Modulation of Regulatory T cell Function Through Specific Molecular Pathways in Transplantation

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Abstract

Regulatory T cells (Tregs) are crucial mediators of immune homeostasis that have shown immense promise as a cellular therapy for immune-mediated pathologies such as transplant rejection. Methods to optimise current Treg-based therapies are critical for its successful and widespread translation to the clinical setting. The aim of this study was to investigate the role of two molecular pathways for the modulation of Treg biology and function.

In the first part of this study, the role of the interleukin-33 (IL-33)/ST2 axis was interrogated in Tregs. This highly pleiotropic pathway has recently been demonstrated to have an important role in Treg biology. Here, it is shown that IL-33-expanded Tregs have enhanced *in vivo* suppressive function in a transplantation model. An in-depth characterization of IL-33-expanded Tregs is presented, providing a detailed quantitative transcriptomic and immunophenotypic analysis. Interestingly, this reveals IL-33-expanded Tregs to harbour a phenotypic signature of enhanced potential for graft-homing.

In the second part of the study, Treg plasticity and the environmental factors which govern their phenotype and function were examined, with a particular focus on the role of hypoxia and the principal regulators of oxygen homeostasis, the HIF/PHD pathway, in modulating Treg biology. Using an array of HIF/PHD2 transgenic mouse models, the study demonstrates that silencing of the critical prolyl hydroxylase domain-containing enzyme, *Phd2,* induces an autoimmune-like

phenotype in mice. This is driven by upregulated HIF-2α signalling, in which the suppressive function of Treg populations is significantly impaired with loss of ability to control graft rejection. Additionally, evidence is presented to demonstrate that HIF-2α may be inhibited to enhance suppressive function of both mouse and human Tregs. These findings are replicated, at least in part, in mice subjected to chronic hypoxic.

While significant progress has been made to unlock the therapeutic potential of Tregs, there remains a great deal more to be learned regarding their behaviour and function for their effective and safe implementation within the clinic. The findings presented in this thesis uncover methods for the molecular manipulation of Tregs to help exploit their versatility for therapeutic use.

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Abbreviations

Activator Protein 1	AP-1
Activation-induced cell death	AICD
Adenosine 5'-triphosphate	ATP
Allogeneic dendritic cells	alloDC
Allogeneic GM-CSF and IL-4-treated dendritic cells	GM-IL-4-DC
Allogeneic GM-CSF and TGF-β-treated dendritic cells	GT-DC
Allogeneic haematopoietic stem cell transplantation	alloHCT
Allogeneic MHC	allo-MHC
Antibody-dependent cell-mediated cytotoxicity	ADCC
Antibody-mediated rejection	AMR
Antigen presenting cell	APC
Autoimmune regulator	AIRE
Autoimmune-Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy	APECED
Basic helix-loop-helix-PAS	bHLH-PAS
Bone marrow transplantation	BMT
C-C chemokine receptor	CCR
C-type lectin receptor	CLR
CD62L-CD44+ T effector cells	T_E
CD62L+CD44- naïve T cells	T_N
CD62L+CD44+ central memory T cells	T_{CM}
Chimeric antigen receptor	CAR
Complementarity determining regions	CDR
Conserved non-coding sequences	CNS
Constitutively active form of inhibitor of NF-κβ kinase β	caIKKβ
CREB binding protein	CBP
Cyclic adenosine monophosphate	cAMP

Cytotoxic T cells	CTL
Cytotoxic T-lymphocyte-associated protein 4	CTLA-4
Damage-associated molecular pattern	DAMP
Delayed-type hypersensitivity	DTH
Dendritic cell	DC
Diacylglycerol	DAG
Donor-specific antibody	DSA
Double negative	DN
Effector T cell	Teff
Erythropoietin hormone	EPO
Experimental allergic encephalomyelitis	EAE
Factor inhibiting HIF	FIH
Forkhead box P3	Foxp3
Germinal centres	GC
Glucose transporter	GLUT
Graft-versus-host disease	GVHD
Granulocyte-macrophage colony-stimulating factor	GM-CSF
Heat shock protein 90	Hsp90
Human leukocyte antigen	HLA
Hypoxia inducible factor	HIF
Hypoxia-responsive element	HRE
IFN	Interferon
IL-2-induced tyrosine kinase	Itk
Immune dysregulation, polendocrinopathy, enteropathy, X-linked syndrome	IPEX
Immunoglobulin superfamily	IgSF
Immunoreceptor tyrosine-based activation motifs	ITAM
In vitro-induced Tregs	iTreg
Indoleamine 2,3-dioxygenase	IDO

Inositol trisphosphate	IP3
Intercellular adhesion molecule-1	ICAM-1
Interleukin	IL
Interleukin-33	IL-33
Interleukin-33-expanded Treg	IL-33-Treg
Intraperitoneal	i.p.
Intravenous	i.v.
Ischaemia/reperfusion	I/R
Knock-out	KO
Knock-down	KD
Latency-associated protein	LAP
Linker for the activation of T cells	LAT
Lymph node	LN
Lymphocyte-activation gene	LAG-3
Lymphocytic choriomeningitis virus	LCMV
Major histocompatibility complex	MHC
Mammalian target of rapamycin	mTOR
Median survival time	MST
Minor histocompatibility antigen	miH
Mitogen-activated protein kinase kinase	MAPKK
Myeloid derived suppressor cell	MDSC
Natural killer cell	NK
Natural killer T cell	NKT
Nitric oxide	NO
Nuclear factor of activated T cell	NFAT
Nuclear factor-κβ	NF-κβ
P-selectin	CD62L
Per-ARNT-Sim	PAS

Peripheral blood	PB
Peripheral blood mononuclear cell	PBMC
Peripherally-derived Treg	pTreg
Phosphatidylinositol 3-kinase	PI3K
Phosphatidylinositol-3,4-trisphosphate	PIP2
Phospholipase C	PLCγ
Prolyl hydroxylase domain	PHD
Protein tyrosine kinase	PTK
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Recombination activating gene	RAG
Regulatory B cell	Breg
Regulatory T cell	Treg
Reverse tetracycline transactivator	rtTA
Serum stimulation 2	ST2
Serum stimulation 2 ligand	ST2L
SH2 containing leukocyte phosphoprotein of 76kDa	SLP-76
Soluble serum stimulation 2	sST2
Spleen	SPL
T cell immunoglobulin and mucin domain-containing protein-3	TIM-3
T cell receptor	TCR
T helper cell	Th
Thymus-derived Treg	tTreg
Tissue-restricted antigens	TRA
Toll-like receptor	TLR
Total body irradiation	TBI
Total lymphoid irradiation	TLI
Transendothelial migration	TEM

Transforming growth factor	TGF
Treg-cell specific demethylation region	TSDR
Tumour necrosis factor receptor superfamily	TNFRS
Tumour necrosis factor-α	TNF-α
Type 1 regulatory cell	Tr1
Type 2 innate lymphoid cell	ILC2
Vascular endothelial growth factor	VEGF
Violet proliferation dye	VPD
von Hippel-Lindau disease	VHL
Zeta-chain-associated protein kinase 70	ZAP-70

1 Introduction

1.1 Brief History of Transplantation

In 1953, a profound interest in transplantation research was ignited upon the first successful human solid organ transplant achieved between identical twins [1, 2]. Since then, gradual progress within the field aided by the development of methods for dialysis, organ preservation/transportation, tissue typing [3], and perhaps most importantly, chemical immunosuppression [4], has made it a reliable and routine therapy for end-stage organ failure. Currently, over 100,000 transplants are performed world-wide ever year. Impressively, one-year graft survival rates of patients receiving kidney transplants, the most commonly transplanted solid organ, has been in excess of 90% within the UK over recent years [5].

Nonetheless, there has not been much progress in long-term (> 1 year) graft survival, which is compounded with the increasing demand for organ donors. Although immunosuppressive agents have been successful at decreasing acute rejection episodes, they have not impacted the rates of chronic graft rejection [6]. Moreover, it is also partially due to the reliance on unspecific immunosuppressive regimens that the long-term prognosis of transplant patients in terms of health has not significantly improved throughout recent years. The most common side-effects of immunosuppressive medications include an increased risk of infection, diabetes, and malignancies.

Therefore, the overarching goal in modern transplantation research has been to optimise and discover methods to improve long-term allograft survival, while reducing the reliance on immunosuppression.

1.2 Transplantation rejection

The immune system is equipped with elaborate mechanisms to protect from infectious pathogens and to also prevent the survival of mutated host cells. These mechanisms are crucial for both initiating signalling to deal with the threat and for also minimizing autoimmune damage to the host. Certain immune cells can detect antigens coated on the surface of harmful microorganisms to distinguish between “self” and “non-self or altered-self” and to respond to “danger” signals released in the presence of pathogens or tissue damage.

Necessarily, it is also due to these complex protective mechanisms that successful engraftment of a transplant requires manipulation of the immune system. Famously in the 1950’s, Peter Medawar published a series of experiments using a rabbit skin transplant model that marked a key breakthrough in transplantation research [7-9]. For one, he demonstrated that rejection was indeed driven by an immunological reaction. His experiments showed that while allogeneic skins were decisively rejected with signs of immune infiltration within the graft, syngeneic grafts survived long-term. Moreover, his studies revealed that subsequent allografts from the same donor were rejected more rapidly after the rejection of the first skin, demonstrating the immune system’s ability to adopt donor-specific memory. This was one of many subsequent findings that further demonstrated the crucial role of immune cells in the rejection of an allograft.

Through these previous findings, it is now known that transplant rejection is a process composed of a complex set of interactions between the innate and adaptive immune system. This complexity makes it difficult to clearly isolate and target mechanisms to prevent rejection.

1.2.1 Innate immune response

The focus of transplantation research since the aforementioned seminal work has necessarily been centred around the adaptive immune system due to findings demonstrating that innate immune cells in most cases may not be enough to drive rejection alone [10]. Nonetheless, recent studies have increasingly emphasised the significant roles that the innate system plays within the process of rejection which cannot be overlooked.

1.2.1.1 Early response to tissue injury and innate recognition

Inevitably, antigen-independent injury both before or after transplantation can initiate proinflammatory stress responses driven by an innate response [11]. Here, it is also important to recognise that early damage to the graft is driven by the innate immune systems of not only the recipient, but also that of the donor. For example, donor organs may be injured in cases of brain or cardiac death, in which inflammatory cytokines and other molecules may be released to activate and recruit immune cells [12, 13]. Furthermore, procurement and transit of the organ cause ischaemic injury and hypoxic stress, which lead to cell injury and death and may also trigger gene and protein expression that lead to inflammation [14]. Finally, subsequent reperfusion injury remains a principal cause of tissue damage and activation of the recipient's innate immune response after transplantation [15].

During a normal physiological state, naïve lymphocytes traffic between the blood and the lymphatic system [16]. However, an inflammatory immune response such as with a transplanted allograft, establishes a chemoattractant gradient towards the graft, in which the graft site produces chemokines that attract activated leukocytes with upregulated chemokine receptor expression [17]. Coupled with upregulation of adhesion and integrin molecules and haematological changes within the inflammatory site, activated leukocytes are systematically guided towards the graft.

Upon transplantation of the allograft, circulating leukocytes are able to exit the blood stream into inflammatory sites in an elaborate process termed leukocyte extravasation. At sites of inflammation, the chemokine gradient and the increased vasodilation with slower blood flow guides activated leukocytes towards the post-capillary venules, increasing their chances of making contact with the endothelial cells of the inflammatory milieu [18]. Then, through a process of “rolling,” leukocytes are gradually slowed down on the surface of endothelial cells through weak interactions between P-selectin (CD62P) on the endothelial cells and selectin ligands on the leukocyte [19]. Eventually, integrin molecules on the leukocytes become activated through interactions with chemokines on the endothelial surface, allowing them to “stop” and bind to endothelial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) with stronger affinity [20]. Finally, the leukocyte then begin to “crawl” across the endothelial cells guided by the chemokine gradient and adhesion and integrin molecules towards the target site in a process termed transendothelial migration (TEM) [18, 21].

Tissue injury, such as those induced by ischaemia/reperfusion (I/R), cause the release of “danger signals” and damage-associated molecular patterns (DAMPs)/alarmins, such as reactive oxygen species (ROS), heat shock proteins, heparin sulphate, and high mobility group-1. The innate immune cells then recognise DAMPs and repeating structural units expressed by pathogens through invariant pathogen-associated pattern receptors (PRRs), such as toll-like receptors (TLRs) and C-type lectin receptors (CLRs) [10, 22]. PRR-triggered responses can then promote activation of adaptive cells and secretion of proinflammatory cytokines [23, 24]. Indeed, TLR engagement through TLR agonists have been shown to induce rejection in mouse models [25, 26]. Moreover, these responses lead to changes within the graft microenvironment, such as in initiating the release of antigens from the graft and modifying the permeability of the endothelial cells lining the vessels, ultimately priming an antigen specific response [27]. Additionally, oxidative stress induced upon I/R injury has also been demonstrated to upregulate TLR2 expression on epithelia and worsen acute kidney injury [28, 29].

1.2.1.2 Innate mechanisms of graft rejection

Upon their activation and recruitment to the graft environment, certain innate cells may inflict damage to the graft themselves. For example, Natural killer (NK) cells are a type of cytotoxic innate lymphocyte cell that mainly function to control tumours and microbial infections by killing target cells (similarly to $CD8^{+}$ cells) and releasing effector cytokines [30]. NK cells are able to recognise and respond to “non-self” cells (also termed “missing self”) through a detection system composed of a series of activating and inhibitory receptors. For example, they express inhibitory receptors that have ligands for self-MHC class I molecules, such as immunoglobulin-like receptors

NKG2A/CD94, which allow them to detect when self-MHC class I molecules are absent on cells, thereby triggering activation. Interestingly, depending on the type of NK cell and the cytokines they release, studies have demonstrated that they can either contribute to tolerance or rejection [31]. It was previously believed that NK cells may not have a significant role in rejection due to reports demonstrating that depletion of NK cells did not alter rat skin or heart allograft outcomes [32, 33]. However, more recent studies have argued that they may have a larger role in both acute and chronic rejection than previously documented. Kroemer et al., demonstrated that IL-15-activated NK cells can directly reject skin allografts in recombination activating gene (RAG)-knockout (KO) mice without adaptive cells [34]. Furthermore, as described by Koenig et al., this may be partially due to an ability for NK cells to directly promote damage to graft endothelial cells, which are unable to send inhibitory signals to recipient NK cells, mimicking the "missing self" phenomenon [35].

Macrophages, which are lymphocytes that detect and phagocytose pathogens, have been shown to comprise 38-60% of infiltrating leukocytes in human transplant biopsies [36, 37]. Depending on the type of macrophage and inflammatory milieu, macrophages have been shown to contribute to both acute and chronic rejection, as well as both cell and antibody-mediated rejection [38, 39]. Allograft-infiltrating macrophages can release ROS and reactive nitrogen species (RNS), which can cause damage to the vascular epithelium and parenchyma [40]. Additionally, they release proinflammatory cytokines within the tissue (i.e. IL-1β, IL-6, TNF-α, and IFN-γ) that can both promote the activation of adaptive immunity and directly accelerate rejection [41].

One of the central initial innate responses post-transplantation comes through the complement system, which is responsible for protecting against pathogens through opsonisation. The complement protein C3 is cleaved and activated through three main pathways (classical, alternative, and lectin), initiating an enzyme cascade that produces a variety of effector molecules. The active products of the complement cascade therefore have been demonstrated to directly damage allografts and promote antigen presentation and adaptive responses [10, 42]. Allografts on murine models with knockout or inhibition of certain complement have been demonstrated to be more protected from damage via ischemia reperfusion injury [43-47]. Nonetheless, the complement system's contribution to rejection may be more significant in its activation of the adaptive immune response. For example, while covalent proteins C3b and C4b can directly bind to target cells and tag them for destruction through opsonisation, the complement proteins or fragments can also be recognised by APCs to promote antigen presentation and T cell activation [10, 48]. Moreover, cleavage of complement proteins such as C5 can release anaphylatoxins (i.e. C5a) that have roles in vasodilation and chemotaxis of T cells to the graft site to further promote rejection [49].

1.2.1.3 Summary

Taken together, recent literature has increasingly uncovered previously underappreciated roles of the innate system in contributing to rejection. Nonetheless, while certain innate immune subsets have potential to damage the graft on their own, the principal feature of innate immunity in rejection appears to be inducing the adaptive response.

1.2.2 Adaptive immune response

Since the seminal rabbit experiments presented by Medawar, the primary focus in the quest for tolerance has justifiably been centred around adaptive immunity. Indeed, this profound interest in the role of adaptive system in the process of rejection have sparked the discovery of critical insights into the processes of allorecognition and signalling and response mechanisms of lymphocytes.

1.2.2.1 T-cell allorecognition

1.2.2.1.1 The Major Histocompatibility Complex (MHC)

The MHC molecules are highly polymorphic glycoproteins, which play a crucial role in the process of graft rejection [50-52]. They function to bind peptide fragments and display them on the cell surface for presentation to T cells [53]. MHCs are located on the short arm of chromosome 17 and chromosome 6 in mice and humans, respectively. In mice, these genes are also known as H-2 genes, while in humans they are known as Human Leukocyte Antigen (HLA) genes. MHC molecules are classified into two groups: MHC class I and MHC class II.

Both classes present antigens to T cells through a peptide-binding groove consisting of a β-pleated sheet and two α-helical regions. MHC class I is composed of non-covalently bound subunits of a membrane-spanning α-chain, encoded by MHC genes, and a β-chain, encoded by the β2-microglobulin gene (outside the MHC gene). Class I molecules are expressed on the surface of all nucleated cells and present endogenous antigens that were degraded within the cytosol to cytotoxic CD8$^+$ T cells (CTL). MHC class II molecules consist of two transmembrane

chains (α and β), which are both encoded by MHC genes, and form a four-domain heterodimeric structure composed of 2α and 2β. Class II molecules are predominantly and constitutively expressed on antigen-presenting cells (APCs), such as dendritic cells (DC), B lymphocytes, monocytes, macrophages, and thymic epithelial cells. However, they can also be acquired and expressed by nonprofessional APCs (i.e. fibroblasts and keratinocytes) and activated human T cells [54]. MHC II glycoproteins mainly present exogenous antigens that have been degraded by immune cells predominantly to $CD4^+$ cells.

Other than MHC molecules, allograft rejection can be the result of other polymorphic molecules, such as minor histocompatibility antigens (miH), which are T-cell epitopes derived from polymorphic proteins [55]. Thus, even when transplant donors and recipients have no MHC-mismatches, transplant rejection can occur through other alloantigens.

1.2.2.1.2 Direct allorecognition

Direct allorecognition is a process by which recipient T cells recognise intact donor MHC-peptide complexes on the surface of donor cells. The passenger leukocyte hypothesis suggests that transplantation induces an inflammatory milieu that causes graft-derived donor APCs to migrate to the recipient's secondary lymphoid tissues, where they activate recipient alloreactive T cells [56]. Studies have demonstrated that depletion of donor DCs leads to loss of immunogenicity and prolonged graft survival within a re-transplantation model of long surviving rat kidney grafts, but could be reversed with adoptive transfer of donor-strain DCs, leading to acute rejection [57].

The precursor frequency of alloreactive T cells can range from 1 to 10%, which is extremely high given that the frequency of T cells that respond to nominal protein antigens is only around $1{:}10^5$ on average. In fact, T cells that recognize alloantigens through the direct pathway can constitute up to 90% of the total alloreactive T cell repertoire. This high frequency may be explained by two hypotheses: (1) the multiple binary complex model, and (2) high determinant density model. The first model, introduced by Matzinger/Bevan in 1977, works within the concept of MHC restriction, in which MHC:peptide complexes are recognised by T cell receptors (TCR) with high specificity [58]. Accordingly, heterogeneity within either the peptide or MHC molecule can result in the activation of T cells. T cells can recognise foreign peptides presented by self-MHC molecules in the presence of pathogens and can mount an immune response. Within the context of transplantation, recipient T cells are presented with an array of foreign peptides by donor MHC molecules, thereby activating a range of T cells. Thus, this model proposes that each of the peptides presented by allogeneic MHC (allo-MHCs) can activate at least one T cell clone [59]. In contrast, the high determinant density model, proposed by Bevan et al. in 1984, attributes the alloresponse to the allo-MHC molecules themselves, rather than the peptides [60]. The model works under the premise that the affinity by which TCRs interact with self-MHCs and allo-MHCs are different, due to differences in the specific polymorphic residues within the TCR docking grooves. It argues that T cells bind allo-MHC complexes with high affinity and recognise the complex as foreign, whereas they are selected to bind self-MHC peptide complexes with low affinity. Moreover, it also suggests that even T cells with low affinity for allo-MHCs can respond due to the high density of MHC molecules within the surface of donor cells.

This high precursor frequency of alloreactive T cells is especially paradoxical given the process of thymic education of T cells, which positively selects for a mature T cell repertoire with self-MHC restriction. This may be explained by the phenomenon of TCR cross-reactivity, in which T cells are thought to be able to respond to an allo-MHC and self-peptide complex if they have a similar conformation to a self-MHC and allo-peptide complex [61]. Because there are no intrinsic mechanisms in place to select against donor antigen reactive cells, these cross-reactive T cells persist within the T cell pool.

While the direct pathway of allorecognition has been demonstrated to have a central role in acute rejection, whether they have persisting roles in later stages is still under question. Earlier reports suggested that the duration of CD4 T cell alloresponses is short-lived due to the limited number and life-span of donor DCs within the allograft [62-64].

1.2.2.1.3 Indirect allorecognition

In 1993, Auchincloss et al. demonstrated findings that suggested that the direct pathway may not be the only form of allorecognition responsible for transplant rejection [65]. In their study, they showed that MHC class I deficient mice, which lack $CD8^+$ T cells, rejected skin from MHC class II deficient donor mice. Within these conditions, direct allorecognition was not possible because there were no recipient $CD8^+$ T cells to recognise donor MHC class I molecules and no allo-MHC class II molecules to present to recipient $CD4^+$ T cells. Thus, it was proposed that donor-derived peptides, including allogeneic MHC molecules, can be processed and presented on recipient MHC molecules to recipient T cells. This is further supported by studies that demonstrate that both self-MHCs and allo-MHCs can process and present MHC molecules within

the environment [66]. It is believed that after transplantation of an organ, inflammatory mediators and chemokines attract recipient leukocytes to the graft where APCs phagocytose debris from the allograft and migrate back to the lymph nodes for presentation.

1.2.2.1.4 Semi-direct allorecognition

More recent studies have supported an alternative process of allorecognition called the semi-direct pathway, or "cross-dressing." In 1999, Bedford et al demonstrated that many recipient APCs within the draining lymphoid organs express intact donor MHC-peptide complexes to present to alloreactive T cells [67]. This potentiates a "three cell model," in which a recipient DC can present allogeneic peptides both directly to $CD8^+$ T cells through an acquired donor MHC class I molecule and indirectly to $CD4^+$ T cells through self-MHC class II. Moreover, $CD8^+$ T cells can also be stimulated by the $CD4^+$ T cell, which is activated by the same DC. Because it is believed that $CD4^+$ T cells can only provide help to $CD8^+$ T cells when they are stimulated by antigens of the same APC [68], this model may provide an explanation for how $CD4^+$ T cells activated through the indirect pathway can also help stimulate $CD8^+$ cells activated through the direct pathway.

Interestingly, although it was thought that the semi-direct pathway was cell-contact dependent, more recent studies have demonstrated that MHC-peptide complexes can be exchanged through extracellular vesicles [69, 70]. Moreover, these studies suggested that the semi-direct pathway may have a more prominent role than previously thought and contradicted the traditional mechanisms of the direct pathway. Rather than donor DCs migrating to recipient draining lymph nodes (LN), they propose that donor MHC molecules are transported to recipient lymphoid organs through exosomes [69, 70]. Moreover, Lakkis's group recently demonstrated that semi-

direct allorecognition were the predominant form of antigen presentation within the allograft itself and a major driver of acute rejection [71].

Taken together, it is clear from recent literature that mechanisms of allorecognition are complex as a whole and the contributions of each pathway to processes of rejection appear to be dependent on various factors.

1.2.2.2 T cell activation

T cells undergo activation and subsequent clonal expansion through the combination of three signals: (1) TCR activation, (2) costimulation, and (3) pro-proliferative cytokines. The first signal comes from antigen-specific binding of the TCR complex to MHC-peptide complex. Then, co-signalling molecules on the T cells interact with ligands on the APCs in a process called costimulation. Finally, cytokines, such as IL-2, within the proximal microenvironment promotes proliferation of the T cells.

1.2.2.2.1 TCR

Since its discovery in 1984 [72], extensive efforts have been dedicated to characterising the complex T cell receptor protein [73]. The majority of TCRs are composed of two membrane-bound, disulphide-linked polypeptide chains (α and β), while a small subset of TCRs express γ and δ chains (approximately 95% $\alpha\beta$ and 5% $\gamma\delta$, in humans). Each of the chains consist of a constant (C) and variable (V) region, where each of the variable regions also contain three hyper-variable domains called complementarity determining regions (CDRs), which are the binding sites of the TCR. The variable domain of immunoglobulins and TCR genes arise through a process called V(D)J

recombination, which occurs in the thymus during early development [74]. V(D)J recombination constitutes a series of DNA cleavage and joining phases to assemble various segments of antigen receptor genes, allowing for the generation of highly diverse repertoire of immunoglobulins and TCRs. Notably, the recombination activating genes, RAG1 and RAG2, are key enzymes critical for initiating the process of V(D)J recombination [75].

After recombination, the chains associate with a CD3 protein, which consist of δ, ε, γ and ζ chain (CD247), through hydrophobic interactions to form the TCR complex [76]. The heterodimers, CD3ε/CD3δ and CD3γ/CD3ε, are located on the sides of the TCR, while a CD247 dimer is located below the TCR. Specifically, for αβ T cells, co-receptors CD4 and CD8 are necessary to initiate TCR signalling for their role in binding non-polymorphic proximal domains of the MHC. Thus, mature αβ T cells are functionally divided into $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, which recognize antigens presented in the context of MHC class II and class I, respectively. When $CD4^+$ T cells become activated, they can differentiate to a variety of cell types and play roles in activating or "helping" immune cells such as B cells and $CD8^+$ T cells, as well as suppressing immune responses. Activated $CD8^+$ T cells have crucial roles as "killer" cells that directly attack infected and malignant cells.

1.2.2.2.2 Costimulation

While TCR engagement leads to initial signal transduction, co-signalling receptors transduce positive or negative signals to modulate TCR signals [77]. In 1970, Bretscher/Cohn proposed a "two-signal model," in which two signals were required for B cell activation [78]. Later, through the discovery of co-stimulating receptors such as CD28 that amplify T cell signalling [79], studies

demonstrated that this also held true for T cells in that they require both (1) TCR stimulation and (2) costimulation. Moreover, T cells cultured with only anti-CD3 antibodies become unresponsive [80, 81], and blocking costimulatory signals rendered them anergic [82]. Thus, after TCR recognizes and engages with an antigen on the MHC complex, binding between co-signalling receptors on the T cells and their ligands on the APCs are necessary for full activation. Most co-signalling molecules fall into two main families of the immunoglobulin superfamily (IgSF) (ex: CD28), or the tumour necrosis factor receptor superfamily (TNFSR) (ex: TNFR1, OX40).

1.2.2.2.3 TCR signal transduction

1.2.2.2.3.1 Proximal signalling

The CD3 proteins contain cytosolic domains with immunoreceptor tyrosine-based activation motifs (ITAMs) [76]. When the TCR is engaged, ITAM is phosphorylated at the tyrosine residues by protein tyrosine kinases (PTKs), which initiates proximal signalling [83]. In this process, members of the Src-family PTKs, such as Lck and Fyn, trigger the phosphorylation of ITAMs. The role that Syk-family kinases play in T cell development and function has been extensively studied, yet still not completely understood. However, unlike Lck [84], Fyn does not seem to be essential for T cell development [85]. After phosphorylation of ITAMs, members of the Syk-family kinases, such as zeta-chain-associated protein kinase 70 (ZAP-70), are able to dock on the ITAMs along with other proteins. ZAP-70 is then phosphorylated by Lck and activates other proteins and recruits adaptors [86].

Linker for the activation of T cells (LAT) and the SH2 containing leukocyte phosphoprotein of 76kDa (SLP-76), which together form a proximal signalling complex, are key adaptors in this stage of signal transduction that play critical roles, such as the activation of Ras (described below) [87, 88]. Phosphorylated LAT recruits Grb2, Gads, phospholipase C (PLCγ)-1, and the p85 subunits of phosphatidylinositol 3-kinase (PI3K), and then SLP-76 binds Gads and PLCγ1 to form a complex. Moreover, phosphorylated SLP76 additionally recruits Vav-1, a GEF, IL-2-induced tyrosine kinase (Itk), as well as other adaptor proteins. In particular, Vav-1 recruitment activates Rac1, a member of the Rho family of GTPases, which plays a role in actin reorganisation [89, 90].

1.2.2.2.3.2 Distal signalling

The proximal signalling processes detailed above function to activate distal signalling pathways. For example, PLCγ hydrolyses phosphatidylinositol-3,4-trisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 initiates release of intracellular Ca^{2+}, which activates calcineurin [91]. This dephosphorylates the nuclear factor of activated T cells (NFAT) transcription factor and allows its translocation to the nucleus, ultimately modulating genes involved in crucial roles within the immune system such as cell differentiation and angiogenesis. Moreover, DAG is also crucial within this process for the activation of T cells [92], as well as in the eventual downstream activation of transcription factor nuclear factor-κβ (NF-κβ) and activator protein 1 (AP-1).

The Ras-Erk1/2-AP1 pathway is another critical signalling process in T cell activation. Ras, a G protein activated in the presence of GTP, activates a serine/threonine kinase, Raf-1 [93]. This leads to the sequential activation and phosphorylation of downstream targets from mitogen-

activated protein kinase kinase (MAPKK, Mek1/2), Erk1/2, to Elk, which induces transcription factor c-fos and ultimately the formation of AP-1 [94]. The role of AP-1 in T cells is still not completely understood, although it is likely it plays a role in T cell development and activation [95, 96].

The PKCθ/IKK/NF-κβ pathway, which is primarily activated by DAG, has been demonstrated to have a variety of roles in T cell activation, survival, and function, as well as Treg development [97]. Many have demonstrated that disruption of this pathway can lead to severe consequences in T cell activation and survival. For example, mice that express a constitutively active form of inhibitor of κB kinase β (caIKKβ) in a T-cell specific manner was demonstrated to promote T cell apoptosis and impair function [98].

Finally, many recent studies have focused on the mammalian target of rapamycin (mTOR) signalling pathway in its regulation of T cell activation and function. mTOR exists in two functionally distinct complexes (mTORC1 and mTORC2) and is activated through the PI3K-Akt and Ras-Erk1/2 pathways [99, 100]. Notably, while elevated levels of mTORC1 can prevent T cell anergy [101], inhibition of mTORC1 with rapamycin induces T cell anergy [102].

1.2.2.3 Adaptive mechanisms of rejection

1.2.2.3.1 Cytotoxic T cell-mediated responses

Naïve $CD8^+$ CTLs are activated by DCs and $CD4^+$ helper T cells. Through the three-cell model (as previously described), $CD4^+$ T cells can promote $CD8^+$ T cell differentiation by direct cell-to-cell contact or by the secretion of effector cytokines, such as IL-2 and interferon (IFN)-γ [10].

Moreover, $CD4^+$ T cells can also condition, or "license", DCs for the indirect activation of CTLs, such as through the CD154/CD40 costimulatory pathway. Whereas CD40 signalling can act as an alternative to $CD4^+$ T cells in priming DCs [68, 103, 104], blockade of this pathway has also been demonstrated to be effective in prolonging allograft survival due to inhibiting DC licensing [105-108]. Once activated, CTLs migrate to the allograft and mount a response upon the recognition of allogeneic MHC class I molecules by releasing granules containing cytotoxic molecules such as perforin or granzyme B, which induce apoptosis of target cells. Moreover, CTLs also upregulate expression of the Fas ligand (FasL), which binds to Fas on target cells to trigger apoptosis.

CTLs have been demonstrated to be able to reject skin allografts independently of $CD4^+$ T cells [109]. Moreover, CTLs isolated from rejecting allografts, which upregulate expression of CTL-associated transcripts (i.e. CD8, perforin, granzymes) [110-112], have been shown to be able to kill donor cells when cultured *in vitro* [113].

1.2.2.3.2 Helper T cell-mediated responses

$CD4^+$ T helper (Th) cells are one of the first adaptive immune cells to respond post-transplantation. Upon alloantigen recognition, $CD4^+$ T cells play a role in allograft destruction through delayed-type hypersensitivity (DTH or Type IV hypersensitivity) [114], in which they release proinflammatory cytokines such as interleukin (IL)-1, tumour necrosis factor-α (TNF-α), and IFN-γ. This leads to the infiltration of leukocytes such as macrophages, which can then be activated to produce mediators with non-specific cytotoxic activity such as nitric oxide (NO) [115]. Moreover, alloantigen-specific Th cells can also stimulate B cells to produce alloreactive antibodies through a process called linked recognition. Interestingly, studies have also identified

populations of cytotoxic $CD4^+$ T cells, which have been shown to kill target cells through the same effector mechanisms as NK and $CD8^+$ cells (Fas/FasL and perforin/granzymes) [116, 117].

Depending on their function and cytokine secretion, $CD4^+$ Th cells have been grouped into different lineages, in which many have been demonstrated to have varying roles in transplant rejection. For example, Th1 cells, which secrete cytokines such as IFN-γ, IL-2, and TNF-α [118], have been demonstrated to inflict allograft damage directly through their high expression of FasL [119]. Moreover, human renal allografts during acute rejection episodes have been shown to have massive infiltration of Th1 cells with high IFN-γ production [120]. Interestingly, Th2 cells, another $CD4^+$ T cell subset, are able to skew the T cell phenotype away from Th1-type through the release of IL-4 and IL-10, and thus may prevent Th1-mediated rejection [121]. Nonetheless, Th2 cells have also been implicated in promoting rejection on their own [122, 123], possibly playing a larger role in chronic rejection [124]. Additionally, there is some evidence to suggest that Th17 cells may have a hand in rejection, due to numerous studies reporting the presence of Th17-secreted cytokines (i.e. IL-17 and IL-21) in rejecting renal transplants [125-127].

1.2.2.3.3 Memory T (secondary) cell responses

In general, memory T cells are generated through two main mechanisms. Firstly, they can be generated during a primary immune response, in which a population of antigen-responsive effector T cells will go on to differentiate into memory cells. Secondly, memory T cells can be generated from the peripheral T cell pool through homeostatic proliferation within a lymphopenic environment.

Alloreactive memory T cells can be generated through sensitisation and prior exposure to donor antigen through blood transfusions, pregnancy, or previous transplants [128]. Nonetheless, they can also be generated without donor antigen sensitisation. The first method is through the homeostatic proliferation of surviving T cell populations after lymphodepletion induced by induction therapies. Secondly, alloreactivity in memory T cells can be established through a mechanism called heterologous immunity, which potentially allows them to respond to a previously unseen foreign antigen due to TCR cross-reactivity [129, 130].

There are several reasons that memory T cells can potentially pose significant issues to graft survival. Throughout the memory differentiation process, TCR and costimulatory signalling cascades are altered, which allows memory T cells to respond to reencountered antigens more rapidly and with greater intensity [131]. This means that memory T cells can continue to survive with limited antigen and costimulation and also respond with a lower activation threshold. Additionally, $CD4^+$ memory T cells provide increased activation of donor-reactive $CD8^+$ T cells [132]. Importantly, commonly used immunosuppressive agents and costimulatory blockade strategies are largely ineffective in depleting memory T cells, highlighting a significant drawback in current regimens [133, 134]. Recent animal and clinical studies have demonstrated efficacy in the use of Alefacept, a fusion protein targeting LFA-3/CD2, in order to selectively deplete effector memory T cells [135, 136]. Nonetheless, Alefacept treatment was not shown to increase patient or graft survival, and further investigation into Alefacept and other immunosuppressive agents directed against memory T cells is necessary.

1.2.2.3.4 B cell response

In the context of transplantation, alloantigen-specific B cells recognise allogeneic antigens such as HLA, which is processed and expressed on the cell surface in conjunction with MHC class II molecules. With the help of $CD4^+$ T cells, alloantigen-loaded B cells then become activated and ultimately induce the formation of germinal centres, in which B cells mature and proliferate. A proportion of B cells will differentiate into plasma cells, which can secrete donor-specific antibodies (DSAs) that elicit antibody-mediated rejection (AMR) [137]. DSA can form before transplantation in sensitized patients and *de novo* post-transplantation. DSAs can promote rejection mainly through injury to the allograft endothelium, through both direct and indirect mechanisms. Direct injury is initiated through the DSA interacting with cell surface antigens on the endothelial cells (i.e. HLA antigens), in which pro-thrombotic and pro-inflammatory molecules are released upon the process of exocytosis [138]. DSAs also elicit endothelial injury indirectly through complement fixation and formation of C5b-C9 (membrane attack complex). C4d is an inactive molecule produced within this process that has been used as a marker in histological staining to detect AMR. Moreover, DSAs can recruit Fc-receptor-expressing molecules, such as NK cells and macrophages, to kill target cells coated by antibody in a process called antibody-dependent cell-mediated cytotoxicity (ADCC) [139].

AMR can be classified into hyperacute, acute, and chronic. Hyperacute AMR is the result of pre-existing antibodies specifically against donor HLA, blood group (ABO)-isoagglutinins, or endothelial cell antigens within the recipient. Cyanosis and necrosis occur within hours after

organ reperfusion, leading to immediate graft loss. However, hyperacute rejection has become increasingly rare due to advanced HLA-matching.

From early mouse studies, it was believed that the humoral response may not have such a significant effect on acute rejection because allograft rejection is not delayed in B-cell deficient mice compared to in control mice [140]. However, subsequent studies also showed that acute rejection was delayed in B-cell deficient mice as compared with controls when T cells were partially suppressed. Moreover, rejection was restored upon the passive transfer of hyperimmune sera [141, 142], indicating that B cells and alloantibodies play a hand in acute rejection. Clinical studies have indicated that acute AMR may occur in patients with sensitised B cells with previous antigen exposure, likely due to pregnancy, blood transfusions, or prior transplants. Acute humoral rejection is most reliably diagnosed with C4d deposition and is said to occur in 20-30% of acute rejection cases [143].

In terms of chronic rejection, clinical studies have indicated a strong correlation between the number of circulating antibodies against HLA and later graft loss [144-146]. Moreover, early AMR and presence of DSAs is highly correlated with eventual renal graft dysfunction or loss [147-149]. Typically, chronic AMR is characterised histologically by transplant glomerulopathy, peritubular capillary multilamination, and positive peritubular capillary C4d staining [150]

Thus, humoral immunity has remained one of the biggest challenges in transplantation, in part due to the manifold documented roles of B cells which may be either detrimental or protective for the allograft. Aside from their role in generating antibodies, B cells may also contribute to alloresponse through many different mechanisms. For example, B cells have been demonstrated

to be involved in the modulation of DCs, in which a recent paper suggests that B cells induce maturation of DCs to mediate Th2 differentiation [151]. Moreover, B cells have also been demonstrated to be able to process antigens and present them to MHC class II-restricted T cells [152]. Certain B cell populations, such as regulatory B cells (Bregs), may also have roles in modulating immune response through the release of cytokines such as IL-10 [153]. Taken together, finding methods to isolate the functions and specific role of B cells during alloresponse have been challenging due to their interactions with other immune cells.

1.2.3 Summary of processes of rejection

In conclusion, tremendous progress has undoubtedly been made to elucidate the roles of various immune cells in contributing to rejection. Nonetheless, the dynamic interplay between both the innate and adaptive immune system has brought challenges in unravelling the critical components necessary to target for the prevention of rejection. Here, it is important to consider that the process of rejection is a spectrum which likely requires a broad lens to understand the whole picture. While the prevention of rejection remains complex, invaluable insights have been gained from investigating the intrinsic immune programs in place, which may be modulated to promote transplant tolerance.

1.3 Mechanisms of self-tolerance

Although the fundamental role of the immune system is to respond and protect the body from foreign pathogens or malignant cells, there are also critical mechanisms in place to restrict immune responses against self-tissue, in a concept termed “self-tolerance.” The importance of these regulatory mechanisms is reflected within autoimmune pathologies, in which one or multiple mechanisms are dysfunctional. The interworking of self-tolerance has naturally guided strategies to promote transplantation tolerance. Overall, the mechanisms of self-tolerance can be broadly classified as “central” for those that occur during T cell development within the thymus or “peripheral” for those acting on mature T cells occurring throughout the rest of the body.

1.3.1 Central tolerance

The T cell repertoire is refined and shaped through a process called thymic education. As described earlier, CD4 and CD8 double-negative (DN) cells first undergo “positive selection,” in which DN cells that fail to bind to self-peptide-MHC complexes are eliminated from the pool. Meanwhile, those with low affinity differentiate into single-positive CD4 or CD8 thymocytes and are recruited to the next checkpoint, termed “negative selection.” Here, developing T cells exhibiting high reactivity towards self-MHC antigens are deleted in the thymus in a process also referred to as “central deletion.” [154].

A protein demonstrated to have an integral role in central tolerance and preventing autoimmunity is autoimmune regulator (AIRE), which promotes the expression of tissue-

restricted antigens (TRAs) to developing thymocytes. Presentation of TRAs during thymocyte development have been demonstrated to eliminate T cells that express TCRs directed against antigens from specific tissue [155, 156]. *AIRE* deficiency has been shown to lead to defects in negative selection [157] and an autoimmune disease called Autoimmune-Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) [158, 159].

1.3.2 Peripheral tolerance

Central deletion is not always a perfect system, in that some self-reactive T cells will avoid deletion and escape the thymus into the periphery. Thus, as another defensive measure to inhibit self-reactive leukocytes and prevent autoimmunity, the immune system has developed various mechanisms of peripheral tolerance, which include peripheral deletion, clonal anergy, ignorance, and immunoregulation [160].

For peripheral deletion, activation-induced cell death (AICD) mediated by the engagement of Fas receptors on self-reactive T cells is demonstrated to be a central process leading to T cell apoptosis [161-164].

Conversely, anergy or inactivation of T cells may occur as a result of antigen encounter, but without costimulation (signal 2). For example, CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4, CD165) is a cell surface protein that interacts with CD80/CD86 ligands on APCs with high affinity to deprive naïve T cells of CD28-mediated costimulation [165]. Studies indicate that although CD80/86 is mainly expressed by APCs, naive T cells can also express these molecules, providing an additional target for CTLA-4-induced anergy [166].

Self-reactive T cells can also be "ignored" and kept physically separate from its antigen due to "immune-privileged sites," in which certain organs tolerate the introduction of antigens that may otherwise cause an unwanted inflammatory response. The most common examples of immune privileged sites are the anterior chamber of the eye and the testes [167].

Finally, immunoregulation of self-reactive lymphocytes through regulatory immune cells is a critical mechanism that protects tolerance, and will be discussed in depth below.

In conclusion, there are myriad mechanisms devoted to maintaining self-tolerance. Thus, finding strategies to investigate and harness these physiological processes have been the principal approach in inducing tolerance towards allografts.

1.4 Experimental and clinical strategies to promote transplant tolerance

Transplantation tolerance can be defined as the specific absence of a destructive immune response to a transplanted tissue in the absence of immunosuppression [160]. Thus, the overarching goal is to not only induce acceptance of the allograft without continued dependence on immunosuppression, but to preserve the recipient's protective immunity against other antigens (i.e. pathogens and malignant cells). The current strategies to approach tolerance can be broadly classified into those depending on deletional mechanisms and others depending on active immunoregulation.

