathology, which led to lethality after approximately 4.5 months of age (Roychoudhuri et al. 2013). Interestingly, the loss of BACH2 resulted in a complete cell-autonomous failure to establish lineage-committed Foxp3^{+} Treg cells. Crucially, the lethal inflammatory phenotype, which was recapitulated in *Rag1*^{KO} mice reconstituted with *Bach2*^{KO} bone marrow, was ameliorated in *Rag1*^{KO} mice receiving both *Bach2*^{KO} bone marrow and wild-type Treg cells. Tconv cells isolated from *Bach2*^{KO} mice and cultured *in vitro* with either Th1, Th2 or Th17 polarizing cytokines showed a greater propensity to differentiate into the respective helper subsets and secrete pro-inflammatory cytokines. These data highlight the critical role that BACH2 plays in establishing functional Treg cells and restraining differentiation toward inflammatory T cell subtypes. The mechanistic details of BACH2 function in T cells were further elaborated, when BACH2 was found to restrain terminal differentiation in CD8^{+} T cells by limiting access of genomic enhancer regions to AP-1 factors (Roychoudhuri et al. 2016). By virtue of their shared sequence binding motifs, BACH2 limited binding of the AP-1 factor, JUND, to genes upregulated following TCR stimulation. In support of these observations, Kuwahara *et al*. observed the capacity of BACH2 to restrict binding of a JUND–BATF–IRF4 heterotrimeric complex to the Th2 cytokine locus control region. Whereas BACH2–BATF heterodimers restrained differentiation of Tconv to Th2 cells, JUND–BATF–IRF4 complexes potentiated the expression of genes required for Th2 cell formation. We thus observe BACH2 functioning in concert with other bZIP domain TFs to moderate inflammatory Teff differentiation and enable the establishment of suppressive Treg cell populations.

BACH2 function appears to be regulated by phosphorylation via the PI3K-AKT-mTOR signalling pathway. Murine $CD8^+$ T cells stimulated *in vitro* show enrichment for BACH2 phosphorylated at the S520 residue, which prevented passage to the nucleus and resulted in increased BACH2 accumulation in the cytoplasm (Roychoudhuri et al. 2016). The corresponding S521 residue in humans fulfils a similar role in chronic myeloid leukaemia cells (Yoshida et al. 2007). In murine pre-B cell lines, nuclear exclusion following BCR stimulation was predominantly related to phosphorylation of the S535 residue, and partially dependant on S509 phosphorylation (Ando et al. 2015). Conversely, hyperactive AKT-mTOR signalling caused by *Pten* ablation results in downregulation of *Bach2* mRNA. In addition, BACH2 phosphorylation can be prevented with the use of pharmacological AKT inhibitors, but not with mTORC1 inhibitors. Thus following antigen receptor engagement, BACH2 function can be controlled by AKT-mTOR signalling, both by post-translational modification and by transcriptional regulation.

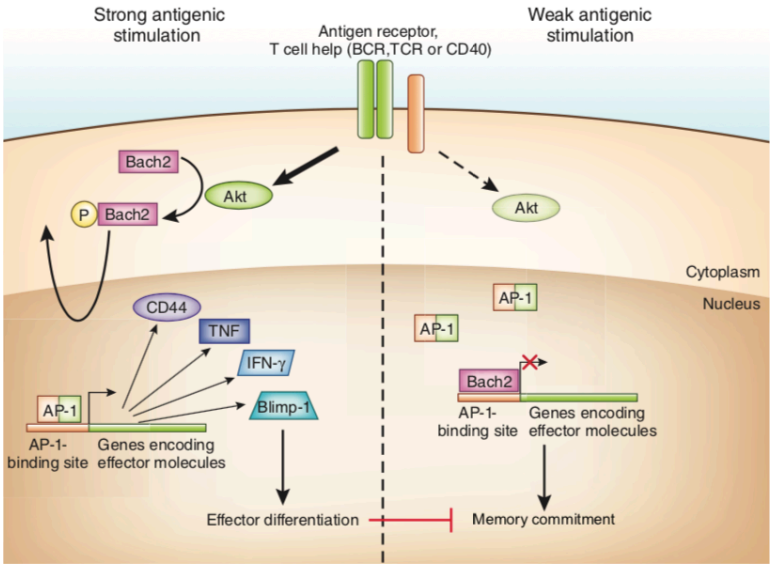

Figure 1.5: BACH2 function is controlled by antigen receptor signalling engagement. Figure adapted from Sidwell and Kallies 2016.

1.7.3 The role of BACH2 in human pathology

Given that BACH2 plays such a fundamental role in the cell types that govern the immune response, one may expect that any circumstance where BACH2 function, expression or regulation is altered would lead to a substantial impact on human health. Indeed, along with the advent of next-generation sequencing, came the finding that single-nucleotide polymorphisms (SNPs) within the *BACH2* locus associate with susceptibility to a plethora of autoimmune and allergic diseases. There are regions of the mammalian genome—referred to linkage disequilibrium blocks—that undergo much less frequent meiotic recombination than is expected from their genomic distances (Reich et al. 2001). A region in high linkage disequilibrium exists in a region that extends from upstream of the transcriptional start site of the *BACH2* locus to intron 4 of the gene (Igarashi, Kurosaki, and Roychoudhuri 2017), where the presence of risk SNPs correlate with susceptibility to asthma (Ferreira et al. 2011), type I diabetes (J. D. Cooper et al. 2008), multiple sclerosis (International Multiple Sclerosis Genetics Consortium et al. 2011), coeliac disease (Dubois et al. 2010), Crohn's disease (Franke et al. 2010), generalised vitiligo (Jin et al. 2012), Hashimoto's thyroiditis and Grave's disease (Medici et al. 2014). Though the direct impact of these risk haplotypes on BACH2 expression remain to be elucidated, these associations with a striking array of diseases suggest a common requirement of BACH2 for appropriate regulation of inflammation.

More directly, BACH2 dysfunction has been implicated in human clinical disease. Whole genome sequencing identified a heterozygous T71C *BACH2* mutation (leading to an L24P amino acid substitution) in an adolescent female patient, who presented with numerous conditions relating to aberrant immunity, such as infancy-onset colitis, non-infectious fever,

splenomegaly, immunoglobulin deficiency, recurrent upper respiratory tract infections and pancytopenia (Afzali et al. 2017). Afzali *et al.* also identified another BACH2 mutation in a different family, consisting of a father and daughter, who shared a G2362A point mutation (leading to an E788K amino acid substitution). Whereas the daughter had unmeasurably low levels of IgA, the father presented with complete immunoglobulin deficiency, and both presented small and large bowel inflammation, recurrent sino-pulmonary infections, bronchiectasis and fibrosis. Both the T71C (causing L24P) and G2362A (causing E788K) mutations were predicted to lead to instability of the BACH2 protein, abrogating its function. Interestingly, some aspects of the cellular phenotypic alterations observed in the *BACH2*^{KO} mouse model were recapitulated in these patients. Here, T cell immune-phenotyping revealed reduced Foxp3 expression in CD4^{+} CD25^{high} CD127^{low} cells and increased expression of T-bet in CD4^{+} Tconv cells isolated from the blood. In addition, the patients presented reduced levels of CD19^{+} CD27^{+} memory B cells. Being hitherto unrecognized in the clinic, this syndrome of *BACH2* haploinsufficiency was termed BACH2-related immunodeficiency and autoimmunity, or BRIDA. Collectively, these genetic and clinical observations contributed to the growing appreciation that BACH2 performs an obligatory role in lymphocyte function, and thus, there is an absolute requirement for BACH2 function in human health.

1.8 Summary

The cells, tissues and organs of the immune system evolved to protect the host from diverse threats. The mammalian immune system evolved to co-exist with micro-organisms—such as the case with microbiota at mucosal surfaces—or to counter pathogenic threats. Mechanisms of defence result in inflammation, which must regulated in order to prevent excessive damage to the host. Immune cells exist in an equilibrium, where those that cause protective inflammation are antagonised in order to prevent excessive cytotoxicity. Treg cells are central

to immune homeostasis, and as all tissues of the body require immunity, Treg cells have evolved diverse immunomodulatory functions throughout the body. The multiplicity of pathogens in nature and diversification of host tissue functions are paralleled by the heterogeneity of Treg cells, and indeed, Treg cells display considerable inter- and intratissue heterogeneity. Transcription factors are central determinants of cellular identity, thus much attention is directed toward those that regulate gene expression necessary to acquire particular phenotypes. BACH2 restrains antigen receptor driven gene expression programs in multiple mature lymphocyte lineages. In constraining lymphocyte differentiation toward terminal effector subsets, and additionally, being required for establishing lineage-committed populations of Treg cells, BACH2 fulfils a central role in shaping the immune response and maintaining immune homeostasis. This absolute requirement is most clearly demonstrated in the human clinical syndrome, BRIDA, and the phenotype of BACH2-deficient mice. Despite BACH2 being required for development of $Foxp3^+$ Treg cells, it remains to be understood whether BACH2 fulfils a function in Treg cells once lineage-commitment has taken place. That is, once $Foxp3^+$ expression has determined Treg lineage identity, does BACH2 serve a continued role in determining Treg cell phenotypes? In this work, we set out to elucidate the role of BACH2 following lineage-commitment of Treg cells. Prior to my doctoral research, experimental models had not been generated to answer such a question. In particular, germline deficiency of BACH2 results in a complete cell-autonomous absence of lineage-committed Treg cells rendering it impossible to study the cell-intrinsic function of BACH2 following Treg lineage commitment. Therefore, we needed to establish a novel model to abrogate BACH2 function strictly after lineage-commitment of Treg cells, which would not interfere with early development of Treg cell populations, and additionally, would avoid loss of BACH2 function in other cell types.

2 Materials and Methods

2.1 Transgenic mice and reagents

The *Bach2*^{tdRFP} and *Bach2*^{flox} mice were provided by Professor Tomohiro Kurosaki at Osaka University (Itoh-Nakadai et al. 2014; Kometani et al. 2013). The *Rosa26*$^{flSTOP\text{-}tdRFP}$ and *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ mice were provided by Dr Michelle Linterman at the Babraham Institute (Luche et al. 2007; Rubtsov et al. 2010). The *Ptprc*^{a} (CD45.1), *RAG2*-deficient, and C57BL/6J mice were provided by the Biological Support Unit (BSU) at the Babraham Institute. The *Foxp3*^{DTR} mouse strain was purchased from the Jackson Laboratory. Littermate controls or age- and sex-matched animals on a C57BL/6 background were used in experiments as indicated. All mice were housed at the Babraham Institute in accordance with UK Home Office guidelines, under project licence PE0D498BB, and all studies were approved by the Babraham Institute Animal Welfare and Ethics Review Board. For mice bearing the *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ mice transgene, induction of Cre-ERT2-mediated recombination was initiated via feeding with tamoxifen-containing chow or corresponding control non-tamoxifen-containing chow (Cat TD130858). All mice were given control non-tamoxifen containing chow for two weeks prior to initiation of treatment due to mitigate neophobic effects.

2.2 Preparing single-cell suspensions from mouse tissues

After mice had been euthanized according to Home Office requirements, whole organs were dissected and stored in phosphate buffered saline (PBS) on ice. Single-cell suspensions were prepared from specific tissues as detailed below.

2.2.1 Dissociation of spleens and thymi

The spleens and thymus were dissociated on 40 μM Falcon™ cell strainers (Thermo Fisher Scientific) using a plunger from a 5 ml syringe (Terumo). Red blood cells were removed from the suspension using 1 ml ACK Lysing Buffer (Gibco) for 45 seconds. After filtering through 40 μM Falcon™ strainers for a second time, single-cell suspensions of splenocytes or thymocytes were stored in PBS at 4 °C until further use.

2.2.2 Dissociation of lymph nodes

The lymph nodes were dissociated on 40 μM Falcon™ cell strainers (Thermo Fisher Scientific) using a plunger from a 5 ml syringe (Terumo) and stored in PBS at 4 °C until further use.

2.2.3 Dissociation of lungs

The lungs were placed in a cold solution of 20 μg/ml DNase solution I (Roche) and 1 mg/ml collagenase (Sigma-Aldrich) and dissociated using scissors. The suspensions were incubated with agitation at 37 °C for 30 minutes, then dissociated again on 40 μM Falcon™ cell strainers (Thermo Fisher Scientific) using a plunger from a 5 ml syringe (Terumo). Red blood cells were removed from the suspension using 1 ml ACK Lysing Buffer (Gibco) for 45 seconds. After filtering through 40 μM cell strainers a second time, single-cell suspensions were stored in PBS at 4 °C until further use.

2.2.4 Isolation of blood lymphocytes

Blood samples were extracted either using tail vain venepuncture on live mice or cardiac bleeds on euthanized mice, by technicians trained according to Home Office requirements, at the Babraham Institute Biological Services Unit. Approximately 50–200 μl of blood was removed and stored in EDTA Microvette tubes (Thermo Fisher Scientific) at room temperature until processing. Red blood cells were removed using 1 ml ACK Lysing Buffer

(Gibco) for 90 seconds and single-cell suspensions were stored in PBS at 4 °C until further use.

2.3 Total or naïve CD4 T cell enrichment

Single-cell suspensions were prepared from particular tissues as detailed in section 2.2. Enrichment of $CD4^+$ T cells was performed using the MagniSort™ Mouse CD4 T cell Enrichment Kit (Invitrogen, Thermo Fisher Scientific). Enrichment of total $CD4^+$ T cells from single-cell suspensions were performed according to the manufacturer's protocol. A more refined enrichment of only naïve $CD4^+$ T cells was achieved by including biotin-conjugated CD44 antibody (0.02 μg per sample) and biotin-conjugated CD25 antibody (2 μg per sample, both Invitrogen, Thermo Fisher Scientific) during labelling with the MagniSort™ Enrichment Antibody Cocktail. Any markers required for cell sorting were stained using flow cytometry cell surface antibodies (detailed in appendix Table 8.1: Flow cytometry antibodies), diluted 1/200, whilst cell suspensions were being labelled with the Enrichment Antibody Cocktail. The cell suspensions were stored in FACS buffer (section 8.3.1 for details) at 4 °C until further use.

2.4 Fluorescence activated cell sorting (FACS)

Enriched single-cell suspensions were prepared as detailed in sections 2.2 and 2.3, filtered through a 30 μM CellTrics™ cell strainer (Sysmex) and re-suspended in RPMI 1640 medium (Invitrogen, Thermo Fisher Scientific) to an approximate concentration of 20 million cells per ml and stored at 4 °C. The suspensions were briefly vortexed and sorted using the BD Influx™ (Becton Dickinson Biosciences), where gates were positioned in accordance with negative controls. Compensation for FACS were generated on software provided for the BD Influx™ using either single-stained UltraComp eBeads™ (Invitrogen, Thermo Fisher

Scientific) or splenocytes. The cells were sorted into solutions of RPMI 1640 medium supplemented with 20% Fetal Bovine Serum (Sigma-Aldrich). After sorting, the data were interpreted using FlowJo® software (LLC).

2.5 DNA isolation for PCR

The single-cell suspensions were prepared as detailed in section 2.2 and treated using the DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacture's protocol.

2.6 Genotyping of mouse lines

2.6.1 Automated genotyping by Transnetyx, Inc

Genotyping of mouse lines was primarily done by outsourcing to Transnetyx, Inc. Tissue samples from transgenic mice and corresponding sequence data were sent to Transnetyx to aid their design of proprietary primer sequences. All future genotyping then involved mouse tissue being biopsied from the ear, placed in Transnetyx 96-well plates and posted to Transnetyx. Genotyping data from Transnetyx were provided within two to three days of receiving the biopsies.

2.6.2 PCR genotyping of genomic DNA for floxed and excised *Bach2*

Single-cell suspensions were prepared as detailed in section 2.2 for whole-tissue analysis or section 2.4 for analysis of specific cell populations. DNA was isolated as detailed in section 2.5 and quantified using a NanoDrop™ (Thermo Fisher Scientific). DNA and primers were prepared for PCR in Quick-Load® *Taq* Master Mix (New England Biolabs) according to manufacturer's protocol. The PCR program and primers used are shown in Table 2.1 and Table 2.2, respectively. All PCR reactions were run on the T100™ Thermal Cycler (Bio-Rad Laboratories).

94°C	2 minutes	
94°C 60°C 72°C	20 seconds 20 seconds 60 seconds	35 x
72°C	3 minutes	
4°C	Indefinitely	

Table 2.1: PCR program for genotyping.

Gene	**Primer sequence**
Bach2^{flox} forward	5'- CCTTACTGGATTCGGATGAGAAGCC-3'
Bach2^{flox} reverse	5'- CTCTGTACACAGTGGGATCCACGGG-3'
Band size:	450 bp
Bach2^{excised} forward	5'- CTCACTATAGGGTTCGAGGAAGT-3'
Bach2^{excised} reverse	5'- GTACAAGAAAGCTGGGTCGG-3'
Band size:	145 bp
TLR9 forward	5'-AGGAAGGTTCTGGGCTCAAT-3'
TLR9 reverse	5'-TCTGTACCCCGTTTCTCTGC- 3'
Band size:	250 bp

Table 2.2: Nucleotide sequences of PCR primers and their product sizes.

2.7 Phenotypic analysis of cells using flow cytometry

2.7.1 Cell surface staining

The single-cell suspensions were prepared as detailed in section 2.2, and cell surface antibodies are detailed in Appendix Table 8.1. Approximately 1 x 10^6 cells were taken from

each suspension for flow cytometry analysis. The cells requiring intracellular staining with antibodies for cytokines were stimulated using phorbol 12-myristate 13-acetate (PMA), ionomycin and brefeldin A (BFA), as detailed in section 2.9. When using Zombie UV™ live/dead dye, cells were first stained with the viability dye alone, at room temperature for 20 minutes, followed by staining with cell surface antibodies (diluted 1/200) in FACS buffer, at 4 °C for 30 minutes. With the eBioscience™ eFluor™ 780 live/dead dye, cells were stained with the viability dye along with cell surface antibodies (diluted 1/200) in PBS, at 4 °C for 30 minutes. Cell surface phosphatidylserine was labelled using the eBioscience™ Annexin V Apoptosis Detection Set PE-Cyanine7 (Invitrogen, Thermo Fisher Scientific), with the dye diluted 1/100, according to the manufacturer's protocol. The samples were either stored in 200 µl of FACS buffer at 4 °C in the dark until flow cytometry analysis, or were then stained intracellularly (section 2.7.2).

2.7.2 Intracellular staining

The cells were stained intracellularly using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Invitrogen, Thermo Fisher Scientific). Reagents from this kit were prepared as detailed in the manufacturer's protocol. The cells were fixed for either one hour or overnight in the eBioscience™ fixation buffer. The cells were stained with intracellular antibodies (diluted 1/200, detailed in Appendix Table 8.1) for one hour in eBioscience™ permeabilisation buffer. The samples were stored in 200 µl of permeabilisation buffer at 4 °C in the dark. The suspensions were briefly vortexed and analysed using the BD Fortessa™ (Becton Dickinson Biosciences) and the data were interpreted using FlowJo® software (LLC).

2.8 Cell stimulation with CD3 antibody and CD28 antibody

96-well plates were coated with 100 µl of CD3e antibody and CD28 antibody (both Invitrogen, Thermo Fisher Scientific), both at 1 µg/ml, in PBS for either four hours at 37 °C or overnight at 4 °C. The plates were washed twice with PBS before adding the single-cell suspensions.

2.9 Cell stimulation with phorbol 12-myristate 13-acetate, ionomycin and brefeldin A

The single-cell suspensions were stimulated in 100 µl of RPMI complete media (detailed in the Appendix) with 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 1 µg/ml ionomycin and 10 µg/ml brefeldin A (BFA, all Sigma-Aldrich) for four hours at 37 °C. The samples were then washed twice with cold PBS and stored at 4 °C until further use.