1.4.1 Mixed chimerism

One of the most promising approaches to transplantation tolerance in recent years has been through the establishment of hematopoietic chimerism ("mixed chimerism") via bone marrow transplantation (BMT), in which a mixture of both donor and recipient haematopoietic progenitor cells is established [168]. This strategy aims to exploit the mechanisms underlying central tolerance, in which the infusion of donor cells may be used to educate developing T cells and delete clones with specificity towards the donor antigen to induce specific alloantigen tolerance. Here, because recipient alloresponsive immune cells (particularly T cells) will resist engraftment of MHC-mismatched bone marrow, conditioning regimens to eliminate or suppress donor reactive immune cells are necessary for successful engraftment [169]. In this respect, reducing the toxicity of the conditioning therapy required for engraftment remains one of the biggest challenges to this strategy.

Recently, there has been much clinical promise in the method of inducing mixed chimerism with the development of less toxic conditioning regimens [160]. In a trial conducted in Massachusetts General Hospital including 5 patients receiving HLA single-haplotype mismatched renal transplants, transient mixed chimerism was achieved in patients by the use of a nonmyeloablative conditioning regimen [170]. 7 of the 10 patients were completely weaned off all maintenance immunosuppression by 14 months post-kidney/BM transplant and maintained good renal function for 5-13 years [171]. 4 patients had continued to remain immunosuppression-free after 5-13 years [172]. Similarly, a Stanford group conducted trials with HLA-matched kidney transplant patients treated with donor $CD34^+$ stem cells and $CD3^+$ T cells

along with a conditioning regime. They reported 17 out of 22 patients were successfully tapered off immunosuppression, with 7 of those exhibiting stable mixed chimerism. Finally, a group from Northwestern University treated 8 HLA-mismatched kidney transplant recipients with donor hematopoietic stem cell treatment [173], along with a slightly more intensive conditioning regimen. This allowed them to induce persistent full chimerism within the recipients, in which recipients were reconstituted with virtually all donor T cells. These clinical trials have been promising for the possibility of long-term allograft tolerance, but further research is necessary to successfully translate it to other organs and to better understand the mechanisms of tolerance, potential side-effects (i.e. graft-versus-host disease, GVHD), and advantages within the different types of chimerisms [174].

1.4.2 Peripheral depletion

Within transplantation research, strategies to induce transplant tolerance through peripheral depletion of donor-reactive T cells had been investigated in animal models with considerable success. A well-defined method is total lymphoid irradiation (TLI), which induces significant lymphocyte depletion. Several animal studies have implemented TLI therapy at the time of transplantation, demonstrating its ability to induce donor-specific hyporesponsiveness and prolonged allograft survival [175-178]. Nonetheless, translation to the clinic has been met with minimal success due to both the inability to sustain peripheral tolerance and the potential complications. Strober et al. published findings of a clinical trial in which 28 renal allograft recipients were treated with a TLI-based therapy, in which only 3 of these patients were

successfully weaned off immunosuppression [179]. To avoid the potential side-effects associated with TBI, another method that has been tested in clinical therapies has been T cell depletion through polyclonal or monoclonal antibodies. In a clinical trial of 31 kidney transplant patients, an anti-CD52 antibody (CD52 is expressed on mature lymphocytes, monocytes and DCs), alemtuzumab (CAMPATH), was administered along with low-dose cyclosporine. 29 patients of this trial maintained normal renal function under low-dose cyclosporine [180]. Nonetheless, a separate trial also revealed that alemtuzumab alone without cyclosporine was not enough to prevent acute rejection [181, 182].

1.4.3 Costimulatory blockade

Alternatively, costimulatory blockade is another method that has been investigated to induce peripheral tolerance, with the premise of blocking signal 2 of T cell activation to induce anergy and/or death. For example, anti-CD154 monoclonal antibodies have been tested but have not been well tolerated in clinical trials due to increased incidences of thromboembolic events [183]. Belatacept, a CTLA-4-Ig, which acts to block CD80/86 ligands on DCs to deprive T cells of CD28-mediated costimulation [165], has shown promise as a therapy for kidney transplantation. Collectively, none of the costimulatory blockade antibodies have demonstrated potential to induce tolerance alone, for adjunctive and sustained immunosuppression have been necessary to prevent rejection.

1.4.4 Regulatory cells

As indicated by the studies leading to the current landscape of transplantation research, the quest for tolerance is still fraught with many challenges, particularly due to the toxic side-effects associated with current treatment methods. Moreover, the mechanisms of action of many of these therapeutic strategies have not been fully elucidated, which have hindered progress in further optimising these therapies. In this regard, the growing knowledge of regulatory cells has significantly improved our understanding of immune tolerance and has paved the way for the implementation of regulatory cell-based therapies in transplantation. Indeed, a number of both innate and adaptive immunoregulatory cell types have already been developed for use within clinical trials [184]. Our group was previously involved in the ONE study consortium, which was a study between multiple European and US centres to assess the safety of regulatory cell infusion in renal transplant recipients.

As demonstrated by the vast amount of resources being invested in researching regulatory cell types in recent years, it is clear the promise that regulatory cell-based therapies now hold within transplantation. However, the subtype that has paved the way for the rest of these cells is the regulatory T cell (Treg), due to its critical role in mediating immune homeostasis. The rest of the introduction will centrally focus on Treg biology and the potential pathways by which it may be modulated.

1.5 Regulatory T cells (Tregs)

The concept of a suppressive population of T cells had been proposed as far back as the 1960's. Nishizuka/Sakakura et al. published a study investigating the pathogenesis of autoimmune oophoritis that developed after neonatal thymectomy in mice [185]. They found that the occurrence of autoimmunity was dependent on the timing of thymectomy. Here, they hypothesised that autoreactive T cells emigrated from the thymus at a certain time point to cause autoimmunity but was suppressed by a different subset of T cells that emigrated days after. Soon after, seminal work by Gershon/Kondo et al. described a population of T cells from animals that accepted allografts that had the capacity to induce dominant tolerance when transferred to another animal [186]. Nonetheless, further characterisation of this population of suppressive T cells remained elusive for many years due to the inability to find a specific marker to distinguish this subset [187].

1.5.1 Phenotype and markers

It was not only until the 1990's when research in suppressive T cells started to gain significant traction. It was in this period that Sakaguchi et al. identified a specific population of $CD4^+$ T cells with high expression of the CD25 marker (IL-2 receptor α chain) that demonstrated potent regulatory properties in self-tolerance and autoimmune suppression [188]. With subsequent studies confirming the suppressive function and phenotype of these cells, this distinct subset was soon identified as the population known today as Tregs [189-191]. It was later described that although they are dependent on the presence of IL-2 for survival, Tregs do not produce their own

IL-2, unlike Teff populations [192]. Nonetheless, the search for defining markers of Tregs did not conclude with the discovery of $CD4^{+}CD25^{high}$ populations. In mice, CD25 largely correlates to a suppressive population of Tregs; however, in human cells, CD25 expression is also upregulated in activated effector T cells, thus not making it a distinguishing marker for human Tregs [187].

The discovery of a X chromosome-encoded transcription factor forkhead box P3 (Foxp3) soon helped to spearhead investigation into the function and role of Tregs. Brunkow et al. demonstrated that disruption or knockout of the *Foxp3* gene led to aggressive autoimmune syndromes that were reversible with restoration of the Treg compartment [193]. In immunodeficient mice, Tregs can also be generated with the addition of the *Foxp3* transgene [194]. In a similar manner, a *FOXP3* mutation in humans leads to a X-linked autoimmune lymphoproliferative disorder called immune dysregulation, polendocrinopathy, enteropathy, X-linked (IPEX) syndrome [187, 195]. Both mice and humans with non-functional *Foxp3* contain populations of non-suppressive $CD4^{+}CD25^{+}$ cells and do not have naturally occurring Tregs [194, 196] [187].

There are certain conditions in which Foxp3 can be expressed within non-Treg populations. For example, in humans, Foxp3 can be transiently expressed within activated and proinflammatory Teffs [197-199]. Moreover, studies have found that there are small populations of mouse T cells that can transiently upregulate Foxp3 during their development, suggesting that Foxp3 expression may not be strictly exclusive to Tregs [200]. Nonetheless, although there are some exceptions, Foxp3 is the single most defining and specific Treg marker to date.

1.5.2 Treg development

Physiologically, Tregs are demonstrated to either develop in the thymus or periphery, and thus may be classified as thymus-derived Tregs (tTregs) and peripherally-derived Tregs (pTregs), respectively. Moreover, Tregs generated *in vitro* may also be classified as *in vitro*-induced Tregs (iTregs) [201].

Similar to other thymocytes, tTregs are generated through thymic selection (positive and negative selection). However, unlike conventional T cells, which are deleted for being too self-reactive, tTregs are able to survive clonal deletion even though they are conventionally thought to be autoreactive. Indeed, the mechanisms underlying the unique ability of tTregs to evade deletion are still under debate. Findings initially provided by Caton et al. suggested that the expression and strength of TCRs specific for self-antigens play a determinant role in tTreg differentiation [202]. Work from Moran et al., in which they developed transgenic mice that reflected the antigen receptor signal, supported this notion, demonstrating that TCR signal strength is stronger in tTregs than in Teffs during development [203]. From these findings, it was hypothesised that tTregs had "intermediate" TCR self-reactivity, in which they had higher affinity than conventional T cells, but not high enough for them to be negatively selected [204]. Nonetheless, other studies have contradicted the idea that TCR self-reactivity is critical for tTreg development, demonstrating that Tregs may not actually be more self-reactive than conventional T cells [205], and that higher affinity does not necessarily correlate with increased Treg selection [206]. Thus, many studies have focused on identifying certain endogenous antigens that may be crucial in tTreg selection [207-209], while others have argued that CD28 costimulation and

certain cytokine signals (i.e. IL-2) are crucial for tTreg development [154, 210-214]. Moreover, others have demonstrated that AIRE may have an integral role in tTreg development by promoting the expression of TRAs to developing thymocytes. Presentation of TRAs during development may influence tTreg developmental rate and/or specific clones of the tTreg repertoire [155, 156]. Taken together, there is still much more that needs to be clarified regarding the factors that dictate Treg generation.

In contrast, pTregs are generated in the periphery from Teffs by TCR stimulation and exogenous antigen under tolerogenic conditions. Certain factors such as tolerogenic DCs and molecules such as transforming growth factor (TGF)-β and retinoic acid have been described to be critical for the development of pTregs [215-217]. As described previously, pTregs not only function to regulate autoreactive T cells that have evaded clonal deletion in the thymus, but are also necessary to prevent hypersensitivity reactions and maintain tolerance to non-pathogenic substances (i.e. environmental antigens and commensal microbiota) [218]. Exogenous antigens within the periphery, such as metabolites produced from the microbiota of the colon, can generate pTreg differentiation. Nonetheless, investigation into the development and function of pTregs has been challenging due to the lack of reliable markers that can distinguish between tTregs and pTregs.

1.5.3 Mechanisms of Treg-mediated suppression

Tregs display significant versatility in their suppressive mechanisms, which can depend on the immune environment, the Treg activation state, and target cell type [219]. In transplantation, for example, the suppressive mechanisms can depend on donor-specificity [220]. Tregs can suppress both directly, through suppressing the target cells themselves, or indirectly, in which they may

induce other cells to suppress the target cell [221]. There are four main processes by which Tregs suppress immune responses: (1) modulation of DC function or maturation, (2) inhibitory cytokine release, (3) cytolysis, and (4) metabolic disruption, [187, 222].

Investigation of specific pathways involving CTLA-4 has demonstrated that Tregs can hinder the activation of effector T cells by modulating DCs. Multiple studies have supported the finding that Tregs may downregulate B7 costimulatory molecules CD80 and CD86 on DCs in a CTLA-4 dependent manner to disrupt the activation of effector T cells by APCs. CTLA-4 interacts with CD80/CD86 ligands with high affinity to deprive naïve T cells of CD28-mediated costimulation [165]. Through CTLA-4 ligation, Tregs may also induce DCs to express the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO), which converts tryptophan to kynurenine, a molecule toxic to T cells [219]. Another relevant molecule expressed on Tregs is lymphocyte-activation gene-3 (LAG-3), which binds to MHC-II molecules to suppress DC maturation [222].

TGF-β, IL-10, and IL-35 are considered to be the main regulatory cytokines released by Tregs. These cytokines are involved in tasks such as the direct suppression of effector T cell signalling, regulation of IFN-γ, the induction of Tregs, and the maintenance of FoxP3 expression [219, 223, 224]. TGF-β was demonstrated to from a complex with latency-associated protein (LAP), which is expressed on activated Tregs, and help suppress Teffs and induce pTregs within the microenvironment [225]. Treg-derived IL-10 has been shown to be crucial for mediation of Th17 cell responses and protection from conditions such as colitis [226]. Finally, IL-35 has additionally been identified as having strong immunosuppressive properties against Teffs and Th17 cells in

particular [227], where IL-35-deficient Tregs were shown to have reduced suppressive function [187, 228].

Tregs may exert suppression through expression of granzymes A/B and perforins that induce programmed cell death. By this mechanism Tregs are able to kill and suppress B cells and possibly hinder effector T cell function through apoptosis [229]. Moreover, similar to cytotoxic $CD8^+$ T cells, Tregs express the Fas ligand on their surface and can induce Fas-dependent apoptosis of $CD8^+$ cells [187, 230].

Metabolic disruption is another potent mechanism of suppression. A number of studies have demonstrated that Tregs can induce apoptosis of effector T cells through their high expression of CD25 that allows them to deprive effector T cells of IL-2 [231]. Moreover, Tregs have been demonstrated to have significant roles in cyclic adenosine monophosphate (cAMP)-dependent pathway and purine catabolism, which regulates the balance between proinflammatory adenosine 5'-triphosphate (ATP) and immunosuppressive adenosine [232]. Elevation of cAMP levels in Teffs inhibits TCR-mediated signalling via ZAP70 phosphorylation and AP-1 activation, which ultimately downregulates IL-2 production [233]. Thus, Tregs may suppress Teffs through cAMP-dependent mechanisms in two separate ways. Firstly, Tregs are known to highly express cell surface ectoenzymes, CD39 and CD73, that convert proinflammatory ATP/ADP into adenosine, which is released into the extracellular space [234-236]. Adenosine then activates A2a receptors on effector T cells and increases intracellular cAMP. Secondly, Tregs can also transfer cAMP to Teffs directly through gap junctions [187, 237, 238].

1.5.4 Tregs in transplantation tolerance

There have been indications that Tregs play a role in the induction of transplant tolerance as far back as the 1970's. Seminal work by Kilshaw et al. demonstrated that splenocytes isolated from mice that accepted skin allografts can be adoptively transferred to confer tolerance to donor skin allografts in other mice, while adoptive transfer of splenocytes from naïve mice accelerated rejection [239]. Similar subsequent studies also corroborated the notion that regulatory lymphocytes can promote transplant tolerance, and only later recognised this phenomenon as being Treg-driven [240-244].

Since then, findings have further cemented Tregs' role in transplantation in both preclinical and clinical models with the development of Treg-based cell therapies. The pretreatment of mice with infusion of donor alloantigen and anti-CD4 mAb can generate alloantigen-specific $CD4^{+}CD25^{+}$ T cells that potently regulate rejection of skin allografts in a CTLA-4 and IL-10 dependent manner [244]. In bone-marrow allograft transplantation mouse models, transfer of either freshly isolated or *ex vivo* expanded Treg cells promotes tolerance of the allograft while hindering GVHD [245, 246].

Throughout the myriad studies focused on developing Treg-based therapies in transplantation, there have been two main approaches: (1) Administration of *ex vivo*-Tregs [247-250], in which Tregs are isolated and expanded in cultures and then infused into the donor **[Figure 1.5]**, or (2) *in vivo* expansion/activation of endogenous Tregs [251-254]. Nonetheless, the current treatments implemented within the clinic have not been fully optimized and still present issues of efficiency, practicality, and safety [187]. Thus, much research has been devoted in refining

these two methods, specifically to expand a greater number of Tregs and/or to increase their suppressive potency. There are many factors to consider in optimizing Treg cell therapy, including antigen-specificity, stability, trafficking, and adjunctive therapy.

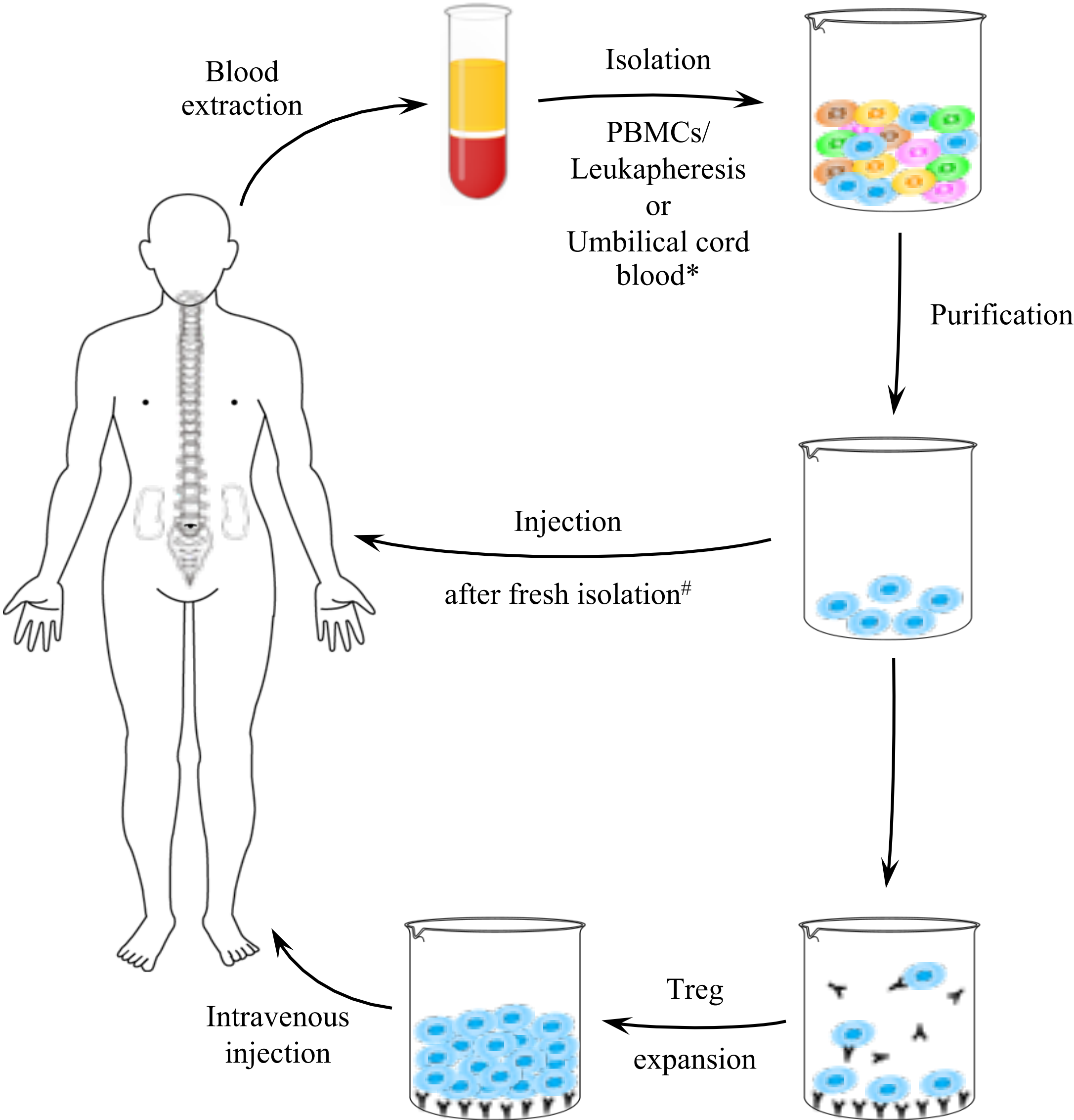


Figure 1.5: Treg isolation/expansion methods [187][1]

[1]Reproduced/adapted with permission from Kawai et al. [187]

1.5.4.1 Antigen-specificity

The antigen specificity of Tregs is an important parameter to consider in transplantation. Currently, most studies focus on the use of polyclonal Tregs, due to the relative ease and reliability in which they can be generated through routine expansion protocols (i.e. anti-CD3/anti-CD28 stimulation and IL-2). Nonetheless, the sheer amount of polyclonal Tregs that are theoretically needed to induce tolerance may be a relevant concern, both practically and in terms of the possibility that it might lead to unwanted global over-immunosuppression (although not proven). Tang et al. estimates that a Treg to effector ratio of 1:1 to 1:2 is needed to regulate rejection in mice [255-257]. Assuming this proportion is consistent for humans, at least 49 to 79 x 10^9 Tregs will be needed to fulfil this requirement in humans [257]. Thus, recent studies have directed their attention to the generation of allospecific Tregs, which have demonstrated significantly higher suppressive potency *in vitro* and greater ability to prolong skin allografts in preclinical models than polyclonal Tregs due to a more focused TCR repertoire [258-263]. Similar to alloreactive $CD4^+$ T cells, there are directly and indirectly alloreactive Tregs. Although Tregs with indirect specificity have been demonstrated to be more suppressive than their direct counterparts, direct alloreactive Tregs are suggested to be much more physiologically abundant and thus more practical to expand [187, 264]. From the work first published by Taylor et al. demonstrating that directly alloreactive Tregs can be generated from polyclonal populations with allogeneic splenocyte stimulation, subsequent groups have continued to build on these protocols [259, 262, 265]. Current studies continue to investigate the balance between the greater suppressive potential and the difficulty in expanding donor-specific Tregs, but alloreactive Tregs may be a more viable option in the future with more well-defined expansion protocols. Notably,

significant advancements have been made in the generation of chimeric antigen receptor (CAR) Tregs, in which nTregs are transduced with a vector containing a chimeric receptor to produce cells that are genetically modified to target donor antigens with high specificity [121]. Although the safety, efficacy, and feasibility of CAR Tregs are still being investigated, they may offer an attractive therapeutic strategy in the future.

1.5.4.2 Treg trafficking

Despite the versatility of mechanisms in which they can exert suppressive functions, Tregs need to be in the vicinity of their targets, making it crucial for Tregs to be able to home to the two sites of suppression, the draining LNs and the graft itself. In these sites, Tregs function to prevent Teff proliferation, cytokine secretion, accumulation, and migration, as well as restricting DC antigen-presentation and migration in a TGF-β and IL-10 manner [266]. Due to the difficulties in establishing a reliable and safe method to track Tregs in humans, knowledge of the trafficking behaviour of human Tregs is sparse; however, *in vivo* pre-clinical studies have provided valuable insight on the molecules and markers, including chemokines and integrins, that dictate Treg migration post-transplantation.

Seminal work by Zhang et al. demonstrated that after islet transplantation, Tregs initially migrate to an islet allograft, guided by expression of C-C chemokine receptor (CCR)-2, CCR4, CCR5, and P/E selectin ligands. Within the allograft, they upregulate effector molecules and subsequently migrate to the LNs, directed by expression of CCR2, CCR5, and CCR7 [266]. Abrogation of the activity of any of these key molecules led to decreased Treg migration to either the allograft or LN and shortened graft survival. Nonetheless, although Tregs with the capacity to migrate to both

sites optimally controlled rejection, they found that migration to the graft site was absolutely necessary to prevent rejection, while homing to the lymph node was less essential.

There has been mounting evidence regarding CXCR3 also having a role in Treg trafficking to the graft, where studies have demonstrated that $CXCR3^{+}$ Tregs can infiltrate the graft better than control Tregs, and its chemokines (CXCL9, CXCL10, CXCL11) are commonly expressed in the urine and graft of kidney transplant patients presented with rejection or early graft loss [267-269].

Our group has previously also demonstrated findings that graft-residing Tregs are able to prolong rejection within a humanised mouse model. Findings revealed that humanised mice receiving human *ex-vivo*-expanded Tregs infiltrated the transplanted skin allograft to mediate peripheral blood mononuclear cell (PBMC) alloresponse and persisted within the graft beyond 100 days post-transplantation. Moreover, when skin allografts from tolerant mice were retransplanted into separate mice that were then challenged with fresh PBMC, the skin re-transplants survived long-term, demonstrating that graft-infiltrating Tregs were sufficient to mediate alloresponse [270].

Taken together, further investigations into Treg trafficking behaviour post-transplantation will guide future therapies in either manipulating the Tregs, the recipient immune systems, or the allograft to allow Tregs to home better to sites of suppression.

1.5.4.3 Treg stability

Previous studies have largely demonstrated the durable stability of Tregs, but there remain findings that suggest that at least some subsets of Tregs display a loss of suppressive function or

FOXP3 expression. There is evidence that a minor population of Tregs, which have lower expression of CD25, will not only lose their suppressive function but also differentiate into effector T cells in certain conditions, particularly when Tregs are adoptively transferred into lymphopenic recipients [271]. Loss of Foxp3 expression after infusion may be attributed to reduced IL-2 production within the host due to the Tregs not being able to produce IL-2 themselves [272]. Some studies have also indicated that Tregs lose their Foxp3 expression through stress signals elicited by proinflammatory cytokines and liposaccharides, where Foxp3 is ubiquinated by the E3 ligase Stub1 [273]. Some of these “ex-Tregs” may be able to regain their suppressive function, but the exact mechanisms and conditions in which this occurs is not yet clearly defined [187, 271].

1.5.5 Clinical trials of Treg cell therapy in transplantation

From the first report of human Treg expansion in 2001 and the subsequent first large-scale expansion of human Tregs in 2004 [274, 275], progress has accelerated swiftly towards clinical trials. Research centres worldwide have now begun to translate their Treg cell therapies to the bedside with varying yet promising results **[Table 1]** [187].

Among the currently running clinical trials, our group is currently a partner in the EU-funded ONE Study (Phase I) and MRC-funded TWO Study (Phase II) trials, which aim to investigate the safety and therapeutic efficacy of Tregs in living donor renal transplantation. The central focus of the ONE study, a consortium coordinated in Regensburg and including centres located in the UK, Germany, France, Italy, and the US, is to test and compare a variety of immunoregulatory cell products in the context of a similar patient population, with a reference group of patients [249].

Both the cellular therapy and reference groups received low dose tacrolimus, MMF, and steroids, with the experimental group receiving regulatory cell therapy and the reference group receiving anti-CD25 antibody induction. These immunosuppressive regimens were held consistent throughout all centres. The manufactured cellular products included polyclonal and alloantigen-specific Tregs, Type 1 regulatory (Tr1) cells, tolerogenic DCs, and regulatory macrophages. The TWO Study aims to assess the efficacy of Tregs in renal transplant recipients. In this randomized controlled trial, patients in the therapy arm will receive expanded derived Tregs and immunosuppression reduced to a monotherapy. The primary outcome measure is biopsy-proven acute rejection within 18 months of transplantation, and patients will also be intensively monitored for detection of changes in immune phenotype [187].

The initial findings within these clinical trials have been invaluable for assessing the safety, efficacy, and practicality of Treg-based treatments. Nonetheless, there is still an immense amount of research required to take the next steps to address concerns still surrounding this form of therapy. In particular, strategies to expand a greater number of Tregs or to enhance Treg suppressive function will be critical in optimising Treg therapy. Notably, one of the main methods that this may be achieved is through the manipulation of specific molecular Treg pathways.

Table 1: Clinical Trials of Transplantation using Tregs [187][1]

Study ID	Trial title [location]	Phase	Intervention	Study Start/ Estimated complete date	Condition [estimated enrolment]	Specificity	Doses
NCT 01634217	Inducible Regulatory T Cells (iTregs) in Non-Myeloablative Sibling Donor Peripheral Blood Stem Cell Transplantation [United States, Minnesota]	I	iTregs	Nov 2013 to Aug 2017	Non-myeloablative HLA-identical sibling donor PBSC Tx [n = 16]	Non-antigen specificity	3, 30, 300, and 1000×10^{6}/kg
NCT 01660607	Phase I/II MAHCT w/ T Cell Depleted Graft w/ Simultaneous Infusion Conventional and Regulatory T Cell [United States, California]	I/II	Fresh nTregs	Dec 2011 to March 2018	Acute GVHD prevention [n = 54]	Non-antigen specificity	1.0×10^{6}/kg (Initial dose)
NCT 01795573	Ex-vivo Expanded Donor Regulatory T Cells for Prevention of Acute Graft-Versus-Host Disease (GVHD) [United States, Florida]	I	Donor alloantigen-reactive Treg (arTreg)	June 2014 to Jun 2019	Acute GVHD prevention [n = 48]	Alloantigen specificity	—
NCT 01903473	Donor Regulatory T Cells Infusion in Patients With Chronic Graft-versus-host Disease [Belgium, Leuven and Liège]	II	Donor Treg	Jul 2013 to Dec 2016	Steroid -refractory chronic GVHD [n = 35]	Non-antigen specificity	0.5×10^{6}/kg
NCT 01911039	Phase 1 Infused Donor T Regulatory Cells in Steroid Dependent/Refractory Chronic GVHD [United States, California]	I	Fresh nTregs (donor Treg)	Jul 2013 to Jul 2016	Steroid-dependent/refractory chronic GVHD [n = 20]	Non-antigen specificity	1, 5, and 15 $\times10^{5}$/kg
NCT 01937468	Trial of Regulatory T-cells Plus Low-Dose Interleukin-2 for Steroid-Refractory Chronic GVHD [United States, Massachusetts]	I	Fresh nTregs and IL-2	Nov 2013 to Nov 2020	Steroid -refractory chronic GVHD [n = 25]	Non-antigen specificity	—
NCT 02385019	A Phase 1/2 Trial of Donor Regulatory T-cells for Steroid-Refractory Chronic GVHD (TREGeneration) [Portugal, Lisboa and Porto]	I/II	Fresh nTregs (donor Treg)	Mar 2015 to Dec 2019	Steroid -refractory chronic GVHD [n = 22]	Non-antigen specificity	0.5, 1, 2, and 3×10^{6}/kg
NCT 02519816	Continuous Alloreactive T Cell Depletion and Regulatory T Cell Expansion for the Treatment of Steroid-refractory or Dependent Chronic GVHD (CARE) [Canada, Quebec]	II	Polyclonal nTregs	Mar 2016 to Aug 2017	Steroid-dependent/refractory chronic GVHD [n = 25]	Non-antigen specificity	—
NCT 02749084	Multiple Donor Treg DLI for Severe Refractory Chronic GVHD (TREG2015001) [Italy, Bologna]	I/II	Fresh nTregs (donor Treg)	Aug 2016 to Mar 2022	Refractory chronic GVHD [n = 20]	Non-antigen specificity	0.5, 1.0, and 2.0×10^{6}/kg

[1]Reproduced/adapted with permission from Kawai et al. [187]

NCT 02991898	Treg Cell With IL-2 to Suppress aGVHD After Umbilical Cord Blood Transplantation [United States, Minnesota]	II	Fresh nTregs and IL-2	Feb 2017 to Jan 2025	Acute GVHD prevention [n = 10]	Non-antigen specificity	—
NCT 01624077	Safety Study of Using Regulatory T Cells Induce Liver Transplantation Tolerance (Treg) [China, Jiangsu]	I	iTregs	Dec 2014 to Dec 2015	Liver Tx [n = 1]	Alloantigen specificity	1×10^6/kg
NCT 02088931	Treg Adoptive Therapy for Subclinical Inflammation in Kidney Transplantation (TASK) [United States, California]	I	Polyclonal nTregs	Mar 2014 to Dec 2016	Living donor kidney Tx [n = 3]	Non-antigen specificity	320×106/ total
NCT 02091232	Infusion of T-Regulatory Cells in Kidney Transplant Recipients (The ONE Study) [United States, Massachusetts]	I	Belatacept-induced nTregs	May 2014 to May 2018	Living donor kidney Tx [n = 8]	Non-antigen specificity	—
NCT 02129881	ONE/TWO Study UK Treg Trial (ONETreg1) [United Kingdom, London and Oxford]	I/II	Polyclonal nTregs	Apr 2014 -	Living donor kidney Tx [n = 12]	Non-antigen specificity	1-10×106/kg
NCT 02145325	Trial of Adoptive Immunotherapy With TRACT to Prevent Rejection in Living Donor Kidney Transplant Recipients (TRACT) [United States, Illinois]	I	Polyclonal nTregs	Apr 2014 to Dec 2021	Living donor kidney Tx [n = 10]	Non-antigen specificity	—
NCT 02166177	Safety and Efficacy Study of Regulatory T Cell Therapy in Liver Transplant Patients (ThRIL) [United Kingdom, London]	I/II	Polyclonal nTregs	Jun 2014 to Jun 2019	Liver Tx [n = 26]	Non-antigen specificity	1 and 4.5×106/ total
NCT 02188719	Donor-Alloantigen-Reactive Regulatory T Cell (darTregs) in Liver Transplantation (deLTa) [United States, California and Minnesota]	I	Donor arTreg	Dec 2014 to Jan 2022	Liver Tx [n = 24]	Alloantigen specificity	50, 200, and 800×106/ total
NCT 02244801	Donor-Alloantigen-Reactive Regulatory T Cell (darTreg) Therapy in Renal Transplantation (The ONE Study) (DART) [United States, California]	I	Donor arTreg	Nov 2014 to Jun 2018	Living donor kidney Tx [n = 16]	Alloantigen specificity	300 and 900×106/ total
NCT 02371434	The ONE Study nTreg Trial (ONEnTreg13) [Germany, Berlin]	I/II	Polyclonal nTregs	Jan 2015 to Dec 2017	Living donor kidney Tx [n = 9]	Non-antigen specificity	0.5, 1.0 and 3.0×106/kg
NCT 02474199	Donor Alloantigen Reactive Tregs (darTregs) for Calcineurin Inhibitor (CNI) Reduction (ARTEMIS) [United States, California, Illinois, and Minnesota]	I/II	Donor arTreg	Sep 2015 to Dec 2018	Liver Tx [n = 18] (Immunosuppression reduction)	Alloantigen specificity	400×106/ total
NCT 02711826	Treg Therapy in Subclinical Inflammation in Kidney Transplantation (TASK) [United States, California]	I/II	I: Polyclonal nTregs II: Donor arTreg	Mar 2014 to Dec 2016	Subclinical inflammation in kidney Tx [n = 3]	Alloantigen specificity	320×106/ total
UMIN 000015789	Tolerance induction by a regulatory T cell-based cell therapy in living donor liver transplantation [Japan, Hokkaido]	I/II	Donor arTreg (iTreg)	Nov 2012 to -	Living donor liver Tx [n = 40]	Alloantigen specificity	1.3-5.4×106/kg

1.5.6 Treg molecular biology

Various modes of transcriptional and epigenetic regulation within Tregs have been identified which may govern and alter their phenotype, stability, and function. In this respect, Tregs appear to be significantly more heterogenous than originally imagined since the discovery of Foxp3. Therefore, identifying and exploiting specific molecular pathways to modulate Treg function remain a promising strategy for improving Treg cell-based therapies.

1.5.6.1 Foxp3 function

Since its discovery, the complex role that Foxp3 plays as a transcription factor in Treg differentiation, function, and maintenance have been thoroughly investigated. Seminal work by Gavin et al. demonstrated that Foxp3 expression is necessary for Treg suppressive function, but dispensable for survival [276]. Here, transcriptional analysis of mice with Foxp3 deficiencies suggested that some of the other defining markers of Tregs, (i.e. upregulated CD25 and CTLA-4) and cytokine secretion patterns are promoted before Foxp3 expression. Thus, subsequent Foxp3 binding may function to exaggerate and stabilise the pre-existing phenotype of Tregs, rather than being a perquisite for initial Treg differentiation [276, 277]. Moreover, Wan et al. showed that the concentration of Foxp3 protein may correlate with suppressive function and may regulate Treg development in a non-binary manner [278]. When Foxp3 protein expression is downregulated approximately 10-fold, Tregs lose suppressive function both *in vitro* and *in vivo* and fail to prevent autoimmunity. However, decreased Foxp3 did not affect thymic development, homeostatic expansion, or TGF-β-dependent *de novo* generation of Foxp3$^+$ cells.

On the contrary, later studies have suggested that there are Foxp3-dependent transcriptional programs that allow them to act as both a transcriptional activator and repressor, suggesting that they are necessary for initial Treg development [102]. Further studies also demonstrated that conditional Foxp3 deletion of fully differentiated peripheral Tregs induced loss of Treg suppressive functions and phenotype, suggesting that sustained Foxp3 expression is indeed necessary for maintenance [279]. Taken together, Foxp3 clearly has extensive roles within Treg biology, but there is still much to be elucidated regarding the full extent of its impact.

1.5.6.1.1 Foxp3 conserved regions and signalling pathways

In both mice and humans, the genomic region of the *Foxp3* locus is composed of four conserved non-coding sequences (CNS), each playing a prominent role in Foxp3 expression. For example, CNS0 has been demonstrated to have roles in initiating Treg super-enhancer activation to induce *Foxp3* expression [280].

CNS1 contains binding sites for the NFAT, which have prominent interactions with Foxp3 in regulating genes. Studies have suggested that Foxp3 and NFAT are in competition for the same binding sites on the IL-2 promoter [281]. Further, several studies have supported the notion that Foxp3 itself forms a complex with NFAT and prevents the formation of a NFAT:AP-1 complex, which is involved in T cell activation, thereby repressing the expression of cytokines such as IL-2 [282, 283].

Moreover, Foxp3 has the ability to physically interact with NF-kβ, which binds to the CNS2 region of *Foxp3* [284]. NF-kβ has far-reaching effects in both innate and adaptive signalling pathways,

many involved in inducing a proinflammatory environment [285]. Bettelli et al. demonstrated that Foxp3-deficient mice have significantly elevated levels of NF-kβ, correlated with increased cytokine gene expression [283]. Foxp3 is able to suppress NF-kβ activity by blocking its ability to induce A20, a well-known NF-kβ target gene. Importantly, TGF-β has been demonstrated to be a mediator of CNS2 demethylation [286]. TGF-β is critical for the induction iTregs and the prevention of Th17 differentiation through interactions with the transcription factor Retinoic-acid Receptor-related Orphan Receptor gamma (RORγt, RORC2 in humans) [287, 288]. STAT5, which is a downstream signalling molecule for IL-2, also binds to CNS2 and has been demonstrated to be required for Treg development [212]. Taken together, CNS2 is a particularly influential region in Foxp3 and Tregs.

Finally, CNS3 has a critical role in Treg biology due to its c-Rel binding element [289]. c-Rel, of the NF-kβ family, has been demonstrated to be indispensable for Treg differentiation [290-292]. Through the use of $CNS^{-/-}$ models, Rudensky's group revealed that CNS3 is vital in the induction of thymic and peripheral Tregs, potentially through binding and formation of a c-Rel containing enhanceosome in cooperation with transcription factors such as NFAT, CREB, p65, and Smad [277, 289, 293].

1.5.6.1.2 TSDR demethylation

Studies from Floess et al. demonstrated that epigenetic changes at CNS2 in particular may be indicative of the stability of the Treg lineage [294]. In characterising mouse $CD4^{+}CD25^{+}$ cells, they found that 95% were $Foxp3^{+}$ and were fully demethylated at CNS2, now also termed the Treg-cell specific demethylation region (TSDR). Moreover, studies have demonstrated that the

methylation status of the TSDR correlated with Foxp3 expression and stability in both mice and humans [294, 295]. In contrast, similar to $CD4^{+}CD25^{neg}$ cells, TGF-β-dependent *in vitro* induced Tregs were found to have almost completely methylated TSDR, thereby calling into question the stability and reliability of *in vitro* induced Tregs for therapeutic purposes [296]. Overall, not much is yet known as to the factors that can modulate TSDR demethylation status, however, it is currently the most reliable marker correlating with Treg stability.

To summarize, it is clear from recent literature that there is significant promise in elucidating the molecular mechanisms within Tregs to uncover and harness their versatile functions. Thus, the rest of the introduction will focus on the investigation of two molecular pathways that have demonstrated intriguing potential in their roles within Treg biology which may be exploited for improving current therapeutics.

1.6 Interleukin-33 (IL-33) / Serum Stimulation 2 (ST2) Axis

1.6.1 Introduction

While significant progress has been made in developing strategies to expand polyclonal Tregs *ex vivo* for therapeutics, there remains clear challenges that will need to be addressed for safe and effective implementation. Notably, as described earlier, there are concerns surrounding the amount of polyclonal Tregs necessary to generate for effective therapy. Additionally, the specificity of infused polyclonal Tregs is still under question, in regard to whether they can be effective in controlling graft rejection while not inducing global immunosuppression. Therefore, strategies to expand a greater number of Tregs or to enhance Treg suppressive function or specificity will be critical in optimising Treg therapy.

In this respect, the Interleukin-33 (IL-33)/serum-stimulation 2 (ST2) pathway may be an intriguing target. Recent findings have shown the potential for IL-33 in modulating Treg biology, in which it has been demonstrated to expand or induce a unique population of Tregs with high expression of its own receptor ST2 [297-299]. Nonetheless, the identity and specific functionality of these Tregs are still under investigation. Moreover, the pleiotropic nature of the axis has made it challenging to tease out its regulatory properties from its well-known inflammatory features [300-302]. Thus, we sought to determine whether we can exploit this pathway to harness its tolerogenic functions and assess the feasibility of this approach for cellular therapy within the context of transplantation.

1.6.2 Interleukin-33 (IL-33)

1.6.2.1 Gene and Protein

In 2003, Baekkevoid et al. visualised a novel endogenous protein and mRNA abundantly expressed in high endothelial venules (HEVs) in humans, hence naming the protein "nuclear factor from high endothelial venules (NF-HEV) [303, 304]. Two years later, seminal work by Schmitz et al. revealed that the carboxy-terminal part of the NF-HEV protein resembled other IL-1 cytokines in conformational fold. Thus, they demonstrated that this protein was a cytokine that induced type 2 immune responses specifically through ST2 and promptly renamed the cytokine as IL-33 [302].

The gene encoding IL-33 is composed of eight exons and located on chromosome 9 and 19, in humans and mice, respectively [302]. The IL-33 protein (270 and 266 residues in human and mice, respectively) is comprised of two evolutionary conserved domains, in which the carboxy-terminal corresponds to the three-dimensional IL-1-like cytokine domain and the N-terminal region contains a chromatin-binding motif and nuclear localisation sequence [303, 304]. IL-33 is most prominently and constitutively expressed in structural and lining cells such as fibroblasts, endothelial, and epithelial cells in both humans and mice [305, 306].

1.6.2.2 *In vivo* secretion and regulation

Under an inactive state, IL-33 is bound to chromatin by a chromatin binding motif and resides in the cell nuclei [307]. Thus, the nuclear localisation sequence within the IL-33 N-terminal is essential for nuclear localisation and chromatin association and may play significant roles in its function or regulation, although evidence conclusively demonstrating its capacity to directly regulate gene expression is lacking [307-309]. Indeed, a knock-in mouse model inducing deletion of the nuclear localisation signal demonstrated ST2-dependent lethal inflammation characterised by eosinophil-dominated immune cell infiltration in multiple organs, along with elevated serum IL-33 levels [310].