2.10 *In vitro* rTreg cell activation assays and iTreg cell differentiation

Resting Treg cells ($CD4^+$ $EGFP^+$ $CD62L^+$) were purified from *Foxp3*$^{eGFP\text{-}Cre\text{-}ERT}$ mice as detailed in section 2.4. The rTreg cells were then cultured at 20,000 cells per well, in a 96-well plate in RPMI 1640 complete media (detailed in the Appendix) with 5 ng/ml TGF-β (R&D Systems, Bio-Techne), 5 ng/ml IL-2 (Peprotech) and 200 nM 4-Hydroxytamoxifen (Sigma-Aldrich) for three days at 37 °C, 5% CO_2. The cells were then transferred to a 96-well plate coated with CD3e and CD28 antibodies (as detailed in section 2.8) and cultured in RPMI complete media with fresh TGF-β, IL-2 and 4-Hydroxytamoxifen (concentrations as before) for a further four days at 37 °C, 5% CO_2.

For *in vitro* differentiation of iTreg cells, naïve $CD44^-$ $CD62L^+$ $CD25^-$ $CD4^+$ T cells were purified by FACS from pre-enriched total $CD4^+$ T cells from spleens and lymph nodes of 8–12 week-old wild type mice, as detailed in section 2.4. The naïve $CD4^+$ T cells were activated

by plate-bound anti-CD3 and soluble anti-CD28 (5 μg/ml each, as detailed in section 2.8) in media for four days in the presence of IL-2 (5 ng/ml, R&D Systems) and TGF-β (5 ng/ml, R&D Systems).

2.11 Preparation of RNA for bulk-sequencing

Purified single-cell suspensions were prepared as detailed in section 2.4 and stored in 40 μl RNA*later*™ Stabilization Solution at -80 °C. The samples were processed using the QIAshredder Kit (Qiagen) according to the manufacturer's protocol. RNA was extracted from the samples using the RNeasy® Plus Mini Kit (Qiagen) according to the manufacturer's protocol. RNA Libraries were prepared using the SMARTer® PCR cDNA Synthesis Kit (Clontech, Takara Bio) according to the manufacturer's protocol and sequenced using the HiSeq® 2500 System (Illumina).

2.12 Analysis of bulk RNA sequencing data

The FastQ files were generated as described in section 2.11. The files underwent analytical quality control with FastQC version 0.11.8, adaptor trimming with Cutadapt version 1.18 and alignment to the NCBIM37 *Mus musculus* genome annotation with hisat2 version 2.1. Cluster Flow was used to pipeline FastQ files through the aforementioned programs (Ewels et al. 2016). Differential gene expression analysis was done using either Cufflinks version 2.2.1 (Trapnell et al. 2012), or DESeq2 version 1.22.1 in R version 3.5.0 (Love, Huber, and Anders 2014). The output from differential gene expression analyses was processed in R.

2.13 Preparation of RNA for single-cell sequencing

Single-cell suspensions of 4000 purified Treg cells in 34 μl RPMI 1640 complete media were prepared as detailed in section 2.4. RNA libraries were prepared for single-cell RNA-

Sequencing (scRNA-seq) using the Chromium Single Cell 3′ Library & Gel Bead Kit v2 (10x Genomics), processed with the Chromium (10x Genomics) and sequenced using the HiSeq® 4000 System (Illumina).

2.14 Analysis of single-cell RNA sequencing data

The raw 10X sequencing data was processed as previously described, except the data were mapped to *mm10*. We confirmed that the cells were sequenced to saturation. Each library was down-sampled to equivalent sequencing depth and were merged with *cell ranger aggr* (version 2.0.2). The merged data were transferred to the R statistical environment for analysis using the package Seurat (Patil et al. 2018; Macosko et al. 2015) version 2.3.4, in R version 3.5.0. Only cells expressing between 200 to 2500 genes, less than 5% mitochondrial-associated transcripts and genes expressed in at least three cells were included in the analysis. The data were then log-normalized and scaled per cell, and the variable genes were detected using the *Findvariablegenes* function in Seurat, as per the default settings. The transcriptomic data from each cell were then further normalized by the number of genes quantified, unique molecular identifier (UMI)-detected and mitochondrial genes to account for technical variation. A principal component analysis was run on the variable genes, and the first five principal components (PCs) were selected for further analyses, based on the standard deviation of the PCs, as determined by an elbow plot in Seurat. The cells were clustered using the *FindClusters* function in Seurat with the default settings, resolution = 0.6 and five PCs. Upon initial analysis, it was identified that the majority of variance was reflective of minor B cell and myeloid contamination. These two clusters were removed (*SubsetData* function), the most variable genes were then re-calculated, the data were re-scaled and the number of principle components for further analysis were re-calculated, as above. The filtered dataset was re-clustered using the *Findclusters* function, using the first seven

principle components, resolution 0.6 (default). A t-distributed stochastic neighbour embedding (tSNE) matrix was calculated using the default perplexity and seven principle components (*RunTSNE* function). For broadly defining the transcriptional features of each cluster, the function *FindAllMarkers* (only.pos = FALSE, min.pct = 0.0, thresh.use = 0) was used and the associated heatmap was generated using the function *DoHeatmap*, using up to the top ten transcripts identified per cluster, as defined by *FindAllMarkers*. The differential expression between cluster Cluster 4 and Cluster 0 was determined using MAST ($q < 0.05$, min.pct = 0, rest as per default, v1.8.0) (Finak et al. 2015). Further visualizations of exported normalized data were generated using the Seurat package and custom R scripts. Down-sampling was achieved using the *SubsetData* function in Seurat to maintain equivalent numbers of cells between KO and WT mice libraries (5046 cells).

2.15 Systemic depletion of Treg cells in the *Foxp3$^{GFP\text{-}DTR}$* mouse model

The systemic ablation of Treg cells was achieved in the *Foxp3$^{GFP\text{-}DTR}$* mouse model by dosing Diphtheria toxin (Sigma-Aldrich) at 50 μg/kg intraperitoneally.

2.16 Statistical analysis

Prism (GraphPad) version 7.05 was used to apply the Student's unpaired two-tailed *t*-test for normally distributed data, or the Mann–Whitney U test for data not normally distributed. Where necessary, the Shapiro–Wilk test was used to test for normality of the underlying sample distribution. Statistical analysis for RNA-Sequencing data is built into the software used for processing the data (see sections 2.12 and 2.14). The q value output, which incorporates correction for multiple testing, was the value used to determine statistical significance. No blinding was necessary, since objective quantitative assays, such as flow cytometry were used. The experimental sample sizes were chosen using power calculations,

using preliminary experiments, or were based on previous experience of variability in similar experiments. The samples which had undergone technical failure during processing were excluded from the analyses.

3 Assessment of BACH2 expression in lineage-committed Treg cells

3.1 Background

Across all different mammalian tissue types, BACH2 expression appears to be highest in B cells, where its peak expression is at the pro-B cell stage and in mature circulating B cells, which have not yet been activated by their cognate antigen (A. Muto 1998). Conversely, its expression in B cells is lower, and undetectable in plasma B cells, which have undergone terminal differentiation following antigen stimulation. Though still substantial, expression of BACH2 is comparatively lower in $CD4^+$ and $CD8^+$ T cells. *Bach2* mRNA is highly expressed in naïve $CD8^+$ T cells and minimally expressed in stimulated effector subsets, whereas intermediate levels of expression are observed in central memory $CD8^+$ T cells (Roychoudhuri et al. 2013, 2016). These observations suggest a model where BACH2 expression is highest in quiescent naive and memory lymphocyte populations, but progressively downregulated by repeated antigen receptor stimulation. In T cells, as BACH2 binds to and obstructs expression of TCR-inducible genes, this gradual stage-specific and signal responsive decline may represent a principal mechanism enabling T cell terminal differentiation.

Although BACH2 expression has been reported in $CD4^+$ T cells, prior to the start of the research reported in this thesis (2015), no published literature had focused specifically on the expression of BACH2 in $CD4^+$ $Foxp3^+$ Treg cell subsets. Similar to B cell populations and $Foxp3^-$ effector T cell populations, Treg cells exist in resting and activated states (section 1.6). Therefore, factors must likewise exist to instruct phenotypic differences. In scRNA-Seq studies published by Zemmour, et al. in 2018, the authors noted that *Bach2* expression was enriched in clusters that expressed *Ccr7*, *Sell* and *Satb1*. Although this gave some indication

of the mRNA expression of *Bach2* in Treg cells, it remained to be understood how BACH2 was expressed in Treg cells at the protein level.

In 2013, Kometani, *et al*. published the use of a novel transgenic mouse strain bearing a red tandem fluorescent protein (tdRFP) inserted in frame into the coding region of the *Bach2* gene (Kometani et al. 2013). This strain thus produced a detectable tdRFP signal, where the expression was driven by the endogenous regulatory elements of the *Bach2* gene. As the tdRFP insertion is disruptive and prevents expression of wild type BACH2, only heterozygous mice were used in their studies. In order to validate that tdRFP expression reflected BACH2 expression in the *Bach2*^{tdRFP} mouse strain, Itoh-Nakadai, *et al*. purified lymphoid progenitor cells expressing either low or high levels of tdRFP by FACS, and quantified the relative levels of Bach2 expression in each population by RT-PCR (Itoh-nakadai et al. 2014). I crossed *Bach2*^{tdRFP} mice with the *Foxp3*$^{EGFP\text{-}DTR}$ mouse strain, which would enable fluorescent tracing of Foxp3^{+} Treg cells—according to GFP expression—and their ablation using DTx (Figure 3.1). I thus generated a novel dual reporter mouse strain, *Bach2*^{tdRFP} *Foxp3*$^{EGFP\text{-}DTR}$, where the levels of tdRFP—serving as a marker indicative of the levels of expression of the *Bach2* gene—could be quantified in Foxp3^{+} Treg cells. The aim of this chapter was to ascertain whether *Bach2* is expressed in lineage-committed Treg cell populations.

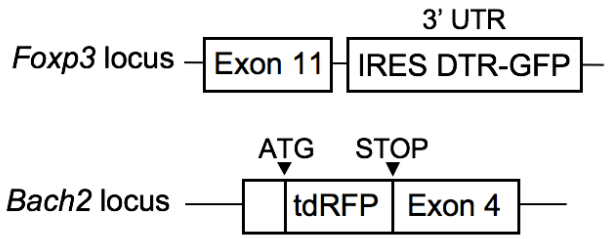

Figure 3.1: Schematic of transgenic loci in *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*^{tdRFP} mouse strain.

3.2 Results

3.2.1 *Bach2* expression in thymic Treg cell precursors

I sought to assess the expression of *Bach2*^{tdRFP} reporter activity in Foxp3^{+} Treg cell precursors developing in the thymus. Thymocytes were extracted from *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*$^{tdRFP/+}$ mice and analysed using flow cytometry. Gating on CD4SP thymocytes revealed that developing Treg cells in the thymus expressed high levels of *Bach2* (Figure 3.1A).

Discerning between different thymocyte populations showed varying *Bach2* reporter expression in relation to developmental stage, with the double-positive (DP) population being negative—comparable to *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*$^{+/+}$ control mice—the Foxp3^{-} CD4SP population exhibiting intermediate *Bach2* reporter expression and the Foxp3^{+} CD4SP population expressing the highest levels (Figure 3.1B). As the frequency of Foxp3^{+} CD4SP cells are comparatively lower in relation to that of other thymocyte types, plotting of the mean fluorescence intensity (MFI) of tdRFP expression further provided useful insight into the relative *Bach2* expression between different thymocyte populations. Thus, developing thymic Treg cells express high levels of *Bach2*, consistent with its role in their development.

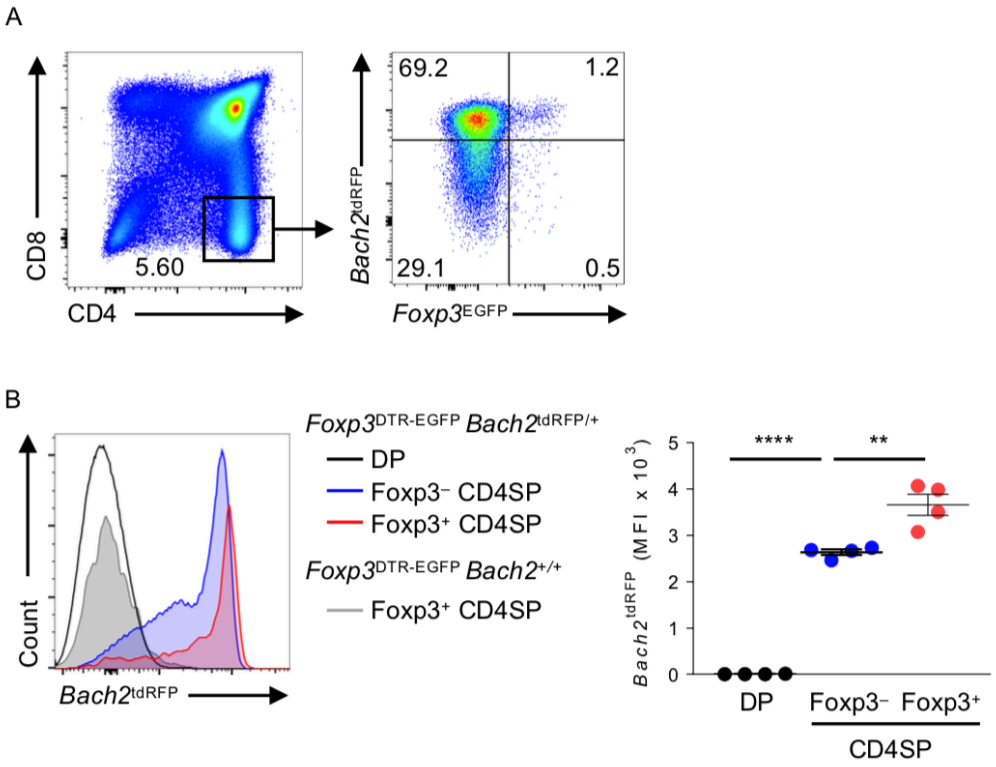

Figure 3.2: *Bach2* is highly expressed during Treg ontogeny.

(**A**) Representative flow cytometry of thymocytes from *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*$^{tdRFP/+}$ mice, with CD4 single-positive (CD4SP) cells gated (left) and the expression of *Bach2*^{tdRFP} and *Foxp3*$^{\text{EGFP-DTR}}$ in these gated cells (right). Gated on live single cells. (**B**) Histograms of representative *Bach2*^{tdRFP} expression (left) and replicate mean fluorescence intensity measurements (MFI, right) of distinct thymocyte populations from *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*$^{tdRFP/+}$ or *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*$^{+/+}$ control mice. Data are representative of two independently repeated experiments with four to five mice per group. **p <0.01, ****P <0.0001; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

3.2.2 *Bach2* expression in peripheral Treg cells under steady-state conditions

As high levels of *Bach2* were observed in thymic Treg cell-precursors, I asked whether high *Bach2* reporter expression was maintained, or differed, upon their emigration from the thymus. Therefore, I isolated blood from *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*$^{\text{tdRFP/+}}$ mice and quantified levels of *Bach2*$^{\text{tdRFP}}$ expression in circulating Treg cells using flow cytometry. In contrast to the unequivocal high *Bach2*$^{\text{tdRFP}}$ expression in thymic Treg cell-precursors, peripheral lineage-committed Treg cells displayed heterogeneous levels of *Bach2*$^{\text{tdRFP}}$ expression (Figure 3.3A). Treg cells expressing high and low levels of *Bach2*$^{\text{tdRFP}}$—hereafter referred to as *Bach2*$^{\text{high}}$ and *Bach2*$^{\text{low}}$ Treg cells, respectively—were clearly discernible. As these Treg cells were isolated from the blood, they could have been of thymic origin or peripherally induced. Nrp1 is considered to represent a marker of tTreg cells under non-inflammatory conditions (discussed in section 1.3.1). I therefore, gated blood Treg cells into Nrp1^{+} or Nrp1^{-} populations and quantified levels of *Bach2* reporter expression. A substantial proportion of Nrp1^{+} Treg cells (~48%), indicative of tTreg cells, were *Bach2*$^{\text{low}}$ (Figure 3.3B). This was especially interesting, given that CD4SP Foxp3^{+} thymocytes predominantly express high levels of the *Bach2* reporter, and thus, one can infer that *Bach2* is downregulated in a subset of thymic Treg cells in the periphery. In comparison, Nrp1^{-} Treg cells—indicative of pTreg cells—were predominantly *Bach2*$^{\text{high}}$ (23% were *Bach2*$^{\text{low}}$). Therefore, these data indicate that a subset of peripheral tTreg cells downregulate *Bach2,* following their emigration from the thymus.

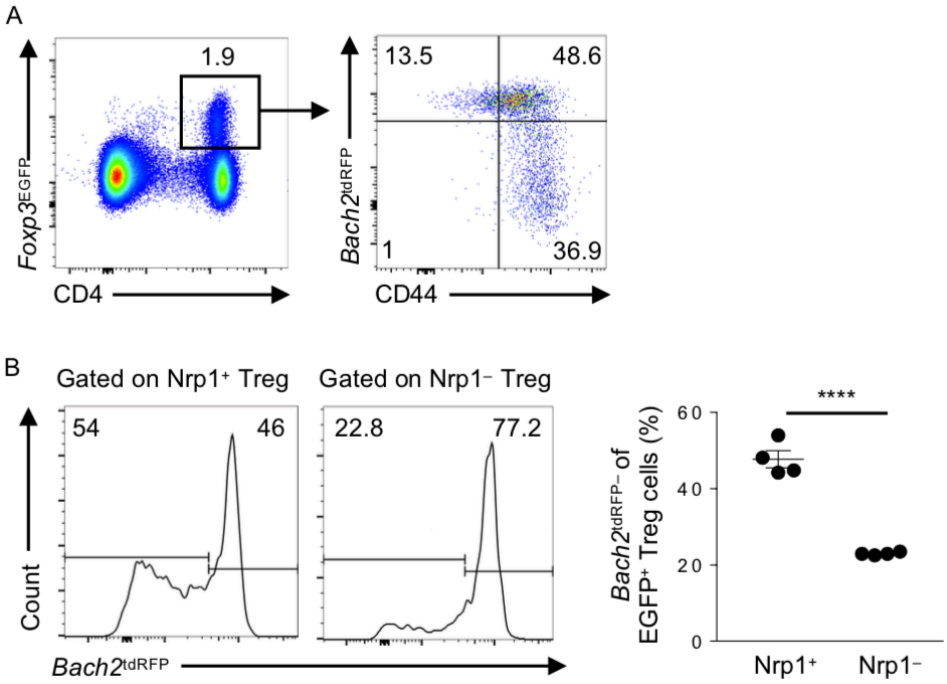

Figure 3.3 Extra-thymic Treg cells express heterogeneous levels of *Bach2*.

(**A**) Representative gating of Treg cells isolated from the blood of *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*$^{\text{tdRFP/+}}$ mice (left) and expression of *Bach2*$^{\text{tdRFP}}$ and CD44 by the gated cells (right). (**B**) Representative histograms (left) and frequency of cells (right) expressing low *Bach2*$^{\text{tdRFP}}$ (*Bach2*$^{\text{low}}$) within Nrp1^{+} and Nrp1^{-} subsets of EGFP^{+} Treg cells from *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*$^{\text{tdRFP/+}}$ mice. Data are representative of two independently repeated experiments with four to five mice per group. ****P <0.0001; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

3.2.3 The downregulation of *Bach2* expression in peripheral Treg cells in response to inflammation

The experiments thus far considered *Bach2* expression during steady-state conditions. That is, the levels of *Bach2*^{tdRFP} expression in a non-inflammatory environment. Being that BACH2 is regulated by antigen receptor and inflammatory signals in B cells and Foxp3^{-} T cells (section 1.7.1), I questioned how levels of *Bach2* would change in Treg cells in response to inflammation. One established approach to induce systemic inflammation and Treg cell activation is the transient incomplete ablation of Treg cells in the *Foxp3*$^{EGFP\text{-}DTR}$ transgenic system (first introduced in section 1.5.5). Here, a single DTx dose causes incomplete transient depletion of Treg cells, which then gradually recover over a period of 10–15 days (J. M. Kim, Rasmussen, and Rudensky 2007). This acute loss results in temporary Teff cell activation and expansion. As I had crossed the *Bach2*^{tdRFP} transgene with *Foxp3*$^{EGFP\text{-}DTR}$ in our dual reporter model, I used the DTx-mediated ablation model to provoke acute inflammation (Figure 3.4).