IL-33 is unique from conventional cytokines in that it lacks a signal sequence and is not actively secreted from cells but instead is released passively in its full length form during cell stress/damage or infection, thus making it initially known for its function as an alarmin [297, 309, 311, 312]. Interestingly, IL-33 has been shown to be bioactive in both its full length and processed, mature form. On one hand, studies have shown that full length IL-33 can induce NF-κβ activity and cytokine production in an ST2 dependent manner [297, 313-315]. On the other hand, in a series of studies, Lefrancais et al. demonstrated that inflammatory serine proteases such as neutrophil cathepsin G, elastase, and proteinase 3 (PR3), mast cell chymase, tryptase, and granzyme B can cleave full length IL-33 into a shorter and mature bioactive form [316, 317]. Moreover, they found that the cleaved, mature forms had significantly increased biological activity, likely making it a form of positive regulation when IL-33 concentration is limited. Studies have noted an elevation in levels of endogenous mature IL-33 *in vivo* upon conditions such as

acute lung injury [317] and airway inflammation [318, 319]. Nonetheless, the functional differences between the immature and mature forms of IL-33 have not been fully elucidated.

IL-33 expression within specific tissues and cell-types can be elevated by pro-inflammatory stimuli. For example, studies have found that TLR ligands, such as LPS, can significantly augment IL-33 expression within many tissues [320-323]. Proinflammatory cytokines such as IL-1β, and TNF-α [304, 324-326] have been shown to induce IL-33 in fibroblasts, while IFN-γ and TNF-α have been shown to do so in skin keratinocytes [327, 328]. Thus, IL-33 has been demonstrated to be induced in inflammatory conditions such as viral infections [329], cigarette smoke [330], allergic lung inflammation following exposure to ovalbumin (OVA), fungal extract or ragweed pollen [331], among many others. Interestingly, proinflammatory stimuli have also been shown to induce IL-33 expression within other cell types in mice, such as macrophages and dendritic cells [321, 322, 332, 333], but not human myeloid cells.

Due to its prevalence within healthy tissues and prominent role in inducing rapid inflammatory processes, mechanisms to suppress IL-33-dependent activity are critical. One of the main mechanisms by which IL-33 activity can be limited is through soluble ST2 and IL-1 receptor accessory protein (IL-1RAcP) (both described in detail below). Similar to their function in processing full length IL-33, certain caspases, such as caspase-1 [313], caspase-3, and caspase-7 [313, 314], have been demonstrated to cleave IL-33 after residue Asp178 (Asp175 in mice) to inactive forms. Finally, IL-33 can also be rapidly terminated within the extracellular environment through oxidation of cysteine residues and formation of two disulphide bridges within the IL-1-like cytokine domain, which ultimately disrupts the ST2 binding site [309, 319].

1.6.3 Serum stimulation-2 (ST2)

1.6.3.1 Gene and protein

In 1992, Tominaga et al. discovered serum stimulation 2 (ST2), a new member of the IL-1 receptor (IL-1R) cytokine family encoded by the gene *Ilrl1*, within mouse fibroblasts primarily as an oncogene-induced gene [334]. However, it remained an orphan receptor for more than a decade until the discovery of IL-33, hindering progress in further elucidating its function at the time.

ST2, encoded by *IL1RL1*, is expressed in the form of four possible transcripts generated through alternative splicing. ST2L (ligand) corresponds to the cell surface membrane embedded receptor, which contains an extracellular region of three Ig-like domains, a transmembrane region, and an intracellular Toll-like/IL-1 receptor domain [308, 335]. Secondly, sST2 (soluble ST2) is a shorter isoform that encodes a soluble decoy protein receptor that acts as one of the major forms of regulation of IL-33-dependent signalling through hoarding IL-33 from ST2L. Finally, ST2V (variant) [336, 337] and ST2LV (ligand variant)[338] are two additional isoforms that have been discovered, although their functions have not yet been heavily studied.

While ST2 was first found to be expressed within Th2 cells [339-342] and mast cells [343-346] as mentioned previously, numerous subsequent studies have revealed that it is expressed within a wide variety of immune cell types, including type 2 innate lymphoid cells (ILC2) [347-351], NK cells [352], Natural killer T (NKT) cells [353, 354], DCs, macrophages, eosinophils [355, 356], and

basophils [355] [353, 357]. Moreover, ST2 expression can also be induced within Th1/cytotoxic T cells [311, 358, 359], and Tregs [298, 360] under specific conditions.

1.6.4 IL-33/ST2 signalling

Once released in the extracellular space, IL-33 can bind to ST2L **[Figure 1.6]**, which subsequently undergoes a conformational change to interact with IL-1 receptor accessory protein (IL-1RAcP) and adjoins their Toll/IL-1 receptor (TIR) domains [322]. This IL-33/ST2/IL-1RAcP complex then recruits proteins such as myeloid differentiation primary response protein 88 (MyD88), IL-1R-associated kinase 1 (IRAK1), IRAK4, and tumour necrosis factor (TNF) receptor associated factor (TRAF6). This leads to the induction of major downstream signalling pathways through the activation of mitogen-activated protein kinases (MAPKs), such as C-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38, and the nuclear factor (NF-κβ) [302, 361]. Ultimately, this induces proliferation, survival, cytokine secretion and amphiregulin (AREG) expression by $ST2^+$ cells [309]. Moreover, MyD88 has been shown to be heavily involved in IL-33-activated innate immune responses, such as the survival, adhesion, and cytokine production of mast cells [362].

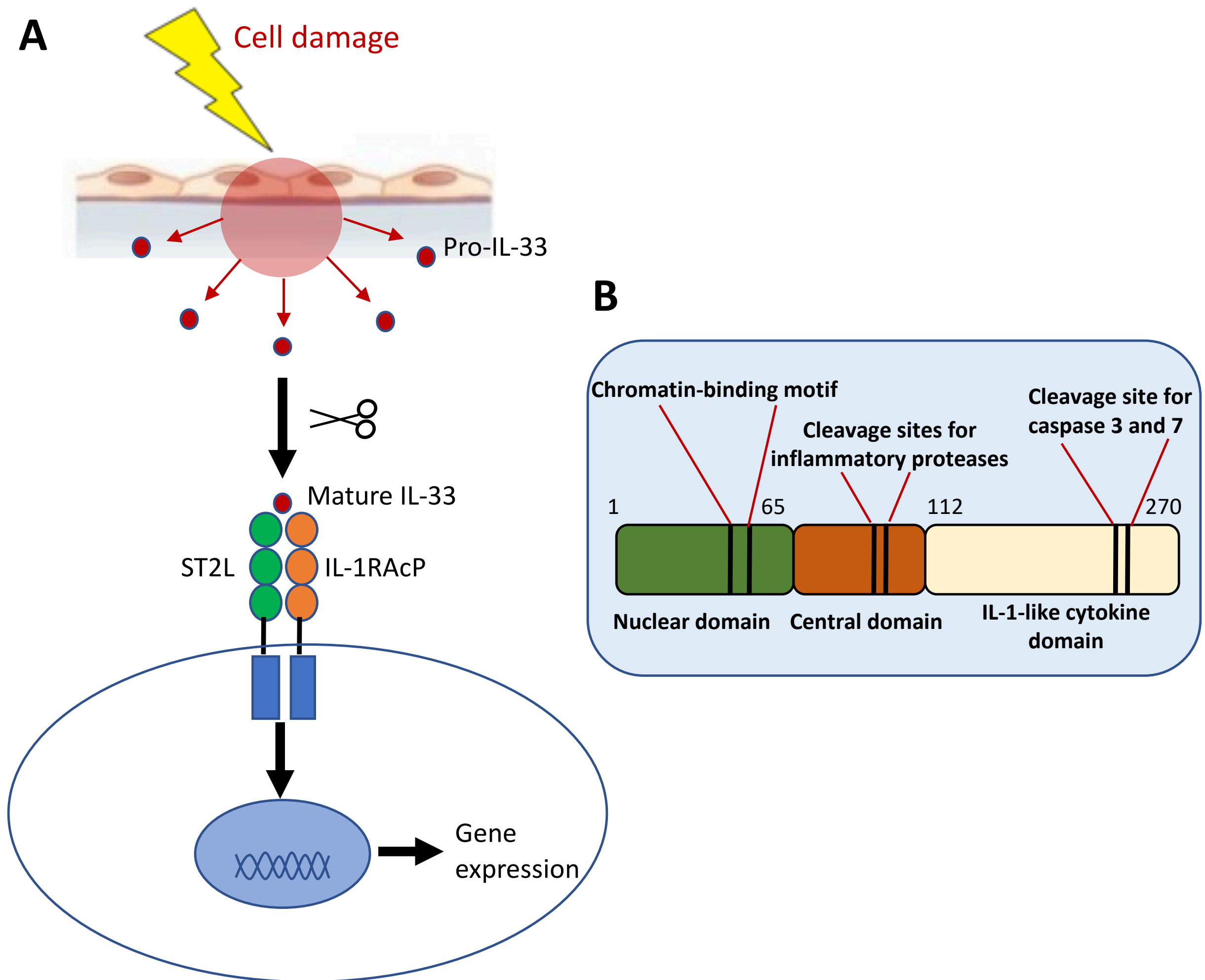


Figure 1.6. IL-33/ST2 pathway. (A) IL-33 was first discovered as an alarmin/DAMP that is released in response to tissue damage or inflammation. Pro-IL-33 can be cleaved by inflammatory proteases or caspase 3/7 to generate mature IL-33 or inactivate it, respectively. Active IL-33 then recruits the IL-1RAcP to form a complex with its receptor ST2L, which is expressed in many types of immune cells. **(B)** Structure of IL-33 polypeptide and its domains and known cleavage sites.

1.6.5 IL-33/ST2 in innate immunity

1.6.5.1 Innate immune cells

Since its discovery, ST2 has been shown to be expressed in a diverse range of innate immune cells. Notably, within eosinophils, IL-33 have roles within their development and activation within bone marrow and tissue [363-365]. ILC2s have high expression of ST2, and IL-33 acts as both an activator and recruiter to inflammatory sites [347, 366]. Additionally, IL-33 has been demonstrated to activate and/or expand mast cells [367], basophils [355, 368], NK cells [352], NKT cells [354], and macrophages [369]. Finally, DCs have also been demonstrated to skew T cells towards a Th2 phenotype and increase Treg proliferation [370, 371].

1.6.6 IL-33/ST2 in adaptive immunity

1.6.6.1 T cells

Many studies have now demonstrated that the IL-33/ST2 axis promotes the differentiation and proliferation of T cells towards effector or memory subsets [372]. Naive T cells do not conventionally express ST2, and thus polarisation and TCR activation is necessary to induce its expression [373]. While only Th2 and Treg cells have been demonstrated to stably express ST2, Th1 and CTLs can transiently express ST2. Within Th2 cells, IL-33 with a STAT5 activator (i.e. IL-2) has been demonstrated to induce IL-13 and IL-5 production *in vitro* in a NF-kβ and p38 dependent manner [374-377]. IL-33 upregulates *Stat5* mRNA expression and phosphorylates GATA-3 and promotes its nuclear translocation. In turn, STAT5 and GATA-3 are recruited to the *Il1rl1* locus to

upregulate ST2 expression [372]. Thus, through this positive feedback loop, IL-33 may upregulate the expression of its own receptor.

Interestingly, similar positive feedback mechanisms may allow Th1 cells to transiently express ST2 as well, in which T-bet [378] and STAT4 [377] bind to the *Ilrl1* locus [359]. Indeed, in a murine model of lymphocytic choriomeningitis virus (LCMV) infection, while transient ST2 expression was detected within Th1 cells of WT mice, those from T-bet or STAT4 knockout mice had no detectable ST2 expression. Moreover, adoptively transferred $ST2^{-/-}$ virus-specific CD4+ T cells demonstrated impaired expansion, Th1 effector differentiation, and antiviral cytokine production [301, 359]. Similarly, IL-33 has been demonstrated to promote clonal expansion, function, and survival of CTLs during LCMV infection and is crucial for the optimal control of infection [311, 359].

1.6.6.2 Tregs

While the bulk of research surrounding this pathway has been focused on Th2 cells, more recent studies have begun to reveal an interesting link between the IL-33/ST2 axis and Treg biology. It is proposed that IL-33-dependent upregulation of Tregs also depends on a positive feedback loop, in which IL-33 recruits both GATA-3 and RNA polymerase II to the *Foxp3* promoter and *Il1rl1* locus in a TGF-β-dependent manner [372, 376]. Thus, IL-33 may indirectly upregulate Foxp3, while also enhancing ST2 expression through GATA-3 and its phosphorylation of STAT5. Moreover, another study showed that *in vitro* IL-33-mediated expansion of hepatic Tregs depended on MyD88

signalling [379]. Nonetheless, the exact pathways whereby the IL-33/ST2 axis acts to enhance Treg function remain unclear, particularly as many of these are shared with Th1 and Th2 cells [359, 380].

IL-33 has been demonstrated to have significant roles within Treg maintenance and stability. *In vivo* recombinant IL-33 injections [298, 299] and *in vitro* expansion with allogeneic DC treatment [370] have both been shown to significantly expand $ST2^+$ Tregs. Phenotypically, $ST2^+$ Tregs display enhanced expression of certain Treg-associated suppressive markers such as Helios, CTLA-4, and CD25 [370]. The majority of $ST2^+$ T cells at steady state are $Foxp3^+$ Tregs, and are preferentially located within the gut [298], lungs [381], and other non-lymphoid tissue [301, 382]. A study by Schiering et al. showed that IL-33 plays a critical role for Treg stability under inflammatory environments such as the gut, in which $ST2^+GATA\text{-}3^+$ Tregs were crucial to prevent T cell mediated colitis [298]. Multiple studies have assessed the suppressive potency of IL-33-expanded Tregs or $ST2^+$ Tregs *in vitro* against effector T cells. While some studies have suggested that $ST2^+$ Tregs are more suppressive than $ST2^{neg}$ Tregs [382] or that IL-33 stimulation enhances Treg suppression [383], others have cited no significant difference [299, 370] or even loss of suppressive function with IL-33 stimulation [384]. Variable experimental conditions between the studies may account for the discrepancies in assessing their suppression *in vitro*.

While the evidence for the role of IL-33/ST2 in Treg biology has thus been promising in mice models, investigations into its translation to human Tregs have also been gradually accumulating. A recent study showed $ST2^+$ Tregs in certain human tissues such as peripheral blood, lungs, and colon in the quiescent state [360]. Moreover, another study detected an increased proportion or

abundance of $ST2^+$ Tregs within the peripheral blood and colon of colorectal cancer patients [385] Recent findings have also demonstrated a role for IL-33 in the expansion of IL-13-secreting Tregs [386].

1.6.7 IL-33/ST2 in transplantation

Due to its demonstrated potential in modulating Treg proliferation and function, a few studies have explored its therapeutic applicability within transplantation. In a mouse GVHD model, both endogenous and exogenous IL-33 was shown to worsen acute GVHD-associated lethality following allogeneic haematopoietic stem cell transplantation (alloHCT) through promotion of type 1 donor alloimmunity [309, 382, 387]. However, another study by Matta et al. using the same GVHD model reported that IL-33 infusion before total body irradiation (TBI)-based conditioning prevented GVHD through the expansion of radiation-resistant $ST2^+$ Tregs in target tissues, thereby arguing that the timing of IL-33 infusion was critical for GVHD pathogenesis [388]. Moreover, Yang et al. recently provided evidence that IL-33-stimulated Tregs have protective functions in GVHD, in part due to higher production of amphiregulin [383].

In solid organ transplant mouse models, *in vivo* IL-33 treatment post-transplantation has been demonstrated to prolong and heart allograft survival and protect against chronic rejection through the expansion of Tregs, myeloid derived suppressor cells (MDSC), and Th2 skewing [299, 389]. Moreover, a study using a semi-allogeneic skin graft model demonstrated that *in vivo* IL-33 injections can prolong survival through induction of MDSCs and peripheral Tregs [390]. Overall,

there is data to support the advantages of IL-33-based therapy in transplantation, however its pleiotropic effects on a wide range of immune cells has made it difficult to pinpoint the mechanisms of its protective effects.

1.6.8 Summary

Taken together, despite its broad involvement on many inflammatory pathways, it is clear that an important role for IL-33/ST2 exists in Treg biology. The main questions we aim to explore will thus be focused on the functionality and phenotypic signature of IL-33-responsive Tregs and whether they can be specifically harnessed within the context of transplantation therapy.

1.7 The Hypoxia inducible factor (HIF) and Prolyl hydroxylase domain (PHD) pathway

1.7.1 Introduction

In addition to elucidating methods to promote Treg expansion or suppression, it is equally vital to improve our understanding of the plasticity and stability of Tregs *in vivo* for optimising Treg cell-based therapy. While Tregs have been considered to be a particularly stable lineage, recent evidence has indicated that certain environmental stressors, such as inflammation, may destabilise their suppressive function or even cause them to lose *Foxp3* expression and convert

to proinflammatory T cells [391]. These findings allude to obvious potential concerns in Treg cell therapy, particularly because Tregs are expected to function within complex inflammatory microenvironments, such as those present in allografts. Interestingly, hypoxic regions are prevalent within inflammatory environments and may be a potential stressor.

To this end, we aimed to investigate how hypoxia may affect Treg biology and whether it can be controlled to modulate their functionality. Namely, we explored the hypoxia inducible factor (HIF) and prolyl hydroxylase domain (PHD) pathway, which are considered to be the master regulators of oxygen homeostasis, and their role within Tregs. The following sections will thus be dedicated to describing the literature surrounding the role of the HIF/PHD pathway within immunology.

1.7.2 HIF

The concept of an oxygen-sensing mechanism was first explored within the context of hormonal control of haematocrit by the erythropoietin hormone (EPO), which was found to be a major component of a feedback loop that controls red blood cell production in response to changes in oxygen levels [392, 393]. Subsequently, HIF, a protein complex that binds to the regulatory DNA sequence at the *EPO* locus, was demonstrated to be the central regulator of this pathway [394, 395].

Since its discovery, HIF has been found to be present in all extant metazoan species that have been analysed [396, 397]. HIF consists of the oxygen sensitive HIF-α subunit and the constitutively expressed HIF-1β subunit, each of which consists of basic helix-loop-helix-PAS

(bHLH-PAS) domains and interacts with each other through a shared Per-ARNT-Sim (PAS) domain [398].

Moreover, there are three isoforms of HIF-α subunits within mammals, HIF-1α (encoded by *HIF1A*), HIF-2α *(EPAS1)*, and HIF-3α (*HIF3A*), each of which can dimerise with HIF-1β to then form the DNA binding complex [392, 399]. While HIF-1α is known to be more widely expressed in different tissues, HIF-2α is more tissue restricted and only found to be expressed in vertebrates [396, 400]. Moreover, while HIF-1α expression is induced more within extreme hypoxic conditions, HIF-2α is expressed in less severe hypoxia with potentially a longer duration of activation [401, 402]. In comparison, the activity and function of HIF-3α is less well-studied, but has been demonstrated to potentially bind to other HIF isoforms and inhibit activity [403].

Taken together, under hypoxic conditions, HIF-α is stabilised and forms the HIF heterodimer with HIF-1β. Once it translocates to the nucleus, it recruits the transcriptional transactivator histone acetyltransferase p300 (p300) and CREB binding protein (CBP), which allows the complex to bind hypoxia-responsive elements (HREs) to activate transcription of target genes.

1.7.3 Prolyl hydroxylase domain (PHD)

In contrast, under normoxic conditions, HIF-α protein is readily degraded mainly through the hydroxylation of prolyl and asparaginyl residues through PHD, a class of enzymes within the 2-oxoglutarate (2-OG)-dependent dioxygenase (2-OG oxygenase) superfamily [404-408]. PHD contains a Fe(II) in its active site and uses O_2 and a-ketoglutarate as substrates (split into CO_2 and succinate) and labels HIF-α subunits through ubiquitination. Subsequently, HIF-α undergoes

proteasomal degradation via the von Hippel-Lindau (VHL) disease suppressor protein, which is the recognition component of a ubiquitin E3 ligase complex [409-411] **[Figure 1.7]**.

PHD enzymes are produced in three isoforms, PHD1 (encoded by *EGLN2*), PHD2 (*EGLN1*), and PHD3 (*EGLN3*), which all have a role in the regulation of HIF. However, while PHD1 and PHD3 seem to have more tissue specific regulation of HIF, PHD2 is thought to be the central regulator of HIF and the most widely expressed. Mice lacking PHD2 (*Egln1*) are found to have placental and heart defects, leading to embryonic death [412]. Other differences between the three isoforms may include sensitivity to oxygen levels for activation [413, 414] and preferences for the hydroxylation of different prolyl residues within HIF substrates [415-417], which may lead to differences in their selectivity against specific HIF isoforms [392]. However, the exact roles and forms of regulation specific to each PHD isoform requires further investigation.

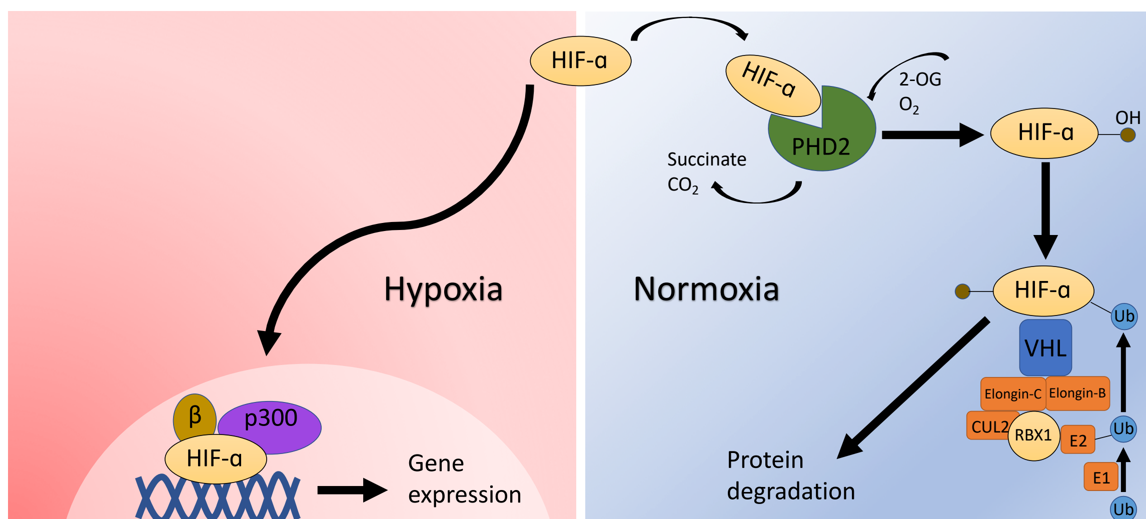


Figure 1.7. The HIF/PHD pathway under hypoxic and normoxic conditions. **(Right)** Under normoxic conditions, HIF-α is readily degraded through different mechanisms. One of which is through PHD2 which uses O_2 and a-ketoglutarate as substrates and labels HIF-α for ubiquitination and VHL-mediated proteasomal degradation. **(Left)** Under hypoxic conditions, the transcription factor HIF-α promotes diverse cellular processes ranging from erythropoiesis, angiogenesis, energy metabolism, and inflammation.

1.7.4 HIF/PHD Signalling

1.7.4.1 Regulation

HIF-1α and HIF-2α are transcriptionally regulated by elements such as STAT3 [418, 419], NF-κβ [420] and DNA methyltransferase 3A (DNMT3A) [421], and HIF-1α is also negatively regulated through a transcriptional feedback loop by an antisense transcript, HIF-1α antisense RNA 2 (*HIFA-AS2*) [422]. Moreover, activation of pro-inflammatory pathways through molecules such as LPS, TNF, IL-6, and IL-18 are also shown to increase transcription of *HIF1A* [423].

Besides the PHD enzymes, another HIF hydroxylase called factor inhibiting HIF (FIH) is a major oxygen-sensitive mechanism that regulates HIF at the post-translational level through a VHL-independent pathway. FIH disrupts the binding between HIF heterodimer and CBP/p300 by targeting an asparagine residue in the C-terminal domain of the HIF-α protein and ultimately inhibits HIF from transactivating genes under normoxia [392, 424, 425]. Moreover, heat shock protein 90 (Hsp90) has been reported to bind to HIF-1α to induce a conformational change to help dimerise with HIF-1β. Hsp90 inhibition was thus demonstrated to reduce HIF-1α transcriptional activity and also promoted more efficient ubiquitination and proteasomal degradation of HIF-1α in an oxygen-independent manner [426]. Other studies have demonstrated that HIF can be mediated post-translation through methylation [427], sumoylation [428], and acetylation [392, 429].

Additionally, mTORC1 is thought to be a central upstream regulator of HIF gene and protein expression, but the exact mechanisms of regulation have been difficult to untangle due to the

two pathways being so intertwined on many levels. Work by Land et al. suggests that HIF-1α activity is enhanced by mTORC1 through overexpression of Rheb, a Ras homologue (Land JBC 2007) [430]. Other papers suggest that 4E-binding protein 1 (4E-BP1) phosphorylation [431], ribosomal protein S6 kinase-1 (S6K1) [432], and/or STAT3 [433] were the central regulators of HIF-1α downstream of mTORC1.

1.7.4.2 HIF dependent gene expression

Among over 1000 genes shown to be directly transactivated by HIF under hypoxic conditions, many encode proteins responsible for increasing O_2 delivery [434]. In particular, HIF has been shown to increase expression of EPO (as previously described) and vascular endothelial growth factor (VEGF), which plays a central role in angiogenesis and neovascularisation [394]. Moreover, HIF also regulates many genes that encode proteins that decrease O_2 consumption, such as glycolytic enzymes responsible for shifting cells towards glycolytic metabolism (i.e. lactate dehydrogenase A and pyruvate dehydrogenase kinase 1) [394, 434-436]. Additionally, HIF regulates expression of the gene encoding glucose transporter 1 (GLUT1, encoded by *Glut1*) [437].

1.7.5 HIF/PHD in innate immunity

HIF has wide-ranging effects on immune cells, especially due to its central role within immunometabolism. Because different cell types have intrinsic differences in their reliance on glycolysis for energy, hypoxia and HIF affect different cell types in various ways. For example,

HIF's effects on granulocytes have been well-documented. Within neutrophils, studies have demonstrated that HIF-1α activation can enhance survival, glycolytic metabolism [438], extracellular bacterial killing [439, 440], and β2 integrin expression [441, 442]. In other granulocytes (basophils, mast cells, and eosinophils), HIF-1α stabilisation has been shown to be critical to their survival and function in both mice and humans [442, 443], as well as the production of certain cytokines (i.e. IL-18, TNF-a, and IL-6 from mast cells) [444]. Studies have demonstrated that HIF-1α inactivation within macrophages can affect their survival, chemotaxis, and other functional properties by inhibiting the production of ATP [440, 445]. Moreover, HIF-1α and HIF-2α activation may affect M1 and M2 macrophage polarisation, respectively [446, 447]. In DCs, HIFs have also been demonstrated to have a variety of roles in survival [448], IFN-y production [449], upregulation of neutrophil-attracting chemokines [450], and T cell stimulation [451]. Finally, HIF-1α has been shown to impact NK cytotoxic function [452], while the VHL-HIF-1α pathway has been shown to play an important role for ILC2 maturation [442, 453].

1.7.6 HIF/PHD2 in adaptive immunity

In HIF-1α$^{-/-}$ Rag2$^{-/-}$ chimeric mice, Kojima et al. found abnormal B cell development and an autoimmune phenotype [454]. A recent study reported that B cells in hypoxic germinal centres (GCs) have reduced proliferation, survival, and impaired antibody class switching [455]. However, during metabolic adaptation, HIF and glycogen synthase kinase 3 (Gsk3) was found to be necessary for GC B cell proliferation in hypoxic conditions [456]. Moreover, HIF-1α was

demonstrated to be a crucial transcription factor for IL-10 producing B cells through the modulation of glycolytic metabolism [457].

The link between HIF and T cells have been of particular interest to many studies, especially in cancer due to the hypoxic microenvironments within tumours. Makino et al. first showed that human peripheral T cells with TCR activation had better survival under hypoxic conditions through a HIF-1α gene product called adrenomedullin [458]. However, a report by Biju et al. showed that pVHL inactivation in the mouse germ line results in embryonic lethality with increased apoptosis in thymocytes, in which simultaneous inactivation of HIF-1 reversed this effect [459]. A recent study by Tyrakis et al. demonstrated that a HIF-1α-dependent mechanism enhanced *in vivo* proliferation, persistence and anti-tumour capacity of adoptively transferred $CD8^+$ T cells [460]. HIF-1α may thus activate $CD8^+$ T cells to increase effector functions and generate memory populations by promoting glycolytic metabolism [442, 461, 462]. Moreover, studies have indicated that the thymus is hypoxic under physiological conditions, suggesting that oxygen tension may be involved in thymocyte development [463]

Interestingly, there have been extensive studies focusing on the HIF pathway's role in the balance between Th17 and Treg differentiation. In particular, Dang et al. reported findings suggesting that HIF-1α skews T cells towards a Th17 transcriptional program, while skewing them away from Treg differentiation [464]. Th17-dependent experimental allergic encephalomyelitis (EAE) could not be induced within HIF-1α$^{-/-}$ mice, which had significantly reduced Th17 cells correlated with increased Treg numbers but not *Foxp3* transcript. Mechanistically, they report that HIF-1α enhances transcriptional activation of ROR-γt by recruiting ROR-γt and p300 to the IL-17

promoters. Additionally, it binds Foxp3 protein and tags it for ubiquitin-dependent proteasomal degradation. This is supported by studies reporting that Th17 cells rely more on the HIF-1α-driven glycolytic pathway than Tregs for survival [465]. Moreover, $CD4^+$ T cells from Itk-deficient mice cultured under Th17 conditions have been shown to have reduced IL-17 expression but increased Foxp3 expression, which was correlated with impaired mTOR/Akt activation and significantly downregulated HIF-1α [466]. Another report by Hsaio et al. demonstrated that suppressive ability is significantly impaired in Tregs that lack expression of DTX1, which promotes the degradation of HIF-1α, and concurrent knockout of HIF-1α restores Foxp3 stability and suppressive ability [467].

Nonetheless, further studies have indicated that the role of the HIF pathway in Treg biology is more complex. For example, reports from Ben-Shoshan et al. showed an increase in Foxp3 expression upon HIF-1α overexpression and enhanced *in vitro* suppression of Tregs under hypoxic conditions, while transfection with short-interfering RNAs for HIF-1α reversed this effect [468]. Clambey et al. found that hypoxia selectively induced *Foxp3* transcription, and Treg-intrinsic HIF-1α expression was necessary to control T-cell-mediated colitis [469]. This suggests that differentiated Tregs may require HIF-1α-driven transcription to function optimally, particularly under inflammatory conditions *in vivo.*

1.7.7 HIF/PHD in transplantation

The HIF/PHD pathway has not been extensively studied within the context of transplant organs and rejection. However, preclinical animal studies suggest that HIF activity may play critical roles in organ survival by increasing perfusion and decreasing fibrosis [470]. Jiang et al. found that pre-treatment of grafts with AdCA5, an adenovirus encoding a constitutively active form of HIF-1α, attenuated airway fibrotic remodelling through increased recruitment of vasoreparative Tie2+ angiogenic cells to the allograft in a model of mouse orthotropic tracheal transplantation. Indeed, Bernhardt et al. found using a rat kidney transplant model that donor PHD inhibitor treatment prior to nephrectomy improved survival of recipients [396, 471]. PHD inhibition in these experiments may have improved transplant outcome due to the accumulation of HIF which induced activation of tissue-protective genes.

1.7.8 Summary

In conclusion, the HIF/PHD pathway has been demonstrated to be involved in a variety of immune pathways. Recent evidence points to a distinct role for the HIF/PHD pathway within Treg biology. Elucidating whether it can be modulated to bolster or enhance Treg stability and function may contribute significant insight into Treg research and improving therapeutics.

1.8 Conclusion

The past few decades have marked an era of promising advances in the quest for clinical tolerance, with much of the progress attributed to the growing knowledge of regulatory T cells (Tregs). With phase I/II clinical trials well underway, it is now more crucial than ever to work to refine and optimize these methods for practicality, efficacy, and safety. Nonetheless, there is still much to learn in the study of Tregs, with obstacles still ahead in the optimisation of Treg-based cellular therapy. Investigating strategies to expand a greater number of Tregs and/or increase their suppressive potency or stability is thus undoubtedly critical. In this respect, the manipulation of specific molecular pathways may be a feasible and fruitful approach.

The IL-33/ST2 and HIF/PHD pathways have respectively become compelling areas of research within immunology in recent years, particularly due to their pervasive effects on myriad signalling pathways. Indeed, their intrinsically pleiotropic nature brings challenges in specifically harnessing the regulatory functions of the pathways. However, further exploration of their effects on regulatory cells and transplantation immunology will aid in unlocking their effects on other immune pathologies as well.

1.9 Aims of Thesis and Objectives

Global aim

To investigate the manipulation of specific molecular pathways in modulating Treg biology and function, for the application of Treg-based cellular therapy within solid-organ transplantation.

Specific aims

i. To explore the role of the IL-33/ST2 pathway modulating Treg biology in mouse models
ii. To assess the efficacy of IL-33-based Treg cellular therapy within the context of transplantation in *in vitro* and *in vivo* mouse models
iii. To investigate the function of PHD2 in Treg biology in mouse models
iv. To examine the effects of chronic hypoxia and HIF-2α inhibition in Treg phenotype and function in mouse and human models

2 Methods

2.1 Reagents

2.1.1 Monoclonal antibodies

Table 2.1: Monoclonal Antibodies used for flow cytometry					
Reactivity	**Specificity**	**Fluorochrome**	**Clone**	**Host/Isotype**	**Supplier**
Mouse	CD3	FITC	145-2C11	Armenian hamster/IgG	eBioscience
Mouse	CD3	PE	17A2	Rat/IgG2b kappa	eBioscience
Mouse	CD4	APC-Cy7/efluor450	GK1.5	Rat/IgG2b kappa	eBioscience
Mouse	CD8	APC-Cy7	53-6.7	Rat/IgG2a kappa	eBioscience
Mouse	B220	APC-Cy7	30-H12	Rat/IgG2a kappa	eBioscience
Mouse	CD45	efluor450	30-F11	Rat/IgG2b kappa	eBioscience
Mouse	Gr-1	APC	RB6-8C5	Rat/IgG2b kappa	eBioscience
Mouse	CD25	PE	PC61.5	Rat/IgG1 lambda	eBioscience
Mouse	CD25	PE-CF594	PC61	Rat/IgG1 lambda	BD
Mouse	Foxp3	FITC/APC/eFluor450	30-F11	Rat/IgG2b kappa	eBioscience
Mouse	CD62L	PE	MEL-16	Rat/IgG2a kappa	BD
Mouse	CD44	PE-Cy7	IM7	Rat/IgG2b kappa	BioLegend
Mouse	IL-17A	PE	ebio17B7	Rat/IgG2a kappa	eBioscience
Mouse	IL-4	PE-Cy7	BVD6-24G2	Rat/IgG1 kappa	eBioscience
Mouse	IFN-y	APC	XMG1.2	Rat/IgG1 kappa	eBioscience
Mouse	IL-2	PE	JES6-5H4	Rat/IgG2b kappa	eBioscience
Mouse	TNF-a	PE-Cy7	MP6-XT22	Rat/IgG1 kappa	eBioscience

Mouse	IL-10	APC	JES5-16E3	Rat/IgG2b kappa	eBioscience
Mouse	GATA-3	PE-CF594	L50-823	Mouse/IgG1 kappa	BD
Mouse	T-bet	PE-Cy7	ebio4b10	Mouse/IgG1 kappa	eBioscience
Mouse	ROR-yt	APC	AFKJS-9	Rat/IgG2a kappa	eBioscience
Mouse	CD39	PE-Cy7	24DMS1	Rat/IgG2b kappa	eBioscience
Mouse	ST2	APC	RMST2-2	Rat/IgG2a kappa	eBioscience
Mouse	CD44	PerCP	IM7	Rat/IgG2b kappa	eBioscience
Mouse	Helios	efluor450	22F6	Armenian hamster/IgG	eBioscience
Mouse	CTLA-4	PE	UC10-4B9	Armenian hamster/IgG	eBioscience
Mouse	CCR4	PE-Cy7	2G12	Armenian hamster/IgG	BioLegend
Mouse	CCR2	BV 421	SA203G11	Rat/IgG2b kappa	Biolegend
Mouse	CCR5	PE	7A4	Armenian hamster/IgG	eBioscience
Mouse	CCR7	APC eFluor 780	4B12	Rat/IgG2a kappa	eBioscience
Mouse	PD-1	PE	RMP1-30	Rat/IgG2b kappa	eBioscience
Mouse	ICOS	PE-Cy7	C398.4A	Armenian hamster/IgG	eBioscience
Mouse	CD69	PE-Cy7	H1.2F3	Armenian hamster/IgG	eBioscience
Human	CD4	PE-efluor 610	RPA-T4	Mouse/IgG1 kappa	eBioscience
Human	CD8	APC-Cy7	SK1	Mouse/IgG1 kappa	BD
Human	HLA-DR	FITC	G46-6	Mouse/IgG2a kappa	BD
Human	CD25	PE-Cy7	M-A251	Mouse/IgG1 kappa	BD

2.1.2 Cytokines

Recombinant murine GM-CSF and IL-4 was purchased from Peprotech EC Ltd (London, UK) and TGF-β was obtained from R&D (Minneapolis, Minnesota, USA). Recombinant murine IL-33 for *in vitro* and *in vivo* experiments were purchased from R&D and BioLegend (San Diego, California, USA), respectively. Recombinant human IL-2 was purchased from Chiron (Proleukin®, Emeryville, California, USA).

2.1.3 Other reagents

7-AAD (7-aminoactinomycin D) for cell viability analysis was purchased from Biolegend. PMA (phorbol myristate acetate) and ionomycin were obtained from Sigma (St. Louis, Missouri, USA). Brefeldin A Solution (1000X) was purchased from eBioscience (Affymetrix, San Diego, California, USA). Doxycycline hyclate was purchased from Sigma.

2.2 In vivo animal procedures

2.2.1 Mice

Mice were housed in the Biomedical Services Unit of the John Radcliffe Hospital (Oxford, UK) and the Functional Genetics Facility of the Wellcome Trust Centre for Human Genetics (Oxford, UK). Animal care was conducted in accordance with the Animals (Scientific Procedures) Act 1986, under PPL numbers 30-3050, 30-2966, P38BE32DE, and P8869535A and PIL number I1D45D6BA.

Table 2.2: Mouse strains used in study							
Strain	Abbreviation	Background strain	H2 haplotype				
			Class I			Class II	
			K	D	L	IA	IE
CBA/Ca	WT CBA	CBA/Ca	k	k	k	k	k
C57BL/6	WT B6	C57BL/6	b	b	b	b	b
CBA/Ca FOXP3^{GFP}-reporter	CBA Foxp3-GFP	CBA/Ca	k	k	k	k	k
C57BL/6 FOXP3^{GFP}-reporter	B6 Foxp3-GFP	C57BL/6	b	b	b	b	b
CBA/Ca Rag$^{-/-}$	CBA Rag	CBA/Ca	k	k	k	k	k
C57BL/6 Rag$^{-/-}$	B6 Rag	C57BL/6	b	b	b	b	b
CAGGrtTA*	Control^{CAG}	C57BL/6	b	b	b	b	b
CAGGrtTA-Phd2miR*	Phd2kd	C57BL/6	b	b	b	b	b
FoxP3-Cre-LSL-CAGGrtTA*	Control^{Foxp3}	C57BL/6	b	b	b	b	b
FoxP3-Cre-LSL-CAGGrtTA-Phd2miR*	Foxp3^{Cre}-Phd2kd	C57BL/6	b	b	b	b	b
CAGGrtTA-Phd2miR/Hif1amiR*	P2H1kd	C57BL/6	b	b	b	b	b
CAGGrtTA-Phd2miR/Hif2amiR*	P2H2kd	C57BL/6	b	b	b	b	b
CAGGrtTA-Hif2amiR*	HIF2akd	C57BL/6	b	b	b	b	b
RosaERTCre*	Control^{Cre}	C57BL/6	b	b	b	b	b
RosaERTCre Hif2a$^{fl/fl}$*	HIF2aKO	C57BL/6	b	b	b	b	b

*Mice used in HIF/PHD2 experiments

2.2.2 Generation of *HIF/PHD2* transgenic mice

All *Hif/Phd2* transgenic mice were generated and bred by Dr Atsushi Yamamoto. Control^{CAG}, Phd2kd, P2H1kd, and P2H2kd were all generated, as described previously [472]. HIF2akd mice were generated through an identical method [472-474]. *Hif/Phd2* KO models, including HIF2aKO mice, were generated, as described previously [472, 475, 476]. Foxp3^{Cre}-Phd2kd mice were generated by crossing Foxp3-Cre mice with mice in which Phd2-miRNA is only transcribed upon the removal of a LoxP-STOP-LoxP sequence. Phd2kd, Hif2akd P2H1kd, and P2H2kd mice allow ubiquitous, reversible knock-down of *Phd2*, *Hif2a*, *Phd2/Hif1a*, and *Phd2/Hif2a*, respectively, upon doxycycline treatment (Control^{CAG} strain was used as their control strain.) Foxp3^{Cre}-Phd2kd mice allow inducible, reversible knock-down of *Phd2* upon doxycycline treatment only in cells expressing Foxp3 (Control^{Foxp3} was used as its control strain.) Hif2aKO strain allows tamoxifen-inducible, irreversible knock-out of *Hif2a* gene (Control^{Cre} strain was used as its control strain.)

2.2.3 Anaesthesia

Surgical procedures were conducted under inhalation anaesthesia, in which IsoFlo® (Zoetis, Parsippany-Troy Hills, New Jersey) was delivered through an anaesthesia machine. For induction amnesia, mice were first placed into an induction chamber. Anaesthetic depth was assessed via a toe pinch, at which point mice were transferred on top of a heating pad with maintenance anaesthesia delivered through a nose cone. Following surgical procedure, mice were transferred to a warm recovery cabinet for 2 hours.

2.2.4 Adoptive transfer of mouse Teffs and Tregs

Cells to be infused were suspended in RPMI-1640 (number of injected cells is detailed in each experiment). B6 Rag or CBA Rag mice were secured in restrainer tubes and their tails were swabbed with ethanol. A 25-gauge syringe with substance (100-200 µl) was prepared and pierced through tail into lateral vein and aspirated. Needle was carefully removed, and pressure was applied to puncture site until bleeding had stopped.

2.2.5 Skin grafting

Skin transplantation was done one day after adoptive cell transfer. Skin grafts were prepared from tail skin of donor sacrificed mice and cut into grafts of approximately 1 cm^2 pieces and placed in saline. Recipient mice (B6 Rag or CBA Rag) were kept under inhalation anaesthesia and incised with graft beds on the left flanks of mice. Grafts were aligned on top of graft beds and secured with Histoacryl® glue (B. Braun, Melsungen, Germany) applied at the corners of graft/graft bed. Non-adhesive iodine-infused dressing and sterile gauze were placed over graft and secured with plasters and autoclave tape wrapped around the abdomen of mice. Dressings were removed 7 days after transplantation. Grafts were regularly monitored for rejection, which was defined as complete destruction of the skin confirmed by two blinded clinicians.

2.2.6 Intraperitoneal (i.p.) injection

Mice were restrained in dorsal recumbent position. A 25-gauge syringe with substance (200-500 µl) was penetrated through the lower abdominal quadrant and aspirated.

2.2.6.1 IL-33 *in vivo* treatment

B6 Foxp3-GFP or CBA Foxp3-GFP mice were injected i.p. with 1µg of recombinant mouse IL-33 (Biolegend) resuspended in 200µl of RPMI per day for six consecutive days.