Eleven days following the administration of DTx to *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*$^{tdRFP/+}$ mice, I isolated splenocytes and assessed the expression of the *Bach2* reporter using flow cytometry. A striking reduction in the expression of *Bach2*^{tdRFP} was observed in splenic Treg cells from DTx-treated mice (Figure 3.5A). Whereas ~63% of splenic Treg cells from the PBS-treated control mice were *Bach2*^{high}, only ~13% were observed in those treated with DTx (Figure 3.5B). Thus, Treg cells downregulate *Bach2* expression in response to inflammation.

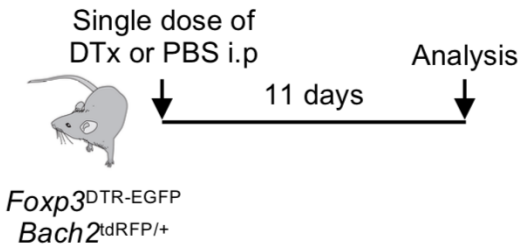

Figure 3.4: Experimental schema of the DTx-mediated acute Treg cell depletion model in the *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*$^{\text{tdRFP/+}}$ transgenic mouse strain.

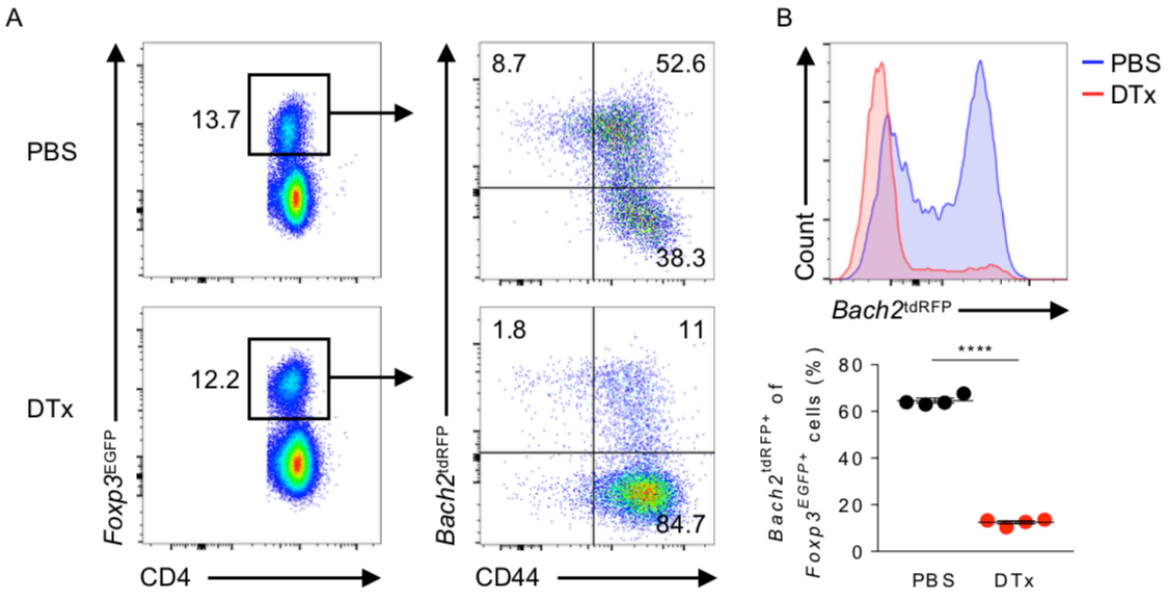

Figure 3.5: Treg cells downregulate *Bach2* expression in response to inflammation.

(**A**) Representative flow cytometry of splenic Treg cells isolated from *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*$^{\text{tdRFP/+}}$ mice 11 days following treatment with PBS control or 50 µg/kg DTx, and their expression of *Bach2*$^{\text{tdRFP}}$ and CD44. (**B**) Representative histograms of *Bach2*$^{\text{tdRFP}}$ expression (top) and replicate measurements (bottom) of *Bach2*$^{\text{tdRFP}}$ expression among EGFP^{+} Treg cells following PBS or DTx treatment. Data are representative of two independently repeated experiments with four to five mice per group. ****P <0.0001; unpaired Student's t-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

3.2.4 The expression of distinct gene sets correlate with expression levels of *Bach2*

I asked whether differences in gene expression could be distinguished between *Bach2*^{low} and *Bach2*^{high} Treg cells. I used FACS to purify EGFP^{+} Treg cells from the spleens of *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*$^{tdRFP/+}$ mice into *Bach2*^{low} and *Bach2*^{high} populations and quantified differential gene expression using RNA-Seq. Unsupervised hierarchical clustering analysis clearly discriminated *Bach2*^{low} and *Bach2*^{high} Treg cells (Figure 3.6A). Within the *Bach2*^{high} population, the expression of genes classically associated with naïve and memory lymphoid-resident T cells, such as *Ccr7* and *Sell*, were enriched. Likewise, genes required for migration to non-lymphoid tissue, such as *Ccr8*, *Ccr2* and *Ccr4* were downregulated. In addition, *Satb1*, which encodes a transcription factor involved in early Treg cell lineage-commitment, was upregulated in *Bach2*^{high} Treg cells. In contrast, within the *Bach2*^{low} population, genes that encode proteins required for Treg cell function, such as *Ebi3* (encodes a subunit for IL-35), *Gzmb* and *Fgl2*, were enriched. Co-stimulatory and co-inhibitory genes, such as *Icos* and *Tigit*, were also found upregulated in *Bach2*^{low} Treg cells. Notably, as a positive control, we observed higher levels of *Bach2* mRNA in *Bach2*^{high} versus *Bach2*^{low} cells, in contrast to Foxp3 mRNA, which was uniformly expressed.

Previous studies have identified gene expression changes in aTreg versus rTreg cells (Luo et al. 2016). Comparing these known changes in these particular genes with those between *Bach2*^{low} and *Bach2*^{high} Treg cells using gene set enrichment analysis (GSEA) revealed that the aTreg gene signature was significantly enriched in *Bach2*^{low} Treg cells (normalised enrichment score; NES = 1.17, $p < 0.05$), as shown in Figure 3.6B. In addition, GSEA analysis identified the enrichment of genes involved in IL-2/STAT5 signalling—a gene set canonically upregulated in TCR-stimulated T cells—in *Bach2*^{low} Treg cells (NES = 1.35, $p < 0.05$). These expression data strongly suggest that rTreg cells are enriched among Treg cells

expressing high *Bach2*, and conversely, aTreg cells are enriched among Treg cells expressing low *Bach2*.

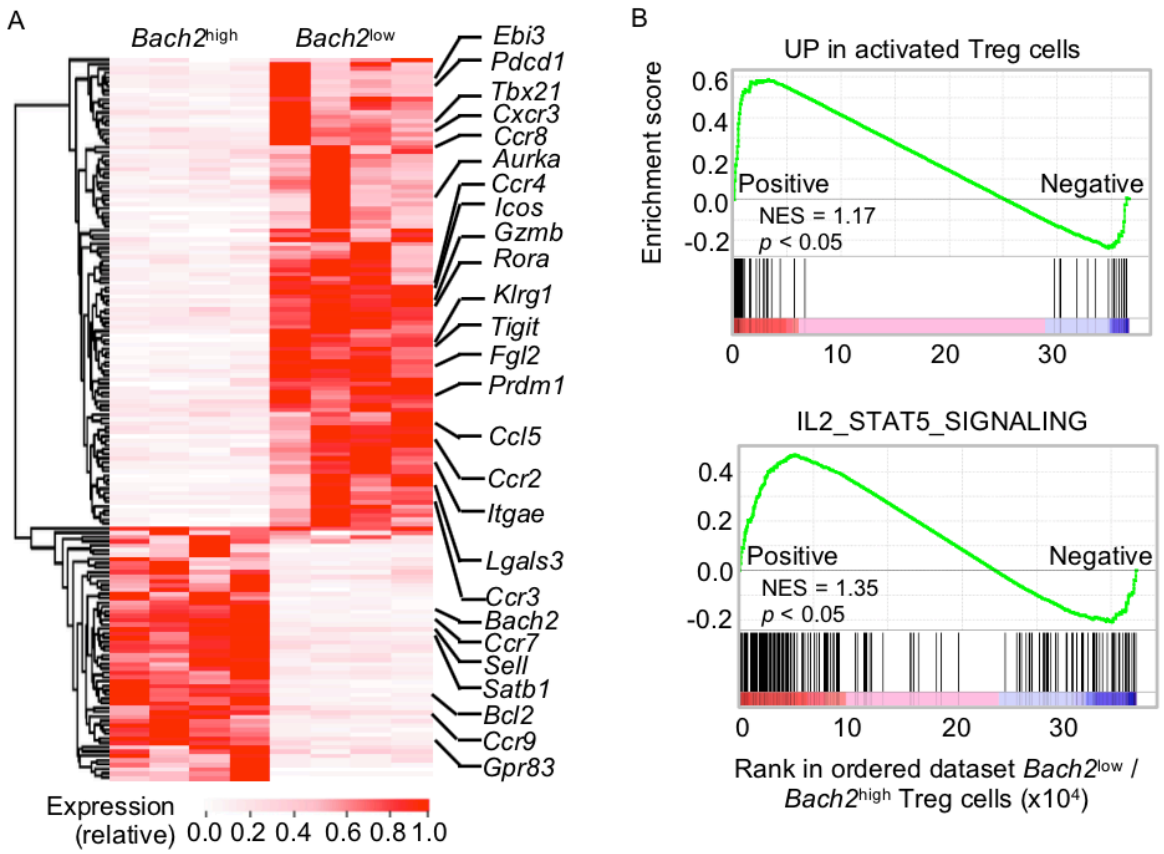

Figure 3.6: Heterogeneous *Bach2* expression in Treg cells identifies distinct populations bearing divergent gene transcription.

(**A**) Unsupervised hierarchical clustering analyses showing differentially expressed genes between *Bach2*^{high} and *Bach2*^{low} Treg cells purified by FACS from spleens of *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*$^{tdRFP/+}$ mice (FC > 2, FDR < 0.05). Replicates and genes are hierarchically clustered on the *x* and *y* axes, respectively. The fragments per kilobase of transcript per million mapped reads (FPKM) values are normalized to row maxima. (**B**) Gene set enrichment analysis (GSEA) of known differentially upregulated genes in aTreg cells versus rTreg cells within the transcriptional differences between *Bach2*^{low} and *Bach2*^{high} Treg cells from *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*$^{tdRFP/+}$ mice (top). GSEA of genes involved in IL-2/STAT5 signalling within the transcriptional differences between *Bach2*^{low} and *Bach2*^{high} Treg cells (bottom). Data are representative of four biological replicates per group.

3.2.5 *Bach2* expression distinguishes different Treg cell phenotypes

I sought to validate the transcriptional data obtained from RNA-Seq experiments at the protein level, and thus used antibody staining to identify several key cell surface phenotypic markers in *Bach2*^{low} and *Bach2*^{high} Treg cells. Flow cytometric analysis of splenic Treg cells from *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*$^{tdRFP/+}$ mice showed that *Bach2*^{low} Treg cells expressed high levels of CD62L and CD25, whereas *Bach2*^{high} Treg cells expressed high levels of KLRG1 and CD44. Although some activation markers, such as CD69, showed no differences in expression, these data were consistent with that obtained from RNA-Seq experiments. Therefore, expression data at the transcriptional and protein level support the idea that high *Bach2* expression demarcates Treg cells with a resting phenotype.

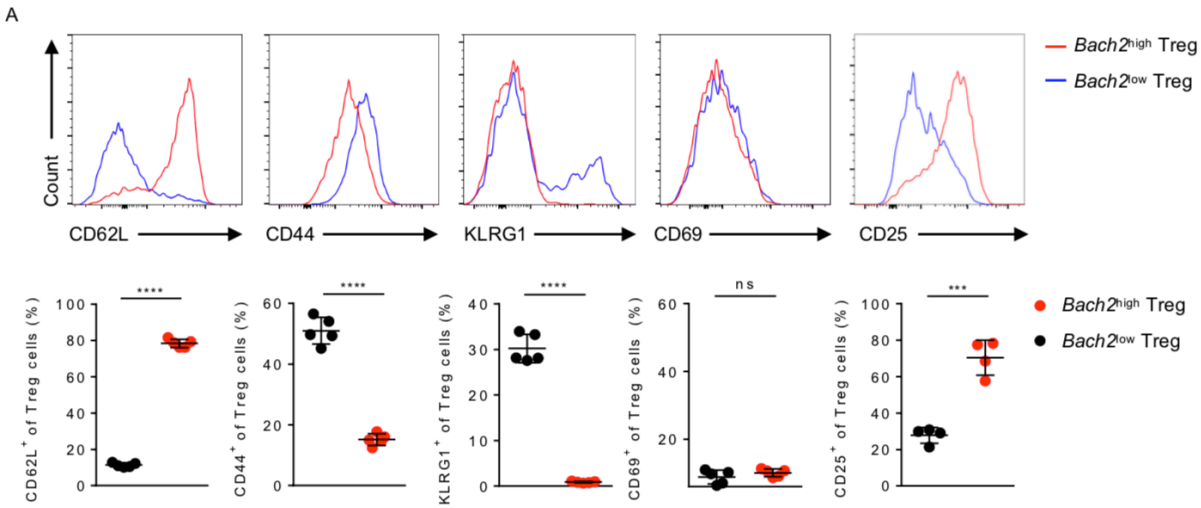

Figure 3.7: *Bach2*^{low} and *Bach2*^{high} Treg cells are phenotypically divergent at the protein level.

(**A**) Representative histograms (top) and replicate measurements (bottom) of the expression of the indicated proteins on the surface of Treg cells expressing high versus low *Bach2*^{tdRFP}, isolated from the spleens of *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*$^{tdRFP/+}$ mice.

3.3 Discussion

3.3.1 Summary

In this chapter, I utilised a novel dual reporter system, the *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*^{tdRFP} mouse strain, to model *Bach2* expression in Treg cells. Flow cytometric measurement of the *Bach2*^{tdRFP} reporter expression within EGFP^{+} Treg yielded novel insights into how *Bach2* expression varies depending on the Treg developmental stage, physiological location and environment. Thymic Foxp3^{+} Treg precursors highly expressed the *Bach2* reporter (Figure 3.2). However, upon their maturation and migration to secondary lymphoid organs, peripheral Treg cells then displayed heterogenous *Bach2*^{tdRFP} expression (Figure 3.3). By leveraging the use of the Diphtheria toxin receptor transgene knock-in at the *Foxp3* locus (Figure 3.4), acute DTx-mediated systemic Treg ablation in the *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*^{tdRFP} revealed that the *Bach2* reporter is downregulated in response to inflammation (Figure 3.5). By comparing global transcriptional differences between Treg cells expressing high versus low levels of the *Bach2* reporter (*Bach2*$^{\text{high}}$ versus *Bach2*$^{\text{low}}$ Treg cells, respectively) using RNA-Seq, I observed enrichment of genes associated with lymphoid residency and quiescence in *Bach2*$^{\text{high}}$ Treg cells (Figure 3.6). Conversely, *Bach2*$^{\text{low}}$ Treg cells were enriched for the aTreg cell gene set and upregulated genes involved in Treg cell function and migration toward non-lymphoid tissue. Indeed, by staining EGFP^{+} Treg cells from *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*^{tdRFP} mice with a panel of hallmark cell surface phenotypic markers, *Bach2*$^{\text{high}}$ Treg cells were CD62L^{+} CD25^{+} CD44^{-} KLRG1^{-}. In contrast, *Bach2*$^{\text{low}}$ Treg cells were CD62L^{-} CD25^{-} CD44^{+} KLRG1^{+} (Figure 3.7). These data represent a comprehensive and novel insight into *Bach2* expression in Treg cells. Collectively, my studies show that heterogeneous *Bach2* expression identifies Treg cells in distinct states of activation. Furthermore, I demonstrated that Treg cells expressing high levels of *Bach2* discriminate rTreg cells, whereas those expressing low levels of *Bach2* distinguish aTreg cells.

3.3.2 Expression of BACH2 in human lineage-committed Treg cells

These data are in agreement with that generated by Zemmour, *et al*., who used scRNA-Seq experiments to show that *Bach2* is downregulated in Treg cells from activated clusters. Interestingly, their analysis of human cells—in accordance with my findings—revealed that high *Bach2* expression coincided with the expression of *CCR7*, *SATB1*, and *SELL*. These findings point toward shared patterns of *Bach2* expression between mouse and human Treg.

3.3.3 The role of BACH2 during thymic Treg cell development

As *Bach2*^{tdRFP} was expressed highly during Treg ontogeny, it is interesting to speculate on the role that *Bach2* fulfils during thymic Treg cell development. Whereas *Bach2*^{tdRFP} expression in DP thymocytes was absent, it was upregulated at the next developmental stage (CD4SP) and peaked in Foxp3^{+} CD4SP cells (Figure 3.2B). Here, it would be interesting to look into what factors propagate *Bach2* expression and are required for its upregulation. In addition, it would be interesting to use highly sensitive techniques to observe where in the genome BACH2 is binding in Foxp3^{+} CD4SP cells, and compare this with the distribution of chromatin accessibility. As absence of BACH2 results in the failure of Foxp3 expression, perhaps these two TFs function in a co-ordinated manner to establish Treg cell populations. However, the role of BACH2 during pre-lineage-commitment of Treg cells is outside the scope of the present work.

3.3.4 Heterogeneous *Bach2* expression in peripheral Treg cells

Whereas developing Treg cells in the thymus were explicitly *Bach2*^{high}, circulating tTreg cells—as identified using Nrp1—expressed *Bach2* at varying levels (Figure 3.3). Interestingly, Treg cells negative for Nrp1—indicating pTreg cells— were predominantly *Bach2*^{high}, which suggested that pTreg cells are less susceptible to *Bach2* downregulation

than tTreg cells under steady-state conditions. It remains to be understood why this may be the case, and further experiments could address if, and why mechanistically, *Bach2* expression is less heterogeneous in Treg cells that are peripherally induced. Further experiments could also consider the relationship between *Bach2* heterogeneity and activation of the PI3K-AKT-mTOR signalling pathway. As BACH2 is phosphorylated downstream of PI3K activation, the levels of activation could be ascertained in *Bach2*^{high} versus *Bach2*^{low} Treg cells from *Foxp3*$^{EGFP\text{-}DTR}$ *Bach2*^{tdRFP} mice. In addition, to further the understanding of my previously mentioned observation, relating to the heterogeneity of *Bach2* expression in circulating tTreg versus pTreg cells, PI3K activation could be measured in circulating Nrp1^{+} versus Nrp1^{-} Treg cells.

4 Validation and phenotyping of the *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*^{flox} mouse strain

4.1 Background

Following on from my observation that levels of *Bach2* expression discriminates Treg cells of distinct phenotypes, I sought to understand the function of BACH2 in lineage-committed Treg cells. As *Bach2* expression is required in Treg cell precursors for upregulation of *Foxp3*—that is, it is required in the developmental stage prior to Treg cell lineage-commitment—use of models, such as *Bach*^{KO} mice, cannot provide a system to study lineage-committed Treg cells (section 1.7.2). Instead, I needed to establish a novel system, where BACH2 function is ablated only after induction of *Foxp3* expression and Treg lineage-commitment has occurred. To achieve this, I crossed mice bearing the *Bach2*^{flox} allele with those bearing a *Foxp3*-driven Cre recombinase transgene. Here, I used two different *Foxp3* transgenes—the *Foxp3*$^{YFP\text{-}Cre}$ and *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ transgenes—in separate crosses. In contrast to the *Bach*^{KO} mouse model—where BACH2 function is abrogated in all cell types from gestation, the use of *Foxp3*-driven Cre recombinase transgenes should restrict excision at the *Bach2*^{flox} locus in cell types only after their expression of *Foxp3* (i.e., after Treg cell lineage-commitment has taken place). Furthermore, as the estrogen receptor is knocked into the Foxp3 locus in the *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ transgene, the protein product will only be driven to the nucleus upon treatment of mice with the estrogen analog tamoxifen. Therefore, use of this particular transgene enables additional temporal control of Cre-mediated excision at the *Bach2*^{flox} transgenic locus. In summary, I generated two novel mouse strains—the *Foxp3*$^{YFP\text{-}Cre}$ *Bach2*^{flox} strain and the *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*^{flox} strain, to understand the function of BACH2 in lineage-committed Treg cells. This chapter concerns the validation and phenotyping of these novel mouse strains.

4.2 Results

4.2.1 *Bach2* excision at the *Bach2*^{flox} locus is not specific to Foxp3^{+} cells in the *Foxp3*$^{YFP\text{-}Cre}$ *Bach2*$^{fl/fl}$ strain

The *Foxp3*$^{YFP\text{-}Cre}$ transgene has been reported in previous studies to result in excision of *loxP*-flanked exons within Foxp3^{+} cells (Rubtsov et al. 2008). I sought to validate that indeed, *Bach2* excision only occurs in Foxp3^{+} Treg cells in the *Foxp3*$^{YFP\text{-}Cre}$ *Bach2*$^{fl/fl}$ mouse strain.

Foxp3^{-} Tconv cells and Foxp3^{+} Treg cells were purified from the spleens of eight-week old *Foxp3*$^{YFP\text{-}Cre}$ *Bach2*$^{fl/fl}$ mice using FACS. Genomic DNA (gDNA) was extracted from these cells and used in PCR with primers that amplify either the *TLR9* gene (control), *Bach2*$^{fl/fl}$ (*loxP*-flanked exon 4), or *Bach2*$^{ex/ex}$ (excised exon 4).

Although unexcised *Bach2*$^{fl/fl}$ was detected in Foxp3^{-} Tconv cells, *Bach2*$^{ex/ex}$ was also detected at similar levels to that from Foxp3^{+} Treg cells, using this semi-quantitative method (Figure 4.2). I concluded that excision of exon 4 is not specific to Foxp3^{+} Treg cells in the *Foxp3*$^{YFP\text{-}Cre}$ *Bach2*$^{fl/fl}$ mouse strain and, therefore, this system was not suitable for understanding the lineage-restricted function of BACH2 in Foxp3^{+} Treg cells.

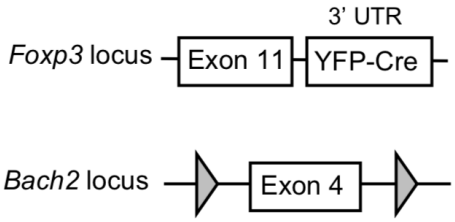

Figure 4.1: Schematic of the transgenic loci in the *Foxp3*$^{YFP\text{-}Cre}$ *Bach2*$^{fl/fl}$ mouse strain.

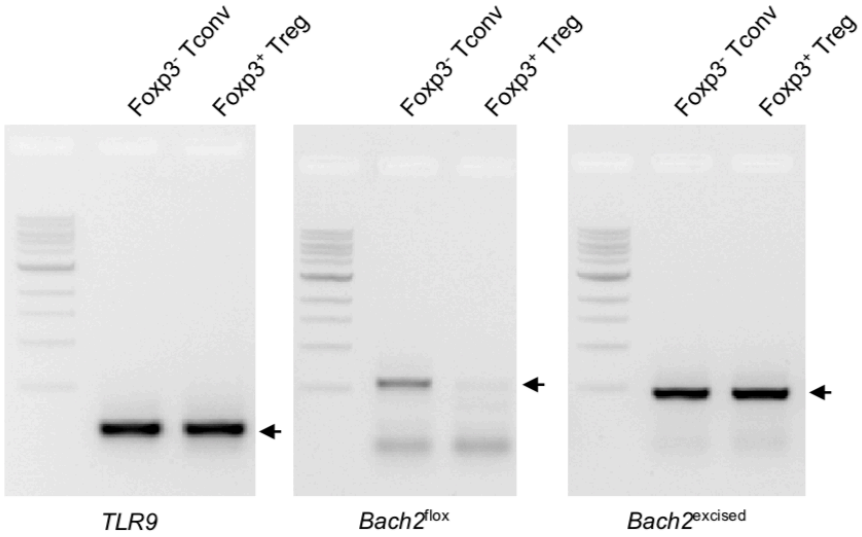

Figure 4.2: Genotyping of splenic Foxp3^{-} Tconv cells and Foxp3^{+} Treg cells from the *Foxp3*$^{YFP\text{-}Cre}$ *Bach2*$^{fl/fl}$ mouse strain.

Primers detect either the *TLR9* gene control locus (band size 450; left panel), *Bach2*$^{fl/fl}$ (*loxP*-flanked exon 4, band size 250; middle panel), or *Bach2*$^{ex/ex}$ (excised exon 4, band size 145; right panel). Primer-amplified products are indicated with black arrows.

4.2.2 Cre-mediated excision in the *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{flox}}$ strain is restricted to Foxp3^{+} Treg cells after tamoxifen treatment

The *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ transgene is an alternative to the *Foxp3*$^{\text{YFP-Cre}}$, which has been suggested to allow a greater level of control—including temporal control—of excision at *loxP*-flanked sites. I crossed mice bearing the *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ transgenes with those bearing *Rosa26*$^{\text{flSTOP-tdRFP}}$—which drives tdRFP expression from the *Rosa26* locus only after Cre-mediated excision of an upstream *loxP*-flanked STOP codon (Figure 4.3A)—to enable indelible marking of any cell that had at any time had been exposed to *Foxp3*-driven Cre expression, using treatment with tamoxifen.

As shown in Figure 4.3B, tdRFP induction from the from *Rosa26*$^{\text{flSTOP-tdRFP}}$ locus was indeed, only seen in cells from mice that had received tamoxifen in the diet for eight weeks. After validating that expression of tdRFP from the *Rosa26* locus was tamoxifen dependant, analysis of individual T cell types isolated from the blood of *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ *Rosa26*$^{\text{flSTOP-tdRFP}}$ mice using flow cytometry showed that tdRFP induction was specific to CD4^{+} T cells (Figure 4.3C). Furthermore, the percentage of *Rosa26*$^{\text{tdRFP}}$-positive CD4^{+} T cells was comparable to the percentage of Foxp3^{+} Treg cells normally found in the blood (14.1%). To confirm that excision of exon 4 in the *Bach2*$^{\text{flox}}$ transgene is specific to Foxp3^{+} Treg cells, and no Cre-mediated excision occurs at this locus in Foxp3^{-} cell types, FACS was used to purify CD4^{+} Foxp3$^{\text{EGFP}}$-negative Tconv cells and CD4^{+} Foxp3$^{\text{EGFP}}$-positive Treg cells from the spleens of tamoxifen treated mice, and the abundance of excised alleles was quantified. Quantitation of the abundance of relative amounts of the *Bach2*$^{\text{excised}}$ transgene showed absent Cre-mediated excision of exon 4 in CD4^{+} Foxp3$^{\text{EGFP}}$-negative Tconv cells from both experimental *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ *Rosa26*$^{\text{flSTOP-tdRFP}}$ mice and control *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ *Rosa26*$^{\text{flSTOP-tdRFP}}$ mice (Figure 4.3D). A positive *Bach2*$^{\text{excised}}$

transgene signal was only detected in the CD4^{+} Foxp3^{EGFP}-positive Treg cell population. This was in contrast to cells isolated from the *Foxp3*$^{YFP\text{-}Cre}$ *Bach2*^{flox} mouse strain, which demonstrated a *Bach2*^{excised} transgene signal in non-Treg cell populations. Therefore, I concluded that Cre-mediated excision events at the *Bach2* and *Rosa26* transgenic loci in the *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ *Rosa26*$^{flSTOP\text{-}tdRFP}$ mouse strain are restricted to Foxp3^{+} Treg cells, after exposure of mice to tamoxifen. As a result of these findings, the *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ conditional knockout (CKO) mouse model was the system chosen for future experiments.

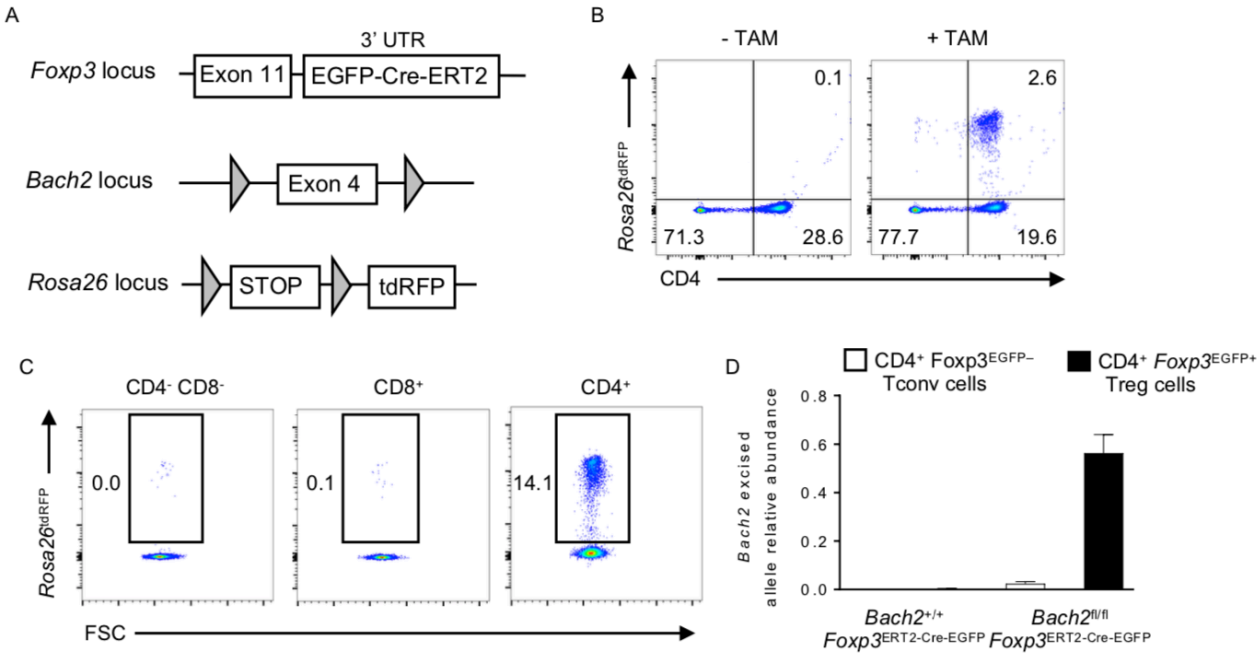

Figure 4.3: Excision of exon 4 in *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ *Rosa26*$^{flSTOP\text{-}tdRFP}$ mice is specific to Foxp3^{+} Treg cells.

(**A**) Schematic of the transgenic loci in the *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ *Rosa26*$^{flSTOP\text{-}tdRFP}$ mouse strain. (**B**) Representative flow cytometry plots showing *Rosa26*$^{flSTOP\text{-}tdRFP}$ induction among circulating CD4^{+} T cells from *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ *Rosa26*$^{flSTOP\text{-}tdRFP}$ mice treated without (-TAM) or with (+TAM) tamoxifen feed for eight weeks. (**C**) Representative flow cytometry plots showing *Rosa26*$^{flSTOP\text{-}tdRFP}$ induction in CD4^{-} CD8^{-} lymphocytes, and CD8^{+} or CD4^{+} T cells from *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ *Rosa26*$^{flSTOP\text{-}tdRFP}$ mice treated with tamoxifen feed for eight weeks. (**D**) Relative quantitation of the abundance of the *Bach2*^{excised} transgene in FACS-purified CD4^{+} Foxp3^{-} Tconv and CD4^{+} Foxp3^{+} Treg cells isolated from the spleens of animals of indicated genotypes treated with tamoxifen for eight weeks. Data

are representative of two individual experiments with six to seven mice per group. *ns*, not significant; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

4.2.3 The function of BACH2 in lineage-committed Treg cells is different to its role prior to lineage-commitment

As Foxp3 induction is dependent upon BACH2 expression during Treg cell development, I asked whether BACH2 is likewise required for the maintenance of Foxp3 after Treg cell lineage-commitment. Cells expressing *Foxp3* in the *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{flox}}$ *Rosa26*$^{\text{flSTOP-tdRFP}}$ mouse strain are indelibly marked upon tamoxifen administration, and subsequently, any cells that have lost *Foxp3* reporter expression can be identified. I examined the ratio of EGFP-negative "ex-Treg cells" within the fraction of tdRFP^{+} CD4^{+} T cells in experimental *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ *Rosa26*$^{\text{flSTOP-tdRFP}}$ mice and control *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ *Rosa26*$^{\text{flSTOP-tdRFP}}$ mice, treated with tamoxifen for eight weeks (Figure 4.4A).

I observed a comparable ratio of ex-Treg cells from experimental and control mice, in both the thymus and spleen (Figure 4.4B). Consistent with the lack of excess lineage-instability caused by the loss of BACH2, splenic Treg cells from tamoxifen-treated *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ mice did not secrete greater amounts of IFN-γ upon *ex vivo* stimulation (Figure 4.4C). In addition, similar percentages and absolute numbers of Foxp3^{+} Treg cells were observed the spleen, mesenteric lymph nodes (mLN) and inguinal lymph nodes (iLN) between tamoxifen-treated *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ and control *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ mice.

Collectively, these date indicate that—in contrast to its function prior to Treg lineage-commitment—BACH2 function following Treg cell lineage-commitment is not required to maintain *Foxp3* expression or restrain inflammatory helper cytokine release.

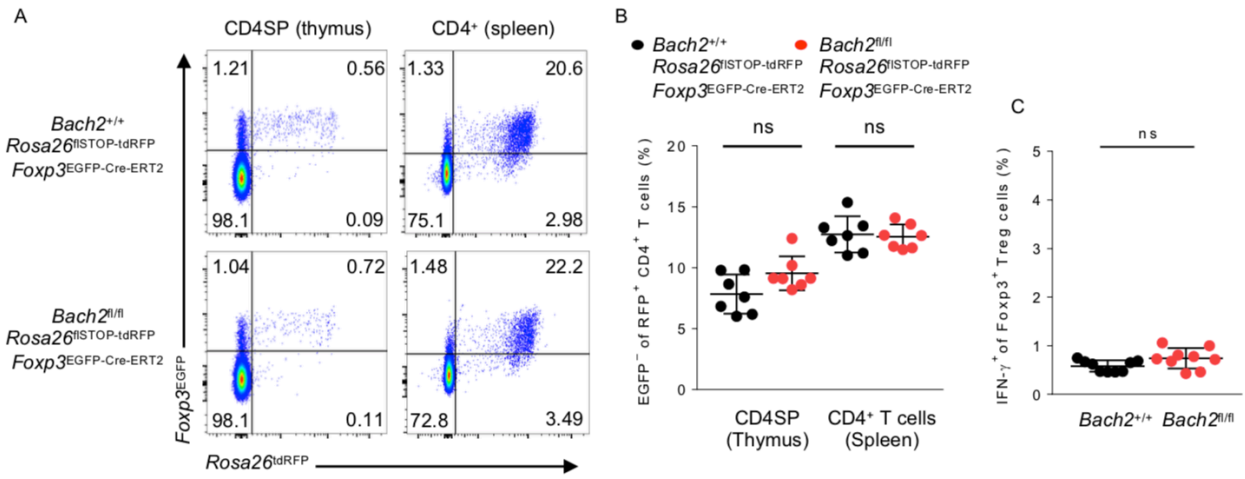

Figure 4.4: The loss of BACH2 function following Treg cell lineage-commitment does not result in Treg lineage instability.

(**A**) Representative flow cytometry and (**B**) replicate measurements of the frequency of EGFP^{-} "ex-Treg" cells within the fraction of *Rosa26*^{tdRFP}-positive CD4SP thymocytes (left) or CD4^{+} splenocytes (right) of mice of indicated genotypes, fed tamoxifen for eight weeks. (**C**) Percentages of splenic IFN-γ^{+} Treg cells isolated *Bach2*$^{+/+}$ *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ mice (*Bach2*$^{+/+}$) and *Bach2*$^{fl/fl}$ *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ mice (*Bach2*$^{fl/fl}$) treated with tamoxifen for eight weeks. Data are representative of two individual experiments with seven mice per group (A and B), or of nine mice per group (C). *ns*, not significant; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

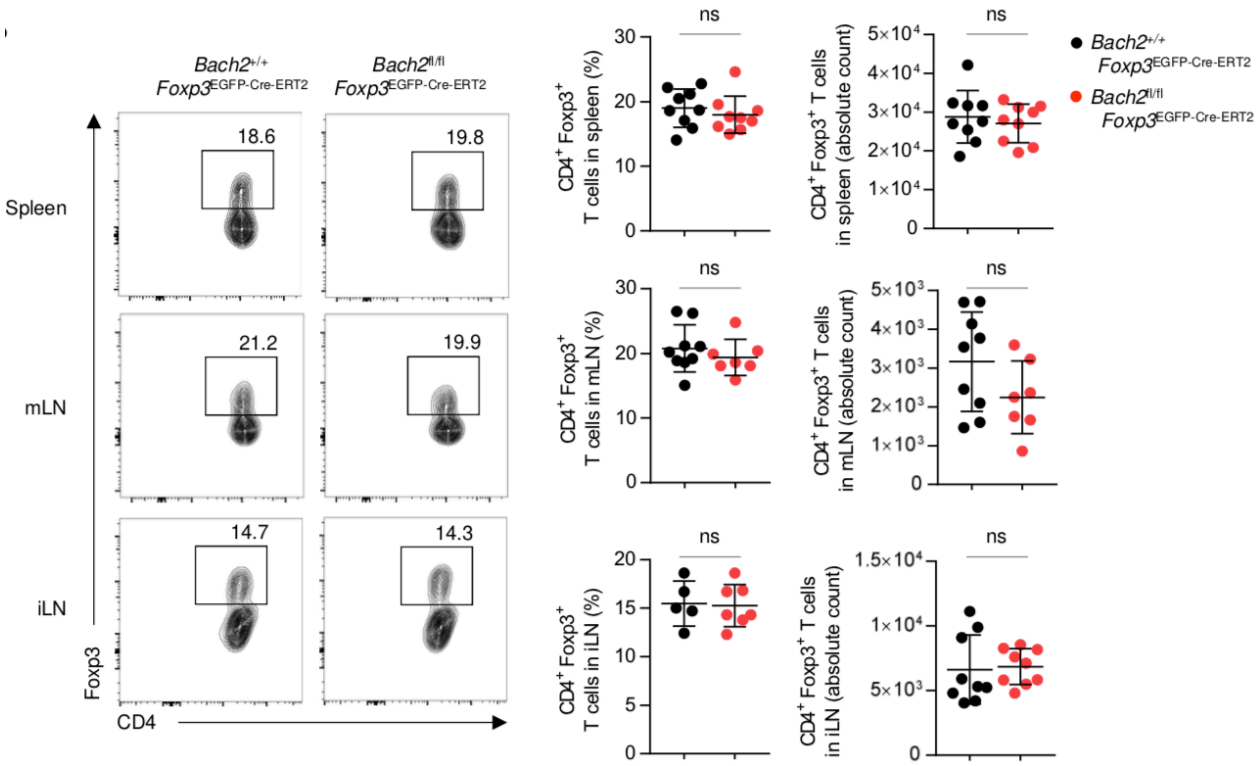

Figure 4.5: Loss of BACH2 function following Treg cell lineage-commitment does not affect the frequency or absolute number of Foxp3$^+$ Treg cells.