2.2.6.2 Rapamycin dosing

Dose and dosing regimen were based on Andrew Whatcott's thesis (University of Oxford, 2016). Rapamune® (Sirolimus/Rapamycin, 1mg/mL oral solution) was purchased through the John Radcliffe Pharmacy, Oxford universities NHS foundation trust, UK. Rapamycin injection solution consisted of 440µL of Rapamune was dissolved in 1mL ethanol, 1mL cremaphore (Sigma), and 18mL sterile PBS. CBA GFP mice were injected i.p. with 1µg of Rapamycin solution per day for three consecutive days.

2.2.7 Tamoxifen treatment

Tamoxifen treatment was administered by Dr Tammie Bishop at the Functional Genetics Facility of the Wellcome Trust Centre for Human Genetics (Oxford, UK). Tamoxifen (20 mg/mL, Sigma) was administered by oral gavage at a dose of 2 mg/day for five consecutive days, as previously described [475, 477].

2.2.8 Chronic hypoxic exposure of mice

Mice in chronic hypoxia experiments were treated and maintained by Dr Tammie Bishop at the Functional Genetics Facility of the Wellcome Trust Centre for Human Genetics (Oxford, UK). Mice

were subjected to chronic hypoxic exposure within normobaric chambers containing 10% O_2 for 4-6 weeks, as described previously [475, 478].

2.3 Flow cytometry

2.3.1 Cell surface marker staining

All cells were stained in 96-well V-bottom plates. Single cell suspensions were stained with monoclonal antibodies (listed in Table 2.1), per manufacturer's recommended concentrations, and then incubated for 30 minutes at 4°C in the dark. Cells were washed once with FACS buffer (PBS solution with 5% FCS and 0.2% sodium azide) and resuspended in 200µL of 2% v/v formaldehyde (Sigma, USA). Cells were analysed on a BD FACSCanto II on the same day or left overnight in 4°C for next day analysis.

2.3.2 Intracellular immunostaining

The Foxp3/Transcription Factor Staining Buffer Set (eBioscience, Invitrogen) was used for intracellular staining, and solutions were prepared per manufacturer's instructions. After cells were stained with cells surface markers and washed, 50µL fixation-permeabilisation buffer from the kit was added per well, in which cells were incubated for 45 minutes (15°C) or overnight (4°C) in the dark. Cells were then washed twice with 200µL of permeabilisation buffer from the kit. Cells were again incubated for 15 minutes at 15°C in the dark with 3µL of rat and/or mouse serum (eBioscience) added per well. Subsequently, cells were stained with monoclonal antibodies, per

manufacturer's recommended concentrations, and incubated for 45 minutes at 15°C in the dark. Cells were washed once with FACS buffer and resuspended in 200µL of 2% v/v formaldehyde (Sigma, USA). Cells were analysed on a BD FACSCanto II on the same day or left overnight in 4°C for next day analysis.

2.3.3 Intracellular cytokine stimulation and staining

For cytokine stimulation, cells were first incubated for 5 hours (37°C and 5% CO_2) in 24-well cell culture plates at concentration of $1x10^6$ per well in complete RPMI culture medium (RPMI-1640, 10% FSC, L-Glutamine, penicillin/streptomycin) supplemented with 1 µg/mL ionomycin (Sigma), 100 ng/mL PMA (Sigma), and 5 µg/mL brefeldin A (eBioscience). Cells were then washed with FACS buffer once and stained with cell surface and intracellular antibodies, as described previously.

2.3.4 VPD labelling

Cells were washed with PBS buffer twice and resuspended at $30x10^6$ cells/mL in PBS. Cells were then incubated at 37 °C for 10 minutes with violet proliferation dye (VPD, BD, San Jose, California) added to a concentration of 1µM. Cells were subsequently washed twice with PBS and resuspended in cultures media for cell counting.

2.3.5 Flow cytometric analysis

All flow cytometric analysis was conducted using FlowJo (ver. 10, Tree Star, Ashland, Oregon, USA).

2.4 Cell isolation

2.4.1 Bead-isolation for mouse T cell populations

Splenocytes were harvested and mashed through sterile 70 µM cell strainers (BD, San Jose, California) with 2mL syringe plungers (BD). Splenocytes were washed through filter with FACS buffer and collected in 50 mL falcon tubes (BD). Cells were then washed once and then resuspended in 3mL of PharmLyse lysing buffer (BD biosciences, Franklin Lakes, NJ, USA) and incubated for 6 minutes. Cells were washed once with PBS and resuspended and cell numbers were counted and $CD4^{+}$ cells were isolated using the Dynabeads Mouse CD4 cells kit (Invitrogen), as per manufacturer's instructions.

To isolate $CD4^{+}CD25^{neg}$ or $CD4^{+}CD25^{+}$ T cell populations, using an anti-CD25 PE antibody (eBioscience) and anti-PE MACS Microbeads (Miltenyi Biotec Ltd., Woking, Surrey, UK, cat 130-048-801). Purity for CD4 and CD25 expression was assessed using BD FACSCanto II.

2.4.2 Cell sorting for mouse Tregs and Teffs

Splenocytes from mice were processed into single cell suspensions and first enriched for $CD4^+$ cells using the Dynabeads Mouse CD4 cells kit (Invitrogen), as per the manufacturer's instructions. $CD4^+$ enriched cells were then stained with fluorochrome-conjugated mAbs and incubated for 30 minutes. Cells were then washed once with FACS buffer, filtered, and then resuspended in FACS. Treg and Teff cell populations were sorted using the FACSAria II (BD Biosciences, Wokingham, Berkshire, UK).

2.5 Mouse cell culture

Mouse culture media (complete RPMI) consisted of RPMI-1640 medium (Merck, Kenilworth, New Jersey) supplemented with penicillin/streptomycin (Sigma Aldrich, Poole, Dorset, UK), L-glutamine (Sigma Aldrich) and 10% heat-inactivated FCS (fetal calf serum, Gibco, Thermofisher, Waltham, Massachusetts). All cell cultures were incubated at 37°C and 5% CO_2.

2.5.1 Generation of mouse bone marrow derived dendritic cells

Femurs were dissected from 6 to 12-week-old WT B6 or WT CBA mice and bone marrow was washed out with a syringe, collected, and counted. Cells were resuspended with culture medium at $1x10^6$/mL and stimulated with either GM-CSF (10ng/mL, Peprotech) and TGF-β (2ng/mL), Peprotech or GM-CSF (10ng/mL) and IL-4 (10ng/mL, Peprotech) and plated onto 24-well plates at 1 mL/well and incubated at 39°C. Half the medium was replaced and cells were restimulated

with cytokines at day 2 and day 4. At day 6, DCs were collected and either used for further cultures or frozen in liquid N_2 for future cultures. DCs precultured with GM-CSF/TGF-β and GM-CSF/IL-4 are referred to as GT-DC and GM-IL-4-DC, respectively.

2.5.2 *In vitro* Treg expansion

2.5.2.1 *In vitro* mouse Treg expansion with GT-DC and IL-33

Spleens were harvested from 6 to 12-week-old B6 Foxp3-GFP or CBA Foxp3-GFP mice and total splenocytes were isolated through mashing the spleens through a 70μm filter and lysed for red blood cells. $CD4^+$, $CD4^+CD25^{neg}$, and $CD4^+CD25^+$ populations were isolated from splenocytes, as described previously in Chapter 2.2.1. $CD4^+$, $CD4^+CD25^-$, and $CD4^+CD25^+$ ($1x10^5$/well) were cultured alone in 96-well U-bottoms plates or with fresh or frozen allogeneic GT-DCs ($2x10^4$/well) and rmIL-33 (R&D, Minneapolis, Minnesota) in varying doses (1, 10, 30 ng/mL) for 14 days at 39°C. At day 7, half of the medium was replaced with new alloDC and/or rmIL-33 in the appropriate wells. At day 14, 3 wells were aggregated together for each sample, which was stained for CD4, CD25, ST2, CD44, CD62L, and FoxP3 and analysed by flow cytometry (FACSCanto II).

2.5.2.2 *In vitro* mouse Treg expansion with GM-IL-4-DC and IL-33

$CD4^+$ cells were isolated from splenocytes as described previously. $CD4^+$ were stained with violet proliferation dye (VPD) and then cultured at $1x10^5$/well with $2x10^4$/well GM-IL-4-DCs and/or rhIL-2 ($1x10^3$U/mL) and/or rmIL-33 (10 ng/mL). At day 5, 3 wells were aggregated for each sample and analysed with flow cytometry (FACSCanto II).

2.5.3 *In vitro* suppression assays

2.5.3.1 Treg suppression assay with bead stimulation

Splenocytes from mice were processed into single cell suspensions. Cells were then enriched for $CD4^+$ cells and subsequently isolated for $CD25^+$ (Treg) and $CD25^{neg}$ (Teff) cells, as previously described. $CD4^+CD25^+$ (Treg) and $CD4^+CD25^{neg}$ (Teff) cell numbers were counted using trypan blue staining, a haematocytometer, and microscope. Teffs were first stained with violet proliferation dye 450 (BD), as described in Chapter 2.3.4. VPD-labelled Teffs were then transferred to round-bottom 96-well plates at a concentration of $1x10^5$ cells/well with anti-CD3/anti-CD28 Dynabeads (Invitrogen, Thermo Fisher) at a concentration of $1x10^5$ beads/well. Control Tregs or IL-33-Tregs were then added at Treg:Teff ratios of 1:1, 1:2, 1:4, and 1:8. Cultures were incubated for 72-96 hrs and harvested. Proliferation by VPD was measured using FACSCanto II and analysed with FlowJo. Division indices, defined as the average number of cell divisions that a cell in original populations has undergone, was calculated in FlowJo through the following formula:

$$\text{Div. Index} = \frac{\#\ \text{cell divisions}}{\#\ \text{precursor cells}} = \frac{\frac{(\#\ \text{cell divisions}_1)}{2^1} + \frac{(\#\ \text{cell divisions}_2)}{2^2} + \cdots + n\frac{(\#\ \text{cell divisions}_n)}{2^n}}{\frac{(\#\ \text{cell divisions}_0)}{2^0} + \frac{(\#\ \text{cell divisions}_1)}{2^1} + \cdots + n\frac{(\#\ \text{cell divisions}_n)}{2^n}}$$

n= # of the division peak

Percentage of suppression was calculated using the following formula:

$$\text{Percent suppression} = \left(1 - \frac{\text{div. index Responders} + \text{Tregs}}{\text{div. index Responders}}\right) * 100$$

2.5.3.2 Treg suppression assays with allogeneic dendritic cell stimulation

In allogeneic dendritic cell (alloDC)-stimulated conditions, WT B6 or WT CBA GM-IL-4-DCs were generated, as previously described. alloDCs were added in *in vitro* suppression cultures at a concentration of $2x10^4$ cells/well with Teff and Tregs and incubated or a total of 114 hrs with 3H thymidine (Perkin Elmer, Beaconsfield, Buckinghamshire, UK) being added in the last 18 hrs. Cell proliferation data was measured in counts per minute (cpm) with a Betaplate reader. Percentage of suppression was calculated using the following formula:

$$\text{Percent suppression} = \left(1 - \frac{(\text{cpm Responders} + \text{Tregs}}{\text{cpm Responders}}\right) * 100$$

2.6 Cell cryopreservation and thawing

Cells were pelleted and resuspended in 225µL RPMI 1640, 225µL heat-inactivated FBS, and 50µL dimethylsulfoxide (DMSO, Sigma) into cryovials. Cryovials were stored in Mr. Frosty^{TM} freezing containers (Thermo Fisher) for 24 hours at -80°C, before being transferred to liquid nitrogen.

For thawing, cryovials were first transferred to -80°C for one hour. Cryovials were thawed in 37°C and immediately transferred into a Corning^{TM} Falcon 50mL tube (Fisher Scientific, Thermo Fisher) containing 10µL of DNase. 10mL of 37°C RPMI was added dropwise to suspension.

2.7 TSDR analysis

CD4$^+$Foxp3$^+$, CD4$^+$Foxp3^{neg}, CD4$^+$Foxp3$^+$CD25$^+$, and CD4$^+$Foxp3$^+$CD25^{neg} populations from doxycycline-treated Control^{CAG} and Phd2kd mice were sorted. Methylation at the TSDR in these populations was analysed by EpigenDx (Hopkinton, Massachusetts) through pyrosequencing of bisulphite-modified DNA, in which four representative CpG residues of the mouse Foxp3 TSDR were analysed using their ADS568-FS2 assay. Demethylation percentage on the active X chromosome was calculated, in which data from female mice were normalised to allow for complete methylation of the TSDR on the inactivated X chromosome.

2.8 Transcriptomic analysis

2.8.1 Cell and RNA isolation

For transcriptomic analysis of splenocytes of PBS or IL-33-treated B6 GFP and CBA GFP mice, 1x10^6 whole splenocytes from each mouse were taken for RNA isolation. For analysis of CD4$^+$GFP$^+$ Tregs from PBS or IL-33-treated mice, all splenic Tregs sorted from each mouse were taken for RNA isolation. RNA was isolated using the RNeasy Mini or Microkit, depending on cell number (Qiagen, Venlo, Netherlands), per manufacturer's instructions.

2.8.2 Data analysis

RNA was analysed with the nCounter® Sprint Profiler (NanoString Technologies, Inc. Seattle, Washington) using the PanCancer Immune Profiling Panel and the nSolver™ Analysis Software (ver. 4.0, Nanostring), per manufacturer's instructions. Data sets were compared using the Advanced Analysis platform (ver. 2.0), in which adjusted *p* value was calculated with control of Benjamini-Yekutieli or Benjamini-Hochberg False Discovery Rate (FDR) (Adjusted p value > 0.05 considered significant).

2.9 Human Treg expansion and assays

2.9.1 PBMC isolation

Human PBMCs were isolated from blood of healthy donors (provided by the National Health Service Blood and Transplant, Oxford, U.K.). Blood was first diluted to 50 mL with PBS. 25 mL of diluted blood was carefully overlaid on top of 15 mL of Ficoll-Paque PLUS (GE Healthcare, Chicago, Illinois) for density gradient centrifugation at 2200 rpm at 15°C for 30 minutes. The lymphocyte later (buffy coat) was collected and washed with PBS. Cells were incubated for 6 minutes with 10mL of PharmLyse lysing buffer (BD biosciences) for red cell lysis and then washed once.

2.9.2 Isolation of human Tregs

$CD25^+$ Tregs were isolated from 2-3x10^8 PBMCs using CD25 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), per manufacturer's instructions, while the rest of the PBMCs were

cryopreserved. Cells were then stained with 7-AAD, anti-CD25 PE-Cy7, ant-CD127 PE, and anti-CD4 PE-Texas Red® antibodies. $CD4^{+}CD25^{+}CD127^{low}$ cells were then flow-sorted using FACSAria II (BD Biosciences).

2.9.3 *In vitro* expansion

Freshly-isolated $CD4^{+}CD25^{+}CD127^{low}$ Tregs were then cultured in complete RPMI medium, supplemented with penicillin/streptomycin (Sigma Aldrich), L- glutamine (Sigma Aldrich), 10% pooled AB human serum (SeraLab, BioIVT, Westbury, New York, USA), 1000U/mL of recombinant human IL-2 (Chiron, Proleukin®) and stimulated with Human T-activator anti-CD3/anti-CD28 Dynabeads (Invitrogen) at a 1:3 Treg to bead ratio. Cells were split and medium with IL-2 (1000U/mL) was replaced, as necessary. After 7 days, beads were removed, and cells were counted and re-plated with new complete medium with IL-2 (1000U/mL) and fresh Dynabeads at a 1:1 Treg to bead ratio. After day 14, beads were again removed, and cells were counted. Tregs were then rested for 2 days in complete medium with IL-2 (200U/mL) without beads. Tregs were subsequently counted and were either used in further assays or cryopreserved.

2.9.4 *In vitro* suppression assay with PT2385

Cells were cultured in 10% FBS complete medium. PBMC (responders) were first stained with VPD (BD Biosciences) and cultured with various Treg:PBMC ratios (1:1 to 1:32) and stimulated with anti-CD3/anti-CD28 Dynabeads (Invitrogen) at a 1:5 bead:PBMC ratio. For DMSO (control) conditions, 0.02% DMSO (Sigma) was added to cultures. For conditions with HIF-2α antagonist, 20µM of PT2385 (Peloton Therapeutics, Inc., Merck, Kenilworth, New Jersey) was added to

cultures. Cell cultures were then incubated for 72-96 hrs and analysed via flow cytometry using FACSCanto II (BD Biosciences). Percentage of suppression was calculated using FlowJo, as described in Chapter 2.5.1.3.

2.10 Statistical analysis

GraphPad Prism software (ver. 6, Graphpad holdings, LLC., San Diego, California) was used for all statistical analysis in this project (with exception to transcriptomic data, see Chapter 2.8). Unpaired, 2-tailed student's *t* test was used to analyse 2 independent groups. Log-rank tests were used to analyse graft survival data. 1-way ANOVA with Tukey's corrections was used for multigroup comparisons. 2-way ANOVA with Sidak's correction for multiple comparison were used when analysing group with 2 independent variables. Repeated measures ANOVA with Tukey's corrections was used to compare groups over time.

2.11 Statement of Contributions and Acknowledgment of Assistance

While the work described in this thesis is entirely my own work, I would like to acknowledge other researchers who have contributed towards the completion of this project. Notably, I would like to acknowledge Dr Atsushi Yamamoto and Dr Chris Pugh for their contributions on the hypoxia project in experimental design and generation of transgenic mice, Dr Tammie Bishop for the maintenance and acquisition of transgenic mice used in the hypoxia project, Dr Ran Li for

assistance with single cell RNA sequencing analysis, and Dr Masateru Uchiyama for assistance in transplantation experiments.

Parts of this thesis (as indicated in specific sections) have been re-used from previously published material (i.e. text and figures) [187, 472] with the permission of co-authors.

3 Effects of IL-33 on Immune Homeostasis and Treg Phenotype

3.1 Introduction

A number of promising clinical trials have highlighted the potential for boosting Treg numbers to rebalance immune function for therapeutic purposes. One of the ways this can be achieved is through the *in vivo* expansion/activation of endogenous Tregs [251-254]. There are now a number of drugs or therapeutic compounds in clinical trials that aim to increase the Treg:Teff ratio by either directly upregulating Treg over Teff populations or by selectively depleting Teffs while allowing Tregs to survive.

The most well-known strategy for the former method has been the use of low-dose IL-2 treatment, which are now being used in clinical trials to enhance Treg stability or function. The premise underlying low-dose IL-2 therapy is that it will selectively activate and expand Treg populations due to their high expression of the IL-2-receptor α (CD25) chain. However, there are still concerns that it can potentially activate other proinflammatory cells [479, 480]. Moreover, while low-dose IL-2 can boost numbers of Tregs, it has not been demonstrated to significantly enhance their suppressive potency or specificity.

Interestingly, recent preclinical studies have demonstrated a similar potential for IL-33, a regulatory alarmin molecule, which has been shown to significantly increase Treg populations

and upregulate the expression of its own receptor, ST2 [298, 299]. Reports have demonstrated that *in vivo* IL-33-based treatments can have protective effects in preventing GVHD [388] and the prolongation of heart [299, 389, 481] or skin [389] allograft survival through the expansion/induction of Tregs and MDSCs, and Th2 skewing. Nonetheless, similar to IL-2, IL-33 is highly pleiotropic and can activate proinflammatory cells. As highlighted by a separate study using the same GVHD model, Reichenbach et al. in contrast found that both endogenous and exogenous IL-33 can have deleterious effects on GVHD severity through the promotion of type 1 donor alloimmunity [309, 387].

It is evident that an important role for IL-33 exists in the control of Treg function and development. It is thus crucial to elucidate further the specific mechanisms and conditions by which IL-33 specifically alters Treg biology and how this affects other proinflammatory cells in order to fully harness its regulatory potential for therapeutic applications. **The goal of this chapter is to investigate the role of IL-33 on Treg biology specifically and its effect on immune homeostasis, with a focus on transplantation**.

Chapter hypothesis and aim

Hypothesis: IL-33 preferentially expands a specific population of suppressive Tregs that promote a tolerogenic environment.

Aim: To investigate the role of IL-33 on the general immune cell environment and Treg biology in *in vitro* and *in vivo* mouse models.

3.2 Results

3.2.1 In vivo IL-33 treatment results in overall downregulation of integral T cell-associated genes

To broadly investigate the effects of *in vivo* IL-33 treatment on the immune phenotype, WT CBA mice were administered recombinant IL-33 (IL-$33_{109\text{-}266}$, 1ug i.p. for 6 consecutive days). In IL-33-treated mice, there was significant splenomegaly and nearly two-fold increase in splenocyte cell numbers compared to PBS-treated control mice (p= 0.0012) **[Figure 3.2.1A]**, consistent with previous reports [298]. Surprisingly however, there were no significant increases in total $CD4^+$ or $CD8^+$ cell counts.

Based on previous reports elucidating the various effects that IL-33 can have on both the innate and adaptive immune cell populations, there was an interest in understanding how exogenous IL-33 may shift the immune cell environment. Thus, to broadly shed light on the effects that *in vivo* IL-33 treatment has within the immune cell environment, Nanostring multiplex gene expression analysis was used to compare the splenocytes of PBS and IL-33 treated mice to determine how the proinflammatory and anti-inflammatory properties of IL-33 manifest in an *in vivo* setting.

The most differentially expressed genes between the PBS and IL-33-treated mice are represented within a volcano plot, with 35 genes that came out as statistically significant (adjusted $p \leq 0.05$). **[Appendix 1** and **Figure 3.2.1B]**. Interestingly, many of the most differentially downregulated genes upon IL-33 treatment were genes for transcription factors and receptors central to the TCR

signalling pathway, including *Cd3e*, *Lck*, *Itk*, and *Zap70* **[Figure 3.2.1C]**. There was also an overall downregulation of genes such as *Jak1*, *Ets1*, *Stat5b*, and *Nfatc3*, and *Ets1* that encode regulators of major pathways within T cells, as well as genes such as *Lgals3*, *Xbp1*, *Pparg* (all upregulated) and *CD55* (downregulated) that have well-documented roles within T cell development and survival **[Figure 3.2.1D]**. Collectively, there was an overall reduction in key genes associated with T cell biology upon IL-33 treatment.

Notably, there was also a shift in gene expression for major cytokine receptors. The most differentially downregulated gene upon IL-33 treatment was *Il7r* (CD127), which is critical for T and B cell maintenance, but lowly expressed on Tregs [482]. Additionally, while there was a reduction in IL-2 receptor (IL-2R) subunit genes *Il2rb* (CD122) and *Il2rg* (CD132), there was a selective increase in expression for *Il2ra* (CD25) **[Figure 3.2.1E]**. As Tregs are the major cell types that highly express IL-2Rα, this suggests that there is an immune shift conducive for preferential IL-2 consumption by Tregs, rather than other cell types that have lower expression of CD25 or those that express the low affinity βγ IL-2R. Thus, this inverse relationship between *Ilra* and *Ilrb/g* may suggest increased IL-2 scavenging by Tregs to deprive IL-2 from the environment and limit Teff activation and proliferation [483, 484].

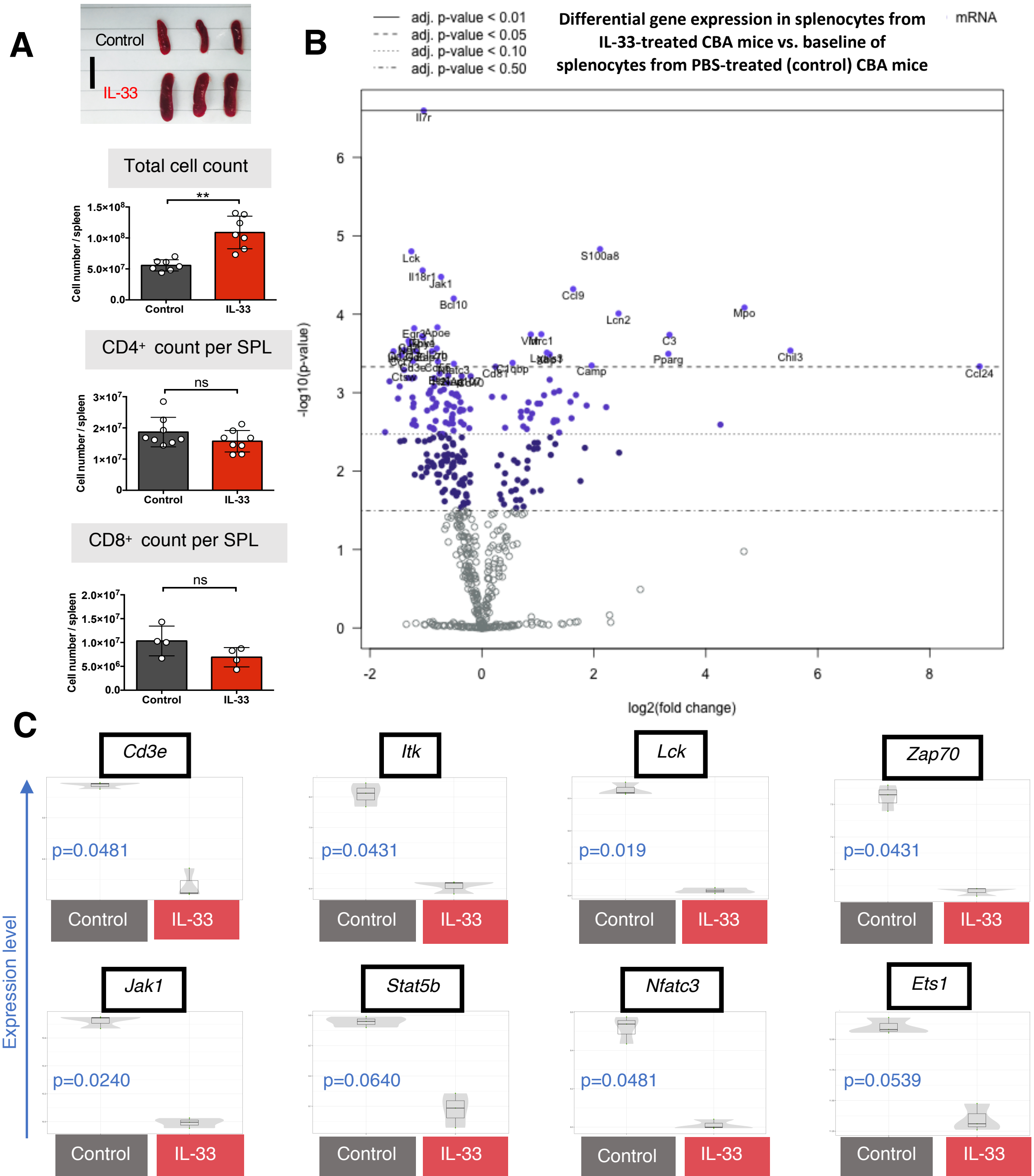


Figure 3.2.1. *In vivo* IL-33 treatment results in an overall downregulation of integral T cell-associated genes. CBA/Ca (H-2^k) mice were injected with either PBS (control, black) or recombinant IL-33 (1ug/day, red) for 6 consecutive days, and spleens (SPL) were analyzed by flow cytometry. RNA from total splenocytes was isolated from PBS (n=3) or IL-33 treated (n=3) H-2^k mice for multiplexed gene expression analysis using Nanostring mouse PanCancer Immune Profiling panel. **(A)** Representative images (scale bar: 1 cm) and graphs of spleens from CBA/Ca PBS- or IL-33-treated mice with total splenocyte count and CD4$^+$ (n=8 per group) and CD8$^+$ (n=4 per group) counts per spleen (unpaired *t* test) (**p<0.01; ns=not significant.) **(B)** Volcano plot revealing the most differentially expressed genes, relative to a baseline of control mice. **(C)** Selected genes associated with T cell signaling are represented in univariate scatter violin plots. **(B-C)** Adjusted *p* value calculated with control of Benjamini-Yekutieli False Discovery Rate (FDR) (Adjusted *p* value >0.05 considered significant, FDR thresholds indicated within volcano plot).

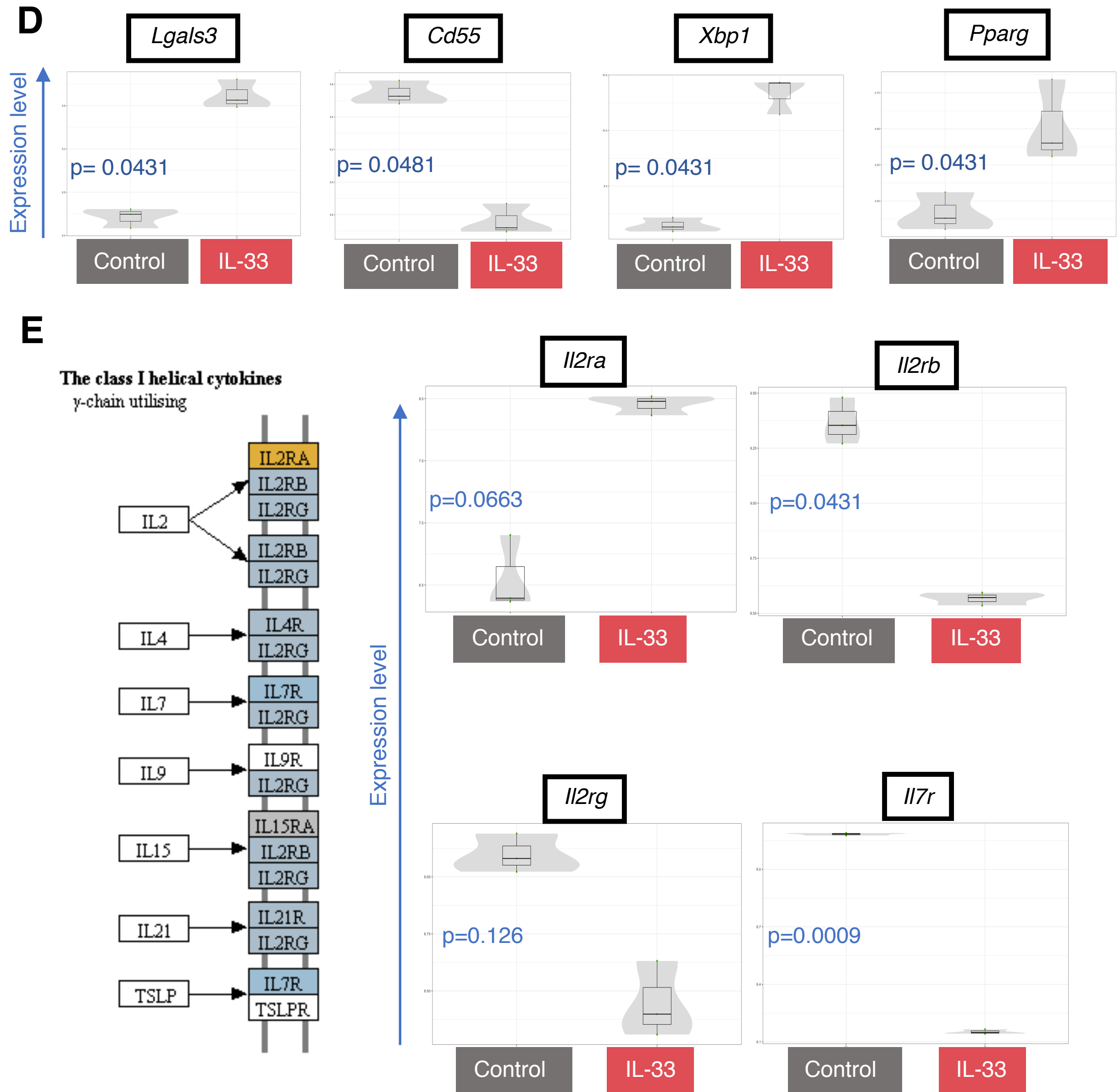


Figure 3.2.1. (continued) CBA/Ca (H-2^k) mice were injected with either PBS (control, black) or recombinant IL-33 (red), as described previously. RNA from total splenocytes was isolated from PBS (n=3) or IL-33 treated (n=3) H-2^k mice for multiplexed gene expression analysis. Selected genes associated with **(D)** T cell development and function and **(E)** cytokine receptor expression are represented in scatter violin plots. **(D-E)** Adjusted *p* value calculated with control of Benjamini-Yekutieli False Discovery Rate (FDR) (Adjusted *p* value >0.05 considered significant, FDR thresholds indicated within volcano plot).

3.2.2 Upregulation of Treg-associated genes and downregulation of effector cell type-associated genes

Overall, there was a substantial downregulation in gene expression profiles within pathways scores upon IL-33 treatment **[Figure 3.2.2A]**. Notably, genes associated with the adaptive response, cell cycle, chemokine & receptors, and cytokine & receptors pathway scores were significantly downregulated**.** Indeed, this is further supported in the cell score analysis, which demonstrated a decreased relative abundance of proinflammatory cell types such as CD8^{+} T cells, NK cells, and Th1 cells, correlating with an increased relative abundance of Tregs, B cells and neutrophils **[Figure 3.2.2B]**. Taken together, IL-33 treatment causes extensive transcriptomic changes that appears to promote a more tolerogenic environment.

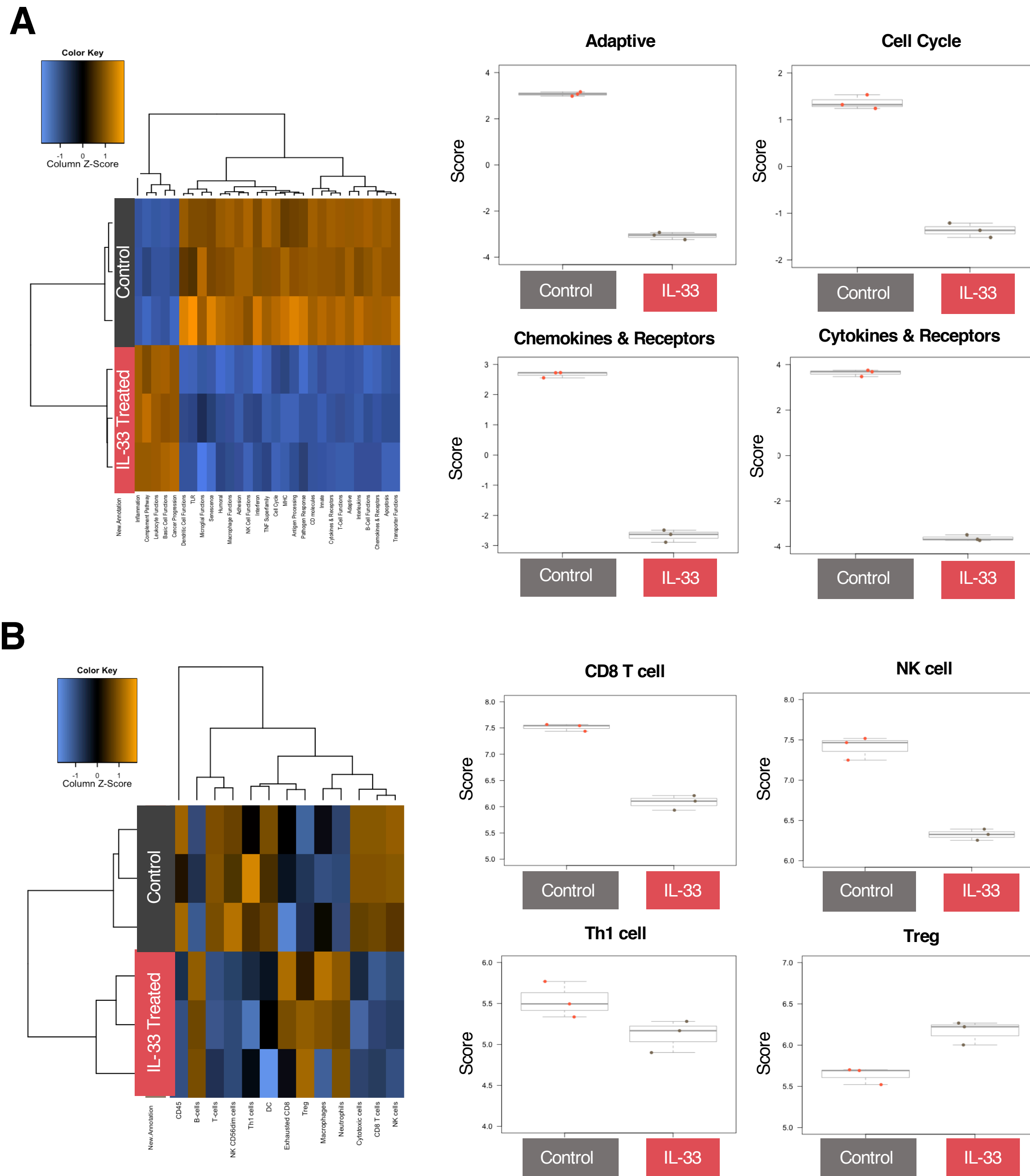


Figure 3.2.2. Upregulation of Treg-associated genes and downregulation of effector cell type-associated genes. CBA/Ca (H-2^k) mice were injected with either PBS (control, black) or recombinant IL-33 (red), as described previously. RNA from total splenocytes was isolated from PBS (n=3) or IL-33 treated (n=3) H-2^k mice for multiplexed gene expression analysis. **(A)** Heat map and dot plots of gene expression profiles of pathway scores , which are fit using the first principal component of each sample based on the individual gene expression levels for all the measured genes within a specific pathway. **(B)** Heat map and dotplots of gene expression profiles of cell type scores, which are displayed on the same scale via a Z-transformation.

3.2.3 Mouse Tregs can be expanded *in vivo* with recombinant IL-33 injections

In order to determine whether recombinant mouse IL-33 (rmIL-33) administration can expand Tregs *in vivo* as previously reported [298] and to investigate phenotypic changes upon stimulation, recombinant mouse IL-33 (rmIL-33) was injected into WT CBA mice (1ug i.p. for 6 consecutive days) and spleens, LN, and peripheral blood were taken for flow cytometric analysis.

Consistent with previous reports [298, 382], there was significant systemic expansion of $CD4^+Foxp3^+$ Tregs within the spleen, lymph nodes, and peripheral blood as reflected in percentage of total $CD4^+$ T cells, in IL-33 treated mice compared to PBS-treated control mice **[Figure 3.2.3A]**. The increase in the absolute number of $Foxp3^+$ Tregs was even more pronounced due to the significant increase in total splenocytes. Additionally, there was a dramatic upregulation of ST2 expression within $Foxp3^+$ Tregs after IL-33 injection **[Figure 3.2.3B]**. Interestingly, in IL-33-treated mice, ST2 expression was very minimal within $Foxp3^{neg}$ cells, and almost exclusively expressed on $Foxp3^+$ Treg populations. Moreover, within these three organs, ST2 expression was also very low in $Foxp3^+$ Tregs from PBS-treated mice under physiological levels.

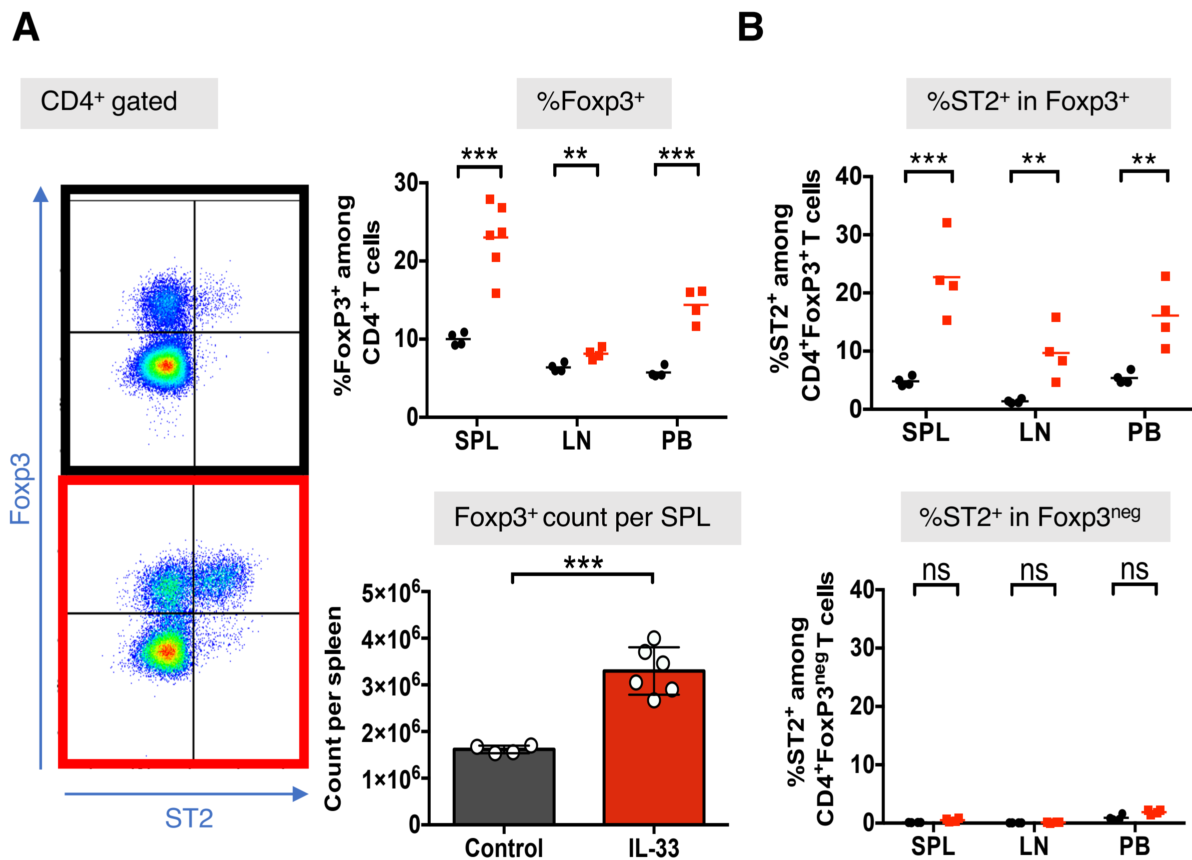


Figure 3.2.3. *In vivo* IL-33 treatment preferentially expands highly activated $ST2^{+}Foxp3^{+}$ Tregs. CBA/Ca (H-2^{k}) mice were injected with either PBS (control, black) or recombinant IL-33 (1ug/day, red) daily for 6 consecutive days (day 1-6, sacrificed on day 7) and spleens (SPL), lymph nodes (LN), and peripheral blood (PB) analysed by flow cytometry. Representative dotplots **(A)** and graphs of **(A)** Foxp3 and **(B)** ST2 expression within SPL, LN, and PB (unpaired t test, n= 3-6 mice) (***p*<0.01; ****p*<0.001; ns=not significant.).