Representative flow cytometry (left) and replicate measurements (right) of Foxp3 expression among CD4+ T cells in indicated organs from tamoxifen-treated animals of the indicated genotypes. Data are representative of two repeated experiments with nine (spleen and iLN) or five to nine (mLN) mice per group. *ns*, not significant; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

4.2.4 BACH2 restrains the expression of aTreg genes in lineage-committed Treg cells

As loss of *Bach2* in lineage-committed Treg did not lead to a loss of Treg cell identity, I asked whether *Bach2* is involved in shaping the phenotypic heterogeneity of lineage-committed Treg cells. Global transcriptional profiling of individual cells—and thus, intercellular heterogeneity at a single-cell level—can be ascertained using scRNA-Seq (described in section 1.6). I purified splenic EGFP^{+} Treg cells from tamoxifen-treated conditional knockout *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ mice and control *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ mice (three biological replicates per genotype)—hereafter referred to as CKO and WT mice, respectively—subjected these cells to scRNA-Seq. The resulting data were analysed principally by Dr James Clarke, with myself, Simon Andrews and Rahul Roychoudhuri guiding the process and providing feedback. Data analysis with Seurat and visualisation using t-distributed stochastic neighbour embedding (tSNE) revealed that Treg cells isolated from CKO and WT mice formed seven transcriptionally distinct clusters (Figure 4.6A–B).

Seurat principal component analysis was used to identify genes whose expression represented the primary sources of heterogeneity across all seven tSNE clusters (Figure 4.6B), highlighting genes such as *Sell*, *Ccr7* and *Cxcr3*. Calculation of relative frequency of WT and CKO Treg cells in each individual cluster—normalised in relation to the total number of cells per cluster and per genotype—revealed a reduction in the frequency of CKO Treg cells in Cluster 0 and Cluster 1. Conversely, a relative increase in the frequency of CKO Treg cells was observed in Cluster 4 (Figure 4.6C). In order to understand these tSNE clusters in greater detail, I examined the differentially expressed genes between Cluster 4 and Cluster 0 (Figure 4.6D). The expression of genes associated with Treg cell activation and known to be downregulated in *Bach2*$^{\text{high}}$ versus *Bach2*$^{\text{low}}$ Treg cells (Figure 3.6A) —such as *Icos*, *Cxcr3*,

Fgl2, *Id2*, *Pdcd1*, and *Casp1*—were significantly elevated in Cluster 4. In contrast, genes associated with lymphocyte quiescence and lymphoid tissue residency, and upregulated in *Bach2*^{high} vs *Bach2*^{low} Treg cells —such as *Sell*, *Ccr7* and *Satb1*—were downregulated in Cluster 4.

To confirm that BACH2 expression underlies the observed differences between WT and CKO Treg cells, I used a GSEA with bulk RNA-Seq data to test whether genes downregulated in *Bach2*^{high} versus *Bach2*^{low} Treg cells were significantly enriched among *Bach2*-deficient CKO Treg cells (Figure 4.7). Unlike scRNA-Seq data, bulk RNA-seq data are not amenable to the exclusion of contaminating cell populations (section 2.14). Therefore, these data cannot be used to draw any solid conclusions.

In summary, these results suggest that BACH2 is a principal regulator of Treg cell transcriptional heterogeneity and is responsible for a component of the gene expression profile associated with rTreg cells.

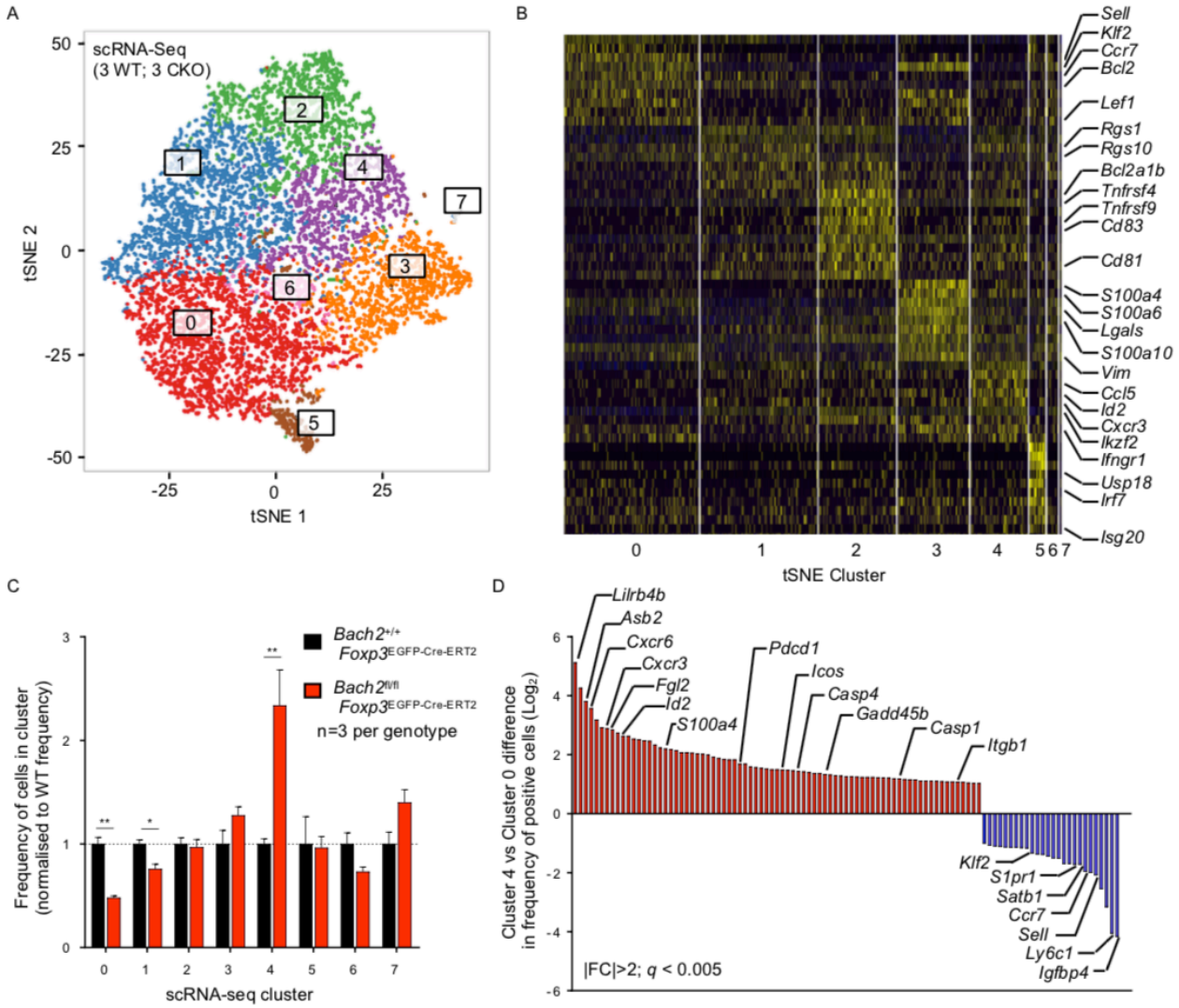

Figure 4.6: *Bach2*-deficient Treg cells are found in clusters that are transcriptionally distinct from *Bach2*-sufficient Treg cells in single-cell RNA sequencing (scRNA-Seq) analyses.

(**A**) T-distributed stochastic neighbour embedding (tSNE) visualisation of single EGFP^{+} Treg cells isolated from the spleens of tamoxifen-treated *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ (WT) mice and *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ (CKO) mice, clustered by gene expression profiles. Three biological replicate cell populations per genotype were subjected to scRNA-Seq. (**B**) Heatmap showing the expression of the genes in the dataset that represent the primary sources of dataset heterogeneity, within each of the seven clusters identified in A. (**C**) The relative frequency of Treg cells from CKO and WT mice within each cluster, normalized to their average ratio among WT replicates. (**D**) The fold change in expression of indicated differentially expressed genes ($p_{adj} < 0.005$ and a log2 fold change > 1) in Treg cells from Cluster 4 versus Cluster 0. Data are from three biological replicates per group.

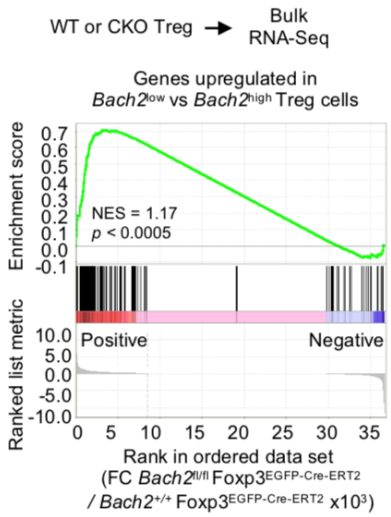

Figure 4.7: Genes that are upregulated in *Bach2*^{low} versus *Bach2*^{high} Treg cells (activated Treg) are significantly enriched among *Bach2*-deficient Treg cells.

Gene set enrichment analysis (GSEA) from bulk RNA-Seq data showing enrichment of genes known to be downregulated in *Bach2*^{high} vs *Bach2*^{low} Treg cells within the transcriptional differences between splenic Treg cells isolated from tamoxifen-treated *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{+/+}$ (WT) mice and *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ (CKO) mice. Data are from three biological replicates per group.

4.2.5 BACH2 restrains the expression of aTreg markers at the protein level in lineage-committed Treg cells

As results from analysis of scRNA-Seq and bulk RNA-Seq data indicated that BACH2 restrains the frequency of aTreg cells in Treg cell populations found in peripheral tissues, I used flow cytometry to examine the expression of key aTreg markers—CD62L, CD44 and ICOS—at the protein level, in Treg cells isolated from several different tissues from tamoxifen-treated conditional knockout *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ (CKO) mice and control *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ (WT) mice. *Bach2*-deficient Treg cells from all tissues examined—the spleen, iLN and mLN—exhibited significantly elevated frequencies of activated Treg cells. Here, loss of *Bach2* in lineage-committed Treg cells resulted in their downregulation of cell surface CD62L and, conversely, their upregulation CD44 (Figure 4.8A, B). In addition, whereas approximately 44% of *Bach2*-sufficient splenic Treg cells expressed cell surface ICOS, a greater proportion of ICOS^{+} splenic Treg cells (approximately 66%) were observed from CKO mice (Figure 4.8C). Therefore, loss of *Bach2* in lineage-committed Treg cells results in greater aTreg differentiation at both the transcriptional and protein level. Collectively, these results suggest that—post lineage-commitment—BACH2 fulfils a central function in maintaining the resting phenotype of Treg cells.

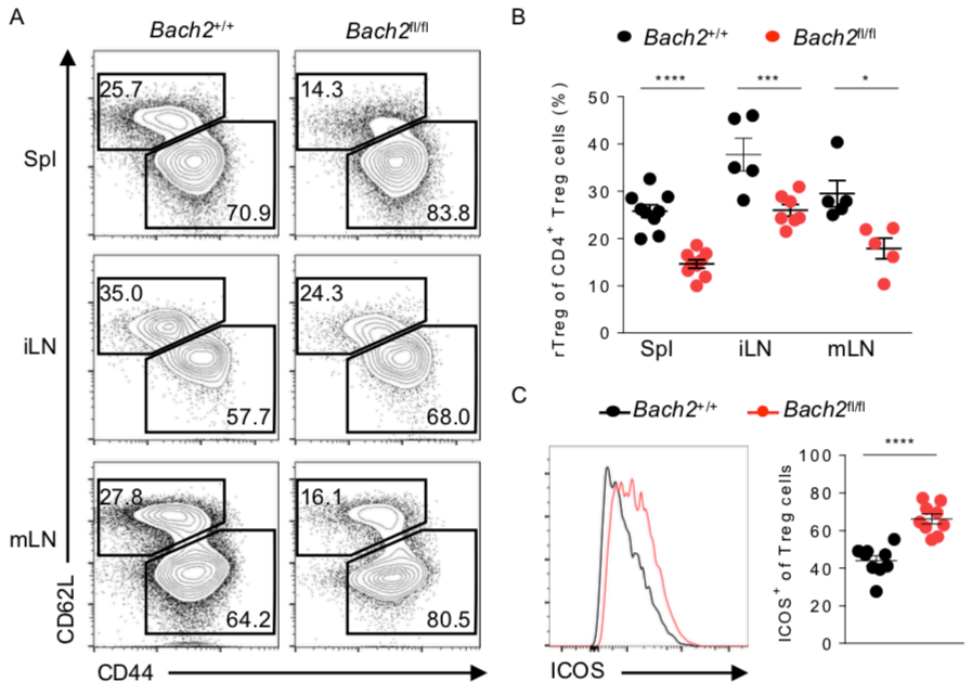

Figure 4.8: *Bach2*-deficient Treg cells from different tissues demonstrate an activated Treg cell phenotype.

(**A**) Representative flow cytometry plots showing cell surface CD62L and CD44 expression by Treg cells isolated from the spleen, mesenteric lymph node (mLN) and inguinal lymph node (iLN) of control *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ mice and *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ and (CKO) mice, treated with tamoxifen for eight weeks. (**B**) Frequencies of CD62L^{+} CD44^{-} resting Treg (rTreg) cells from mice described in **A** within indicated tissues. (**C**) Representative histogram showing the expression (left) and replicate measurements (right) of ICOS on splenic Treg cells from control (black) and CKO (red) mice treated with tamoxifen. Data are representative of two experiments with nine mice per group (spleen and iLN) or four to five mice per group (mLN). *P <0.05, ***P <0.001, ****P <0.0001; unpaired Student's t-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

4.3 Discussion

4.3.1 Summary

In this chapter, I validated a novel animal model to interrogate the function of BACH2 in lineage-committed Treg cells. In the *Foxp3*$^{\text{YFP-Cre}}$ *Bach2*$^{\text{flox}}$ mouse strain, Cre-mediated excision of *loxP*-flanked exon 4 in the *Bach2* locus was observed in *Foxp3*-negative cell types (Figure 4.2). In contrast, Cre-mediated excision events were induced specifically in *Foxp3*-positive Treg cells in the *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ mouse strain—following tamoxifen administration—as demonstrated using genotyping and constitutive reporter labelling with the *Rosa26*$^{\text{flSTOP-tdRFP}}$ transgene (Figure 4.3). Upon confirmation of the *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ model as a robust system, the addition of the *Rosa26*$^{\text{flSTOP-tdRFP}}$ transgene in the system for lineage tracking revealed that no Treg lineage instability resulted from the loss of BACH2 function in lineage-committed Treg cells (Figure 4.4). Concordantly, no decrease in the percentage of EGFP^{+} Treg cells was observed in different tissues examined in conditional knockout *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ versus control *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ mice (Figure 4.5). However, dimensionality reduction analyses of scRNA-Seq data showed that CKO Treg cells expressed transcriptional profiles distinct from WT Treg cells. Whereas *Bach2*-sufficient splenic Treg cells isolated from control mice grouped in a cluster representing canonical rTreg cell genes, *Bach2*-deficient splenic Treg cells isolated from CKO mice grouped in clusters whose genes associate with the aTreg cell phenotype (Figure 4.6). In addition, analysis of bulk RNA-Seq data of CKO versus WT Treg cells using GSEA with genes associated aTreg cells—as identified in section 0 and shown in Figure 3.6—with that aTreg cell genes were enriched in *Bach2*-deficient splenic Treg cells (Figure 4.7). Consistently, flow cytometry analysis of key cell surface activation markers confirmed that at the protein level, CKO Treg cells likewise display an aTreg cell phenotype (Figure 4.8). These data suggest that loss of *Bach2* in lineage-committed Treg cells results in

their greater predisposition to acquire an activated phenotype and that BACH2 functions to maintain resting Treg cells.

4.3.2 Promiscuous Cre-deletion caused by non-Foxp3-specific expression of the *Foxp3*$^{YFP\text{-}Cre}$ transgene

The finding that non-specific deletion of *loxP*-flanked genes occurs in the *Foxp3*$^{YFP\text{-}Cre}$ system—as shown in Figure 4.2—is surprising, given the widespread use of this transgene in the community and the substantial number of publications on its use. Informal discussions within the Laboratory of Lymphocyte Signalling and Development at the Babraham Institute support this finding, where the same observation has been made independently, by different scientists with different alleles. This promiscuous Cre-mediated deletion caused by non-specific expression—or 'leaky' expression—of the *Foxp3*$^{YFP\text{-}Cre}$ transgene, was also reported by Franckaert *et al*., who utilized these leaky excision events to understand the biology of CD28 signalling in Foxp3^{+} versus Foxp3^{-} T cells (Franckaert et al. 2015). Informal discussions with other scientists indicate that the level to which leaky excision events impact the system also depend on the region flanked by *loxP* sites. Under such a scenario, some opt to conduct genotyping for all individual mice and then exclude those demonstrating leaky excision. Given the extent to which leaky excision events are known in the *Foxp3*$^{YFP\text{-}Cre}$ system it is important that a broader awareness is generated in the field about the potential artefacts that could result from use of this strain in experiments without testing of the specificity of Cre-mediated excision for all alleles used.

4.3.3 BACH2 is repurposed following Treg cell lineage-commitment

Given that BACH2 acts in Treg cell precursors to establish a population of Foxp3^{+} lineage-committed Treg cells (as discussed in section 1.7.2), it was unexpected to find that following

Treg cell lineage-commitment, BACH2 no longer functions in the maintenance of Foxp3^{+} T cell populations. Prior to conducting the lineage tracking studies—utilizing the *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{flox}}$ conditional knockout system with the *Rosa26*$^{\text{flSTOP-tdRFP}}$ transgene —I expected to observe a loss of Treg cell identity in Treg cells that had lost BACH2 function following lineage-commitment. In contrast, no increase in ex-Treg cell frequencies were observed from CKO *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ *Rosa26*$^{\text{flSTOP-tdRFP}}$ mice on comparison with controls. These findings indicate that BACH2 fulfils different functions depending on the developmental stage of the T cell; whereas BACH2 acts in Treg cell precursors to establish Foxp3^{+} Treg cells, BACH2 functions to direct phenotypic heterogeneity in lineage-committed Treg cells. This repurposing of the function of BACH2 is not uncommon to transcription factors and has been reported in other instances, such as for Th-POK (Carpenter et al. 2017).

4.3.4 scRNA-Seq studies discern considerable transcriptional heterogeneity from splenic Treg cells

The dimensionality reduction analyses of splenic *Bach2*-sufficient and -deficient Treg cells—as illustrated in Figure 4.6—were in agreement with other studies that have similarly found considerable transcriptional heterogeneity within Foxp3^{+} Treg cells isolated from the spleen (Zemmour et al. 2018). These data (Figure 4.6) support the notion that Foxp3^{+} Treg cells are not a uniform population, which employ the same suppressive mechanisms. Indeed, seven transcriptionally distinct clusters were observed. In addition, as the commonly used marker genes of the two defined Treg cell states—rTreg and aTreg—did not definitively associate with just two separate clusters, these data suggest transcriptomic disparity even within resting or activated Treg cells. This is especially pertinent, given that only splenic Treg cells (i.e., from one tissue type) were analysed. Further advances in technology would enable greater insight into Treg cell heterogeneity between different tissues.

The expression of genes such as *Sell*, *Satb1* and *Ccr7*, represented the principal sources of heterogeneity within this dataset (Figure 4.6B). This finding was reassuring, given these genes and their corresponding proteins are known to delineate Treg cell populations into subtypes (see section 1.6). Indeed, I in part, utilized measurement of cell surface CD62L (the product of *Sell*) to confirm that Treg cells isolated from CKO mice were predisposed toward the aTreg cell phenotype (Figure 4.8). Further incorporated into this analysis, was the measurement of cell surface CD44 and ICOS expression (Figure 4.8C), which have also been proposed as markers to discriminate activated Treg cells (Firan 2006; Bollyky et al. 2007; Zhang et al. 2017). The data generated from this principal component analysis (Figure 4.6B) provide additional marker genes that could be incorporated in analyses to define Treg cell subtypes from heterogeneous Treg cell populations.