3.2.4 IL-33 treatment preferentially activates Tregs over Teffs

Having demonstrated increase in Tregs frequency and number upon IL-33 *in vivo* treatment, its effects on cell activation (CD44 vs. CD62L expression) was further explored. Interestingly, upon IL-33 treatment there was an overall shift within $Foxp3^+$ Tregs in general towards an effector phenotype with upregulated expression of CD44 and downregulated CD62L **[Figure 3.2.4A].** Importantly, although there was a similar shift in CD44 and CD62L expression within $Foxp3^{neg}$ populations after IL-33 treatment, the increases of $Foxp3^{neg}$ $CD62L^{neg}CD44^+$ T effector (T_E) populations in both percentage and absolute count were not statistically significant, and not nearly as accentuated as within $Foxp3^+$ populations **[Figure 3.2.4B]**. ST2 expression was largely limited to $CD44^+Foxp3^+$ cells, making it a surrogate marker for Treg activation **[Figure 3.2.4C].**

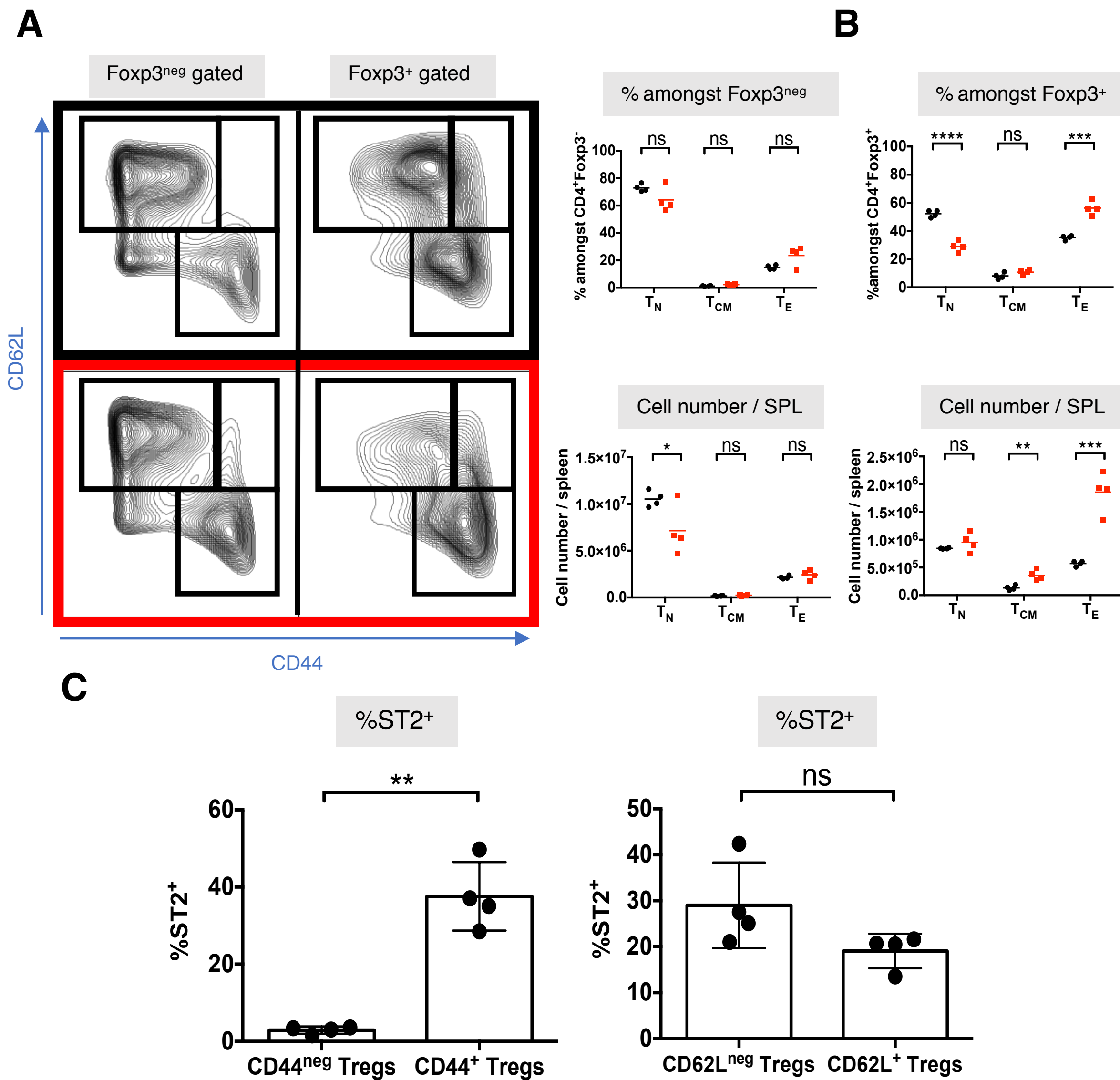


Figure 3.2.4. IL-33 treatment preferentially activates Tregs over Teffs. CBA/Ca (H-2^k) mice were injected with either PBS (control, black) or recombinant IL-33 (red), as described previously and spleens were taken for analysed by flow cytometry. Representative dotplots and graphs of CD44 and CD62L expression and populations of naïve (T_N, CD62L$^+$CD44^{neg}), central memory (T_{CM}, CD62L$^+$CD44$^+$), and effector/effector memory T cells within **(A)** Foxp3^{neg} and **(B)** Foxp3$^+$ populations and **(C)** their correlation with ST2 expression within Foxp3$^+$ Treg populations (unpaired *t* test, n=4)(**p*<0.05;***p*<0.01;****p*<0.001;*****p*<0.0001; ns=not significant.)

3.2.5 IL-33-treated mice stably maintain upregulated Foxp3 and ST2 expression levels within the peripheral blood

To assess how IL-33 treatment affects Foxp3/ST2 expression dynamics *in vivo* over time, the peripheral blood of PBS and IL-33-treated mice at multiple time points during and after cessation of treatment was analysed. Here, Foxp3 **[Figure 3.2.5A]** and ST2 **[Figure 3.2.5B]** expression peaked the day after treatment was stopped (d7), followed by a drop in Foxp3 expression soon after (d12). Nonetheless, the Foxp3 and ST2 expression levels remained significantly upregulated above physiological levels even up to two weeks post-treatment. The drop in Foxp3$^+$ Tregs within the peripheral blood may suggest their migration elsewhere or a requirement for continual IL-33 stimulation for survival. Moreover, when IL-33 was re-administered (d23-28), mice remained responsive to subsequent IL-33 activation, and Foxp3 and ST2 expression was upregulated to levels comparable with the primary treatment. The persistent effect on Foxp3 and ST2 levels within the peripheral blood even after IL-33 treatment was discontinued suggests that ST2$^+$ Tregs remain able to persist peripherally, despite being most commonly located within tissues [485-487].

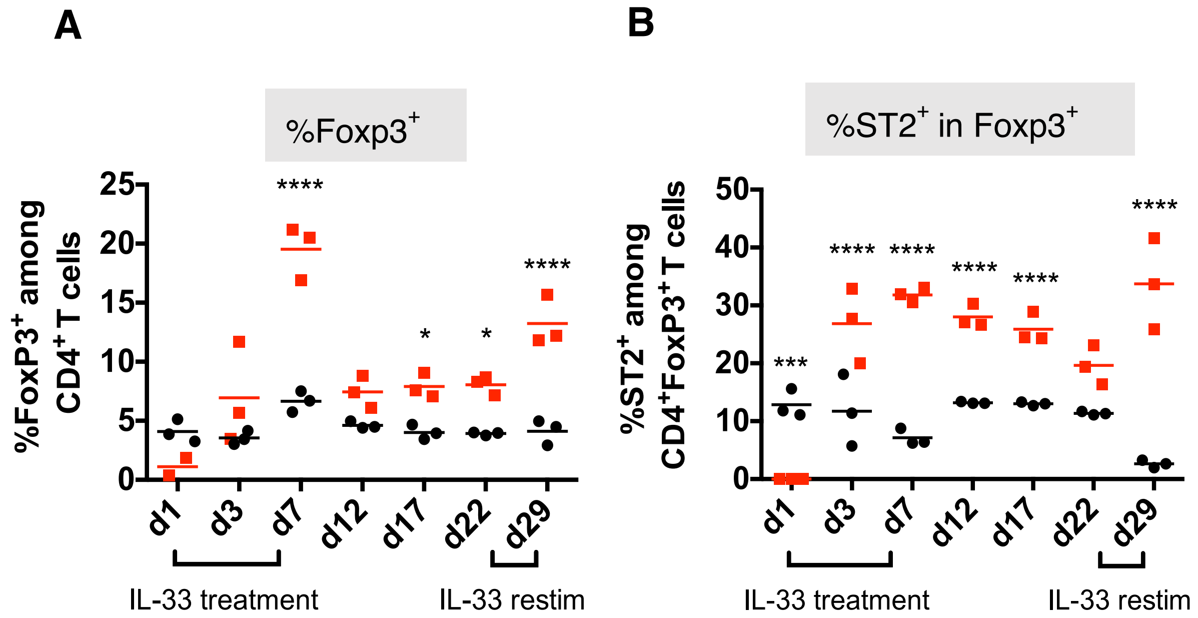


Figure 3.2.5. IL-33-treated mice stably maintain upregulated Foxp3 and ST2 expression levels within the peripheral blood. Peripheral blood time-course analysis of **(A)** Foxp3 and **(B)** ST2 expression from PBS or IL-33-injected CBA (H-2^{k})) mice. Injections were given on days 1 to 6 and days 23 to 28 (2-way ANOVA with Sidak's corrections for multiple comparisons, n=3) (****p*<0.001; *****p*<0.0001; ns=not significant.)

3.2.6 IL-33-Tregs upregulate suppressive markers

To explore the phenotype of IL-33-Tregs, expression of markers associated with Treg-mediated function was measured. IL-33-Tregs significantly upregulated their expression of the costimulatory/coinhibitory molecules CTLA-4, ICOS, and PD-1 **[Figure 3.2.6A]**. The ectonucleotidase molecules CD39 and CD73 were also upregulated **[Figure 3.2.6B]**, suggesting enhanced activity through the generation of adenosine [234, 488, 489] and/or cAMP transfer [233, 490]. CD25 expression levels were also increased **[Figure 3.2.6C]**, correlating with transcriptomic data **[Figure 3.2.1E]**. Finally, ST2 expression within each of the respective Foxp3$^+$ populations co-expressing Treg-associated markers was significantly higher in IL-33-Tregs **[Figure 3.2.6A-C]**. This high correlation between the expression of ST2 and other suppressive markers may be an indication that IL-33 has a direct effect in enhancing Treg suppression.

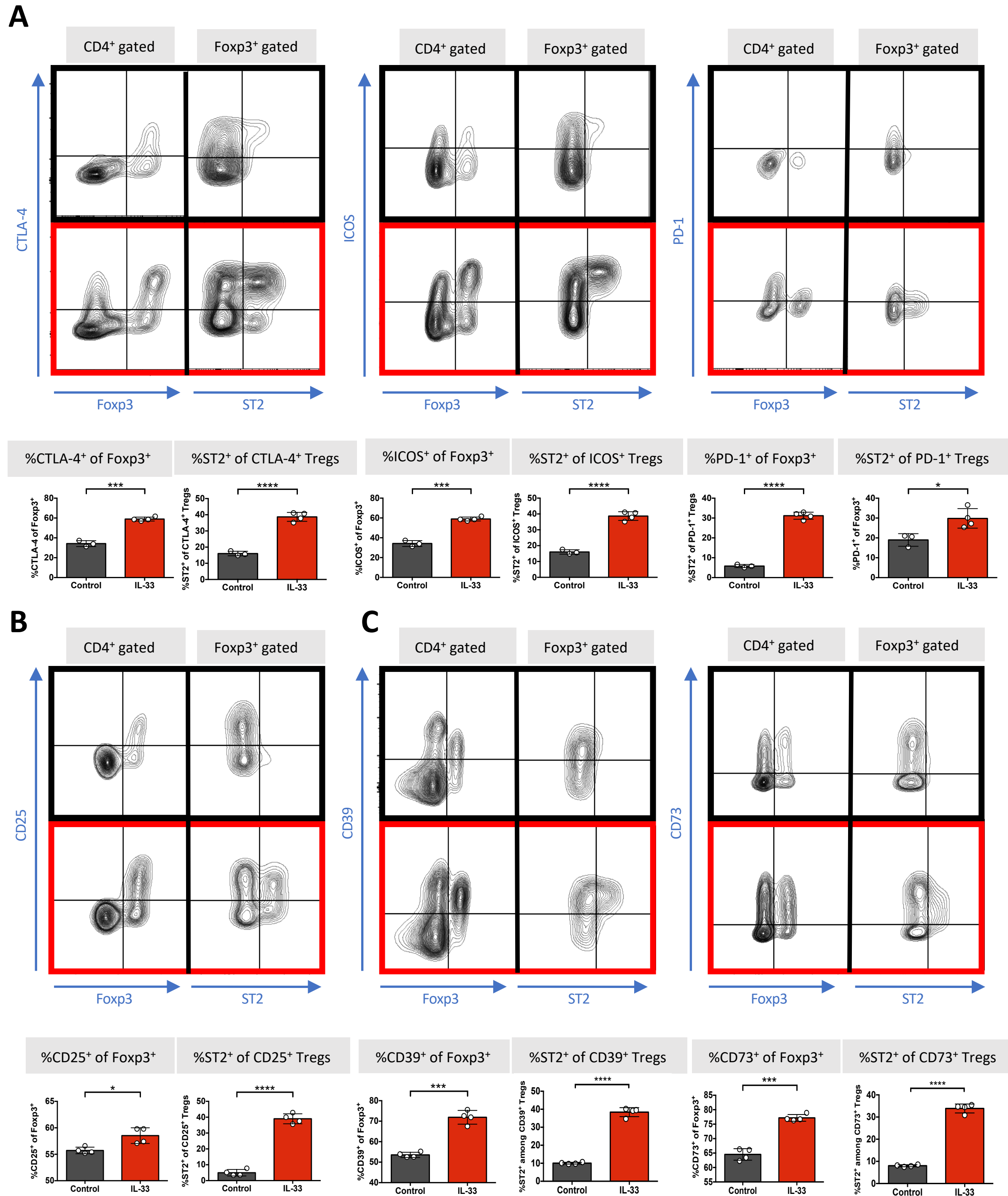


Figure 3.2.6. IL-33-Tregs upregulate Treg-associated functional molecules. CBA/Ca (H-2^k) mice were injected with either PBS (control, black) or recombinant IL-33 (red), as described previously. Representative dotplots and graphs of expression of **(A)** the costimulatory markers CTLA-4, ICOS, and PD-1, **(B)** CD25, and **(C)** the ectonucleotidases CD39 and CD73, all within Foxp3$^+$ populations and expression of ST2 within their respective Treg populations (unpaired *t* test, n=4) (**p*<0.05; ****p*<0.001; *****p*<0.0001; ns=not significant.)

3.2.7 IL-33 treatment with concurrent rapamycin treatment demonstrate enhanced Treg expansion

Additionally, IL-33 treatment was used in combination with clinically-relevant forms of treatment that are known to promote Treg populations in order to determine whether combined therapy will yield an additive effect. Here, the effects of a mTOR inhibitor, rapamycin, with IL-33 treatment was investigated. Rapamycin has been demonstrated to both hinder activation of Teffs and to expand/induce Tregs and enhance their survival [491]. Thus, the Treg-dominant response towards IL-33 stimulation suggested that this feature can be further accentuated and optimised with rapamycin.

Here, WT CBA mice were injected with PBS or IL-33 as previously described in one group (d1-6), with other groups received only rapamycin (d1-3) or a combination of both (Dose and dosing regimen based on Andrew Whatcott's thesis, 2016) **[Figure 3.2.7A]**. As expected, Foxp3^{+} expression within CD4^{+} was not significantly increased upon rapamycin treatment alone within the spleens and peripheral blood, while IL-33 treatment alone enhanced Foxp3 expression significantly more than either PBS or rapamycin alone **[Figure 3.2.7B]**. This was consistent with ST2 expression within Foxp3^{+} Tregs. Interestingly, there was a significantly enhanced effect on Foxp3 and ST2 expression with combined IL-33 and rapamycin treatment in both the spleen and peripheral blood compared with IL-33 treatment alone. Moreover, to investigate the effect of rapamycin and IL-33 on conventional T cells, CD44 and CD62L expression was measured to determine the relative frequency of naïve and effector subsets. Although not significant, in agreement with our previous data, there was a slight increase in CD62L^{neg}CD44^{+} T_E within non-

Treg populations after IL-33 treatment **[Figure 3.2.7C]**. However, this increase in the non-Treg T_E population was minimised when rapamycin was used simultaneously with IL-33.

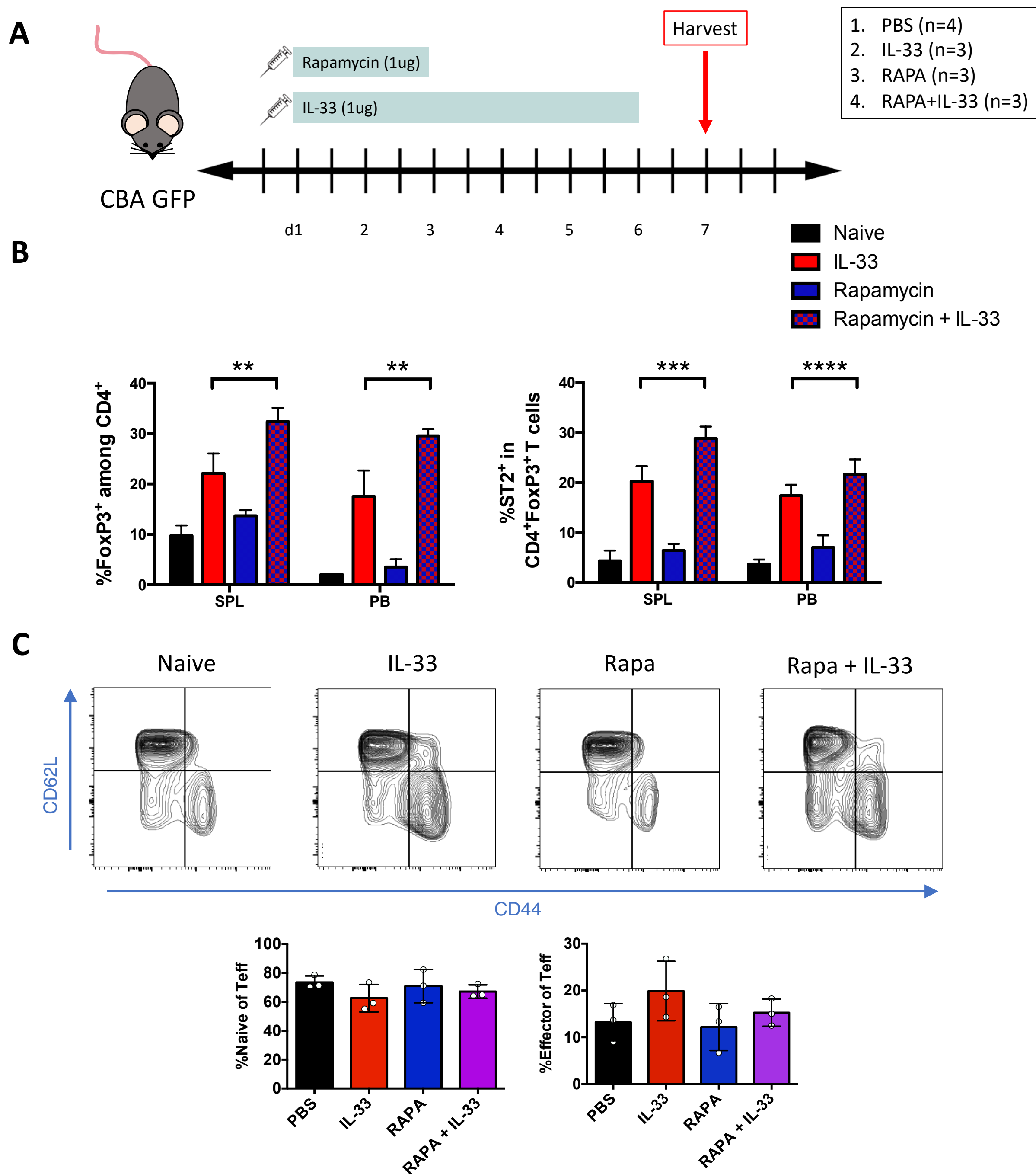


Figure 3.2.7. IL-33 treatment with concurrent rapamycin treatment demonstrate enhanced Treg expansion. (A) CBA/Ca (H-2^k) mice were injected with either PBS (black, d1-6), recombinant IL-33 (d1-6, 1ug/day, red), rapamycin (d1-3, 1ug/day, blue), or both IL-33 and rapamycin (purple)) and spleens (SPL) were analyzed by flow cytometry. Graphs show **(B)** expression of Foxp3 in CD4$^+$ T cells and expression of ST2 in Foxp3$^+$ Tregs. **(CC)** Representative dotplots and graphs show percentage of naïve (CD62L$^+$CD44^{neg}) and effector (CD62L^{beg}CD44$^+$) T cells within non-Treg population (gated as CD4$^+$Foxp3^{neg}). (A-C) n=3-4 per group. (A/C) Statistics analysed with 1-way ANOVA with Tukey's corrections for multiple comparisons (**p<0.01; ***p<0.001; ****p<0.0001.)

3.2.8 IL-33 with tolerogenic allogeneic dendritic cells can expand, but not induce Tregs

Further experiments were next conducted to investigate whether Treg can similarly be expanded with IL-33 stimulation *in vitro* for two central reasons. For one, it was necessary to further elucidate the origin and proliferative capacity of IL-33-responsive Tregs. Secondly, developing a reliable IL-33-dependent method for Treg expansion could have potential for clinical application. Currently, there are now well-established GMP-approved expansion protocols for the generation of Tregs for use in clinical trials. However, there are indeed still questions regarding the cell product's effectiveness, stability, and practicality. Thus, methods to expand Tregs in greater numbers or those that generate more potent or specific Treg populations are in demand.

In order to first broadly investigate the impact of IL-33 on mouse $CD4^+$ populations under allogeneic stimulation, *in vitro* co-culture models were utilised **[Figure 3.2.8A]**. Our group has previously developed a protocol for mouse Treg generation utilizing allogeneic tolerogenic GM-CSF and TGF-β-treated dendritic cells (GT-DCs) and recombinant cytokines (i.e. IFN-γ) [492]. First, the role of IL-33 in the *in vitro* expansion or generation of mouse Tregs was investigated. Total $CD4^+$, $CD4^+CD25^{neg}$ (Treg-depleted, T effector group), or $CD4^+CD25^+$ cells (Treg group) were isolated from splenocytes from WT CBA mice, and then stimulated with WT B6 alloDCs (GT-DCs) with IL-33 (10 ng/mL).

While the addition of IL-33 significantly expanded $Foxp3^+$ Tregs within both $CD4^+$ and $CD4^+CD25^+$ populations, there were no significant increases when culturing $CD4^+CD25^{neg}$ populations under

similar conditions **[Figure 3.2.8B-C]**. This indicated that IL-33 may have a greater impact on Treg expansion than induction. Additionally, treatment with IL-33 significantly increased ST2 expression within $Foxp3^+$ populations on all starting populations, even within the $CD4^+CD25^{neg}$ cultures in which Treg populations were minimal. As indicated through staining $CD4^+CD25^+$ cultures with a violet proliferation dye (VPD), $Foxp3^+$ Tregs expanded significantly more with IL-33 stimulation than with alloDC treatment alone **[Figure 3.2.8D].** Finally, consistent with the *in vivo* data, Tregs within these cultures shifted from a naïve to an effector phenotype, with upregulated CD44 and downregulated CD62L **[Figure 3.2.8E]**. Taken together, these data demonstrate that IL-33 can synergise with tolerogenic dendritic cells to expand, but not induce, $Foxp3^+$ Tregs with high ST2 expression *in vitro*.

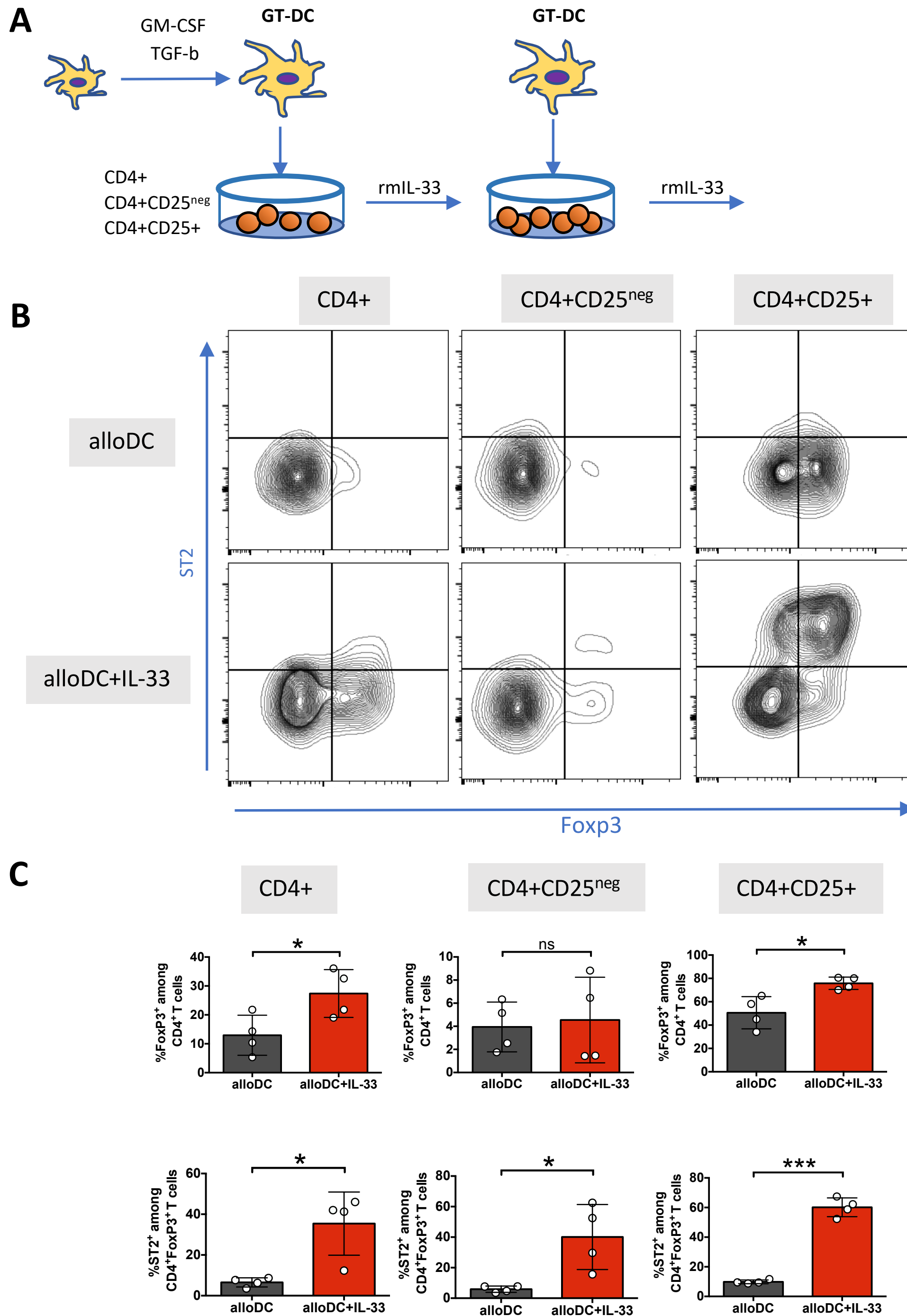


Figure 3.2.8. IL-33 with tolerogenic allogeneic dendritic cells can expand, but not induce Tregs. (A) $CD4^+$, $CD4^+CD25^{neg}$, and $CD4^+CD25^+$ T cells were isolated from CBA/Ca (H-2^k) spleens, stained with violet proliferation dye (VPD), and stimulated with IL-33 (10ng/mL) and/or C57BL/6 (H-2^b) allogeneic DCs (GT-DC), which were generated in the presence of GM-CSF (10ng/mL) and TGF-β (2ng/mL). Cultures were restimulated with new GT-DC and IL-33 after one week and were harvested for flow cytometric analysis after two weeks. **(B-C)** Representative dot plots and graphs of expression of Foxp3 and ST2 of $CD4^+$, $CD4^+CD25^{neg}$, $CD4^+CD25^+$ cultures after stimulation with IL-33 and GT-DC. (unpaired *t* test, n=4) (**p*<0.05; ****p*<0.001; ns=not significant.)

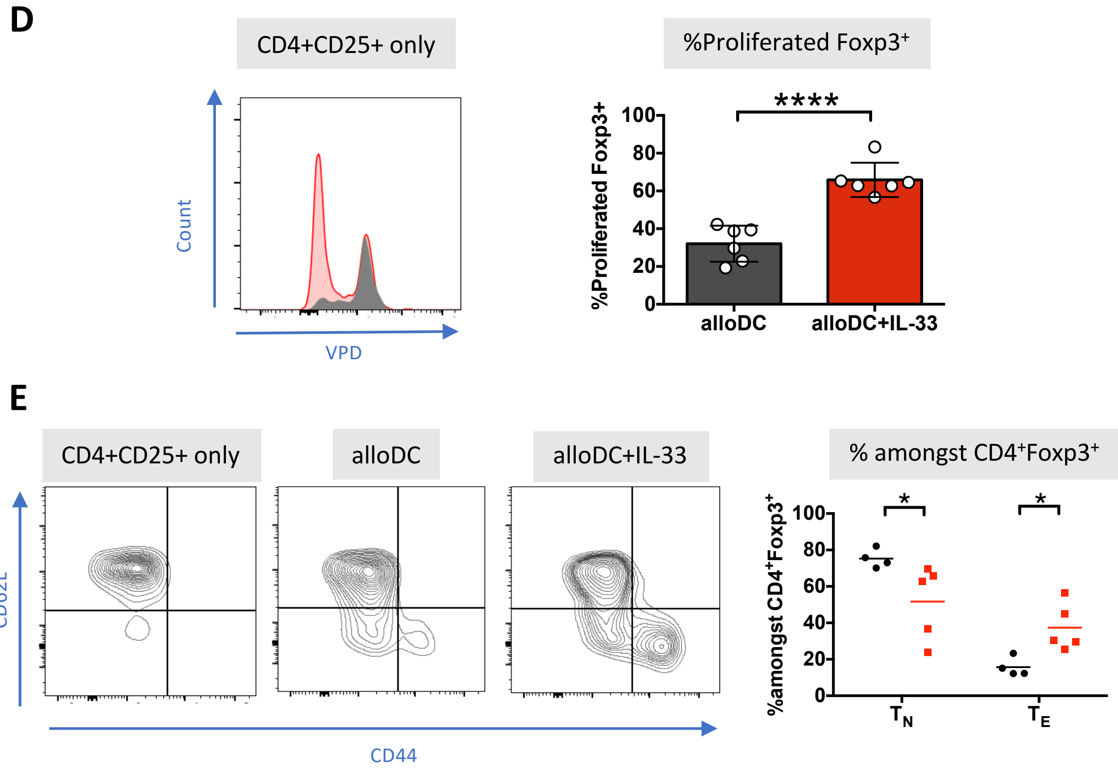


Figure 3.2.8. (continued) IL-33 with tolerogenic allogeneic dendritic cells can expand, but not induce Tregs. (D) Histogram and graph of Foxp3^{+} cells proliferation within CD4^{+}CD25^{+} cultures. **(E)** Representative dot plots and graph of CD44 and CD62L expression within CD4^{+}CD25^{+} cultures. (unpaired *t* test, n=4) (**p*<0.05; *****p*<0.0001; ns=not significant.)

3.2.9 Tregs can be expanded significantly with immunogenic alloDCs with IL-33

Based on the significant expansion of Tregs with tolerogenic DCs, it was next investigated whether similar IL-33-dependent Treg expansion and phenotypic changes would be observed using more conventionally immunogenic DCs. Thus, an adapted *in vitro* model for Treg expansion with immunogenic DCs [370] was used **[Figure 3.2.9A]**. Bone marrow-derived DCs from WT B6 mice were generated in the presence of GM-CSF and IL-4 (GM-IL-4-DCs) instead of TGF-β. GM-IL-4-DCs and IL-33 were cultured with $CD4^+$-isolated WT CBA-derived splenocytes, which were stained with antibodies (CD4, CD25, Foxp3, ST2) and violet proliferation dye (VPD) to measure both Treg proliferation and suppression of effector T cell populations.

Here, $CD4^+$ T cells cultured with GM-IL-4-DC stimulation alone significantly upregulated their expression of Foxp3, along with ST2 expression of $Foxp3^+$ Tregs **[Figure 3.2.9B]**. However, concurrent IL-33 treatment significantly enhanced this effect, consistent with the cultures using tolerogenic dendritic cells. Moreover, in measuring VPD dilution, a significantly larger proportion of $Foxp3^+$ Tregs proliferated with IL-33 and alloDC treatment than alloDC treatment alone **[Figure 3.2.9C]**. Conversely, $Foxp3^{neg}$ cells demonstrated less proliferative capacity in the presence of IL-33.

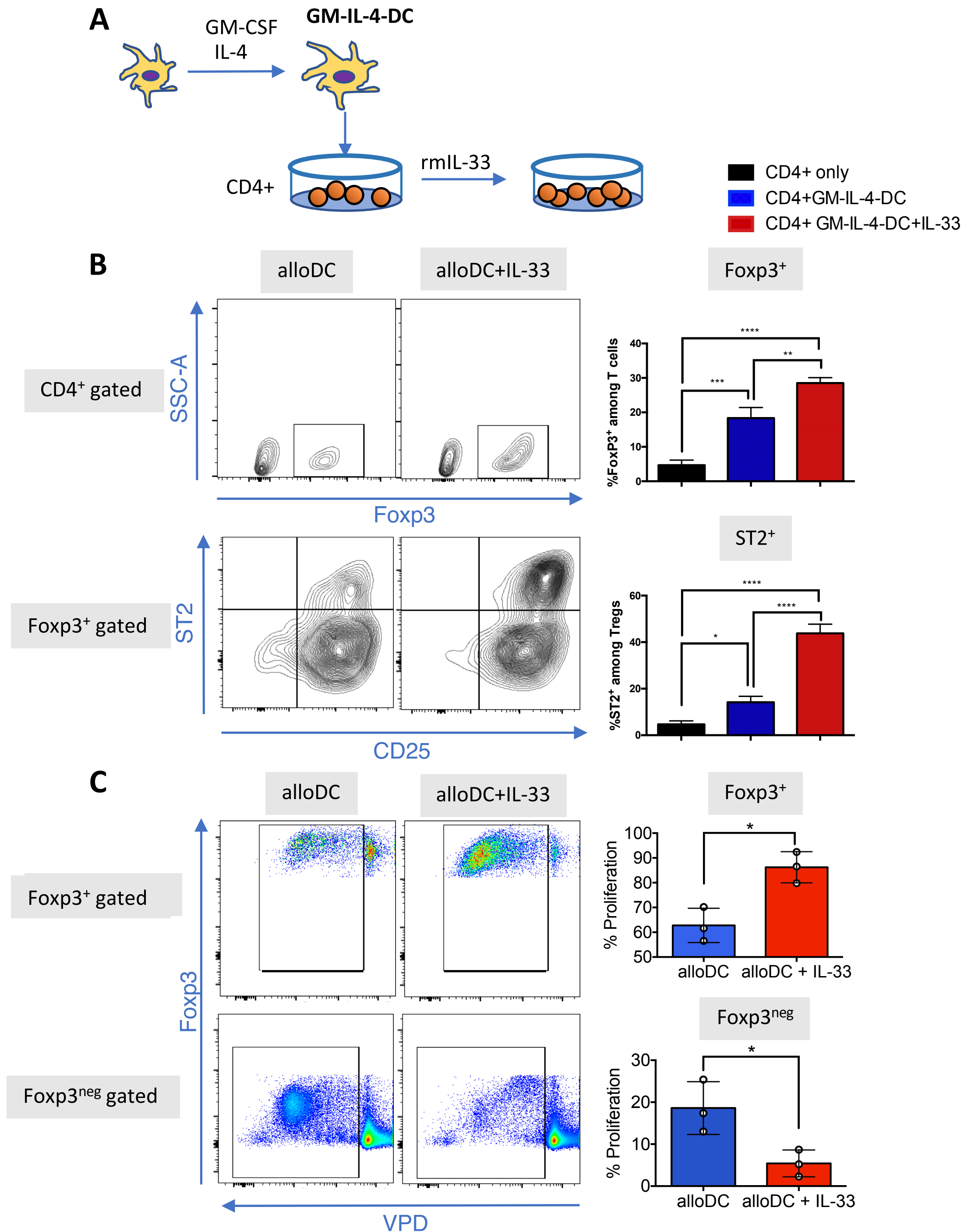


Figure 3.2.9. IL-33 with immunogenic dendritic cells can preferentially expand Foxp3⁺ populations over Foxp3ⁿᵉᵍ. (A) CD4⁺ T cells were isolated from CBA/Ca (H-2ᵏ) spleens, stained with violet proliferation dye (VPD), and stimulated with IL-33 (10ng/mL) and/or C57BL/6 (H-2ᵇ) allogeneic DCs (GM-IL-4-DC), which were generated with GM-CSF (10ng/mL) and IL-4 (10ng/mL). Cultures were incubated for five days and harvested for flow cytometric analysis. Representative dot plots and graphs of expression of **(B)** Foxp3 and ST2 and **(C)** proliferation of Foxp3⁺ and Foxp3ⁿᵉᵍ populations after GM-IL-4-DC with/without IL-33 stimulation. (B) Analysed with 1-way ANOVA with Tukey's corrections for multiple comparisons) (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.) (C) Analysed with unpaired students *t* test (*p<0.05).

3.3 Discussion

This chapter sheds light on the effects of IL-33 treatment on the general immune environment with a focus on Tregs in particular. Based on these findings, IL-33 preferentially expands, but does not induce, a population of Tregs. This distinct population of IL-33-expanded Tregs had high expression of ST2 and suppressive Treg markers with a shift towards an effector phenotype. The upregulation in Treg populations and Treg-associated genes correlated with an overall downregulation in genes associated with proinflammatory cell types. These observations will help provide guidance in assessing the feasibility of IL-33-based treatments in regard to their potential undesirable off-target proinflammatory effects.

While other studies have demonstrated the effects of exogenous IL-33 treatment on isolated immune populations, an understanding of how Tregs and other immune cell populations collectively change in response to IL-33 has previously been difficult to characterise *in vivo*. Our broad examination using transcriptomic analysis presents a snapshot of the effects of *in vivo* IL-33 treatment on the immune cells. The significant overall downregulation of integral T cell genes **[Figure 3.2.1]** and other genes associated with many proinflammatory cell types (i.e. NK cells, CD8 T cells, and Th1 cells), coupled with an upregulation of Treg-associated genes **[Figure 3.2.2]**, suggests that cell types such as Tregs are more responsive towards IL-33 *in vivo*. Indeed, this is consistent with previous studies that demonstrate that cells such as Tregs have stable expression of ST2, while cytotoxic T cells and Th1 cells only transiently express ST2 under certain conditions [377, 378]. Moreover, the selective increase in *Il2ra* (CD25) and decrease of the other IL-2R subunits may suggest that there is an immune shift conducive for preferential IL-2 consumption

by Tregs, rather than other cell types that have lower expression of CD25 or those that express the low affinity $\beta\gamma$ IL-2R [493]. This may be an indication of Tregs exerting suppression against other T cells by depriving CD25 from the environment [231].

The immunophenotyping data presented here is consistent with previous findings suggesting that there is a preferential activation or expansion of Treg populations after IL-33 treatment. ST2 expression was very lowly expressed in Foxp3^{neg} populations in control mice, but minimally expressed within Foxp3^{+} populations **[Figure 3.2.3.]**. This is again corroborated by our transcriptomic data suggesting that Tregs may be more responsive to IL-33 than some other immune populations at the physiological state. Additionally, the finding that IL-33 treatment significantly upregulates its own receptor within Foxp3^{+} but not within Foxp3^{neg} populations may be important in regards to how the IL-33/ST2 axis is regulated at the molecular level**.** With respect to the idea that ST2 is self-amplified through a positive feedback loop [372], the population that has higher expression of ST2 or is more responsive to IL-33 may then upregulate ST2 at a higher rate than populations that begin with a lower expression of ST2. Although not demonstrated from previous studies, this may allow for populations highly expressing ST2 to continuously hoard IL-33 from the environment similarly to how Tregs may deprive IL-2 from Tconvs with their high expression of CD25. In a similar manner, the significant increase in T_E populations (CD62L^{lo}CD44^{+}) within Foxp3^{+} but not Foxp3^{neg} populations after IL-33 treatment **[Figure 3.2.4.]** suggests that there is selective activation of Tregs within the CD4^{+} population.

Assessment of Foxp3/ST2 expression dynamics *in vivo* over time after IL-33 treatment also gives valuable insight regarding the activity of ST2^{+} Tregs *in vivo* and the duration of the effect of IL-33

on Tregs **[Figure 3.2.5.]**. Given that ST2$^+$ Tregs are lowly expressed within peripheral blood and are most commonly identified as tissue-resident Tregs, it was surprising that Foxp3 and ST2 expression remained upregulated above physiological levels within the peripheral blood for up to two weeks after cessation of treatment. In particular, many recent studies describe important roles for IL-33 and ST2$^+$ Tregs in regulating local inflammation and maintaining metabolic homeostasis within peripheral tissues, which have much higher frequencies of ST2$^+$ Tregs than in secondary lymphoid organs or blood [485-487]. Indeed, Delacher et al. found that almost all fat-derived Foxp3$^+$ Tregs are ST2$^+$ [487], suggesting that ST2$^+$ Tregs have clearly defined roles within certain peripheral tissues. Thus, this brings into question the function of ST2$^+$ Tregs within other tissues that lowly express them. While this question will be discussed further in later chapters, the data presented in this chapter demonstrate that ST2$^+$ Tregs can persist within the peripheral blood, suggesting that they may hold other functions separate from those described in specialised tissues.

While the mechanisms by which specific Treg populations exert their suppressive functions are highly variable, expression of certain Treg-associated markers provides an indication of their preferred mechanisms **[Figure 3.2.6.]**. These findings are in agreement with previous studies that have observed an upregulation of suppressive markers in ST2$^+$ Tregs after IL-33 treatment [370]. Specifically, in this study there was enhanced expression of costimulatory markers (CTLA-4, ICOS, PD-1) as well as of CD25 after IL-33 treatment. The upregulation of ectoenzyme CD39/CD73 markers and their positive correlation with ST2 expression within Tregs may form part of a larger facet to IL-33-Treg biology. These molecules can augment the ability of IL-33-Tregs to inhibit Teff responses through the production of adenosine from ATP. Similar to IL-33, extracellular ATP can

act as a danger signal in response to cell damage [494], with reports suggesting that IL-33 and ATP may act to induce the release of one another under inflammatory conditions [495, 496]. Thus, coupled with reports that there is substantial release of IL-33, ATP, and other alarmin/DAMPS after transplantation, IL-33-Tregs may have a unique potential in modulating inflammation within an allograft microenvironment [497-499].

Furthermore, establishing an *in vitro* method to upregulate Tregs with IL-33 stimulation allowed us to further explore mechanisms and features of IL-33-dependent expansion. *In vitro* IL-33-expanded Tregs using either tolerogenic **[Figure 3.2.8.]** or immunogenic alloDCs **[Figure 3.2.9.]** had similar phenotypic features of those observed *in vivo*, including high expression of ST2 and CD25, and a skew towards an effector phenotype. Moreover, the preferential proliferation of $Foxp3^{+}$ over $Foxp3^{neg}$ T cells supported our *in vivo* findings. Interestingly, IL-33 stimulation significantly expanded Tregs, but was not able to induce Tregs**.** This is consistent with previous literature, in which a report by Delacher et al. using methylome analysis found that the distinct epigenetic profile of ST2-expressing Tregs was only present within thymic Tregs but not peripherally induced Tregs [487]. Another recent study by Yang et al. traced adoptively transferred WT T cells and WT or $ST2^{-/-}$ Tregs in mice with acute GVHD and reported that the dominant population of Tregs within the gut were from thymic origin. Meanwhile, ST2 inhibition within thymic donor Tregs reduced Treg frequency in the gut within these models [383]. Taken together, this strongly suggests that IL-33-dependent enhancement of Tregs works through the expansion of existing thymic Tregs, rather than the conversion of $Foxp3^{neg}$ Teffs into peripherally-induced Tregs, which previous studies have suggested may be less stable than thymic Tregs. Thus, this finding may have several implications on the applications of IL-33-based therapy.