4.3.5 Molecular mechanisms of BACH2-mediated constraint of aTreg cell differentiation

Pertinent questions remain regarding how, at the molecular level, BACH2 fulfils its role as a regulator of Treg cell heterogeneity. Being a transcription factor, where does BACH2 bind in the genome in Treg cells and what are these sites (e.g., promoters, enhancers) that BACH2 binds to? Does the binding of BACH2 to different genomic loci affect the expression of modules of genes associated with specific T cell activation processes, such as responses to cytokines or TCR signalling? Does BACH2 co-localise with any other transcription factors, or perhaps associate with any epigenetic regulators, to regulate gene expression? Such questions are beyond the scope of this thesis, and indeed, form the basis of a thesis by another student in the Roychoudhuri laboratory at the Babraham Institute, Firas Sadiyah.

5 Assessing the cell-intrinsic function of BACH2 in Treg cells

5.1 Background

Although previous findings in this thesis identify BACH2 as a factor required by lineage-committed Treg cells for the maintenance of the resting phenotype, uncertainty regarding (1) how this is achieved, and (2) whether this function is cell-intrinsic, remain to be addressed.

Regarding the first point, studies of the BACH2 in other lymphoid cell lineages clearly implicate its action in response to antigen receptor activation. This topic is discussed in detail in section 1.7 and illustrated in Figure 1.5, of the thesis introduction. As demonstrated by Levine *et al.*, the transition of rTreg cells to the aTreg phenotype—a process where BACH2 function was identified as a central factor—is dependent upon continued TCR signalling (Levine et al. 2014). Hypothetically then, it is plausible that—similar to other lymphoid lineages—BACH2 acts in response to antigen receptor activation in lineage-committed Treg cells. The initial results section of this chapter aims to address whether this indeed, is the case.

Regarding the second point, the conditional knockout model utilized in the results section from the previous chapter does not address the cell-intrinsic function of BACH2. As all—or at least, the majority—of Treg cells in tamoxifen-treated *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ mice are *Bach2*-deficient, it is impossible to discriminate the cell-intrinsic and cell-extrinsic consequences of lineage-restricted BACH2 loss in Treg cells. To state this with a hypothetical example, say that a particular TF exists, whose function in a given cell type to regulate the expression of genes involved in the release of a cytokine required for their proliferation. In a system where this TF is globally ablated in all cells of this particular cell

type, one would observe the failure of this cell type to proliferate. However, one would not be able to ascertain whether the function of this TF is cell-intrinsic (i.e., it regulates the expression of genes involved in cellular proliferation, such as those required for cell cycle), or the function of this TF is cell-extrinsic (i.e., it regulates the expression of genes involved in the synthesis and secretion of a lymphoproliferative cytokine). The separation of cell-intrinsic versus cell-extrinsic function of BACH2 can, therefore, only be ascertained in systems containing both *Bach2*-deficient and *Bach2*-sufficient Treg cells simultaneously. In such a system, any phenotype observed in the *Bach2*-deficient Treg cell compartment—but not in the *Bach2*-sufficient Treg cell compartment—could be attributed to the cell-intrinsic function of BACH2. The results section of this chapter aims uses a natural chimeric mouse system to address this.

5.2 Results

5.2.1 BACH2 restrains TCR-driven stimulation in lineage-committed Treg cells

I sought to determine whether BACH2 acted to control antigen-receptor driven aTreg differentiation in lineage-committed Treg cells. rTreg cells were FACS purified from experimental *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ and control *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ mice (hereafter referred to as CKO and WT mice, respectively)—on the basis of high CD62L expression—cultured for three days in 4-hydroxytamoxifen (4-OHT), then transferred to anti-CD3 plus anti-CD28 antibody-coated plates and cultured for a further four days (where a plate with no antibodies served as a control), as depicted in Figure 5.1. Whereas both WT and CKO Treg cells maintained a rTreg cell phenotype after seven days of culture without TCR stimulation (Figure 5.1B, top panels), increased aTreg differentiation was observed among CKO Treg cells following anti-CD3/28 stimulation (Figure 5.1B, bottom panels). TCR stimulation resulted in an average of 18.8% of CD62L^{-} CD44^{+} aTreg from control *Bach2*-sufficient mice versus 38.8% from CKO *Bach2*-deficient mice (Figure 5.1C, top right plot). Consistent with increased *in vitro* activation, a greater percentage of CTLA-4$^{\text{high}}$ Treg cells were also observed among the CKO cells (76%, versus 65% from the WT group; Figure 5.1C, top left plot). In support of previous findings of a lack of excessive lineage instability following the loss of BACH2 function after Treg lineage commitment (Figure 4.4), I observed no differences in the frequency of Foxp3^{+} T cells of live CD4^{+} T cells from WT and CKO groups at the end of the assay (Figure 5.1C, bottom right plot). These data suggest that BACH2 acts restraining TCR-driven stimulation in lineage-committed Treg cells to regulate differentiation of rTreg into aTreg cells.

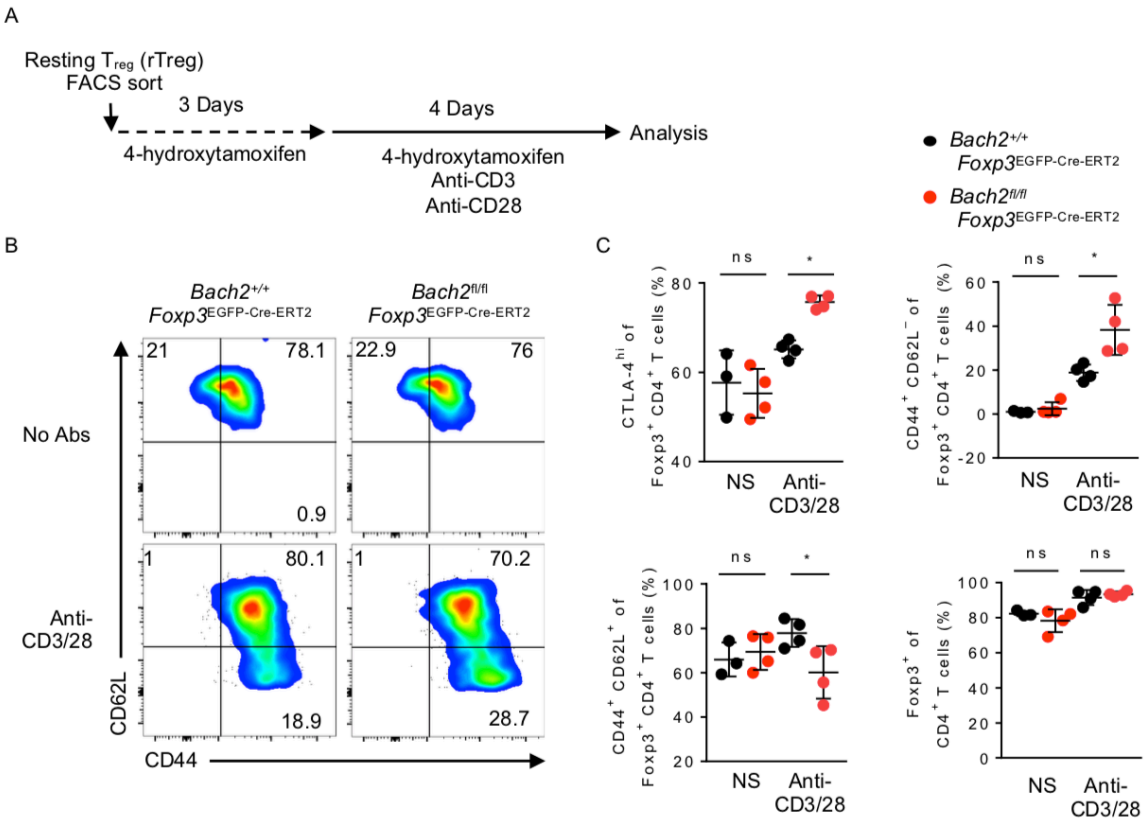

Figure 5.1: BACH2 restrains the differentiation of rTreg to aTreg by restraining TCR-driven stimulation in lineage-committed Treg cells.

(**A**) Experimental schema of the rTreg activation assay. Resting CD62L^{+} EGFP^{+} Treg cells were purified by FACS from spleens of experimental *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ and control *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{+/+}$ mice and cultured in the indicated conditions. (**B**) Representative flow cytometry plots showing the cell surface expression of CD62L and CD44 by Treg cells of indicated genotypes, with or without stimulation using plate-bound anti-CD3 plus anti-CD28 monoclonal antibodies. (**C**) Replicate measurements of the phenotypic Treg cell markers at the end of the culture assay. Data are representative of two individual experiments with eight mice per group, pooled in culture to constitute two biological replicates per group, with each replicate tested in duplicate or triplicate (B and C). Data shown in (C) are all four biological replicates per group from two individual experiments, where the average of the technical replicates (duplicate or triplicate) was used. *ns,* not significant*;* **P* < 0.05; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

5.2.2 BACH2 is required for cell-intrinsic maintenance of Treg cell populations

Previous studies indicate that rTreg cells are long-lived (Cheng et al. 2012). As BACH2 was required for the maintenance of rTreg cells, I questioned whether the loss of rTreg cell maintenance—in the absence of BACH2 function—is associated with the cell-intrinsic loss survival of Treg cell populations over time. By virtue of the *Foxp3* locus on the X chromosome, female mice heterozygous for the *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ transgene are natural chimeras, as random X-inactivation during development results in the production of both EGFP-Cre-ERT2-expressing and non-expressing cells (Figure 5.2A). Therefore, the *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$ mouse strain is a system, whereby both *Bach2*-sufficient and *Bach2*-deficient lineage-committed Treg cells are generated, which permits the analysis of the cell-intrinsic effects resulting from ablation of BACH2 function to be assessed.

Flow cytometric analysis of chimeric *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$ and control *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{+/+}$ mice treated for two weeks with oral tamoxifen revealed a decrease in the proportion of CD62L$^{\text{high}}$ CD44$^{\text{low}}$ rTreg cells and concomitant increase in ICOS expression in the EGFP^{+} Treg cell compartment (Figure 5.2B and C). By eight weeks of oral tamoxifen treatment, although no differences in the proportion and frequency of developing EGFP^{+} Treg cells were observed in chimeric mice in the thymus (Figure 5.3A, top panel), I observed a significant reduction in the proportion and frequency of EGFP^{+} Treg cells in chimeric mice in the spleen and blood (Figure 5.3A, bottom panel). Subsequently, time-course studies using repeated flow cytometry analysis revealed a gradual attrition in EGFP^{+} Treg cell percentages in chimeric *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$, compared to controls, over a period of eight weeks post-administration of oral tamoxifen (Figure 5.3B and C). Importantly, this phenotype is distinct from that observed in chimeric mice where *Bach2* is ablated in a proportion of cells

prior to Treg cell lineage-commitment, where a complete cell-autonomous defect in the generation of Treg cells, including in the thymus, is observed among *Bach2*-deficient cells.

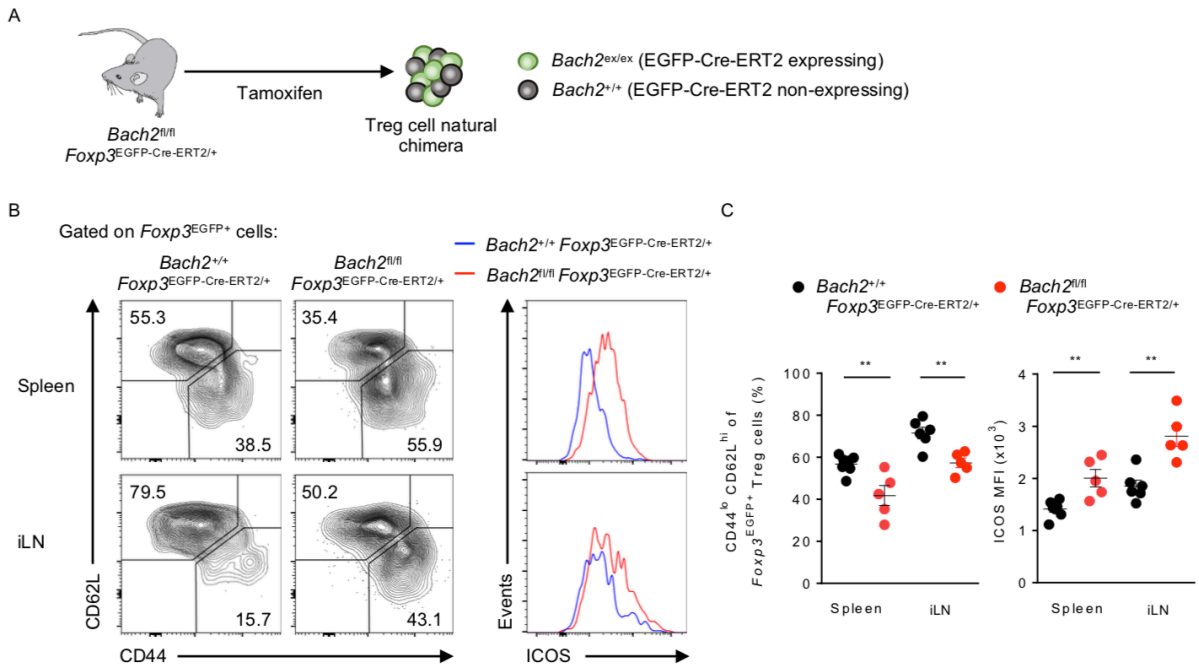

Figure 5.2: The *in vivo* cell-intrinsic function of BACH2 is to restrain aTreg cell differentiation.

(**A**) Illustration of the genetic chimerism that results from X-inactivation in Treg cells isolated from tamoxifen-treated female *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$ mice. (**B**) Representative flow cytometry plots and (**C**) replicate measurements of cell surface CD62L, CD44 and ICOS expression on EGFP^{+} Treg cells isolated from the spleen and iLN of chimeric female *Foxp3*$^{\text{eGFP-Cre-ERT/+}}$ *Bach2*$^{\text{fl/fl}}$ or control *Foxp3*$^{\text{eGFP-Cre-ERT/+}}$ *Bach2*$^{+/+}$ mice treated with tamoxifen for two weeks. Data are representative of two experiments with four to five mice per group. **P* <0.05, ***P* <0.01; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

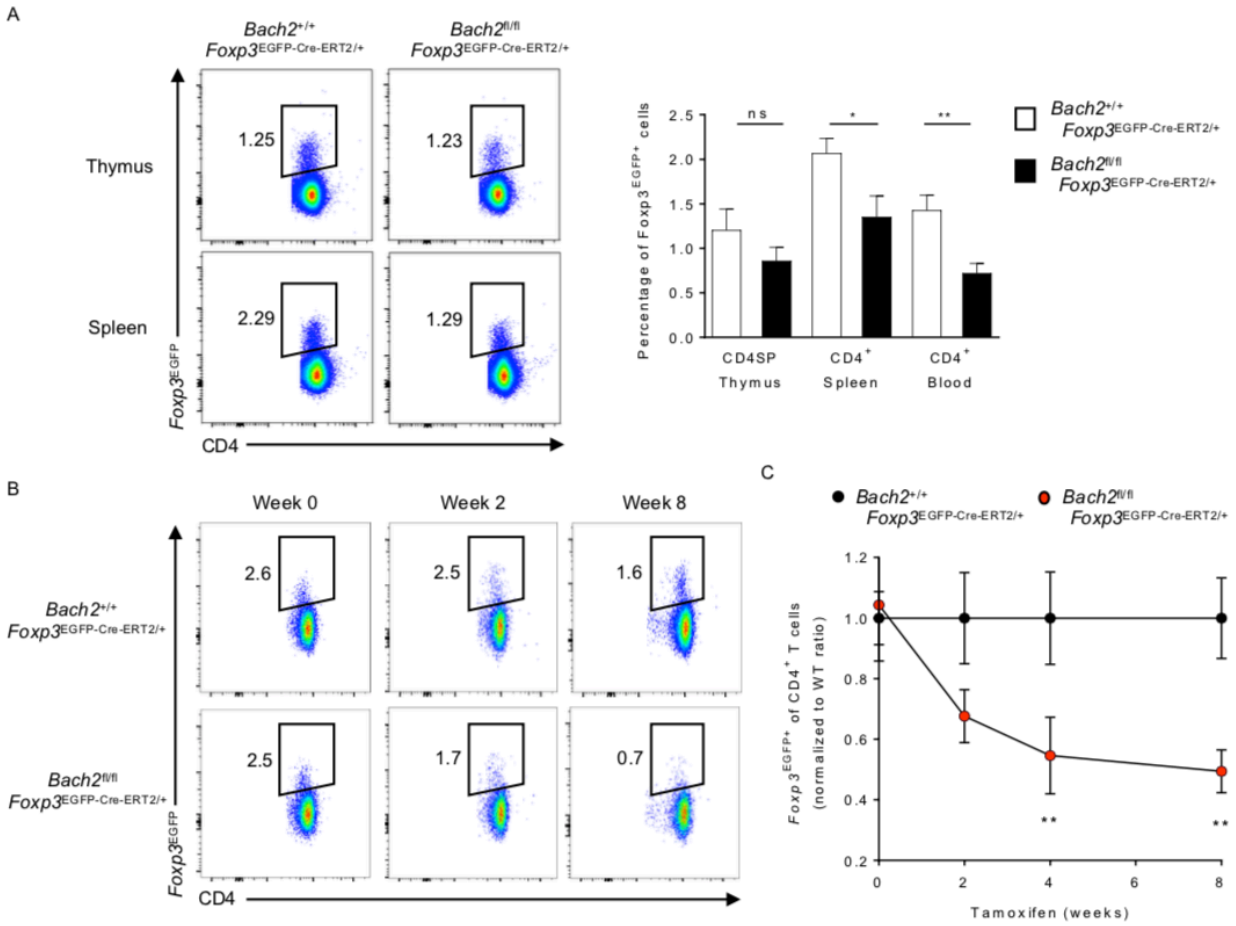

Figure 5.3: BACH2 is required for long-term maintenance of Treg cell populations.

(**A**) Representative flow cytometry (left) and the percentages (right) of EGFP$^+$ Treg cells from the CD4SP thymocyte or splenic CD4$^+$ T cell population from animals of the indicated genotypes fed tamoxifen for eight weeks. (**B**) Representative flow cytometry showing the frequency of EGFP$^+$ Treg cells of total CD4$^+$ T cells in the blood of animals at indicated time points following the initiation of tamoxifen treatment. (**C**) Replicate measurements of the EGFP$^+$ Treg cell percentages shown in B, normalized to the mean values measured in *Foxp3*$^{\text{eGFP-Cre- ERT/+}}$ *Bach2*$^{+/+}$ mice. Data are representative of three experiments with five to seven mice per group. *ns,* not significant*;* **P* <0.05, ***P* <0.01; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

A cell-autonomous defect in the maintenance of EGFP^{+} Treg cells in the periphery was observed in female *Foxp3*$^{\text{eGFP-Cre- ERT/+}}$ *Bach2*$^{\text{fl/fl}}$ chimeric mice, whereas the thymic frequency of Treg cells was unchanged. This is distinct from the complete cell-autonomous loss of Treg cells observed upon germline disruption of BACH2 in *Bach2*-knockout animals. I sought to confirm that this phenotype resulted from the differences in the timing of *Bach2* ablation relative to *Foxp3* expression and not to inherent differences between the *Bach2*$^{\text{KO}}$ and *Bach2*$^{\text{flox}}$ alleles used. Therefore, I sought to test whether the cell-intrinsic phenotype driven by germline excision of the Bach2-flox allele is similar to that of the *Bach2*-knockout allele, where there is a complete loss of thymic Treg differentiation. To examine whether this was the case, mice bearing germline excision of the *Bach2*$^{\text{flox}}$ transgene were generated by transiently administering tamoxifen to male *Bach2*$^{\text{fl/+}}$ *Rosa26*$^{\text{Cre-ERT2}}$ mice, which after three months were bred with WT C57BL/6 female mice. The subsequent F1 progeny, bearing germline excision of the *Bach2*$^{\text{flox}}$ transgene, were then intercrossed to generate *Bach2*$^{\text{ex/ex}}$ (homozygous for the *Bach2*$^{\textit{excised}}$ transgene) female mice. As a means to test cell-intrinsic function, as illustrated in Figure 5.4, lethally irradiated mice (*Ptprc*$^{\text{a/a}}$, or CD45.1^{+}) were reconstituted with bone marrow (BM) cell mixtures comprising wild type BM (*Ptprc*$^{\text{a/b}}$, or CD45.1/2^{+}) in a ~1:1 ratio with BM derived from either: (1) *Bach2*$^{+/+}$, (2) *Bach2*$^{-/-}$, or (3) *Bach2*$^{\text{ex/ex}}$ mice (all *Ptprc*$^{\text{b/b}}$, or CD45.2^{+}). Here, *Bach2*$^{+/+}$ refers to mice homozygous for the wild type *Bach2* allele, whereas *Bach2*$^{-/-}$ refers to mice homozygous for the *Bach2*$^{\text{KO}}$ transgene. Three months after reconstitution, tissues were extracted and analyzed using flow cytometry.