Taken together, these findings that illustrate that there is preferential expansion or activation of Treg populations have implications in terms of the feasibility of *in vivo* IL-33 treatments. The primary concern for *in vivo* IL-33 treatment is the potential off-target proinflammatory effects given its highly pleiotropic nature. This is a similar concern to potential side effects caused by *in vivo* IL-2 treatment, which may activate proinflammatory cells along with Tregs [479, 480]. Nonetheless, both our transcriptomic and immunophenotyping data suggest that IL-33 promotes a more tolerogenic environment overall. It is promising that IL-33 treatment-induced activation was largely restricted to Foxp3^{+} cells, including the selective upregulation of ST2 within Foxp3^{+} populations. In particular, upregulated ST2 expression within Foxp3^{neg} populations can have enhanced proinflammatory effects [380]. This indicates that i.p. IL-33 treatment may not elicit a Teff-driven proinflammatory response. Moreover, the upregulation of ST2 and Foxp3 after a second course of stimulation two weeks after the cessation of the first course **[Figure 3.2.5]** also suggests that circulating ST2^{+} Tregs are responsive to restimulation by IL-33. This may be an important consideration in the context of transplantation, in which ST2^{+} Tregs may again respond and potentially be restimulated by IL-33 released in the allograft microenvironment [498, 500].

Nonetheless, it is critical to keep in mind that these observations reflect changes in immune landscape upon IL-33 treatment in a normal physiological state. Indeed, exogenous IL-33 in other contexts may elicit induction of proinflammatory response that can exacerbate various conditions. Although the IL-33 treatment regimen has been well tolerated by mice with no salient changes in health or outward appearance, changes within non-lymphoid organs upon IL-33 treatment was not closely monitored in this study. However, other studies have observed that i.p. IL-33 treatment can indeed elicit features of inflammation within organs such as the lung,

small intestine, and esophagus, in which they detected infiltration of immune cells such as eosinophils and neutrophils. [302]. Coupled with reports that expression of IL-33 can induce conditions such as eosinophil-driven airway inflammation in the lungs [501], IL-33 administration may not be well-tolerated in certain settings due to its role in activating innate immunity. Additionally, another report has demonstrated that IL-33 injections can also trigger B cell expansion and autoantibody formation, which may exacerbate or induce autoimmune conditions [502]. Indeed, our transcriptomic analysis of splenocytes from IL-33-treated mice support this in regard to an upregulation of these immune subsets. Cell score analysis revealed that there was an increase in the relative abundance of genes associated with B cells and neutrophils **[Figure 3.2.2]**. Moreover, among the most differentially upregulated genes in IL-33-treated mice was *Ccl24*, which has a major role in eosinophil and neutrophil chemotaxis [503]. Therefore, while IL-33 administration has been demonstrated to be well-tolerated and may enhance tolerogenic properties in certain conditions, it is necessary to heed caution in its direct use *in vivo*.

Collectively, from the phenotyping data, it is difficult to determine whether IL-33 has a direct role in downregulating proinflammatory immune cells and/or an indirect role in dampening the overall immune system through the selective expansion of regulatory cells. Thus, it is necessary to also measure the suppressive function of IL-33-expanded Tregs to help investigate this question. This will be addressed in the following chapter.

In conclusion, the results in this chapter demonstrate a shift in balance of immune cell populations in response to IL-33 stimulation with a specific effect on Treg number and phenotype. From these data and previous literature, there are evident concerns for the *in vivo*

administration of IL-33 as a treatment method due to its potential off-target effects. Thus, subsequent experiments will focus on assessing the suppressive function of IL-33-expanded Tregs and exploring their application within adoptive Treg cell therapy.

4 Manipulation of the IL-33/ST2 axis for Enhanced Treg Function

4.1 Introduction

One of the main focuses of recent research and clinical trials in Treg cell therapy has been on the adoptive transfer of *ex vivo*-expanded Tregs. Our group is currently a partner in the EU-funded ONE Study (Phase I) and MRC-funded TWO Study (Phase IIb) clinical trials, which aim to investigate the safety and therapeutic efficacy of Tregs in living donor renal transplantation. In these trials and a number of others, adoptive Treg therapy has been well-tolerated in patients with no significant adverse effects.

Nonetheless, there are limitations in current strategies that require optimisation. For example, standard expansion protocols (commonly with anti-CD3/anti-CD28 beads, IL-2, and rapamycin) with repetitive stimulation can potentially yield final cell products that lose potency or stability, which can result in effector T cell overgrowth [504-506]. Moreover, most current trials expand polyclonal Tregs, which could elicit non-specific immunosuppressive responses. It has been estimated that a polyclonal Treg to Tconv ratio of 1:1-1:2 is sufficient to prevent allograft rejection through mouse studies [187, 255-257]. The likely high number of cells therefore required in humans brings significant challenges. Additionally, infusion of polyclonal Tregs can

theoretically carry the risk of global over-immunosuppression [507]. Methods to enhance Treg proliferation, stability, or function may therefore help to optimise current strategies.

Harnessing the tolerogenic properties of IL-33 may thus offer a novel and practical strategy. As described in prior literature and our results outlined in the previous chapter, IL-33 can significantly expand effector Treg populations that upregulate suppressive markers. However, there are concerns of IL-33 also activating immunogenic cells. **Thus, in this chapter, the goal is to further characterise IL-33-expanded Tregs and assess their functionality within the context of transplantation. By isolating IL-33-expanded Tregs, we aim to exploit IL-33-associated protective features, while avoiding its proinflammatory effects.**

Chapter hypothesis and aims

Hypothesis: The IL-33/ST2 axis plays a protective role in transplantation through a Treg-dependent mechanism.

Aim 1: To determine the suppressive capacity of IL-33 expanded Tregs in the context of transplantation.

Aim 2: To assess strain to strain mouse differences in IL-33 responsiveness.

4.2 Results

4.2.1 Mouse IL-33-expanded Tregs do not demonstrate greater suppressive function *in vitro*

Building on the findings in Chapter 3 that reveal the suppressive phenotype of IL-33-expanded Tregs (IL-33-Tregs), we investigated whether these molecular changes resulted in enhanced functional activity *in vitro*. Data from previous studies are conflicting, with some showing enhanced Treg function with the addition of exogenous IL-33 into suppression cultures [383], and others showing loss of suppression [384]. IL-33-induced Tregs have also previously been shown to have no advantage over non-IL-33 treated Tregs [299, 370]. Moreover, the majority of these previous studies were performed using C57BL/6 or BALB/C strains.

The suppressive capacity of IL-33 Tregs from CBA mice was therefore assessed compared to control Tregs against bead-stimulated control $CD4^{+}CD25^{neg}$ Teffs at multiple Treg:Teff ratios. Surprisingly, there was no significance when using bead-stimulated conditions, and in fact IL-33-Tregs were found to trend towards promoting proliferation at the lowest Treg:Teff (1:4) ratios **[Figure 4.2.1A/B]**. However, this may be attributed to greater access of cells to anti-CD3/anti-CD28 beads, leading to overstimulation of already highly phenotypically activated IL-33-Tregs.

Subsequently, Tregs were also assessed within suppression assays using allogeneic immunogenic dendritic cells (allo-DCs) (generated with GM-CSF and IL-4), which provide weaker stimulation than beads and are more physiologically-relevant. Similarly, no significant differences in suppression between control and IL-33-Tregs were observed under these conditions either

[Figure 4.2.1A/C]. Taken together, although they upregulated many markers central to major Treg mechanisms of suppression, IL-33-Tregs did not demonstrate functional advantages *in vitro.*

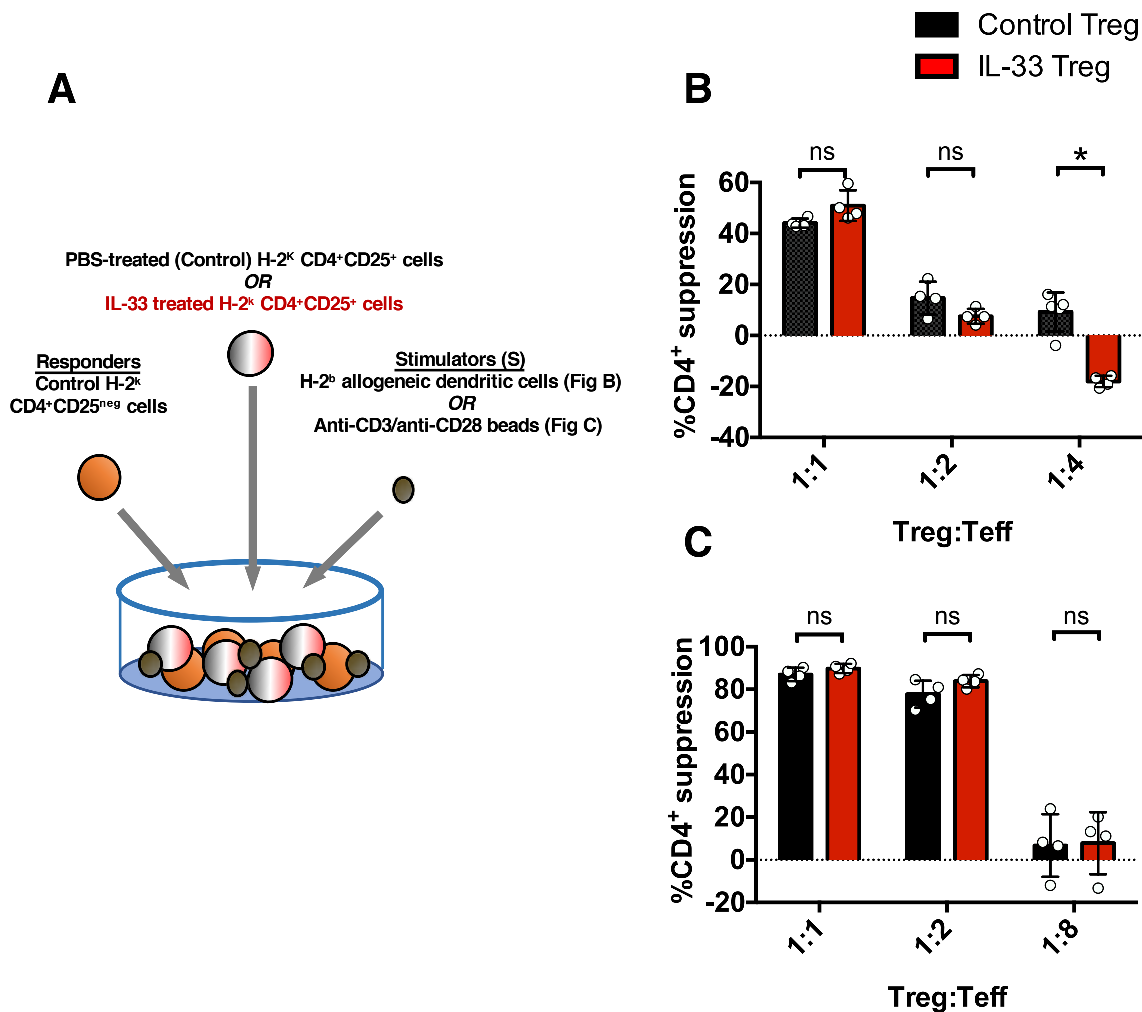
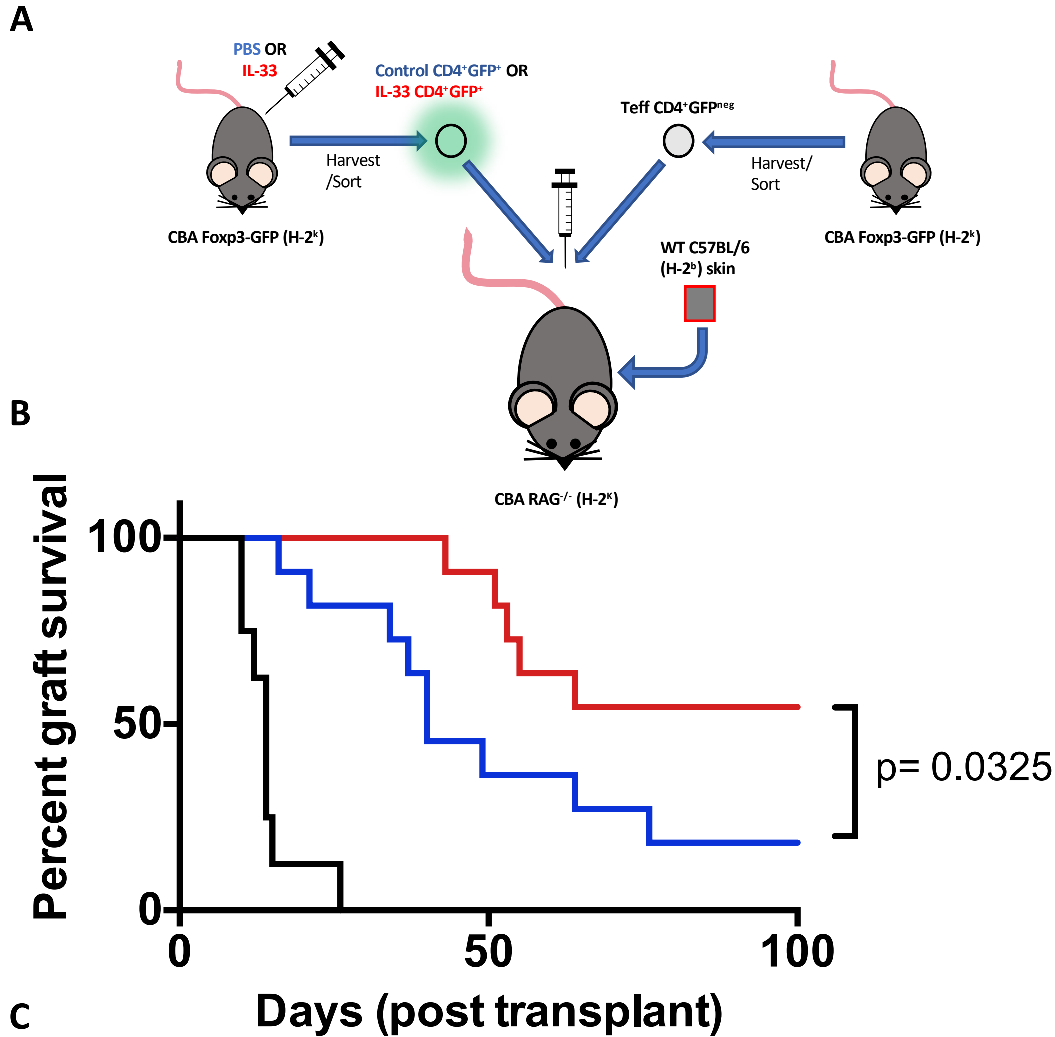


Figure 4.2.1. Mouse IL-33-expanded Tregs do not demonstrate greater suppressive function in vitro. **(A)** Schematic of *in vitro* suppression assay design. $CD4^+CD25^+$ Tregs isolated from splenocytes of PBS- or IL-33-treated CBA mice were cultured with $CD4^+CD25^{neg}$ Teff responders stimulated with **(B)** anti-CD3/anti-CD28 beads or **(C)** H-2^b allogeneic DC cells to assess their suppressive potency *in vitro*. **(B-C)** Data are shown as means ± SD. Unpaired, independent groups (1:1, 1:2, 1:4, and 1:8) of 2 were analysed using unpaired *t* tests. (*p<0.05; ns=not significant.)

4.2.2 IL-33-expanded Tregs are better able to prolong skin allograft tolerance

In vitro assays cannot fully recapitulate the full spectrum of elements that may be relevant to Treg activity. Indeed, previous studies have demonstrated that ST2^{+} Tregs can sustain more stable expression of Foxp3 than their ST2$^{-/-}$ counterparts through IL-33 signalling under inflammatory environments [298]. Moreover, other studies have demonstrated that exogenous IL-33 treatment can prolong allogeneic heart and semi-allogeneic skin graft survival [299, 390].

Therefore, it was hypothesised that IL-33-Tregs may hold functional advantages *in vivo* that may not be reflected in the *in vitro* suppression models. Moreover, no studies have yet assessed the *in vivo* function of Tregs isolated from IL-33-treated mice. Thus, to address this, we used a well-characterised skin transplantation model in which CBA (H-2^{k}) RAG$^{-/-}$ mice receive a fully MHC-mismatched WT C57BL/6 (H-2^{b}) skin allograft and an adoptive transfer of H-2^{k} effector CD4^{+} cells that results in graft rejection with a median survival time (MST) of 14 days. Mice were treated with Tregs at ratios that are known to only moderately extend graft survival **(Figure 4.2.2A)**. As expected, treatment with control Tregs resulted in a modest extension of allograft survival (MST 40 days, **Figure 4.2.2B/C)**. Remarkably, IL-33-Tregs demonstrated enhanced activity in comparison to control Tregs, resulting in long term engraftment (MST >100 days, p=0.036).



C

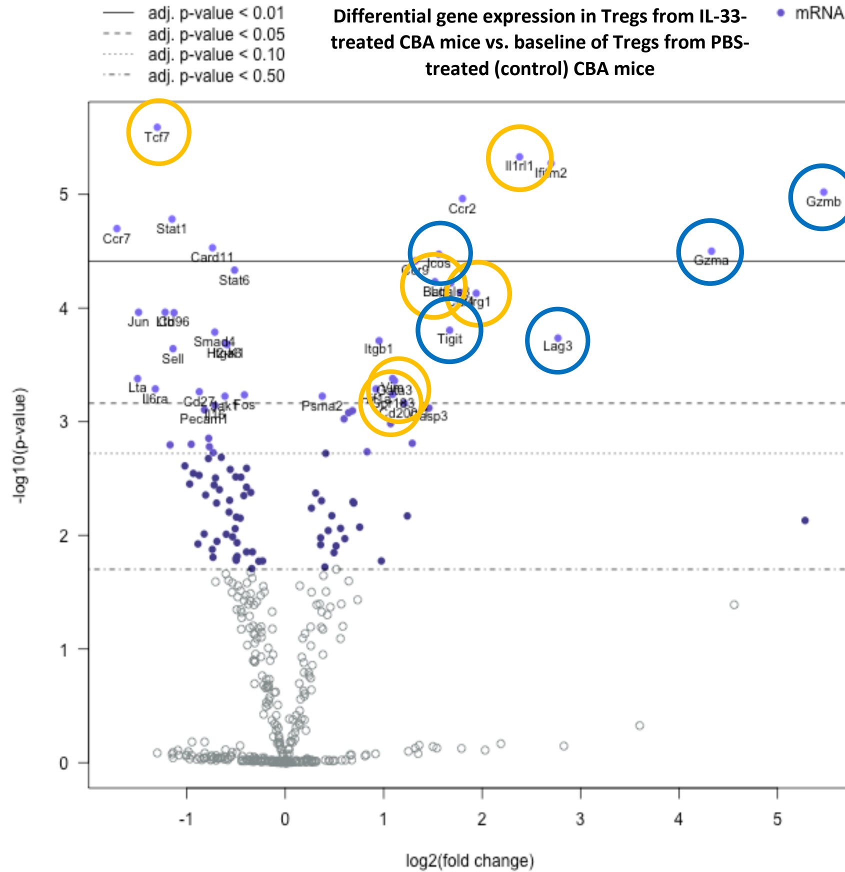
Condition	n	Survival (days)	MST
WT Teff only	8	10,10,12,14,14,14,15,26	14
WT Teff + Control Treg	11	16,21,34,37,40,40,49,64,76,100,100	40
WT Teff + IL-33-Treg	11	43,51,53,55,64,100,100,100,100,100,100	>100

Figure 4.2.2. IL-33-expanded Tregs are better able to prolong skin allograft tolerance. (A) Schematic of experimental skin transplant model design. CBA Rag1$^{-/-}$ (H-2^k) received 5x10^4 CD4$^+$GFP^{neg} effector T cells (Teff) with or without 2.5x10^4 CD4$^+$GFP$^+$ Tregs from H-2^k Foxp3-GFP mice that were treated with PBS or IL-33, as previously described. One day later, mice received an allogeneic fully-MHC mismatched C57BL/6 (H-2^b) skin allograft, which was monitored for rejection for 100 days post-transplantation. **(B)** Graft survival curve (two independent assays) of three groups of mice receiving WT Teffs only (n=8, black), WT Teffs + control Tregs (n=11, blue), and WT Teffs + IL-33-Tregs (n=11, red). **(C)** Survival data were analysed using log-rank test (p value > 0.05 considered significant).

4.2.3 IL-33-expanded Tregs demonstrate distinct transcriptomic shifts in suppressive markers

To elucidate the underlying functional advantages of IL-33-Tregs *in vivo* versus *in vitro*, changes in gene expression between control and IL-33-treated Tregs were measured. Thus, Tregs were flow sorted from IL-33-treated and control WT CBA mice and subjected to a multiplexed quantitative transcriptomic analysis, as described previously.

The most differentially expressed genes between the control and IL-33-Tregs are represented within a volcano plot, with 37 genes that came out as statistically significant (adjusted $p \leq 0.05$) **[Appendix 2** and **Figure 4.2.3A]**. As expected, two of the most differentially regulated genes, *Il1rl1* and *Gata3,* were signature genes associated with IL-33-Tregs **[Figure 4.2.3B].** In addition, *Tcf7* (downregulated), *Batf, Cd200r1*, and *Klgr1* (all upregulated) were also among the most differentially expressed genes, which have recently been identified as signature genes within $ST2^+$ tissue-resident Tregs [487]. Consistent with our immunophenotyping, many of the most differentially upregulated genes were those commonly associated with Treg suppressive function such as *TIGIT*, *ICOS*, and *LAG-3* **[Figure 4.2.3C]**. Two of the most upregulated genes in IL-33-Tregs were the granzyme genes *Gzma* and *Gzmb*, both molecules important for Treg activity [229]. Interestingly, granzyme B is also known to cleave IL-33 into more mature and potent forms, thus also acting as a feedback mechanism for tonic IL-33 activation of $ST2^+$ cells [316, 317].



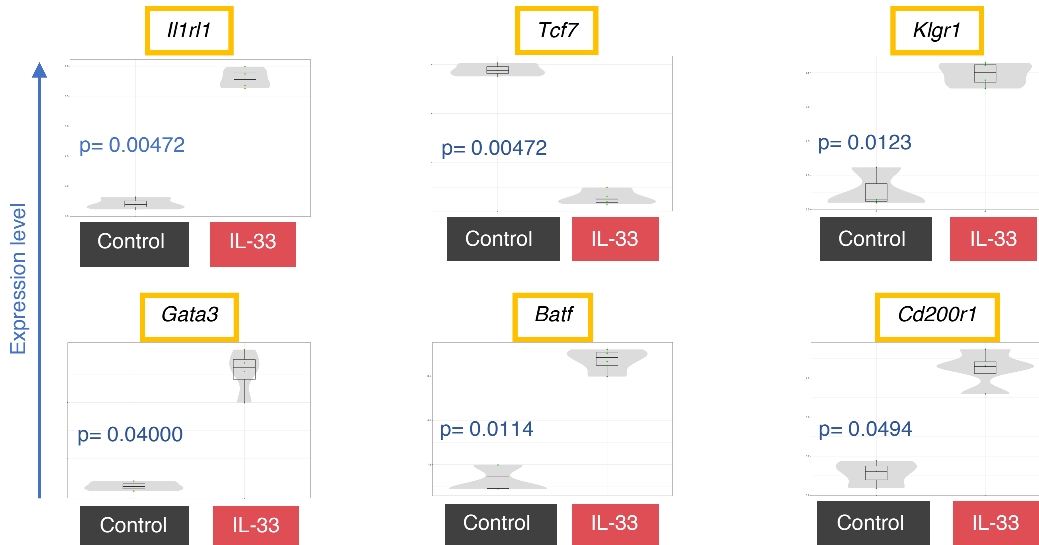


Figure 4.2.3. IL-33-expanded Tregs demonstrate distinct transcriptomic shifts in suppressive markers. RNA from splenocyte-derived FACS-sorted CD4⁺CD25⁺ Tregs were isolated from PBS (n=3) or IL-33 treated (n=4) H-2^{k} mice, as previously described, for gene expression analysis. **(A)** Volcano plot reveals the most differentially expressed genes, relative to a baseline of control mice. Among significantly differentiated genes, those associated with ST2⁺ and tissue-derived Tregs **(B, yellow)** are represented in scatter violin plots. Adjusted *p* value calculated with control of Benjamini-Yekutieli False Discovery Rate (FDR) (Adjusted *p* value > 0.05 considered significant, FDR thresholds indicated within volcano plot.)

Figure 4.2.3. (cont.) IL-33-expanded Tregs demonstrate distinct transcriptomic shifts in suppressive markers. (C, blue) Genes associated with Treg suppressive function and development are represented in scatter violin plots. Adjusted *p* value calculated with control of Benjamini-Yekutieli False Discovery Rate (FDR) (Adjusted *p* value > 0.05 considered significant, FDR thresholds indicated within volcano plot.)

4.2.4 IL-33-expanded Tregs adopt graft-homing phenotype

Interestingly, examination of chemokine receptor genes revealed a highly significant differential upregulation of *Ccr2* and *Ccr4* **[Figure 4.2.4A]**. CCR2 and CCR4 are both critical to the migration of Tregs to the allograft [266, 508-510], and are also associated with enhanced regulatory function [511, 512]. Two of the most differentially downregulated genes in IL-33-Tregs were *Sell* and *Ccr7*, both of which are strongly associated with Treg homing to LNs [266, 513, 514]. This was consistent with the downregulation of CD62L upon IL-33 treatment **[Figure 3.2.4B]**. Moreover, the lymphotoxin genes *Lta* and *Ltb*, which guide Tregs from the allograft to the lymph node via afferent lymphatics [515], were also two of the most differentially downregulated genes.

Changes in chemokine transcripts were confirmed by flow cytometry, demonstrating that cells expressed the expected pattern of homing molecules on their cell surface **[Figure 4.2.4B-E]**. In addition to CCR2 and CCR4, expression of chemokine receptor CCR5, which has also been demonstrated to be crucial for homing to the allograft [266, 516, 517], was also enhanced within IL-33-Tregs. Although the percentage of $CCR7^+$ cells was reduced within IL-33-Tregs, CCR7 MFI and proportion of cells within $CD4^+$ cells that were $CCR7^+Foxp3^+$ was increased within IL-33-Tregs. Collectively, our findings suggest that IL-33-Tregs, through their expression of CCR4, CCR5, and CCR2, are likely to migrate to the allograft to suppress alloresponses locally. This specific enhancement of chemokine receptor expression provides a basis for their enhanced *in vivo* activity in spite of equivocal *in vitro* activity.

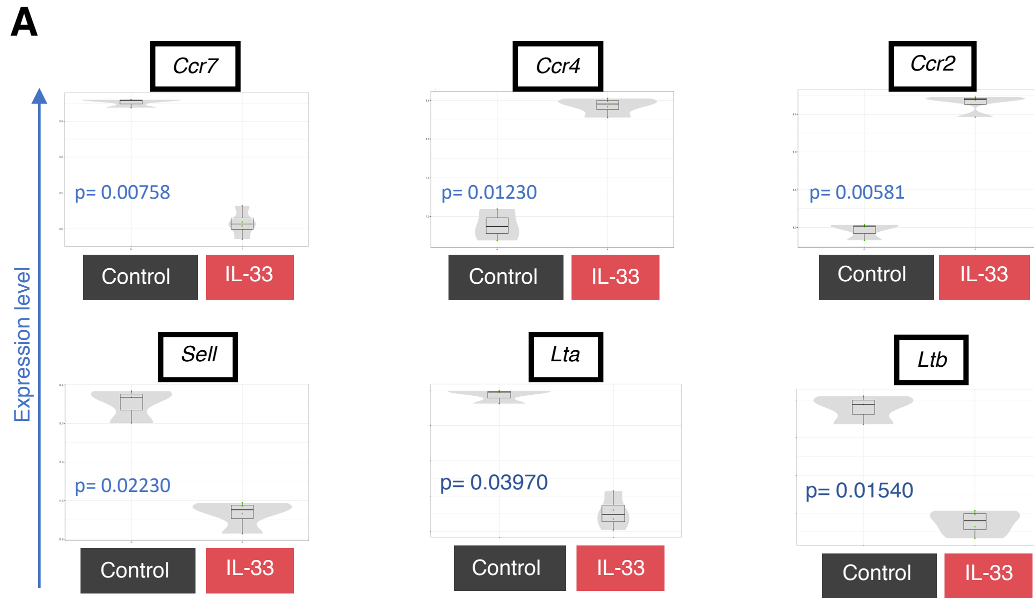


Figure 4.3.4. IL-33-expanded Tregs adopt graft-homing phenotype. RNA from splenocyte-derived FACS-sorted CD4⁺CD25⁺ Tregs were isolated from PBS (n=3) or IL-33 treated (n=4) CBA mice, as previously described, for gene expression analysis. **(A)** Genes associated with graft and lymph node homing are represented in scatter violin plots. Splenocytes from CBA mice treated with PBS or IL-33 were taken for flow cytometric analysis. Adjusted *p* value calculated with control of Benjamini-Yekutieli False Discovery Rate (FDR) (Adjusted *p* value > 0.05 considered significant, FDR thresholds indicated within volcano plot.)

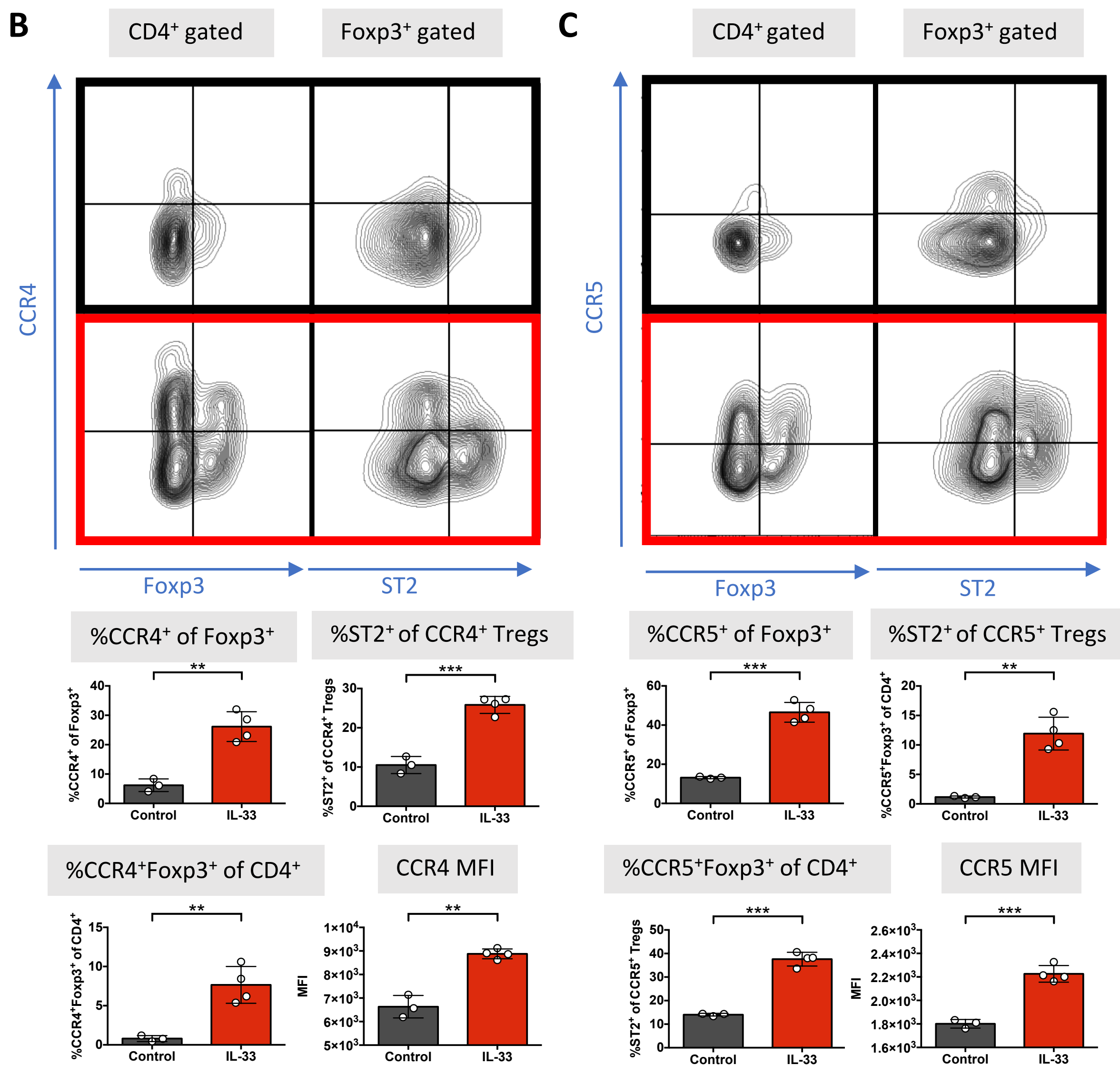


Figure 4.3.4. (cont.) IL-33-expanded Tregs adopt graft-homing phenotype. (B) Representative dotplots show CCR4 versus Foxp3 in CD4+ gated populations and CCR4 versus ST2 expression in Foxp3+ gated populations. Graphs depict percentage of CCR4+ of Foxp3+ populations, percentage of ST2+ of CCR4+ Tregs, percentage of CCR4+Foxp3+ of CD4+ cells, and CCR4 MFI within Tregs. Data are also shown for **(C)** CCR5. (unpaired *t* test, n=4) (**p*<0.05; **p<0.01; ****p*<0.001; *****p*<0.0001; ns=not significant.)

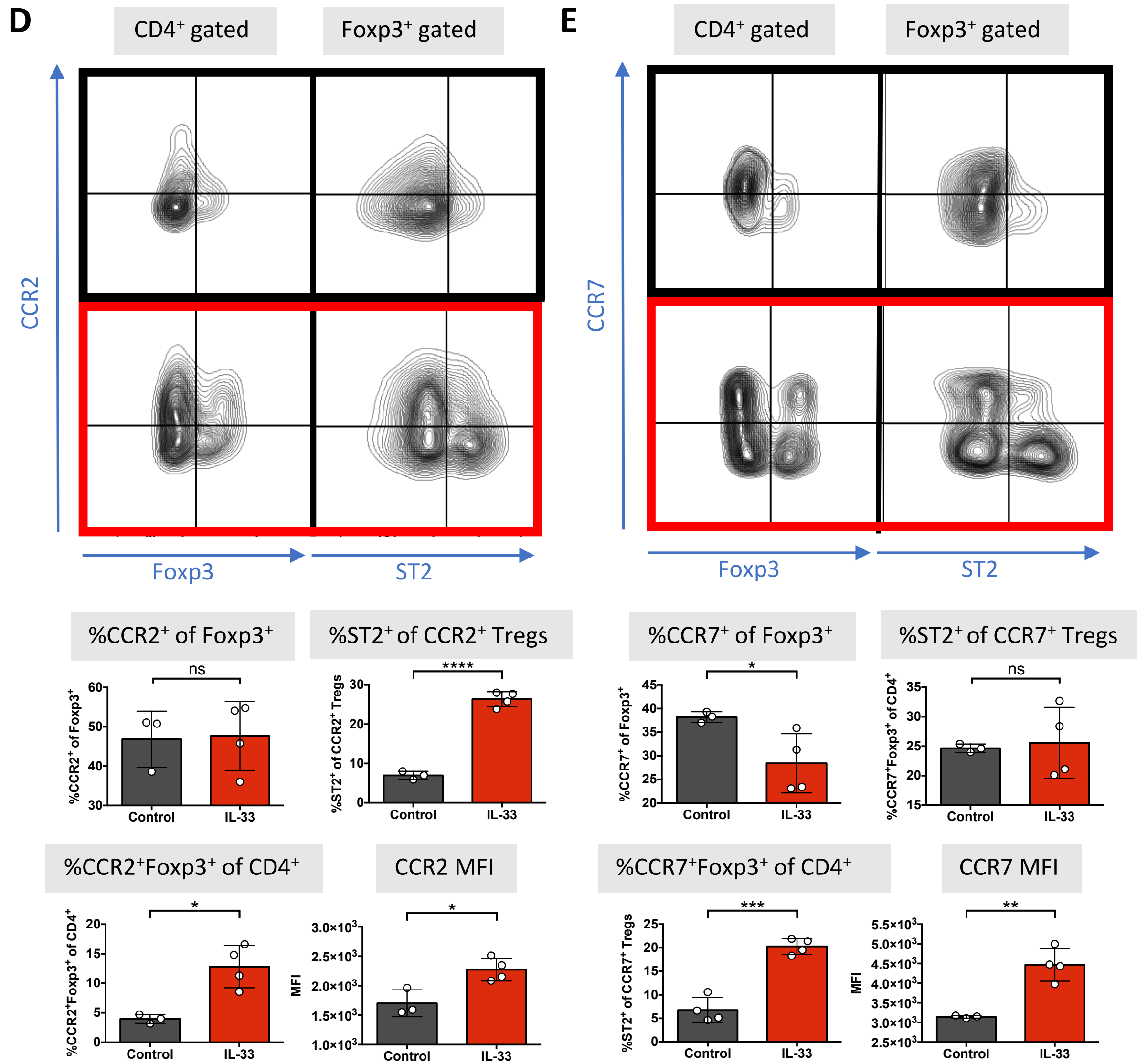


Figure 4.3.4. (cont.) IL-33-expanded Tregs adopt graft-homing phenotype. Similarly to shown in **(B/C)**, data are also shown for **(D)** CCR2, and **(E)** CCR7 (unpaired *t* test, n=4) (**p*<0.05; **p<0.01; ****p*<0.001; *****p*<0.0001; ns=not significant.)

4.2.5 IL-33 treatment expands Tregs in C57BL/6 mice

We next sought to assess whether IL-33 demonstrates similar effects *in vivo* within different strains of mice. Specifically, we were curious to investigate differences in responsiveness to IL-33 in C57BL/6 models, which were used as the donors in prior experiments. Previous studies have alluded to C57BL/6 and CBA mice having different immune responses intrinsically, with C57BL/6 mice being more strongly Th1 biased [518-520].

WT C57BL/6 mice that were treated with the same course of IL-33 injections demonstrated similar phenotypic changes as the IL-33-treated CBA mice. This included upregulation of Foxp3^{+} Tregs **[Figure 4.2.5A]** and ST2 expression **[Figure 4.2.5B]** within the SPL, LN, and PB. Moreover, there was significant decrease in T_N (CD62L^{+}CD44^{neg}) and increase of T_E (CD62L^{neg}CD44^{+}) and T_{CM} (CD62L^{+}CD44^{+}) proportions in both Foxp3^{+} and Foxp3^{neg} populations **[Figure 4.2.5C]**. Notably, in contrast to the CBA mice, ST2^{+} and T_E proportions also increased significantly within Foxp3^{neg} populations after IL-33 treatment **[Figure 4.2.5B/C)**. Moreover, while upregulation of CD25 in IL-33-Tregs was observed in CBA mice, CD25 expression was downregulated in C57BL/6 mice **(Figure 4.2.5D).**

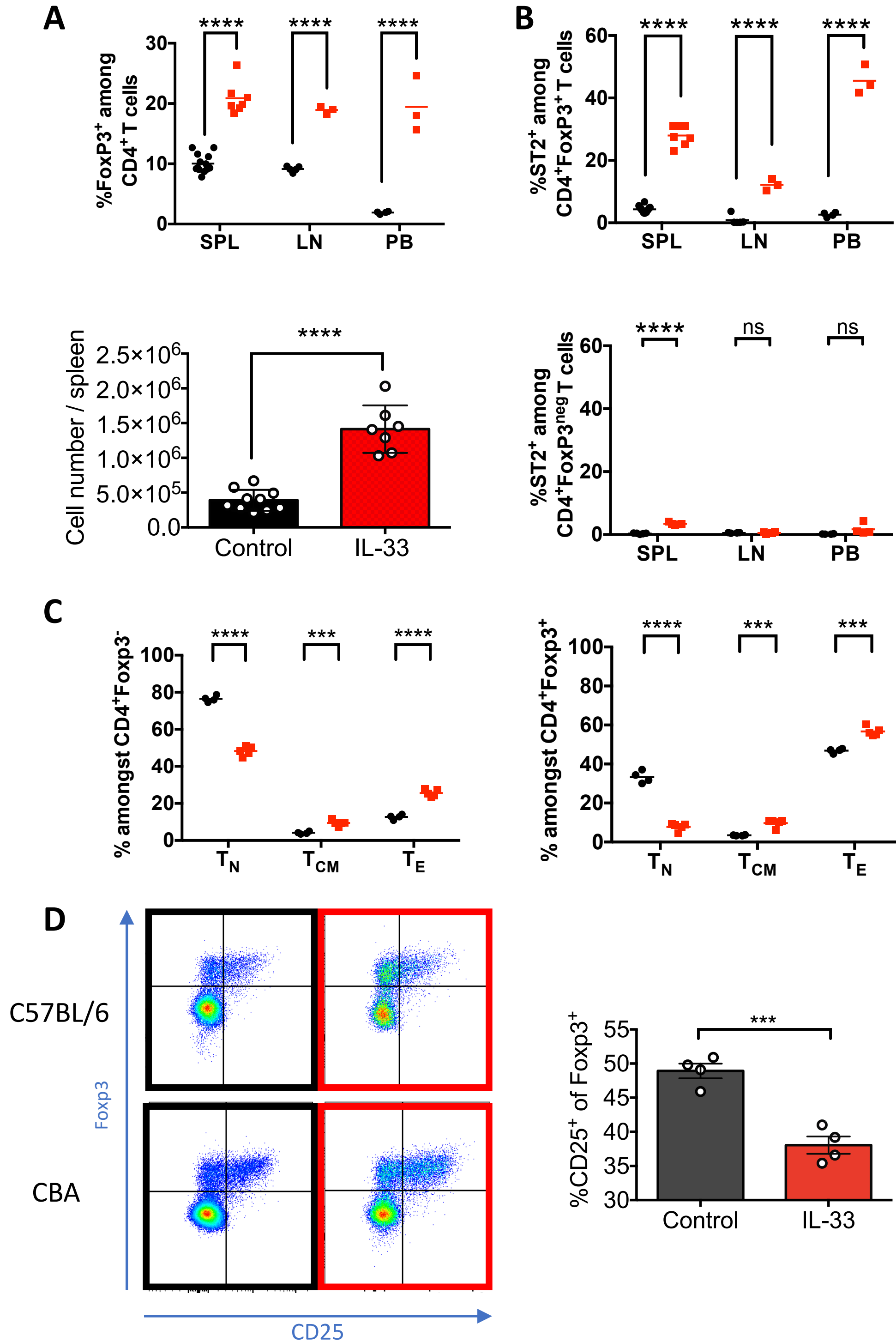
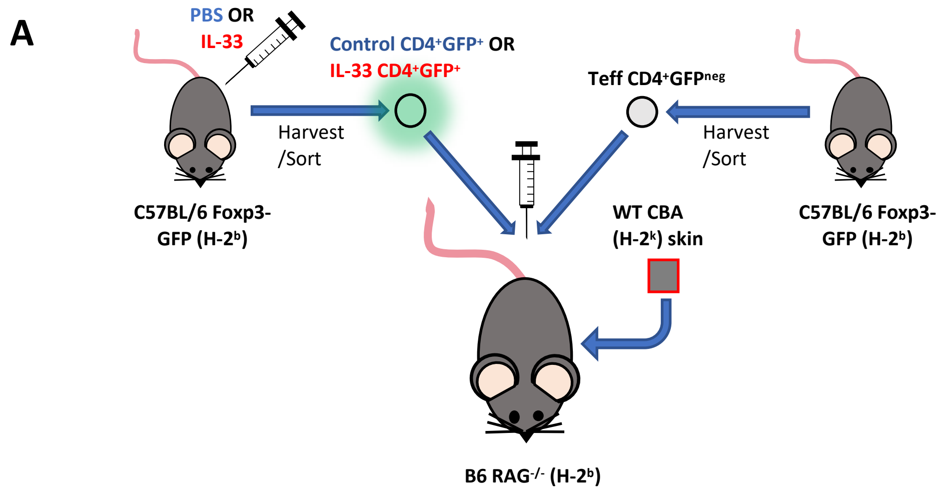
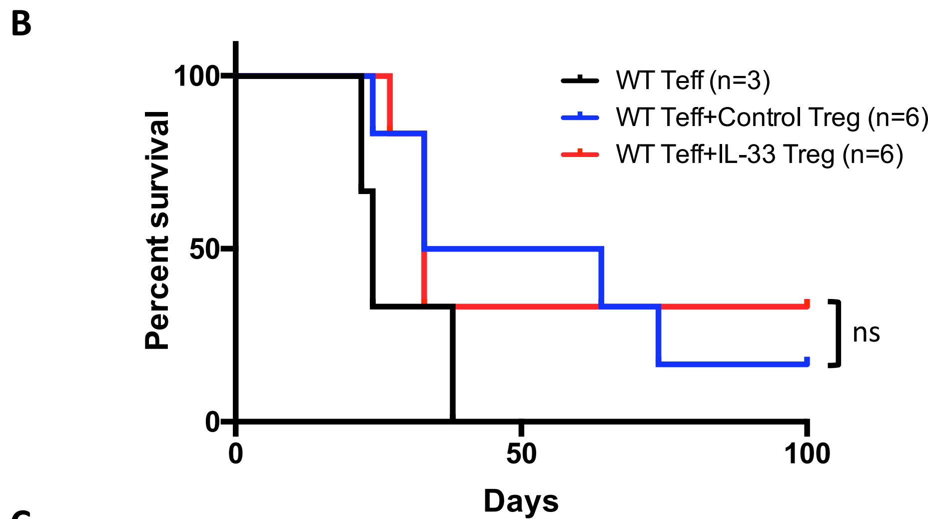


Figure 4.2.5. IL-33 treatment has similar effects on Tregs in C57BL/6 mice. C57BL/6 mice were injected with either PBS (control, black) or recombinant IL-33 (1ug/day, red) for 6 consecutive days, and spleens (SPL), lymph nodes (LN), and peripheral blood (PB) analyzed by flow cytometry. Representative dotplots and graphs of **(A)** Foxp3 and **(B)** ST2 expression within SPL, LN, and PB (unpaired t test, n= 3-9 mice). Representative dotplots and graphs of **(C)** CD44/CD62L and **(D)** CD25 within SPL (unpaired t test, n= 4 mice).