Analysis of CD45.2^{+} CD4^{+} T cells within the thymus (Figure 5.5A, top panel) and spleen (Figure 5.5A, bottom panel) of recipient mice revealed a complete cell-intrinsic defect in the generation of Foxp3^{+} cells by either cells bearing the *Bach2* knockout transgene (*Bach2*$^{-/-}$) or

the germline-excised *Bach2*^{flox} transgene (*Bach2*$^{ex/ex}$). In contrast, Foxp3^{+} T cells were produced by wild type (*Bach2*$^{+/+}$) cells. Therefore, the germline-excised *Bach2*^{flox} transgene phenocopies the *Bach2* knockout transgene and recapitulates the complete cell-autonomous defect in Treg cell generation observed upon germline *Bach2* ablation. These data indicate that the cell-autonomous phenotype observed in the *Foxp3*$^{eGFP\text{-}Cre\text{-}ERT}$ *Bach2*^{flox} system are a consequence of the timing of *Bach2* ablation relative to Foxp3 induction, rather than allele-specific differences.

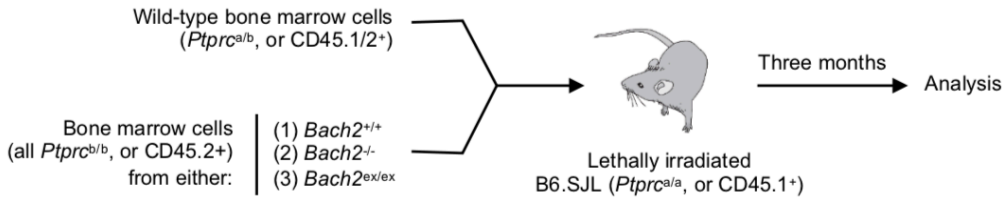

Figure 5.4: Experimental schema of cell transfer experiment.

Mice were lethally irradiated (*Ptprc*$^{a/a}$, or CD45.1^{+}) and reconstituted with bone marrow (BM) cell mixtures consisting of wild type BM (*Ptprc*$^{a/b}$, or CD45.1/2^{+}) in a ~1:1 ratio with BM derived from either: (1) *Bach2*$^{+/+}$, (2) *Bach2*$^{-/-}$, or (3) *Bach2*$^{ex/ex}$ mice (all *Ptprc*$^{b/b}$, or CD45.2^{+}).

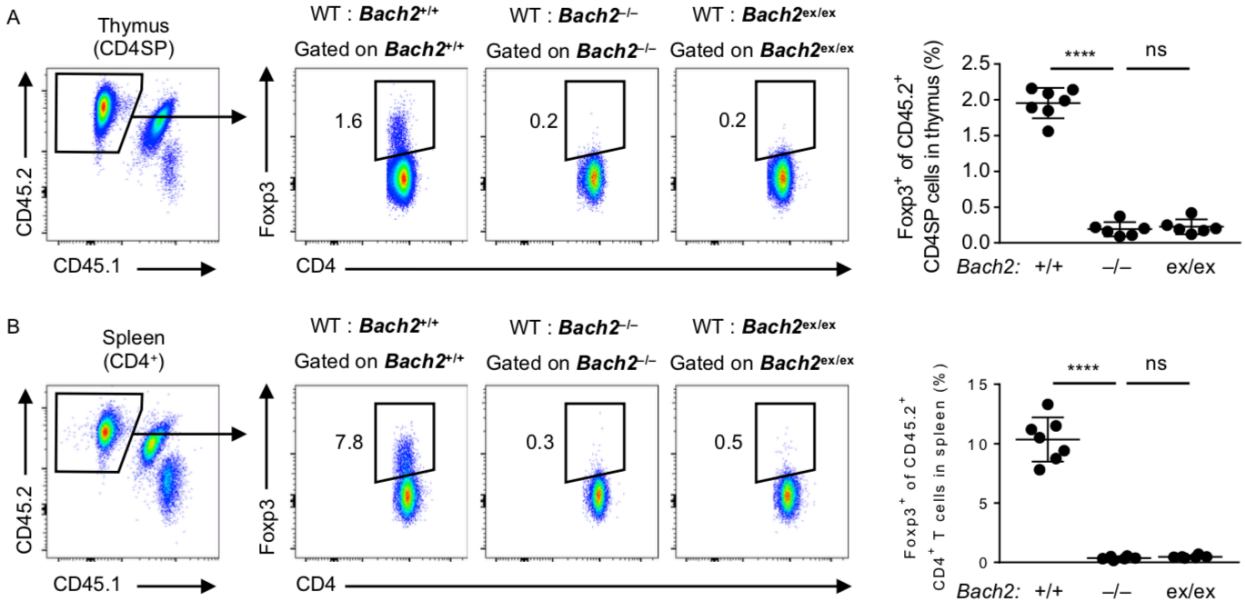

Figure 5.5**:** Germline excision of the *Bach2*^{flox} transgene results in a complete cell-intrinsic defect in the generation of Foxp3^{+} Treg cells.

Representative flow cytometry (left) and replicate measurements (right) showing the frequency of Foxp3^{+} Treg cells in the thymus (**A**) and spleen (**B**) within the CD45.2^{+} compartment of reconstituted B6.SJL mice (as detailed in Figure 5.4). Data are representative of six mice per group. *ns*, not significant; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

5.2.3 The expression of BACH2 is required in lineage-committed Treg cells for maintenance of immune homeostasis

I asked whether loss of rTreg cell populations has an impact on immune homeostasis during the steady-state. Eight weeks following initiation of tamoxifen treatment, flow cytometry was used to examine the cell surface markers expressed by conventional $CD8^+$ and $CD4^+$ $Foxp3^-$ T cells in *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ (CKO) mice. Within the $CD8^+$ T cell population, the analysis revealed a decrease in the percentage of $CD62^+$ $CD44^-$ naïve cells and increase in $CD62^+$ $CD44^+$ central memory cells in both the spleen (Figure 5.6A–B, top panel) and inguinal lymph nodes (Figure 5.6D–E, top panel) of CKO mice. Whereas an increase in $CD62^-$ $CD44^+$ effector $CD8^+$ T cells was observed in the iLN of CKO mice, no increase was seen in the spleen. In contrast to the $CD8^+$ T cell population, within the $CD4^+$ $Foxp3^-$ T cell compartment, the analysis did not reveal any difference in the proportions of naïve and central memory cells in either the spleen (Figure 5.6A–B, bottom panel) or iLN (Figure 5.6D–E, bottom panel) of CKO mice. However, a mild increase in cell surface ICOS expression was observed in CKO versus control mice (a mean value of 43% versus 37%, respectively) by splenic $CD4^+$ $Foxp3^-$ T cells (Figure 5.6C).

Consistent with altered proportions of naïve and central memory $CD8^+$ T cells, flow cytometry analysis following intracellular cytokine staining revealed an upregulation of IFN-γ by splenic $CD8^+$ T cells, but not by $Foxp3^–$ $CD4^+$ Tconv cells, from tamoxifen-treated CKO mice (Figure 5.7). Collectively, these studies demonstrate that the expression of BACH2 following Treg lineage-commitment is necessary for the maintenance of immune homeostasis under steady-state conditions.

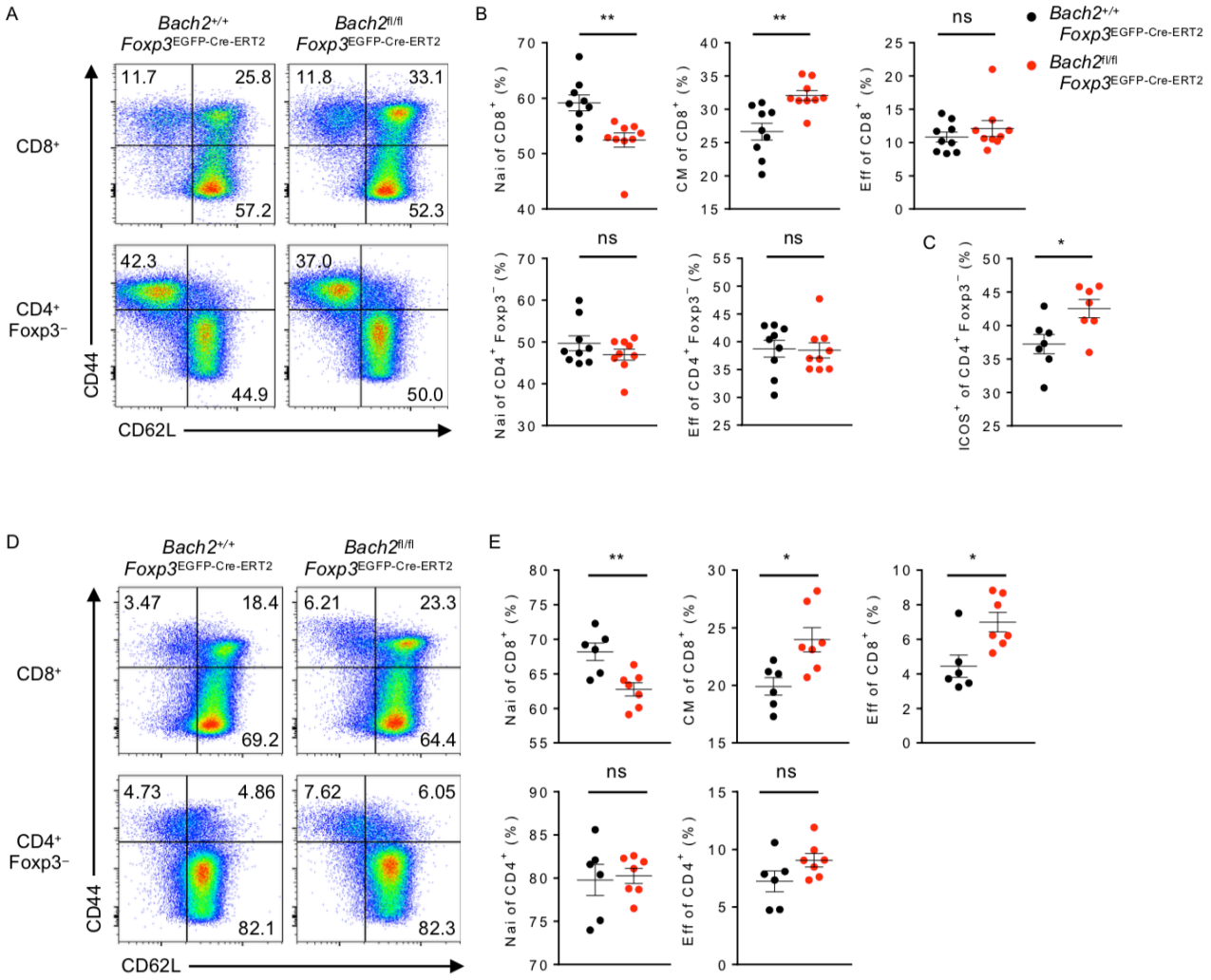

Figure 5.6: Loss of *Bach2* in lineage-committed Treg cells results in an alteration in the phenotype of conventional T cells.

Representative flow cytometry showing cell surface CD44 and CD62L expression on gated $CD8^+$ T cells and $Foxp3^-$ $CD4^+$ Tconv cells in the spleen (**A**) and iLN (**D**) from tamoxifen-treated experimental *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ and control *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ mice. Replicate measurements of the percentages of naïve, central memory and effector cells within gated $CD8^+$ T cells and $Foxp3^-$ $CD4^+$ Tconv cells in the spleen (**B**) and iLN (**E**) from A and D, respectively, and percentages of $ICOS^+$ $Foxp3^-$ $CD4^+$ Tconv cells (**C**), from mice of indicated genotypes. Data are representative of two repeated experiments with six to nine mice per group. *ns*, not significant. **P* <0.05, ***P* <0.01; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

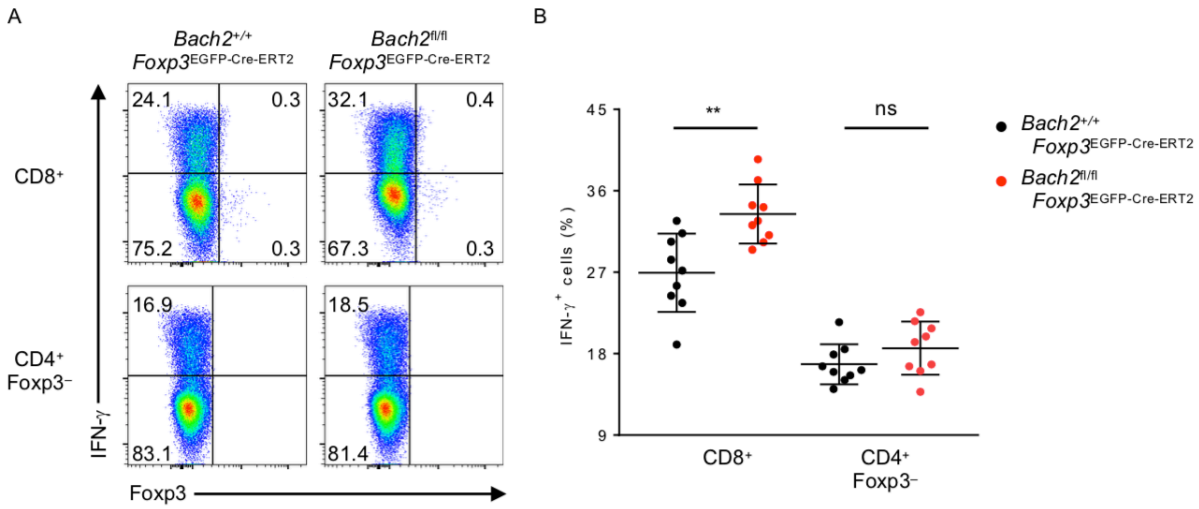

Figure 5.7: Loss of *Bach2* in lineage-committed Treg cells results in increased expression of cytosolic IFN-γ in splenic $CD8^+$ T cells.

(**A**) Representative flow cytometry (**B**) and replicate measurements of IFN-γ and Foxp3 expression by splenic $CD8^+$ and $Foxp3^-$ $CD4^+$ T cells from spleens of experimental *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ (CKO) and control *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2}$ *Bach2*$^{fl/fl}$ mice treated with tamoxifen for eight weeks. Data are representative of two repeated experiments with six to nine mice per group. *ns*, not significant, $^{**}P < 0.01$; unpaired Student's *t*-test. Numbers in gates show percentages. Bars and error show mean and s.e.m.

5.3 Discussion

5.3.1 Summary

In this chapter, I show that BACH2 restrains antigen receptor stimulation in lineage-committed rTreg cells to regulate their differentiation into aTreg cells (Figure 5.1). By using a novel chimeric *in vivo* system—the female heterozygous *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$ mouse strain—I found that the restraint of rTreg cell differentiation to aTreg by BACH2 is cell-intrinsic (Figure 5.2). Furthermore, the gradual attrition of Treg cell populations in this chimeric model demonstrated that BACH2-mediated restraint of aTreg cell differentiation is required in a cell-autonomous fashion to maintain Treg cell populations in the periphery over time (Figure 5.3). I then sought to examine the functional significance of BACH2 in lineage-committed Treg cells under steady-state conditions. As shown in Figure 5.6, alterations in the proportions of naïve and central memory cells were observed in the $CD8^+$ T cell population from the *Bach2* conditional knockout mouse model. In addition to effect on $CD8^+$ T cell activation status, the loss of BACH2 function in lineage-committed Treg cells lead to a greater production of the pro-inflammatory cytokine IFN-γ, by the $CD8^+$ T cell population (Figure 5.7).

5.3.2 The TCR-mediated modulation of BACH2 function in rTreg cells

The studies described in section 5.2.1 highlight how BACH2 mechanistically operates following TCR engagement in lineage-committed Treg cells to restrain the differentiation of resting Treg cells toward an activated Treg cell phenotype. The *in vitro* culture method used in these studies resulted from substantial optimization of Treg cell culture conditions, and consequently represented a useful system, whereby the role of antigenic stimulation, specifically, could be delineated. As discussed in the chapter introduction, BACH2 acts restrains antigen receptor stimulation in other lymphocyte lineages. From this finding, I can

conclude that this is conserved in Treg cells. Much attention has been drawn toward the role of TCR stimulation in guiding Treg cell fate and function, and therefore, BACH2 function should now be considered an important factor in guiding the outcome of antigen receptor stimulation in Treg cells. Similar to other lymphocyte lineages, it is highly likely that—within lineage-committed Treg cells—TCR stimulation leads to phosphorylation of BACH2 via the PI3K-AKT-mTOR signalling pathway (as described in section 1.7.2). Future studies could specifically address the role of PI3K-AKT-mTOR signalling in modulating BACH2 function, specifically within the Foxp3^{+} Treg cell lineage.

5.3.3 BACH2-mediated restraint of aTreg cell differentiation is required for cell-intrinsic maintenance of Treg cell populations

The studies in section 5.2.2 identified that, in the absence of BACH2 function in lineage-committed Treg cells, there was a cell-intrinsic failure of the ability to maintain the rTreg cell phenotype, which resulted in a loss of Treg cell populations. Data are shown from analyses conducted on T cells from the thymus, spleen, iLN and blood (Figure 5.2 and Figure 5.3), extracted from the female heterozygous *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$ mouse strain. It would be insightful to determine whether this phenotype is also true of tissue-resident Treg cells (such as those described in section 1.6), as opposed to just lymphoid-resident and circulating T cell populations. However, methods of Treg cell extraction from non-lymphoid tissue tend to be aggressive, in terms of the tissue dissociation protocols, which often result in high cell death and few Treg cells extracted. In addition, in the system I used, it was necessary to use EGFP—from the heterozygous *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ transgene—as a marker to identify Treg cells where random X-inactivation had resulted in *Cre* expression, and therefore, *Bach2* ablation. The signal resulting from *EGFP* expression in this system was low and it was at times, challenging to discern the *Foxp3*$^{\text{EGFP}}$-positive Treg cell population. These two factors

would made it difficult to examine the phenotypes of tissue-resident Treg cells in the chimeric system. In order to reliably determine the *Foxp3*^{EGFP}-positive Treg cell population in the tissues that I analysed (thymus, spleen, iLN and blood), I always included a negative control to set flow cytometry gates (a sample derived from a female *Foxp3*$^{+/+}$ mouse), and included as many experimental samples as possible. However, generating mice of experimental and control genotypes was a lengthy process, often taking up to 2–3 months between each experiment. In order to confidently identify the Treg cell attrition phenotype in experimental chimeric mice, I repeated the analysis three separate times (Figure 5.3). Given enough time, future studies could optimize a tissue Treg cell extraction protocol to elucidate whether the identified phenotype in the chimeric system is similarly demonstrated by tissue-resident lineage-committed Treg cells.

Given the decline in the proportion of Foxp3^{+} Treg cells in female *Foxp3*$^{EGFP\text{-}Cre\text{-}ERT2/+}$ *Bach2*$^{fl/fl}$ mice treated with tamoxifen for eight weeks, efforts were directed toward understanding the fate of these absent Treg cells. Going on the premise that, perhaps, these Treg cells were undergoing apoptosis, initial studies involved taking serial blood samples each week following initiation of tamoxifen treatment and using flow cytometry to quantify the proportion of phosphatidylserine expression with annexin V staining. After one full study, no differences in Treg cell apoptosis was observed. Following on from conversations with David Tough, at GlaxoSmithKline, who suggested that apoptotic T cells would be cleared from the circulation from the spleen, and thus, the blood was not the appropriate tissue to be sampled for testing, analysis of splenic Treg cells were conducted. Another full study was conducted, where at biweekly intervals following the initiation of tamoxifen treatment, a group of mice were sacrificed from the cohort to provide splenocytes for analysis. Data from this one study, with few biological replicates per group at each timepoint, revealed a very

mild increase in phosphatidylserine expression of Foxp3^{+} Treg cells from experimental *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$ mice versus controls (data not shown). Repeating this observation proved to be very challenging, due to the aforementioned difficulties presented by this particular system (described in previous paragraph). Attempts at repeating this observation were either unsuccessful at a technical level (too few Foxp3^{+} Treg cells could be identified by flow cytometry) or yielded contradictory results (no differences in the proportion of annexin V positive Foxp3^{+} Treg cells were observed between experimental and control groups). For these reasons, the data generated from these studies were not included in this chapter. Future studies could be directed toward understanding whether Treg cell attrition in the chimeric system is a result of increased apoptosis of *Bach2*-deficient Treg cells, and address the exact fate of the Treg cells that are absent at eight weeks post-initiation of tamoxifen treatment.

In the *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$ chimeric system, a constant supply of Treg cells develop in the thymus. It would be insightful to re-examine cell-intrinsic function of BACH2 in lineage-committed Treg cells in the chimeric system, in the absence of any new thymic input. Here, thymectomy could be used to prevent a constant supply of developing Treg cells. Another approach could be to utilize the *Rosa26*$^{\text{tdRFP}}$-tracking system detailed in section 4.2.2 (Figure 4.3A). Here, female *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ *Rosa26*$^{\text{flSTOP-tdRFP}}$ mice could be generated heterozygous at the *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ transgene and transiently treated with tamoxifen to pulse label lineage-committed Treg cells. *Rosa26*$^{\text{RFP}}$-positive Treg cells could then be examined by flow cytometry after a given time period to understand how these cells were maintained, irrelevant of new thymic contribution to the Treg cell pool.

5.3.4 The loss of immune homeostasis caused by cell-specific ablation of BACH2 function

The studies in section 5.2.3 identified that the ablation of *Bach2* in lineage-committed Treg cells resulted in a reduced proportion of naïve $CD8^+$ T cells, increased proportion of central memory $CD8^+$ T cells, and increased proportion of $CD8^+$ IFN-γ^+ T cells, during steady-state immune regulation. It is interesting to note that this loss of immune homeostasis was predominantly restricted to the $CD8^+$ T cell lineage, as this phenotype was not observed in the $CD4^+$ $Foxp3^-$ Tconv cell lineage. Functionally, $CD8^+$ T cells are geared toward production of IFN-γ^+. Perhaps excessive activation had been initiated in $CD4^+$ $Foxp3^-$ T cells (the activation threshold had been surpassed) and were simply not identified with the particular set of experiments that I had conducted, consistent with increased ICOS expression. Future experiments could comprise of intracellular staining for the hallmark lineage-specifying transcription factors T-bet, GATA-3 and RORγt to examine whether other mechanisms of $CD4^+$ $Foxp3^-$ T cell activation exist in the CKO mouse model.

It is interesting to note that loss of rTreg cell populations not only resulted in the loss of immune homeostasis in $CD8^+$ T cells, but more specifically, it primarily manifested with an increase of the central memory population (Figure 5.6). It is unclear why loss of rTreg cell populations would result in the failure to restrain memory $CD8^+$ T cells, as opposed to effector $CD8^+$ T cell differentiation. Perhaps the aTreg cells present in the CKO system, are functionally capable of restraining terminal $CD8^+$ T cell differentiation by preventing the threshold of activation—required for effector $CD8^+$ T cell differentiation—from being surpassed. Another possible explanation may relate to co-migration of rTreg and $CD8^+$ memory cells, due to the expression of shared migratory molecules. Collectively taking all these points into consideration—that rTreg cells are required to restrain naïve to memory

$CD8^+$ T cell differentiation, whereas aTreg cells restrain the terminal differentiation of naïve or memory $CD8^+$ T cells to effector cells—suggest that rTreg and aTreg cells regulate distinct stages of effector cell differentiation. This observation has not been discussed in the literature to date and requires further clarification in future studies. Disease models, such as influenza recall responses, could perhaps be utilized to study memory $CD8^+$ T cell responses in the CKO model. In addition, being that greater IFN-γ is produced by $CD8^+$ T cells in the CKO model, understanding if tumour growth is impacted would be important. At the time of submission of this thesis, studies are underway using the MC38 colon carcinoma tumour model in the CKO mouse model. It would also be interesting to explore what specifically—in terms of the functional mechanisms exerted by lineage-committed Treg cells—is responsible for the restraint of $CD8^+$ T cell differentiation from naïve to central memory cells, versus naïve or central memory to terminally differentiated cells. Perhaps a specific suppressive molecule, or set of suppressive functions, are employed by Treg cells at each stage of $CD8^+$ T cell differentiation. This, however, would be difficult to interrogate in the CKO system, as numerous functional differences are evident between rTreg cells and aTreg cells (Figure 3.6).

5.3.5 Differences in phenotype between the conditional knockout system and heterozygous chimeric system

Collectively, consideration of the phenotypes of the conditional knockout (*Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$) strain and chimeric CKO (*Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$) strain yields important insights into cell-intrinsic function of BACH2 in lineage-committed Treg cells during steady-state immunoregulation. In the chimeric CKO *Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$ system, where both *Bach2*-sufficient and *Bach2*-deficient lineage-committed Treg cells are generated following tamoxifen administration, a competitive defect in survival became evident in the Treg population that lacked BACH2 function. Here, the competitive advantage of BACH2

function in *Bach2*-sufficient Treg cells resulted in their predominance in the Treg cell pool. In contrast, in the *Foxp3*$^{\text{EGFP-Cre-ERT2/EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ mouse strain, the majority of Treg cells undergo Cre-mediated *Bach2* ablation and thus, all Treg cells have the same competitive disadvantage. Here, compensatory expansion of the Treg cell pool in response to the increased Tconv cell activation may preclude observation of the Treg attrition phenotype. Therefore, competitive systems—such as that generated in the chimeric female heterozygous mice—are necessary to elucidate the cell-intrinsic function of BACH2 in lineage-committed Treg cells.

6 Thesis Discussion

6.1 Summary of key findings

Chapter 3 describes a novel dual reporter system—the *Foxp3*$^{\text{EGFP-DTR}}$ *Bach2*$^{\text{tdRFP}}$ mouse strain—to measure *Bach2* and Foxp3 expression at a single cell level using flow cytometry in T cells. This model was used to determine that thymic Treg cell precursors express high levels of *Bach2* during development. However, *Bach2* is heterogeneously expressed by mature extra-thymic Treg cells, where levels of *Bach2* are downregulated by inflammation. Treg cells that express low levels of *Bach2* present gene transcriptional profiles and cell surface markers consistent with activated Treg cells.

These findings were further elaborated in Chapter 4, where I generated a novel conditional knockout system—the *Foxp3*$^{\text{EGFP-Cre-ERT2}}$ *Bach2*$^{\text{fl/fl}}$ mouse strain—to explore the function of BACH2 in lineage-committed Treg cells. Following the validation of this model as a robust system, fate tracking studies revealed that expression of *Bach2* is not required to maintain the Treg cell identity in lineage-committed Treg cells. However, analysis of gene transcription at a single-cell level and hallmark cell surface markers revealed that BACH2 function is necessary for lineage-committed Treg cells to maintain a resting Treg cell expression phenotype.

In Chapter 5, I determined that BACH2 acts following TCR engagement to restrain the differentiation of rTreg cells to aTreg cells. By utilizing CKO mice that are heterozygous at the *Foxp3* locus—*Foxp3*$^{\text{EGFP-Cre-ERT2/+}}$ *Bach2*$^{\text{fl/fl}}$ mice—I generated a chimeric system, which revealed that BACH2 restraint of the rTreg to aTreg cell transition is cell-intrinsic, and is necessary to maintain Treg cell populations during steady-state immunoregulation. Finally, I

demonstrated that BACH2-mediated constraint of aTreg cell differentiation is required to prevent excessive differentiation of naïve $CD8^+$ T cells to memory cells, and their production of the pro-inflammatory cytokine IFN-γ, under steady-state immune homeostasis.

These data represent a hitherto unknown body of findings; they have not yet been published in any peer-reviewed journals or formally reported by any other research groups. These findings form the basis for a manuscript, which at the time of submission of this thesis, is under review at the *Journal of Experimental Medicine*. These findings provide a broader significance of the transcription factor BACH2 in Treg cell biology and the molecular basis for Treg cell heterogeneity and maintenance.

6.2 Future directions

6.2.1 The function of BACH2 in human lineage-committed Treg cells

The findings presented in this thesis have highlighted many more questions relating to Treg cell biology and the function of BACH2, which require addressing in future studies. These studies detailed in this thesis centred upon use of mouse models, thus one pertinent question is, do human Treg cells demonstrate similar patterns of BACH2 expression at the protein level? Examining human lineage-committed Treg cells is partially confounded by lack of reliable markers (discussed in section 1.3.2). In addition, BACH2 antibodies do not yet exist for flow cytometric analysis. However, future studies could utilize the BACH2 antibody appropriate for Western blotting, to interrogate BACH2 expression in $CD4^+$ $CD25^+$ IL-$7R^-$ human T cells (Liu et al. 2006). Likewise, does BACH2 restrain aTreg cell differentiation in humans? The relationship between *Bach2* expression and Treg cell activation in human single-cell Treg transcriptomes bear striking similarities to murine Treg cell transcriptomes, which is suggestive that BACH2 may share functional homology in Treg cells across species

(Zemmour et al. 2018). Future studies could perhaps answer this by extraction of Treg cells from human blood, ablation of *Bach2* using siRNA knockdown, and phenotypic analysis with flow cytometry.

6.2.2 The role of BACH2 in determining tissue-resident Treg cell phenotypes

The data described in this thesis were primarily generated from murine Treg cells extracted from primary or secondary lymphoid tissues. Previous studies highlight the critical immunoregulatory and non-immune functions of tissue resident Treg cells, however, this Treg cell population remains poorly characterized. These populations are typically examined based on their expression of hallmark lineage-defining molecules, such as PPAR-γ for adipose tissue Treg cells and AREG for muscle Treg cells. Do these tissue resident Treg cells display heterogeneous BACH2 expression and can BACH2 expression be used to determine their activation status? As discussed previously, analysis of tissue resident Treg cells from murine models can be challenging (section 5.3.3), and thus, answering these questions would certainly require focused efforts in future studies.

6.2.3 The modulation of BACH2 function in response to TCR signalling in Treg cells

The observation that BACH2 functions following TCR engagement was an interesting finding that could be further elaborated. Use of the *Nur77* expression as a proxy for antigen receptor stimulation demonstrated that rTreg cells do continually encounter cognate antigen, and as BACH2 is required to constrain the transition of rTreg to aTreg cells, it would be interesting to examine if any relationship between the expression of Nur77 and BACH2 exist with relevance to Treg cell phenotypes. The dynamics of BACH2 phosphorylation that result from TCR engagement— with regard to the PI3K-AKT-mTOR signalling pathway—could also be explored. Despite this pathway performing a well-known role in the modulation of

BACH2 function via its attenuation in the cytosol in other lymphocyte lineages, this remains to be confirmed in lineage-committed Treg cells.

6.2.4 The molecular mechanisms of BACH2-mediated constraint of aTreg cell differentiation

Much is left unanswered with regard to the molecular mechanisms that underpin BACH2-mediated restraint of aTreg cell differentiation, which was discussed in section 4.3.5. Future studies should utilize ChIP-Seq experiments to determine where BACH2 binds in the genome of Treg cells. As BACH2 is predominantly found at enhancer regions in other lymphocyte lineages, ChIP-Seq measurements could determine if this is likewise true in the Treg cell genome. Use of ATAC-Seq (assay for transposase-accessible chromatin using sequencing) on Bach2-*deficient* Treg cells extracted from the BACH2 CKO model versus *Bach2*-sufficient WT Treg cells could highlight loci where BACH2 is involved in modulating genome-wide chromatin accessibility. As BACH2 is known to prohibit other members of the bZIP transcription factor family—notably IRF4 and JUND—from binding certain loci, the functional relationships between BACH2 and other bZIP TFs should be addressed.

6.2.5 The fate of *Bach2*-deficient Foxp3$^+$ Treg cell populations in the chimeric CKO mouse model

As described in section 5.2.2, loss of BACH2 in lineage-committed Treg cells in a competitive system results in a cell-intrinsic failure of the maintenance of Treg cell populations. As detailed in section 5.3.3, preliminary studies were performed in an attempt to understand the fate of these *Bach2*-deficient Treg cells. As it is unlikely that these Treg cells lose their suppressive identity (section 4.2.3), it remains plausible that these *Bach2*-deficient Treg cells are lost from the Treg cell population in a slow, gradual decline via apoptosis,

which is difficult to detect experimentally (described in section 5.3.3). Future studies should address whether this is, indeed, the case in the chimeric mouse model. Detection of markers for apoptosis, other than annexin V, may represent an alternative. However, owing to the need to preserve EGFP signal in *Bach2*-deficient Treg cells, the commonly used alternative intracellular markers— such as caspase-3/7, caspase-9, or Bcl-2—are not feasible. Future studies should focus on examining Treg cells extracted from secondary lymphoid tissue (not the blood) and should utilize a large number of biological replicates.

6.2.6 The relationship between Treg cell activation state and pathology

The consequence of BACH2 ablation in lineage-committed Treg cells is the failure to maintain immune homeostasis during the steady-state (discussed in 5.3.4). Here, loss of BACH2 function in Treg cells leads to increased memory $CD8^+$ T cell differentiation and increased IFN-γ production by $CD8^+$ T cells upon TCR stimulation. As *Bach2*-deficient Treg cells are predominantly of an aTreg cell phenotype, and these aTreg were able to prevent excessive $CD8^+$ T cell terminal differentiation in the spleen, these findings suggest that rTreg cell populations fulfil an essential role of constraining memory $CD8^+$ T cell differentiation. How this is achieved remains to be explored. Perhaps it is merely a reflection of similar cell surface homing molecules–such as CD62L and CCR7—that are jointly expressed by both rTreg cells and naïve $CD8^+$ T cells. Or perhaps, distinct functional mechanisms, or a repertoire of suppressive actions, are employed by rTreg cells to restrain memory $CD8^+$ T cell differentiation. Further work could explore this concept further, and in addition, its bearing on disease should be examined. As $CD8^+$ T cell differentiation plays a central role in many disease pathologies, factors that influence it may present an important role in human disease. For example, memory $CD8^+$ T cells perform important functions during re-exposure to infectious pathogens, and terminally differentiated $CD8^+$ T cells perform important

functions during anti-tumour immune responses. At the time of submission of this thesis, one study had been conducted using the BACH2 CKO strain in the MC38 colon carcinoma model. Additional studies are currently ongoing.

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8 Appendices

8.1 Original page numbering for thesis submitted July 2019

8.2 List of minor revisions made to thesis for November 2019 submission

8.2.1 Changes to formatting

- Figures in results sections placed on separate page to text.
- Text in discussion placed on continuous pages.
- Bibliography reformatted to include all authors.

8.2.2 Changes to text and responses to examination comments

Page and line references refer to that of the revised thesis submitted November 2019.

- Removal of text “which was later realised to comprise the Treg cell immunosuppressive repertoire” (page 26, line 2).
- Revision of text to “CTLA-4 juxtacrine signalling” (page 29, line 8).
- Section of introduction added: Contribution of other suppressive lymphocytes to immune homeostasis (page 36).
- Revision of text in figure legend to “Gated on live single cells” (page 65, line 3).
- Regarding Figure 3.5 (page 69), the examiners questioned whether absolute numbers were recorded during flow cytometry analysis. The original data show that no absolute numbers were recorded during analysis.
- Removal of text “and visualized using the Southern blotting technique” (page 79, line 8).
- Revision of text so that genotypes are referred to, as opposed to alleles (page 79 and 80).
- Revision of text to “I examined the percentage of” (page 84, line 5).
- Revision of text to mention contamination of B cells in bulk RNA-Seq data (page 88, line 8).

- Regarding Figure 4.8 (page 92), the examiners questioned whether any markers of maturation or other molecules detected in the RNA-Seq data were included in the analysis. The original data did not include any additional markers than those already shown.
- Regarding Figure 5.3 (page 104), the examiners questioned whether the absolute number of Treg cells was quantified during the flow cytometry analysis. The original data did include flow cytometry count beads and although the mean absolute number of *Bach2*-deficient Treg cells was numerically lower than that of the *Bach2*-sufficient Treg cell population, the individual data points were quite variable and the differences between groups were not statistically significant.

8.3 Buffers

8.3.1 FACS buffer

PBS (provided by the Babraham Institute technicians)

3% Fetal Bovine Serum (Sigma-Aldrich)

20 mM EDTA (Sigma-Aldrich)

8.3.2 Complete media

One 500 ml bottle of RPMI or DMEM is supplemented with the following:

50 ml heat inactivated FBS (Sigma-Aldrich)

5 ml Pen/Strep (Gibco, Thermo Fisher Scientific)

5 ml Minimum essential medium non-essential amino acids (Gibco, Thermo Fisher Scientific)

5 ml Sodium Pyruvate (Gibco, Thermo Fisher Scientific)

500 µl 2-Mercaptoethanol (Gibco, Thermo Fisher Scientific)

500 µl Fungizone (Gibco, Thermo Fisher Scientific)

500 µl Gentamycin (Sigma-Aldrich)

8.4 Flow cytometry antibody details

Marker	Fluorochrome	Cat. Number	Clone
B220 (CD45R)	BV510	103247	RA3-6B2
B220 (CD45R)	FITC	11-0452-85	RA3-6B2
CD11b	PE	12-0112-83	M1/70
CD11c	FITC	11-0114-85	N418
CD152 (CTLA-4)	APC	17-1522-80	UC10-4B9
CD223 (LAG-3)	APC-eFluor 780	15-0691-81	C9B7W
CD25	BV786	564023	PC61
CD278 (ICOS)	PE	12-9949-81	C398.4A
CD4	APC	553051	RM4-5
CD4	APC-eFluor 780	47-0042-82	RM4-6
CD4	PE-Cyanine7	25-0042-82	RM4-7
CD44	BV786	563736	IM7
CD44	PerCP-Cyanine5.5	45-0441-82	IM7
CD45.1	PE	12-0453-82	A20
CD45.2	eFluor 506	69-0454-82	104
CD45.2	PerCP-Cyanine5.5	45-0454-82	104
CD62L	eFluor 450	48-0621-82	MEL-14
CD62L (L-Selectin)	PE	12-0621-82	MEL-14
CD62L (L-Selectin)	PerCP-Cyanine5.5	45-0621-82	MEL-14
CD8a	APC	17-0081-81	53-6.7
CD8a	APC-eFluor 780	47-0081-82	53-6.7
CD8a	BV510	100751	53-6.7

CD8a	eFluor 450	48-0081-82	53-6.7
CD8a	PE	12-0081-81	53-6.7
F4/80	APC	17-4801-80	BM8
Foxp3	APC	17-5773-82	FJK-16s
Foxp3	eFluor 450	48-5773-82	FJK-16s
Foxp3	PE	12-5773-80	FJK-16s
Foxp3	FITC	53-5773-82	FJK-16s
GITR	PE	12-5874-82	DTA-1
IFN-gamma	PerCP-Cyanine5.5	45-7311-82	XMG1.2
IL-2	PE	12-7021-82	JES6-5H4
Ki67	PE	12-5698-82	SolA15
KLRG1	APC	17-5893-81	2F1
KLRG1	PE-Cyanine7	25-5893-82	2F1
Ly-6C	PE-Cyanine7	25-5932-82	HK1.4
Ly-6G (Gr-1)	eFluor 450	2-9668-80	1A8-Ly6g

Table 8.1: Flow cytometry antibodies