4.2.6 C57BL/6 (H-2^b) IL-33-expanded Tregs do not demonstrate enhanced ability to prolong CBA (H-2^k) skin allograft

C57BL/6 IL-33-Tregs were next assessed for *in vivo* functional advantages. To investigate this, C57BL/6 Rag$^{-/-}$ mice were transplanted with CBA skin allografts. Relative to CBA mice, C57BL/6 mice are immunogenic and required a 10-fold increase in Treg:Teff ratio (1:2 for CBA versus 5:1 for C57BL/6) upon adoptive transfer to prolong allografts to a comparable MST **[Figure 4.2.6A]**. Nonetheless, when C57BL/6 Foxp3$^+$ Tregs from PBS or IL-33 treated mice were adoptively transferred into C57BL/6 Rag$^{-/-}$ recipients with WT C57BL/6 Teffs, IL-33-Tregs demonstrated no advantage in prolonging the allograft **[Figure 4.2.6B/C].**





C

Condition	n	Survival (days)	MST
WT Teff only	3	22, 24, 38	24
WT Teff + Control Treg	6	24, 33, 33, 64, 74, 100,	48.5
WT Teff + IL-33-Treg	6	27, 33, 33, 33, 100, 100	33

Figure 4.2.6. C57BL/6 (H-2^b) IL-33-expanded Tregs do not demonstrate enhanced ability to prolong CBA (H-2^k) skin allograft. (A) Schematic of experimental skin transplant model design. C57BL/6 Rag1$^{-/-}$ (H-2^b) received 1x10^4 CD4$^+$GFP^{neg} effector T cells (Teff) with or without 5x10^4 CD4$^+$GFP$^+$ Tregs from H-2^b Foxp3-GFP mice that were treated with PBS or IL-33, as previously described. One day later, mice received an allogeneic fully-MHC mismatched H-2^k skin allograft, which was monitored for rejection for 100 days post-transplantation. **(B)** Graft survival graph (two independent assays) of three groups of mice receiving WT Teffs only (n=3, black), WT Teffs + control Tregs (n=6, blue), and WT Teffs + IL-33-Tregs (n=6, red). **(C)** Survival data were analysed using log-rank test (p value > 0.05 considered significant, ns= no significance).

4.2.7 Effects of IL-33 treatment are minimal at the transcriptomic level in C57BL/6 mice

To determine whether IL-33 treatment induces similar transcriptomic shifts in C57BL/6 as in CBA mice, spleens of PBS (n=3) and IL-33-treated C57BL/6 mice (n=3) were analysed using Nanostring transcriptomic analysis. Surprisingly, IL-33 treatment did not induce significant differences in gene expression, relative to the evident shifts observed in IL-33-treated CBA mice **[Figure 4.2.7A]**. The only differentially expressed gene between C57BL/6 control and C57BL/6 IL-33-treated groups that was identified as significant (adjusted $p < 0.05$) was *Casp8* (adj. $p = 0.0281$), which was downregulated upon IL-33 treatment.

However, IL-33-treated C57BL/6 mice appeared to have an overall dampened immune response compared to C57BL/6 controls, similar to the changes observed in CBA mice. Cell type scoring analysis identified Treg-type genes as the only cell type upregulated in IL-33-treated groups **[Figure 4.2.7B]**. Similarly, all immune Pathway Scores were downregulated with IL-33 treatment, with the exception of the complement pathway score **[Figure 4.2.7C]**. Overall, transcriptomic analysis suggests that there is an overall dampening of the immune system coupled with the promotion of Treg-associated genes in both C57BL/6 and CBA mice treated with IL-33, but the transcriptomic shifts in the former appear to be weaker.

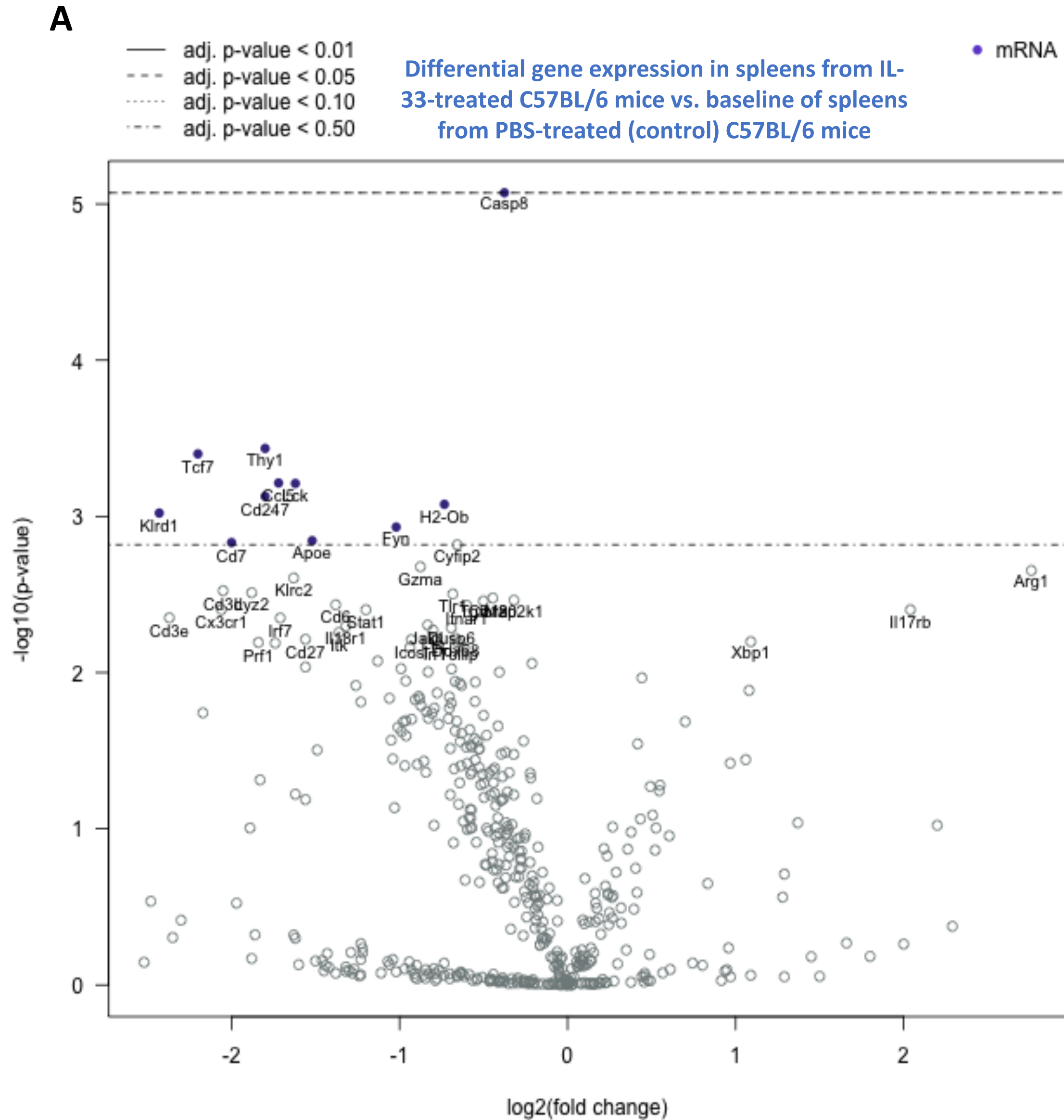
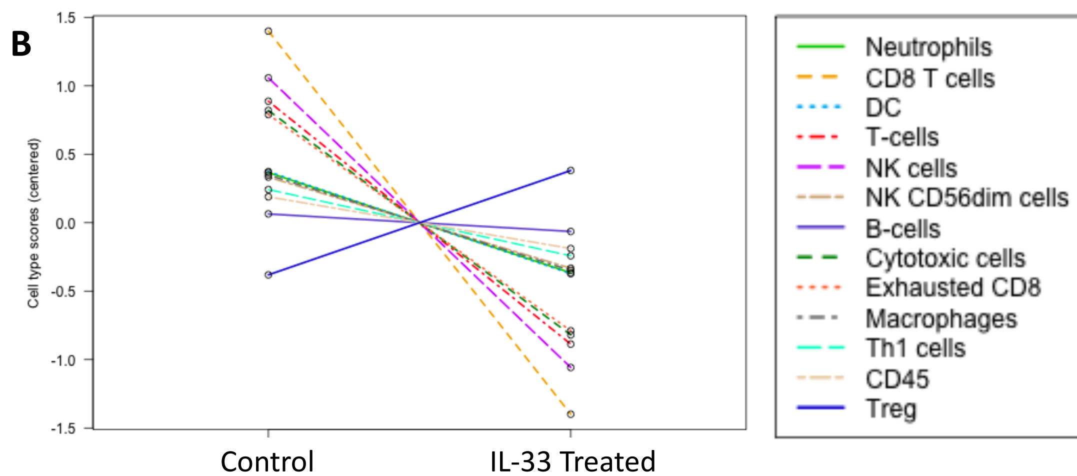


Figure 4.2.7. Effects of IL-33 treatment are minimal at the transcriptomic level in C57BL/6 mice. C57BL/6 mice were injected with either PBS (control, n=3) or recombinant IL-33 (n=3), as described previously, and RNA from total splenocytes isolated for multiplexed gene expression analysis. **(A)** Volcano plot reveals the most differentially expressed genes, relative to a baseline of control mice. Adjusted *p* value calculated with control of Benjamini-Yekutieli False Discovery Rate (FDR) (Adjusted *p* value > 0.05 considered significant, FDR thresholds indicated within volcano plot.)

B



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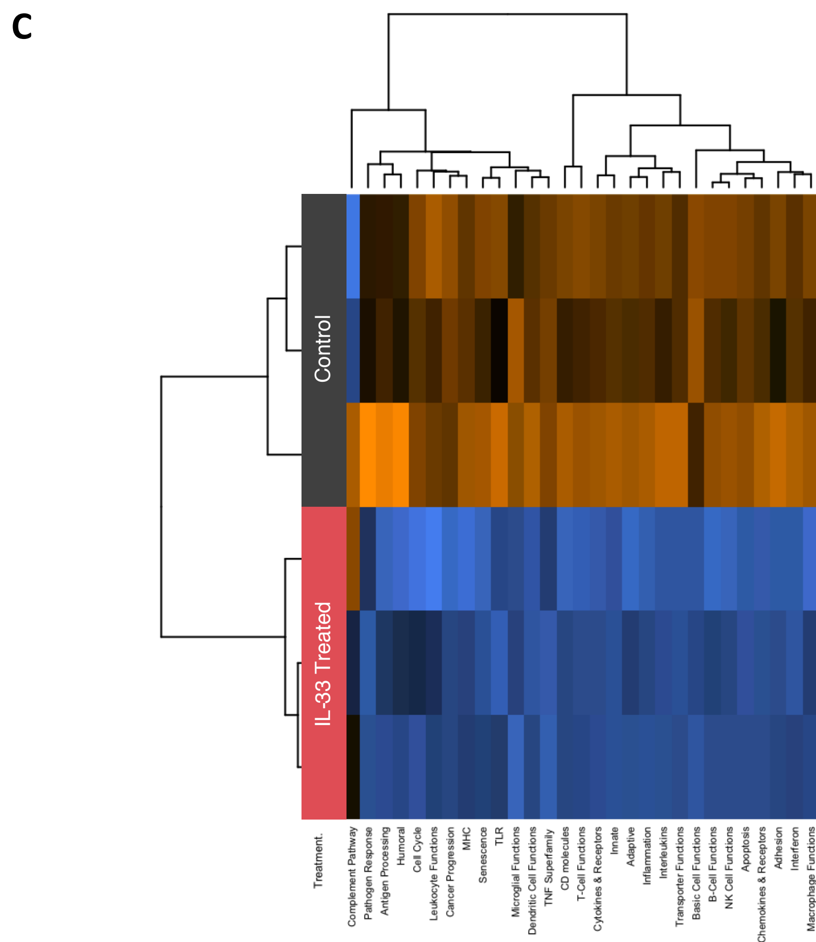


Figure 4.2.7. (cont.) Effects of IL-33 treatment are minimal at the transcriptomic level in C57BL/6 mice. (B) Graph of gene expression profiles of cell type scores, which are displayed on the same scale via a Z-transformation. **(C)** Heat map of gene expression profiles of pathway scores, which are fit using the first principal component of each sample based on the individual gene expression levels for all the measured genes within a specific pathway.

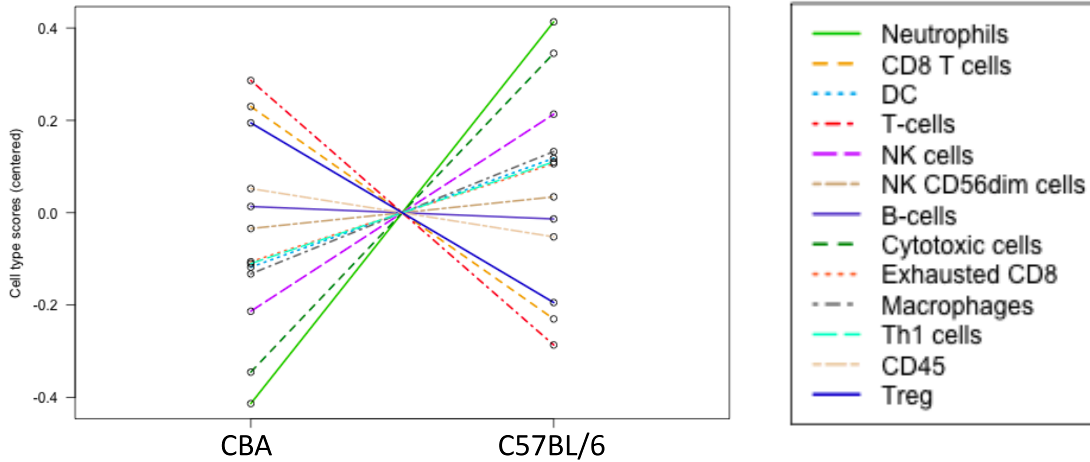
4.2.8 C57BL/6 and CBA mice have divergent transcriptomic profiles

To understand the differences in responsiveness to IL-33 stimulation between C57BL/6 and CBA mice, spleens from C57BL/6 and CBA control mice were compared using transcriptomic analysis. Here, 20 significantly differentially expressed genes (adj. $p < 0.05$) were identified between the two strains, with many being MHC class II genes, as expected. Nonetheless, the most differentially upregulated gene in C57BL/6 mice was the *Havcr2* gene (adj. p= 0.00067), which encodes for T cell immunoglobulin and mucin domain-containing protein-3 (TIM-3) **[Figure 4.2.8A]**. TIM-3 is widely known as a central regulator of Th1 immunity and a selective marker for IFN-γ-secreting Th1 cells but is not expressed on Th2 cells [521, 522]. Moreover, other genes such as *Cd38* (adj. p= 0.0493) [523], *Klrc2* (adj. p= 0.0493) [524, 525], and *Klrk1* (adj. p=0.0604)[526] have established roles within Th1 polarisation or function. This was consistent in the Cell Type Scoring analysis, notably in which there were increased Th1 and cytotoxic cell associated genes and decreased Treg cell associated genes in C57BL/6 compared to CBA mice **[Figure 4.2.8B]**. Interestingly, C57BL/6 mice demonstrated upregulation of most immune Pathway Scores **[Figure 4.2.8C]**. Taken together, this data demonstrate that C57BL/6 mice may have a more Th1-biased phenotype than CBA mice. This potentially explains why C57BL/6 immune cells are less responsive to IL-33 treatment, which may reflect inherent functional differences.

A

Differential gene expression in spleens from PBS-treated C57BL/6 mice vs. baseline of spleens from PBS-treated CBA mice

Figure 4.2.8. C57BL/6 and CBA mice have divergent transcriptomic profiles. RNA was isolated from total splenocytes from C57BL/6 (n=3) and CBA (n=3) for multiplexed gene expression analysis. **(A)** Volcano plot reveals the most differentially expressed genes, relative to a baseline of control mice. Adjusted p value calculated with control of Benjamini-Hochberg False Discovery Rate (FDR) (Adjusted p value > 0.05 considered significant, FDR thresholds indicated within volcano plot.) **(B)** Graph of gene expression profiles of cell type scores, which are displayed on the same scale via a Z-transformation. **(C)** Heat map of gene expression profiles of pathway scores, which are fit using the first principal component of each sample based on the individual gene expression levels for all the measured genes within a specific pathway.



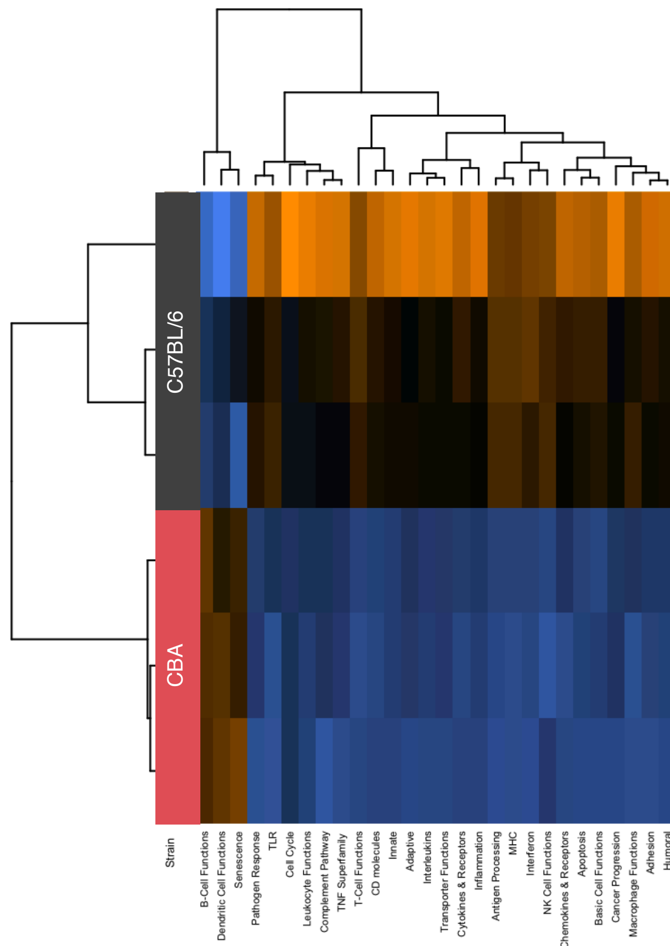


Figure 4.2.8. (cont.) C57BL/6 and CBA mice have divergent transcriptomic profiles. (B) Graph of gene expression profiles of cell type scores, which are displayed on the same scale via a Z-transformation. **(C)** Heat map of gene expression profiles of pathway scores, which are fit using the first principal component of each sample based on the individual gene expression levels for all the measured genes within a specific pathway.

4.3 Discussion

In this chapter, the tolerogenic properties of the IL-33/ST2 axis were harnessed for use in Treg cell therapy by specifically isolating IL-33-expanded Tregs. Adoptively transferred IL-33-Tregs demonstrated a dramatic functional advantage in prolonging allograft survival. The in-depth characterisation of IL-33-Tregs provides evidence of their potentially superior graft-homing capabilities. Previous studies have demonstrated the therapeutic benefit of direct *in vivo* IL-33 treatment in GVHD [388] and transplantation models [299, 389, 390]. In these studies, protection was mediated through a number of mechanisms that include the induction of Tregs, MDSCs, and Th2 skewing. Our ability to tease apart these consequences through adoptive transfer assays emphasises the integral role that IL-33-expanded Tregs have in isolation.

Prior studies that have applied *in vitro* suppression assays to study the role of the IL-33/ST2 axis in modulating Treg functional suppression have been conflicting. However, the inconclusive findings may be related to variations in experimental design. The differences between the models range from the assessment of $ST2^{+}$ versus $ST2^{neg}$ Tregs [299, 370, 382], pre-IL-33 stimulation [299, 370] versus incorporation of IL-33 directly within the culture [383, 384], anti-CD3/anti-CD28 bead [299, 370, 384] versus APC-stimulated conditions [382], and strain differences between C57BL/6 [299, 370, 382, 383] versus BALB/c [384]. In an effort to understand how IL-33 affects overall immune balance, we sought to assess the suppressive capacity of the total Treg population that develops after IL-33 treatment, instead of focusing specifically on $ST2^{+}$ versus $ST2^{neg}$ Tregs **[Figure 4.2.1.]**.

Moreover, the discrepancy observed in the suppressive function of IL-33-Tregs *in vitro* versus *in vivo* highlights the importance of assessing cellular therapies *in vivo*, where 'whole animal'-related mechanisms of function and migration may be otherwise lost. Although Tregs demonstrate significant versatility in suppressive mechanisms, their graft-protective functions are primarily exerted locally within the allograft or draining LNs, making sufficient homing capabilities as critical as suppressive potency *in vivo*. Notably, $CCR2^{-/-}$ and $CCR5^{-/-}$ Tregs have been demonstrated to have completely impaired ability in prolonging islet allograft survival in mice [266]. On one hand, the upregulation of chemokines receptor markers such as CCR4, CCR5, and CCR2 within IL-33-Tregs will make it more likely that they will successfully home to the allograft from the peripheral blood upon infusion [266, 510]. On the other hand, the downregulation of CD62L and CCR7 suggest that IL-33-Tregs will also have decreased ability to home to the LNs [513, 527]. Notably, however, Zhang et al. demonstrated that while Treg migration to both the allograft and draining LNs are beneficial for optimal suppression, only graft-homing, and not LN-homing Tregs, were absolutely critical for prolongation of the allograft [266]. Additionally, it is important to consider that IL-33 treatment also upregulates graft-homing markers on $Foxp3^{neg}$ populations, potentially neutralising the enhanced graft-homing potential of $Foxp3^{+}$ Tregs. Thus, this is possibly another limitation of treatment using direct *in vivo* IL-33 administration. In the current study, our assays are limited by an inability to precisely identify the timing of migration of Tregs to the site of the alloresponse. Moreover, it is necessary to assess in future studies whether the therapeutic benefits of adoptive therapy of IL-33-Tregs is applicable to other types of allografts, as chemokine receptors such as CCR4 are known to have crucial roles in homing to the skin specifically [528].

Aside from the shifts observed in genes involved in cell homing, the transcriptomic data provide an insight into the distinct identity of IL-33-Tregs. Notably, splenocyte-derived IL-33-Tregs demonstrated a transcriptomic profile akin to skin or fat Tregs **[Figure 4.2.3]**. In an elegant study, Vasanthakumar et al., demonstrated that Tregs from visceral adipose tissue (VAT-Tregs) have a high expression of ST2 and depend on transcriptional regulators BATF and IRF4 for their differentiation [485]. This is supported by a study by Delacher et al. in which they identified specific patterns of epigenetic modification within tissue-resident $ST2^+$ Tregs that may confer them with specialised tissue functions and distinguish them from conventional Tregs found in lymphatic organs [487]. Interestingly, they found that among the most differentially expressed genes in tissue-resident $ST2^+$ Tregs relative to conventional lymphoid organ Tregs were *Il1rl1*, *Batf*, *Klrg1*, *Cd220r1*, and *Gata3* (all upregulated), and *Tcf7* (downregulated), which were also among the most differentially expressed genes in IL-33-Tregs within this study. In particular, they identified BATF as an essential transcriptional regulator of tissue-resident $ST2^+$ Tregs. Moreover, they found that the small percentage of $ST2^+$ Tregs that reside in spleens have the same distinct signature as those found in tissues. Therefore, IL-33-Tregs used in this study most likely represent a heterogenous population in which a large proportion closely resembles the phenotypic profile of tissue-resident $ST2^+$ Tregs.

These data bring into question the source and function of $ST2^+$ Tregs. Given previous literature and the findings in this study, $ST2^+$ Tregs most likely arise from conventional $Foxp3^+$ Tregs and not from $Foxp3^{neg}$ T cells **[Figure 4.2.3]**. There is a possibility that each organ has their own ability to induce $ST2^+$ Treg programming, as discussed by Delacher et al. [487]. Another possibility is that $ST2^+$ Tregs are sourced from distinct organs such as the skin or fat, which produces and

recirculates them to other organs. This seems plausible considering Tregs from some organs such as fat have extremely high expression of ST2 compared to lymphoid organs that have very minimal at the physiological level. However, another study suggests that there is insignificant circulation of Tregs between peripheral blood and fat [487, 529]. Regardless of the source, even the minimal presence of $ST2^{+}$ Tregs in lymphoid tissues at the physiological level and their continued persistence within the blood after IL-33 treatment **[Figure 3.2.5.]**, suggest that there may be a specialised function for these cells outside of tissues.

The strain-to-strain differences observed in the suppressive function of IL-33-Tregs *in vivo* **[Figure 4.2.6]** coupled with differences in responsiveness to IL-33 treatment at the transcriptomic level **[Figure 4.2.7]** was surprising but may be correlated to intrinsic baseline differences within these two strains. Within the context of transplantation, the skin allograft models suggest that there is higher immunogenicity in the adoptively transferred C57BL/6 cells, whereby C57BL/6 recipients require a much higher Treg:Teff ratio to control graft rejection in an adoptive cell transfer model than CBA recipients. While a Treg:Teff ratio of 1:2 is required in CBA mice to prolong the a fully MHC-mismatched allograft to a median survival time (MST) of approximately 40 days, a ratio of 5:1 is required in C57BL/6 mice (personal communication with Stephen Juvet, University of Toronto). Additionally, the immunophenotyping data demonstrated that there was significant enhancement of ST2 and CD44 within non-Treg populations in C57BL/6 splenocytes after treatment **[Figure 4.2.5.]**, suggesting that it may elicit a stronger Teff-driven proinflammatory response relative to within CBA mice. The downregulation of CD25 within C57BL/6 IL-33-Tregs also suggest that they may have become apoptotic or stressed [530].

Prior studies have also reported significant differences in immune response between mice with CBA and C57BL/6 backgrounds. For example, Houpt's group demonstrated that CBA mice are much more susceptible to amoebic intestinal infection of *Entamoeba histolytica* compared to C57BL/6 mice because of a propensity to mount a strong Th2 response, in which increased IL-4 production suppresses protective IFN-γ [518, 531]. Moreover, another study comparing the cytokine expression patterns between C57BL/6 and CBA mice following infection with *Salmonella typhimurium*, found that C57BL/6 mice expressed IFN-γ mRNA at higher and more sustained levels than CBA mice [520]. Our transcriptomic data comparing C57BL/6 and CBA lymphoid cells were consistent with these findings. In particular, aside from the MHC II protein-encoding genes, *Havcr2* (encoding Tim-3) was the most significantly differentially upregulated gene coupled with the biggest fold change in C57BL/6 mice **[Figure 4.2.8.]**. Tim-3 is expressed on fully differentiated mouse IFN-γ-secreting Th1 cells (but not other $CD4^+$ T cell subsets) and acts as a central inhibitory molecule that modulates strength of Th1 responses [522, 532, 533]. Although *Ifng* (IFN-γ) was not among the significantly differentiated genes, the upregulation of *Havcr2* and other Th1-associated genes may be an indication of a more Th1-skewed immune environment. As ST2 is highly expressed on Th2 cells but only transiently in Th1 cells, differences in Th1/Th2 balance may have an effect on the responsiveness to IL-33.

Moreover, shifts in the balance of Th1/Th2 and the cytokine milieu are key determinants of the various effects that IL-33 can have within the microenvironment. For example, in the presence of IL-12, IL-33 has been demonstrated to induce production of IFN-γ in T cells and Th1 differentiation [534, 535], which may then form a regulatory loop in which IFN-γ induces IL-33 to

then induce more IFN-γ [536]. Therefore, the particular strain or pathology needs to be taken into account with IL-33-based treatments. Studies using BALB/c mice, which are considered to be more conventionally Th2-biased, may be beneficial for comparison. Taken together, the pleiotropic functions of IL-33 and intrinsic differences between C57BL/6 and CBA mice may potentially account for differences in responsiveness to IL-33 and *in vivo* function of IL-33-Tregs, though future experiments will need to further elucidate the mechanisms underlying these differences.

In conclusion, this chapter sheds light on the effects of IL-33-based treatments in shifting the balance of the immune regulatory state and Treg biology specifically. The specific characterisation and isolation of IL-33-expanded Tregs to harness the tolerogenic properties of the IL-33/ST2 axis provides a potentially promising novel therapeutic approach for transplantation and other immune pathologies.

5 Role of PHD2 on Immune Phenotype and Treg Function

5.1 Introduction

As immune cells are frequently recruited to regions of environmental stress in response to injury or infection, they have necessarily developed complex compensatory mechanisms to adapt and function properly within many of these environments. Recent studies have highlighted the presence of hypoxic regions in inflammatory sites and the role of hypoxic stress on immune cell function [455, 537, 538][434]. Notably, the hypoxia inducible factor (HIF) and prolyl hydroxylase domain (PHD), which were first recognised as regulators of red blood cell production relative to oxygen levels, are now also considered to have prominent roles in immune cells. Indeed, the HIF protein has been demonstrated to be widely expressed in all types of immune cells, including Tregs [539]. In terms of HIF regulation, PHDs are oxygen-dependent enzymes demonstrated to be its central inhibitors. In particular, PHD2, the most ubiquitous PHD isoform, may have prominent features in modulating adaptive immunity [540].

Tregs commonly migrate and function within hypoxic regions, such as in infection, tumour, and allograft microenvironments. Nonetheless, the role of the HIF/PHD pathway in Treg populations has not yet been extensively studied. Therefore, to investigate these questions with a particular focus on its effects on Tregs, we used novel global conditional *Phd2* knockdown and Foxp3-restricted *Phd2* knockdown mice models. While previous studies have used lineage specific

promoter driven knockout models to assess the effects of inhibiting components of this pathway, a knockdown system may be more reflective of physiological or pathological conditions and may better translate clinically for use in pharmacological inhibition. Additionally, this model will allow for enhanced control or modulation of the PHD2 activity and permit the study of reversibility of PHD2 inhibition.

Chapter hypothesis and aim

Hypothesis: PHD2 inhibition improves Treg function

Aim: To investigate the phenotypic and functional changes in Tregs upon the silencing of PHD2

Figure 5.1.1. in this chapter has been reproduced/adapted with permission from Yamamoto et al. [472] but was not generated by me and is data leading up to this thesis.
Figures 5.2.1, 5.2.2, 5.2.3, 5.2.4, 5.2.5, 5.2.6, and 5.2.8 in this chapter have also been reproduced/adapted with permission from Yamamoto et al. [472] and are entirely my own work.

5.1.1 Data leading up to thesis

Previous experiments conducted by our group in collaboration with the Hypoxia Biology Group from the Nuffield Department of Medicine provided the groundwork for the experiments and results presented in Chapters 5 and 6.

To first investigate the general immune effects of the inhibition of *Phd2*, global *Phd2* knockdown (CAGGrtTA-Phd2miR, Phd2kd) mice with C57BL/6 (H-2^b) background were generated using mice that expressed a tetracycline (tet)-dependent CAGG promoted reverse tetracycline transactivator (rtTA) **[Figure 5.1.1A]**. rtTA heterodimerises with doxycycline (dox) and induces transcription of an mRNA encoding a silencing microRNA (miR) carrying a shRNA targeting *Phd2* and an mRNA encoding eGFP [472]. Control mice ($Control^{CAG}$) used in the following experiments possess the CAGG rtTA but not the microRNA/eGFP cassette.

Upon the knockdown of *Phd2* in mice undergoing 4 weeks of dox treatment (2mg/mL in drinking water), a number of phenotypic changes were apparent. Among the salient features were alopecia with greasiness of hair **[Figure 5.1.1B]**, significant weight loss **[Figure 5.1.1C]**, and enlarged peripheral LNs **[Figure 5.1.1D]** and spleens **[Figure 5.1.1E]**. Additionally, there were clear shifts in the general immune cell composition within the peripheral lymph nodes with significant increases $CD45^+$, $CD3^+$, $B220^+$, and Gr-1^+ populations **[Figure 5.1.1F]**, as reflected in absolute number but without any changes in relative percentage of any particular population.

Taken together, these results suggested that *Phd2* inhibition led to distinct changes within the immune phenotype and required further investigation.

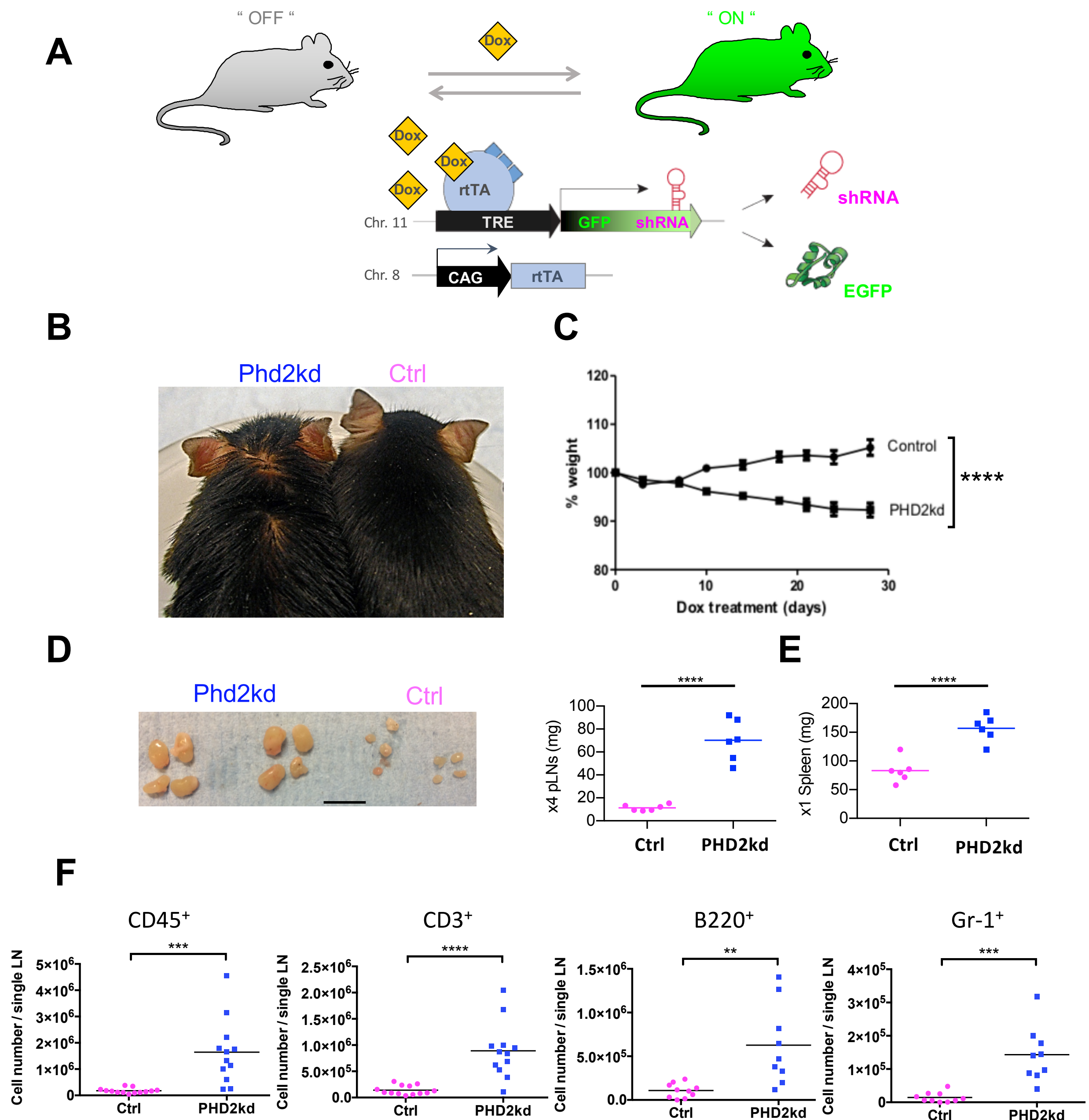


Figure 5.1.1. Data leading up to thesis. (A) Schematic of *Phd2* knockdown mouse model. Global *Phd2* knockdown (Phd2kd) mice with C57BL/6 (H-2^b) background were generated through mice that expressed a doxycycline (dox)-dependent CAGG promoted reverse tetracycline transactivator (rtTA). Activation with doxycycline induces transcription of an mRNA encoding a silencing microcRNA (miR) carrying a shRNA targeting *Phd2* and an mRNA encoding eGFP. Control mice used in the following experiments possess the CAGG rtTA but not the microRNA/eGFP cassette. Phd2kd and Control^{CAG} (CAGG rtTA) mice were generated by Atsushi Yamamoto. **(B)** Representative image of control (pink) and Phd2kd mice (blue) treated with dox (2 mg/mL with 30% sucrose drinking water ad libitum) for 4 weeks. **(C)** Mean percentage of body weight changes relative to d0 (day dox treatment was started) was recorded for the duration of doxycycline treatment (2-way ANOVA, n=7-8) (****p<0.0001.) Representative image and tissue weights (mg) of **(D)** LNs and **(E)** spleens taken from control and Phd2kd mice after 4 weeks dox (unpaired *t* test, n=6) (****p<0.0001.) **(F)** Graphs of CD45$^+$, CD3$^+$, and Gr-1$^+$ cell numbers per LN from control and Ph2dkd mice (unpaired *t* test, n=10-14)(**p<0.01, ***p<0.001, ****p<0.0001)

5.2 Results

5.2.1 Phd2kd mice display a dysregulated immune phenotype

The data leading up to this thesis **[Figure 5.1.1]** demonstrating an overall increase in various immune cell populations upon the knockdown of *Phd2* suggested that this may at least be partially driven by immune dysregulation. Thus, to further investigate this, changes specifically within T cell populations were examined within Control^{CAG} and Phd2kd mice. Within the LNs of Phd2kd mice, there were significant increases in $CD4^{+}CD8^{neg}$ and $CD8^{+}CD4^{neg}$ populations in cell numbers but no alterations in relative ratios compared to control mice **[Figure 5.2.1A]**. There were no significant increases in $CD4^{+}CD8^{+}$ and $CD4^{neg}CD8^{neg}$ cells, which have been associated with lymphoproliferation in autoimmune conditions [541, 542]. These changes in CD4 and CD8 populations were less pronounced within the spleen**.**

On further phenotyping of the $CD4^{+}$ subsets, there was a significant increase in $CD4^{+}Foxp3^{+}$ Treg populations as reflected both in percentage and absolute counts in both the peripheral LNs and spleens **[Figure 5.2.1B]**. This was surprising given the immune dysregulation phenotype, as the proportion of regulatory populations was expected to be to be inversely correlated with that of other immune cell populations. In summary, the silencing of *Phd2* induces an autoimmune phenotype characterised by lymphoproliferation.

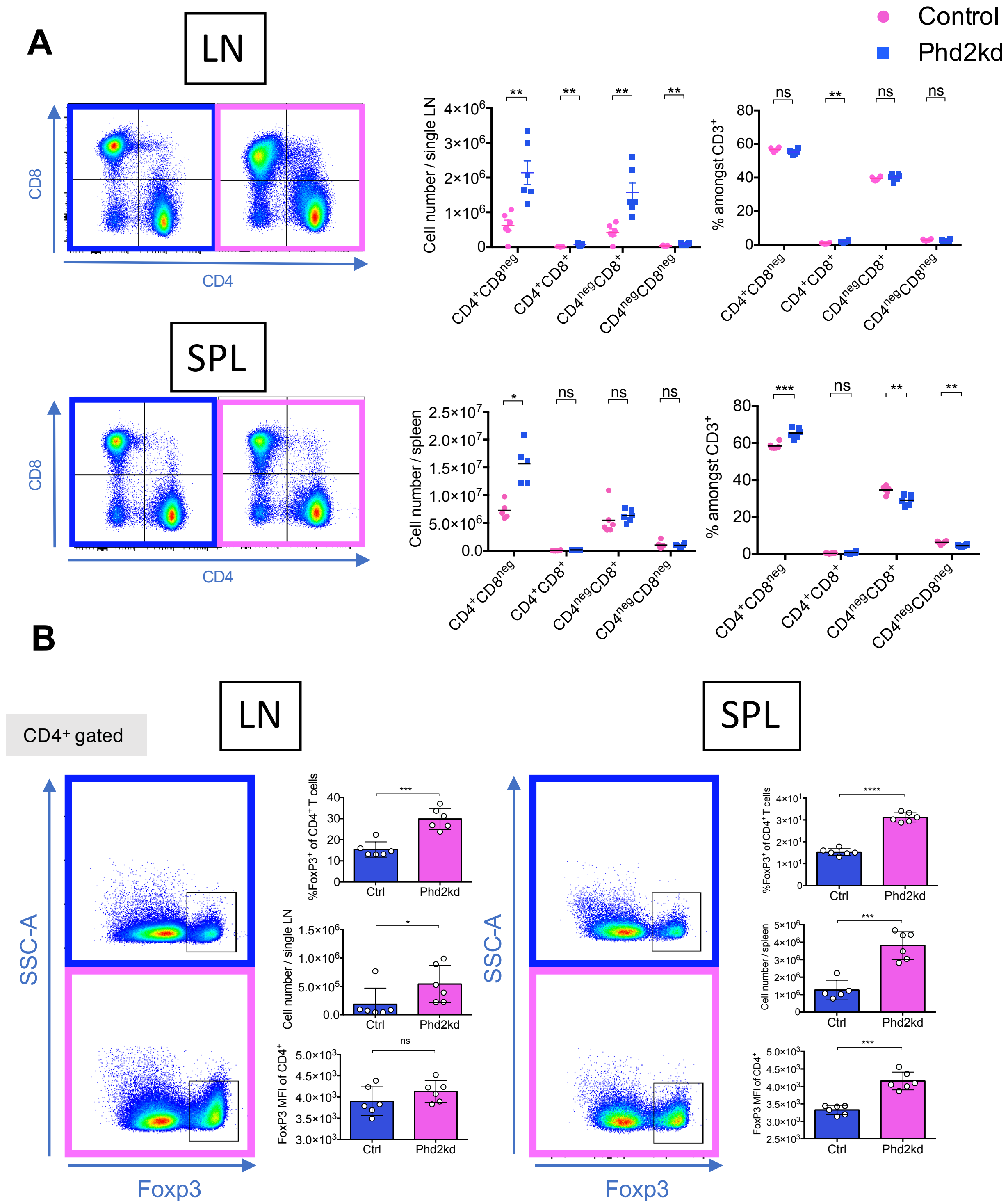


Figure 5.2.1. Phd2kd mice display a dysregulated immune phenotype. Control (pink) and Phd2kd mice (blue) were treated with dox (2 mg/mL with 30% sucrose drinking water ad libitum) for 4 weeks and LN and SPL were taken for flow cytometric analysis. **(A)** Representative dot plots and graphs show cell number and percentage of $CD4^{+}CD8^{neg}$, $CD4^{+}CD8^{+}$, $CD4^{neg}CD8^{+}$, and $CD4^{neg}CD8^{neg}$ populations from LN and spleens (**p*<0.05,***p*<0.01, ****p*<0.001, n=5). **(B)** Representative dot plots and graphs show Foxp3 expression in both LNs and spleens, as reflected in percentage, cell number, and median fluorescent intensity (MFI) (unpaired *t* test, n=5-6) (**p*<0.05, ****p*<0.001, *****p*<0.0001).

5.2.2 Phd2kd Tregs have impaired *in vivo* suppressive function

Upon observing the inflammatory phenotype with increased Treg populations, we next sought to assess their suppressive potency. Here, an adoptive cell transfer and skin transplantation model was used. WT C57BL/6 $CD4^+CD25^{neg}$ Teffs were adoptively transferred with or without $CD4^+CD25^+$ Tregs from spleens of $Control^{CAG}$ or Phd2kd mice to C57BL/6 Rag recipients, which were transplanted a day later with a full-MHC mismatched CBA ($H\text{-}2^k$) skin allograft **[Figure 5.2.2A]**.

In the first skin transplantation experiment, control and Phd2kd mice were pre-treated with dox for 1-week (2 mg/ml in drinking water, ad libitum) before their cells were harvested for adoptive transfer in order to assess the functionality of cells with inhibited *Phd2* at the point of transfer. C57BL/6 Rag recipients were then maintained on dox for the duration of the experiment. After 60 days post-transplantation, mice receiving control Tregs were found to be able to modulate Teff response and prolong allograft significantly longer than those receiving only Teffs, as expected **[Figure 5.2.2B]**. However, mice receiving Phd2kd Tregs instead demonstrated completely impaired ability to prolong graft survival and had comparable survival rates to the Teff only group.

Nonetheless, it was necessary to consider that pre-treatment of mice with dox may have contributed to the impairment of Phd2kd Tregs, in which the isolated Tregs from control and Phd2kd Tregs may have not been identical at the point of transfer. To account for this, an identical skin transplantation experiment was conducted, but this time dox treatment was initiated a day after transplantation. In agreement with the first skin transplant experiment, mice receiving

Phd2kd Tregs again demonstrated significant impairment in prolonging graft survival **[Figure 5.2.2C].**

In conclusion, Phd2kd Tregs exhibited significant loss of suppression *in vivo*. Thus, the following experiments aimed to specifically examine the phenotypic changes that occurred upon *Phd2* silencing within both $CD4^+Foxp3^{neg}$ and $CD4^+Foxp3^+$ populations.

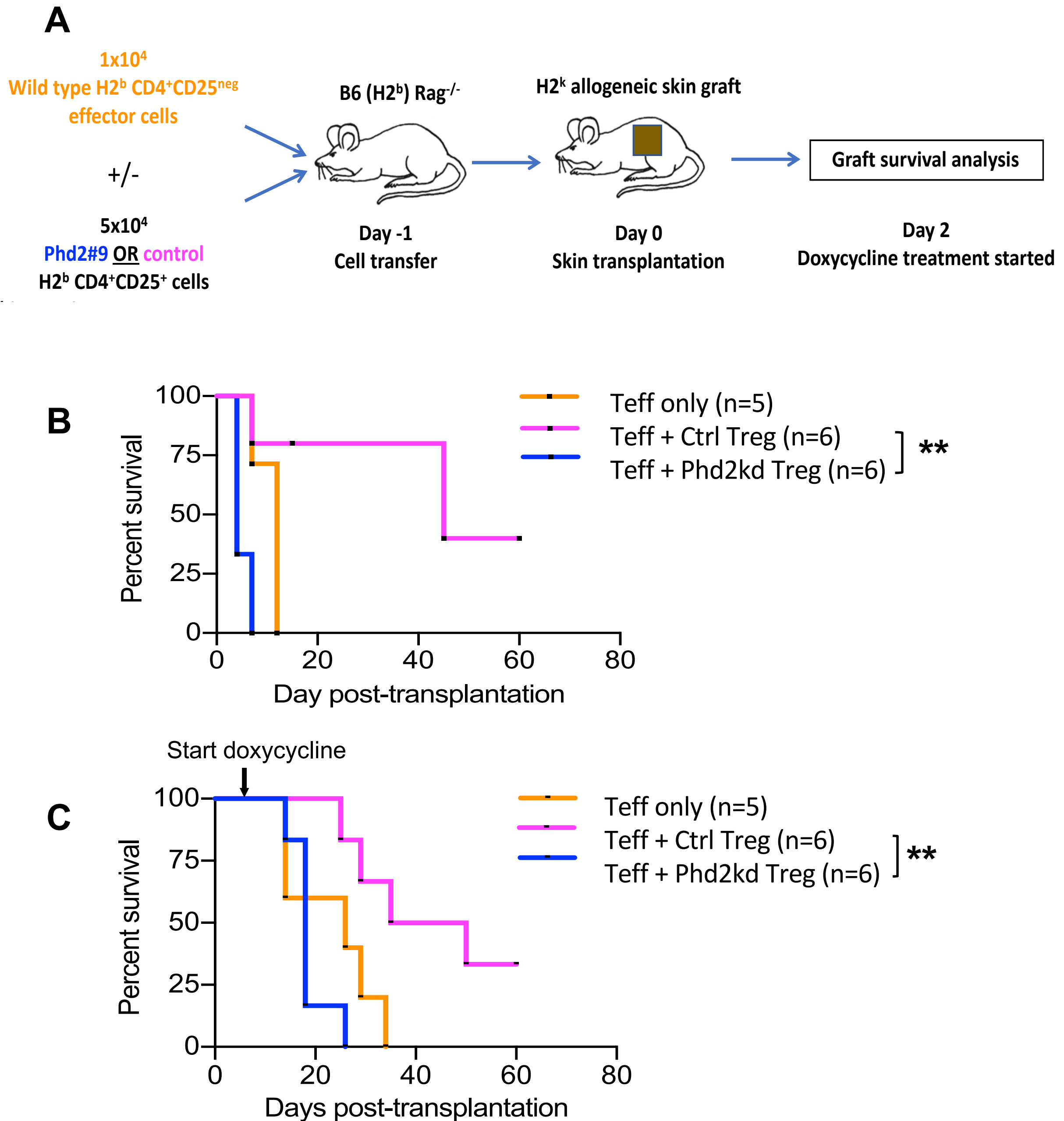


Figure 5.2.2. Phd2kd Tregs have impaired *in vivo* suppressive function. (A) Schematic of experimental skin transplant model design. **(B)** C57BL/6 *Rag1*$^{-/-}$ (H-2^b) received WT (H-2^b) CD4$^+$CD25^{neg} effector T cells (Teff) with or without CD4$^+$CD25$^+$ Tregs from either H-2^b control or Phd2kd mice, which were pre-treated with doxycycline for 1 week (2mg/mL in drinking water, ad libitum). Graph shows skin survival of three groups of mice receiving WT Teffs only (n=5, orange), WT Teffs + control Tregs (n=5, pink), and WT Teffs + IL-33-Tregs (n=5, blue). **(C)** Graph survival data of a subsequent experiment with identical design, except doxycycline treatment (2mg/mL in drinking water, ad libitum) was initiated a day after transplantation. **(B/C)** Survival data were analysed using log-rank test (**p<0.01).

5.2.3 Upregulation of activation and memory markers in cells from Phd2kd mice

To explore the changes in activation and memory markers between control and Phd2kd mice, expression of CD44 and CD62L was measured. Upon silencing PHD2, there was a dramatic shift from $CD62L^{+}CD44^{neg}$ naïve T cell populations (T_N) to $CD62L^{neg}CD44^{+}$ effector/effector memory T cell populations (T_E) in both $Foxp3^{neg}$ and $Foxp3^{+}$ populations in LNs and spleens **[Figure 5.2.3]**. Within the LNs, the shift in changes from T_N to T_{EM} was more dramatic in $Foxp3^{+}$ than in $Foxp3^{neg}$ populations. T_{CM} populations in Phd2kd mice were low and not significantly different to control mice. Again, observing an increase in a population of highly activated Tregs within Phd2kd mice was unexpected given the observed immune dysregulated phenotype [193].

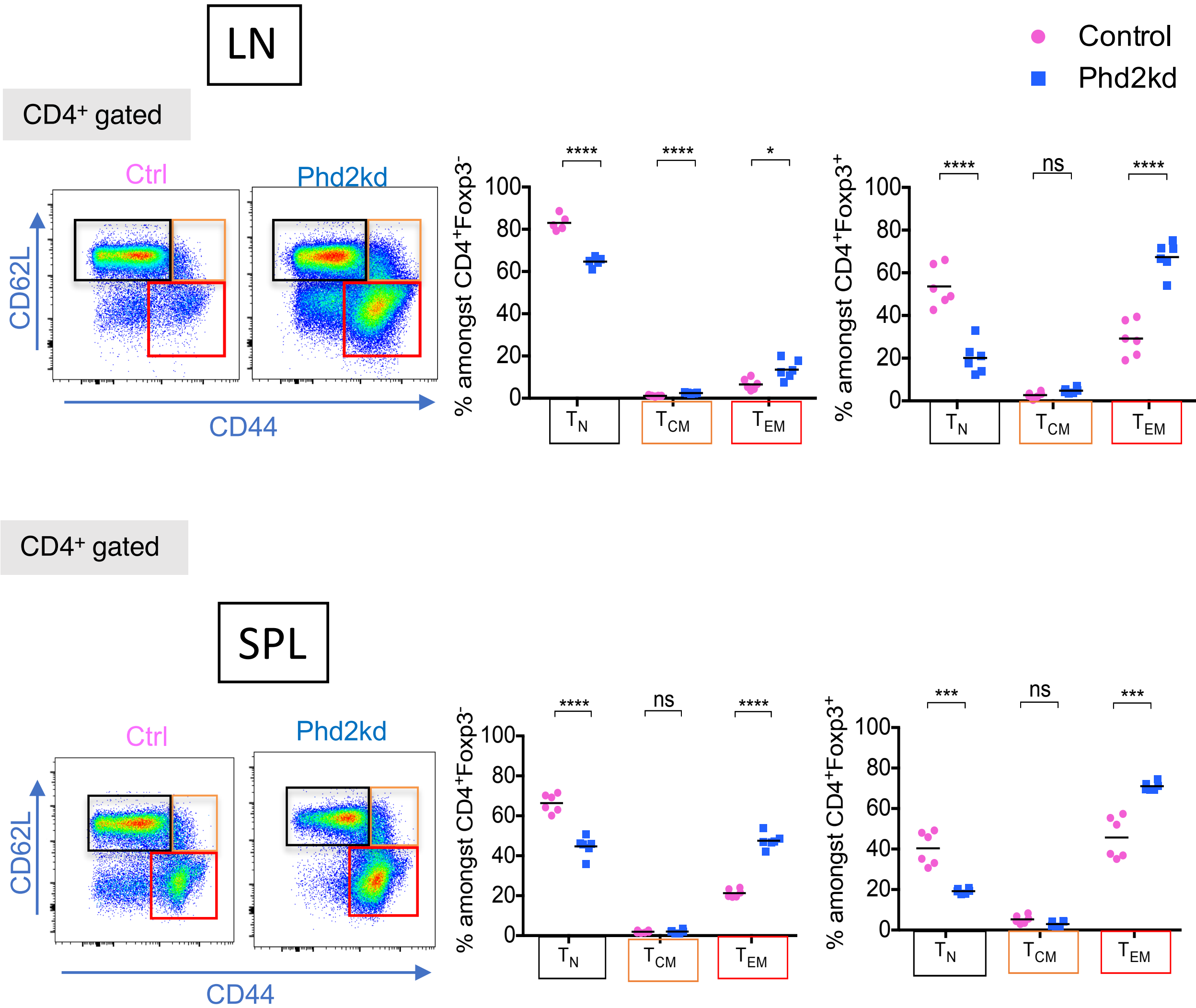


Figure 5.2.3. Upregulation of activation and memory markers in cells from Phd2kd mice. Control (pink) and Phd2kd mice (blue) were treated with dox (2 mg/mL with 30% sucrose drinking water ad libitum) for 4 weeks and LNs and spleens were harvested for flow cytometric analysis. Representative dot plots and graphs of proportion of naïve (T_N, CD62L$^+$CD44^{neg}), central memory (T_{CM}, CD62L$^+$CD44$^+$), and effector/effector memory (T_{EM}, CD62L^{neg}CD44$^+$) populations within Foxp3$^+$ or Foxp3^{neg} cells of LNs and spleens (unpaired *t* test, n=6)(**p*<0.05, ****p*<0.001, *****p*<0.0001.)

5.2.4 Upregulation of Th1-associated molecules in Phd2kd mice

To further characterise the changes within Teff ($CD4^{+}Foxp3^{neg}$) and Treg ($CD4^{+}Foxp3^{+}$) populations in Phd2kd mice, the expression of an array of transcription factors and intracellular cytokine markers were measured. Although there were no significant changes in many of these markers upon the silencing of Phd2, there was a dramatic shift in the expression of Th1-associated markers, T-bet and TNF-α, in both $Foxp3^{neg}$ and $Foxp3^{+}$ populations within pLNs **[Figure 5.2.4A]**. While there was no increase in expression of IFN-γ in Treg populations, there was a significant upregulation within Teff populations.

To assess whether Tregs may have converted into proinflammatory Th17 [506, 543, 544] or IL-2-producing effector T cells [282, 545], IL-17/ROR-γt and IL-2 expression were also examined, respectively. Neither Teffs nor Tregs populations demonstrated a shift towards a Th17 phenotype and there was no increase in expression of IL-2. Data were broadly similar in cells obtained from the spleen **[Figure 5.2.4B]**.

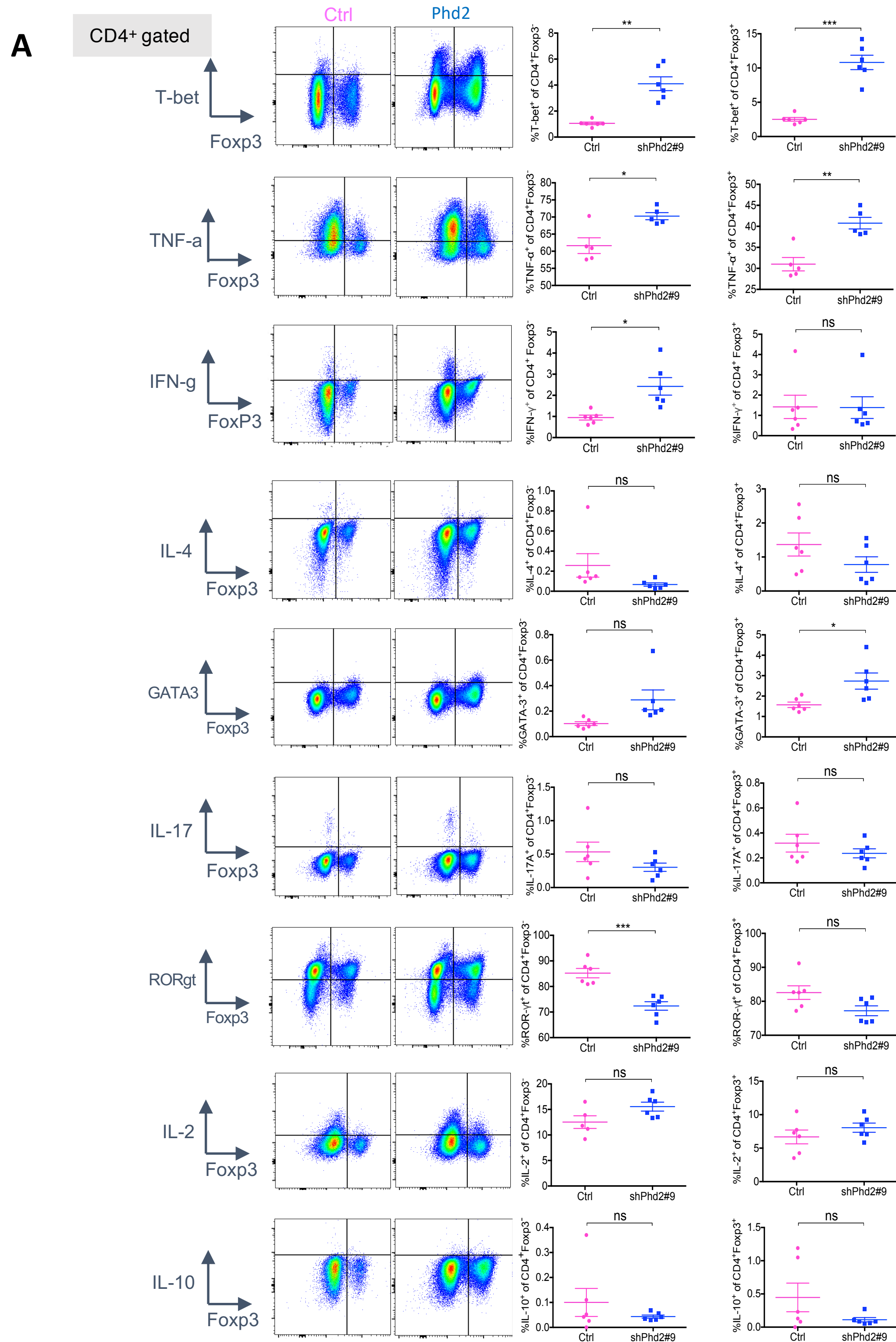


Figure 5.2.4. Upregulation of Th1-associated molecules in Phd2kd mice. Control (pink) and Phd2kd mice (blue) were treated with dox (2 mg/mL with 30% sucrose drinking water ad libitum) for 4 weeks and LNs and spleens were harvested for flow cytometric analysis. Representative dot plots and graphs of expression of Th1 markers and other transcription factors and cytokines from both **(A)** LNs (unpaired *t* test, n=6)(**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.)

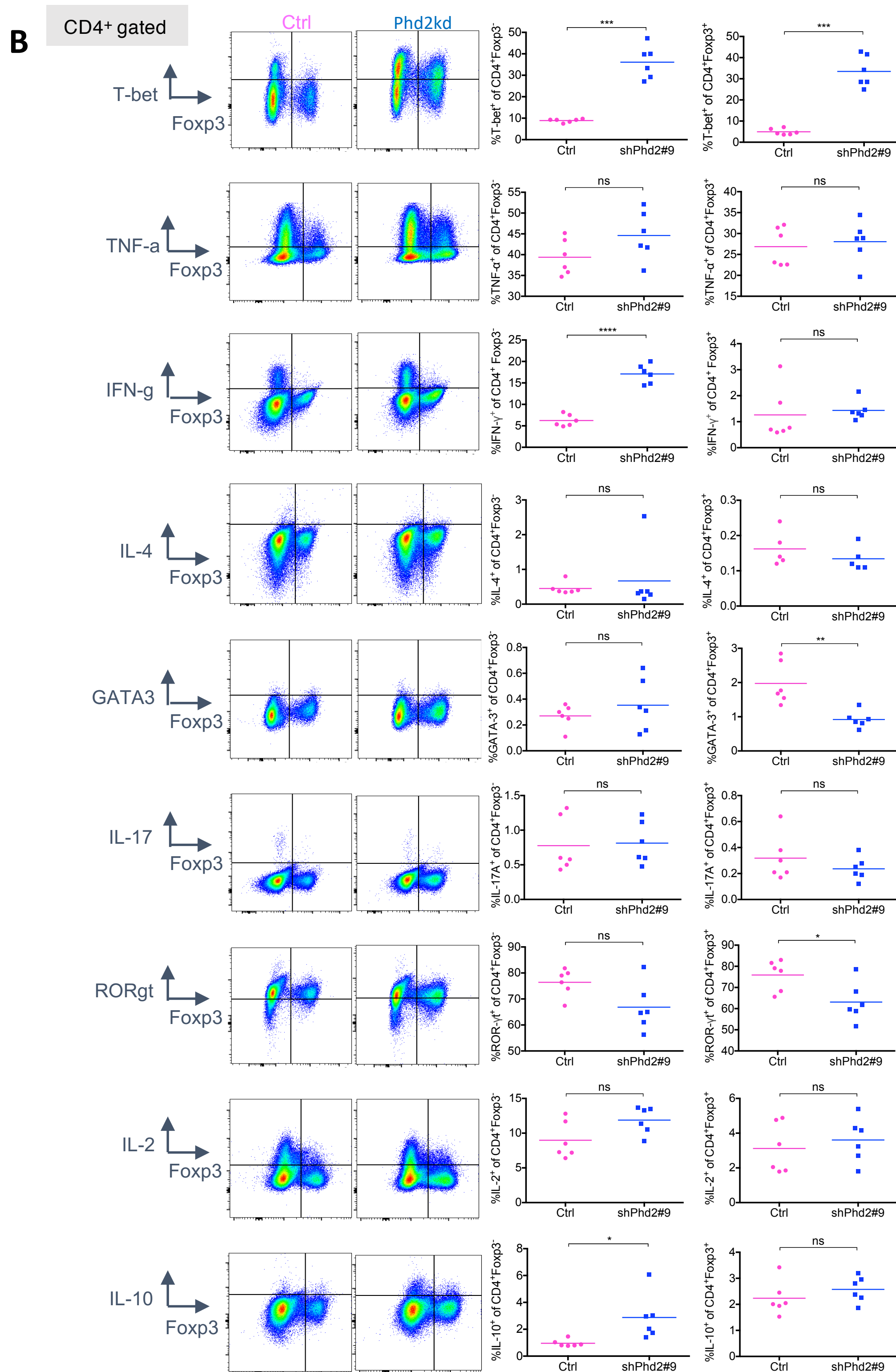


Figure 5.2.4. (cont.) Upregulation of Th1-associated molecules in Phd2kd mice. (B) Representative dot plots and graphs of expression of Th1 markers and other transcription factors and cytokines from spleens (unpaired *t* test, n=6)(**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.)

5.2.5 Downregulation of CD25 in Foxp3$^+$ Treg populations

Between control and Phd2kd mice, there were no significant differences in the proportions of CD4$^+$CD25$^+$Foxp3^{neg} T cells, which are representative of activated Teff populations. Nonetheless, Phd2kd Foxp3$^+$ Tregs significantly downregulated CD25 (IL-2R) expression compared to control Foxp3$^+$ Tregs within both the LNs **[Figure 5.2.5A]** and spleens **[Figure 5.2.5B]**. In mice, high expression of CD25 is a relatively reliable marker of Treg populations, as is Foxp3 expression. The percentage of Foxp3$^+$ cells within CD4$^+$CD25$^+$ populations is generally very high within spleens and LNs in WT mice [546]. The rare expansion of the CD4$^+$Foxp3$^+$CD25^{neg} thus brings into question whether this population expanded from Teff cells or CD4$^+$Foxp3$^+$ cells that had lost CD25 expression. Taken together, PHD2 silencing appears to result in the expansion of a unique population of effector CD4$^+$Foxp3$^+$CD25^{neg} Treg cell populations, which may be related to the overall impaired function of Tregs in these mice.

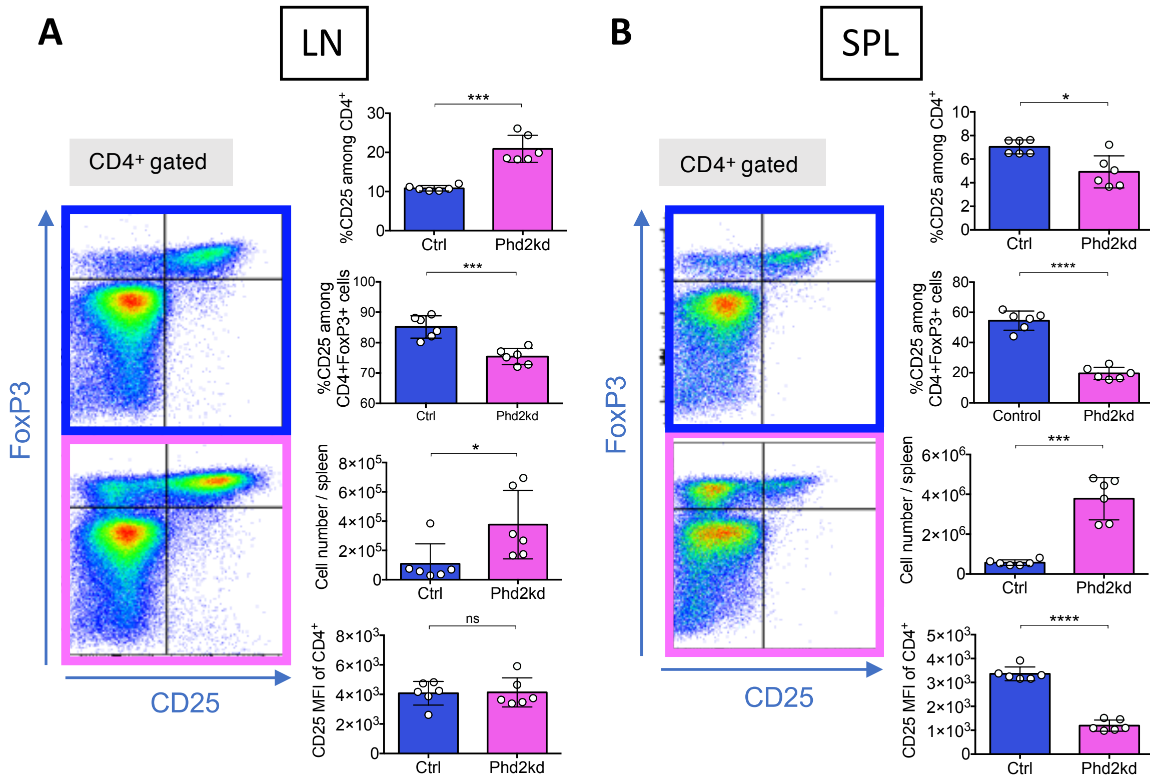


Figure 5.2.5. Downregulation of CD25 in Foxp3+ Treg populations. Control (pink) and Phd2kd mice (blue) were treated with dox (2 mg/mL with 30% sucrose drinking water ad libitum) for 4 weeks and LNs and spleens were harvested for flow cytometric analysis. Representative dot plots and graphs showing expression of CD25, as represented by percentage within $CD4^+$ and $CD4^+Foxp3^+$ populations, cell number, and MFI, of **(A)** LNs and **(B)** spleens (unpaired *t* test, n=6) (**p*<0.05, ****p*<0.001, *****p*<0.0001.)

5.2.6 Phd2kd Tregs have a fully demethylated Treg-specific demethylated region

Given the loss of suppressive function, upregulation of Th1-type markers, and the downregulation of CD25, we sought to investigate whether Phd2kd Tregs still maintained a conventional Treg phenotype. In humans, Foxp3 expression is known to be transiently expressed on activated Teffs [199]. However, unlike nTregs, which have an almost entirely demethylated Treg-specific demethylated region (TSDR), Teffs are strongly methylated. Thus, demethylation levels of the TSDR within control versus Phd2kd Tregs were analysed.

Interestingly, Phd2kd Tregs were strongly demethylated, similar to that of the controls **[Figure 5.2.6A]**. Moreover, to investigate whether the CD25 downregulation specifically had any effect on the demethylation levels, the $Foxp3^{+}CD25^{neg}$ and $Foxp3^{+}CD25^{+}$ subsets of control and Phd2kd Tregs were compared as well. There were again no major differences in TSDR demethylation levels within these populations, regardless of expression of CD25 **[Figure 5.2.6B]**. Therefore, while Phd2kd Tregs demonstrate a unique phenotype with loss of suppression, the high TSDR demethylation levels suggest that they maintain a Treg lineage.

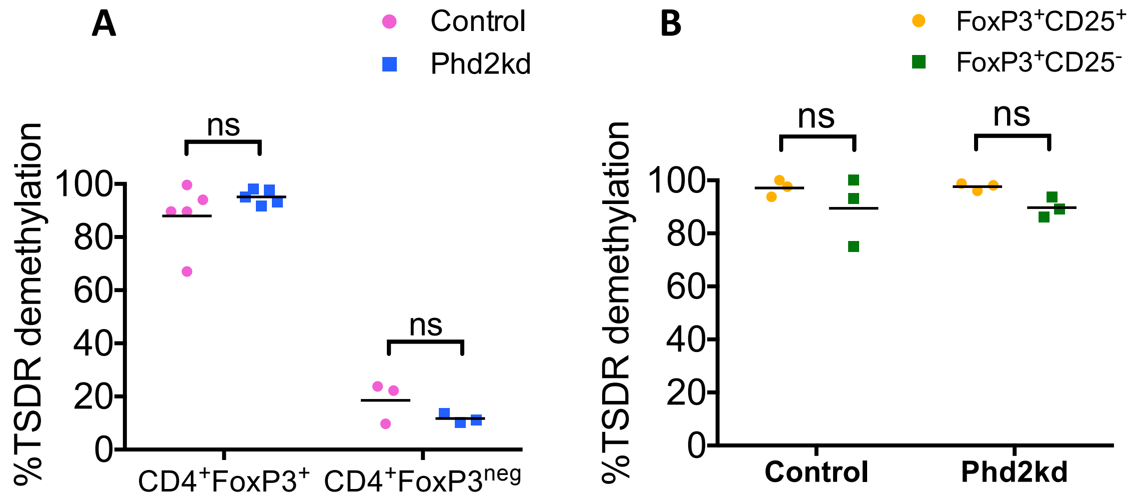


Figure 5.2.6. Phd2kd Tregs have a fully demethylated Treg-specific demethylated region. Control (pink) and Phd2kd mice (blue) were treated with dox (2 mg/mL with 30% sucrose drinking water ad libitum) for 4 weeks. **(A)** $CD4^+Foxp3^+$ and $CD4^+Foxp3^{neg}$ from spleens of control and Phd2kd mice were flow-sorted. Percentage of demethylation of TSDR on active X chromosome was analysed by pyrosequencing. **(B)** $CD4^+Foxp3^+CD25^+$ (yellow) and $CD4^+Foxp3^+CD25^{neg}$ (green) cells were also flow sorted and percentage of demethylation of TSDR was calculated with the same method. (unpaired *t* test, n=3-5).

5.2.7 Phenotypic changes of Phd2 silencing follow a time-dependent response

In order to explore how early the immune cell changes manifest after initiation of doxycycline and their time-course, Control^{CAG} and Phd2kd mice were treated for different durations with doxycycline (1, 2, 3, and 4 weeks) **[Figure 5.2.7A]**. Interestingly, the absolute count of CD3^{+}, CD4^{+}, CD8^{+}, CD25^{+}, **[Figure 5.2.7B]** and Foxp3^{+} **[Figure 5.2.7D]** cells followed a time-dependent response, with numbers peaking around 4 weeks. Moreover, activation and memory markers, such as CD69 (early T cell activation marker) and CD62L/CD44 were also examined. Even with only a single week of doxycycline treatment, the immune effects of Phd2 silencing were clearly observed in terms of upregulation of CD4^{+}CD69^{+} cells, downregulation of naïve T cells, and an increase in effector T cells **[Figure 5.2.7C]**. Collectively, these data show that the emergence of phenotypic shifts upon Phd2 knockdown are rapid and dependent on the amount of time that *Phd2* remains silenced. The rapid induction of changes in immune phenotype upon inhibition of *Phd2* further suggests that it plays a fundamental role in maintaining immune homeostasis.

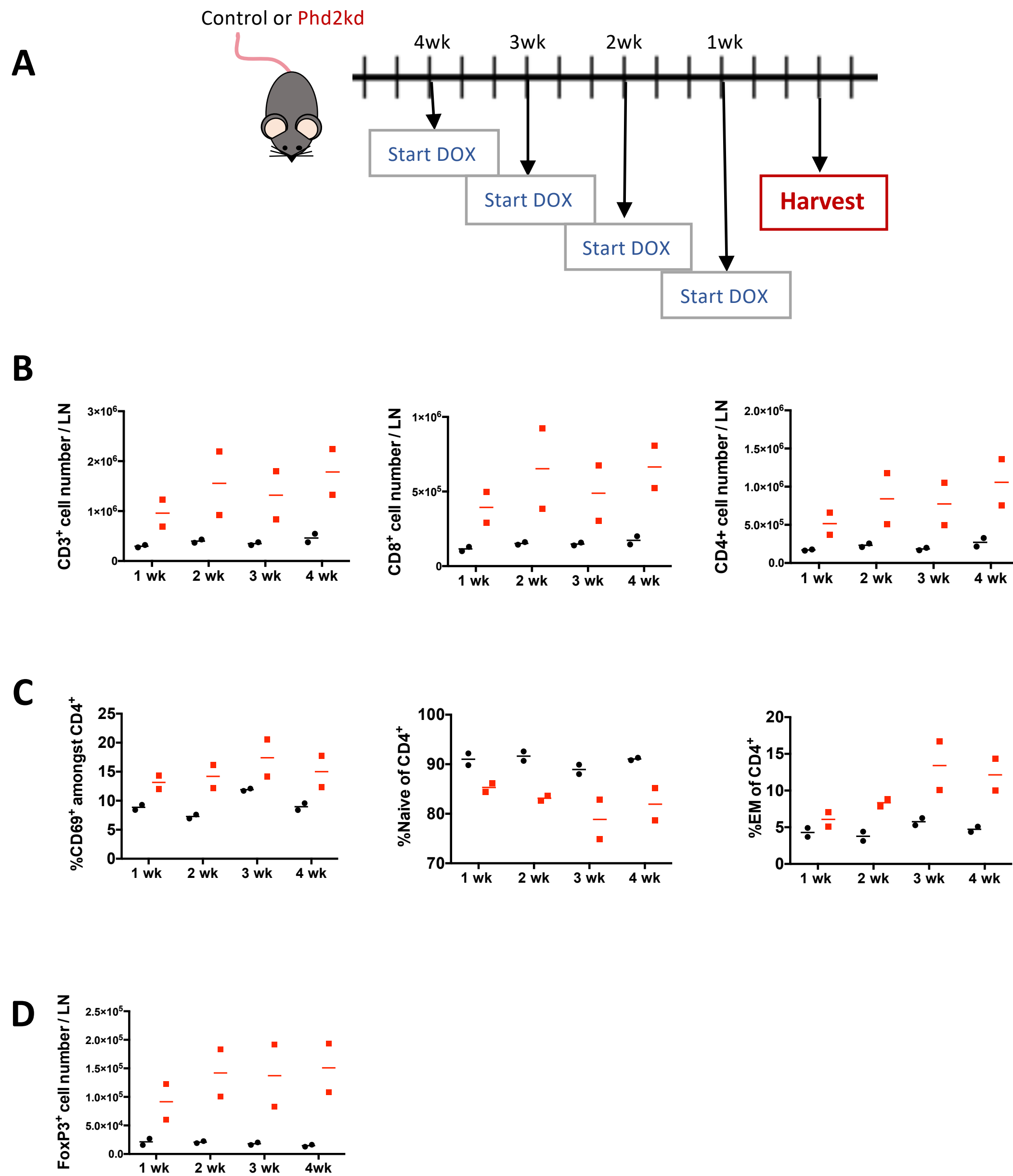


Figure 5.2.7. Phenotypic changes of Phd2 silencing follow a time-dependent response. (A) Schematic of experimental plan. Separate groups of control (pink) and Phd2kd mice (blue) mice were started on a course of doxycycline (2 mg/mL with 30% sucrose drinking water, ad libitum) either 1, 2, 3, or 4 weeks before they were harvested for pLNs. Flow cytometric analysis of **(B)** cell numbers of T cell subsets, and expression of **(C)** activation/memory markers and **(D)** Foxp3 (n=2 per group).

5.2.8 Phd2 knockdown-induced phenotype can be fully reversed upon the removal of doxycycline

In order to establish whether the phenotype induced by *Phd2* silencing can be reversed upon reestablishment of Phd2 expression, mice were treated with dox for 3-4 weeks and analysed (ON group), while others were taken off dox treatment after 3-4 weeks of treatment and analysed 7 weeks later (ON/OFF group). While the ON group developed lymphadenopathy and developed weight loss as predicted, those in the ON/OFF group eventually recovered to a normal phenotype. By the 7th week after doxycycline withdrawal, ON/OFF mice had regained body weight **[Figure 5.2.8A]** and reduced lymphadenopathy **[Figure 5.2.8B]**. Immune cell populations within lymph nodes were reduced to numbers comparable to control levels **[Figure 5.2.8C]**.

Furthermore, key markers that had distinctive shifts upon the silencing of *Phd2* were additionally analysed. Here, T-bet expression was significantly downregulated in Phd2kd ON/OFF mice relative to Phd2kd ON mice and back to levels comparable to those of control mice **[Figure 5.2.8D]**. Moreover, CD25 expression within Foxp3$^+$ cells was re-upregulated upon the removal of dox, replenishing the Foxp3$^+$CD25$^+$ population **[Figure 5.2.8E]**. Collectively, these experiments demonstrate that the phenotype induced by the silencing of *Phd2* was neither a permanent phenotypic change in cell populations nor an irredeemable loss of cell functions.

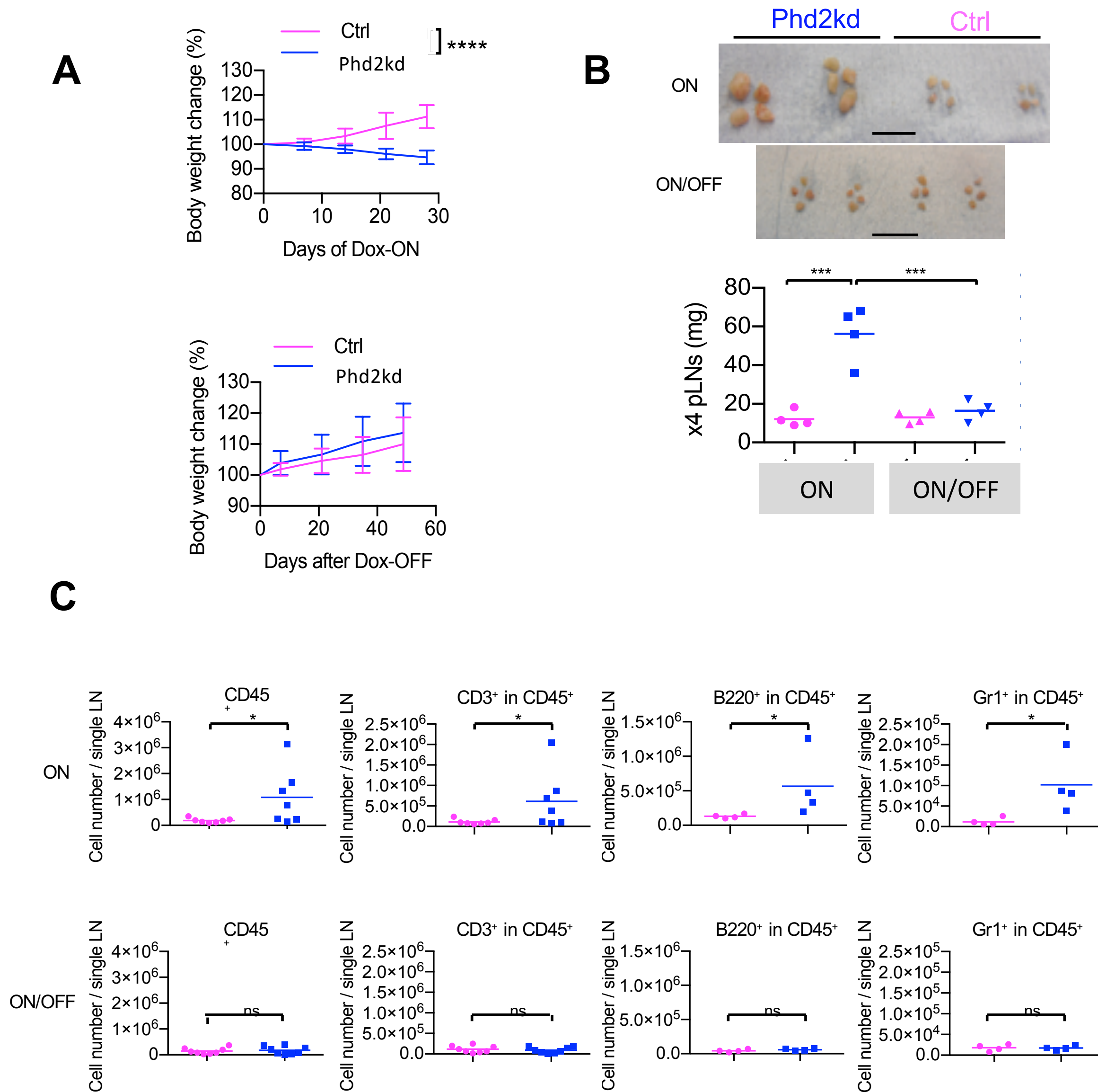


Figure 5.2.8. Phd2 knockdown-induced phenotype can be fully reversed upon the removal of doxycycline. Control (pink) and Phd2kd mice (blue) were treated with dox (2 mg/mL with 30% sucrose drinking water ad libitum) for 4 weeks. One group was harvested after 3-4 weeks of dox (ON), while the other group was taken off dox treatment (after 3-4 weeks) for 7 weeks before being harvested (ON/OFF). **(A)** Mean percentage of body weight changes relative to d0 (day dox treatment was started) was recorded for the duration of doxycycline treatment (2-way ANOVA, n=7-8) (****p<0.0001.) **(B)** Representative image and tissue weights (mg) of pLNs taken from control and Phd2kd mice after 4 weeks dox (unpaired t test, n=6) (***p<0.001.) **(C)** Graphs of $CD45^+$, $CD3^+$, $B220^+$, and Gr-1^+ cell numbers per LN from control and Ph2dkd mice from ON and ON/OFF groups (unpaired t test, n= 4-9)(*p<0.05.)

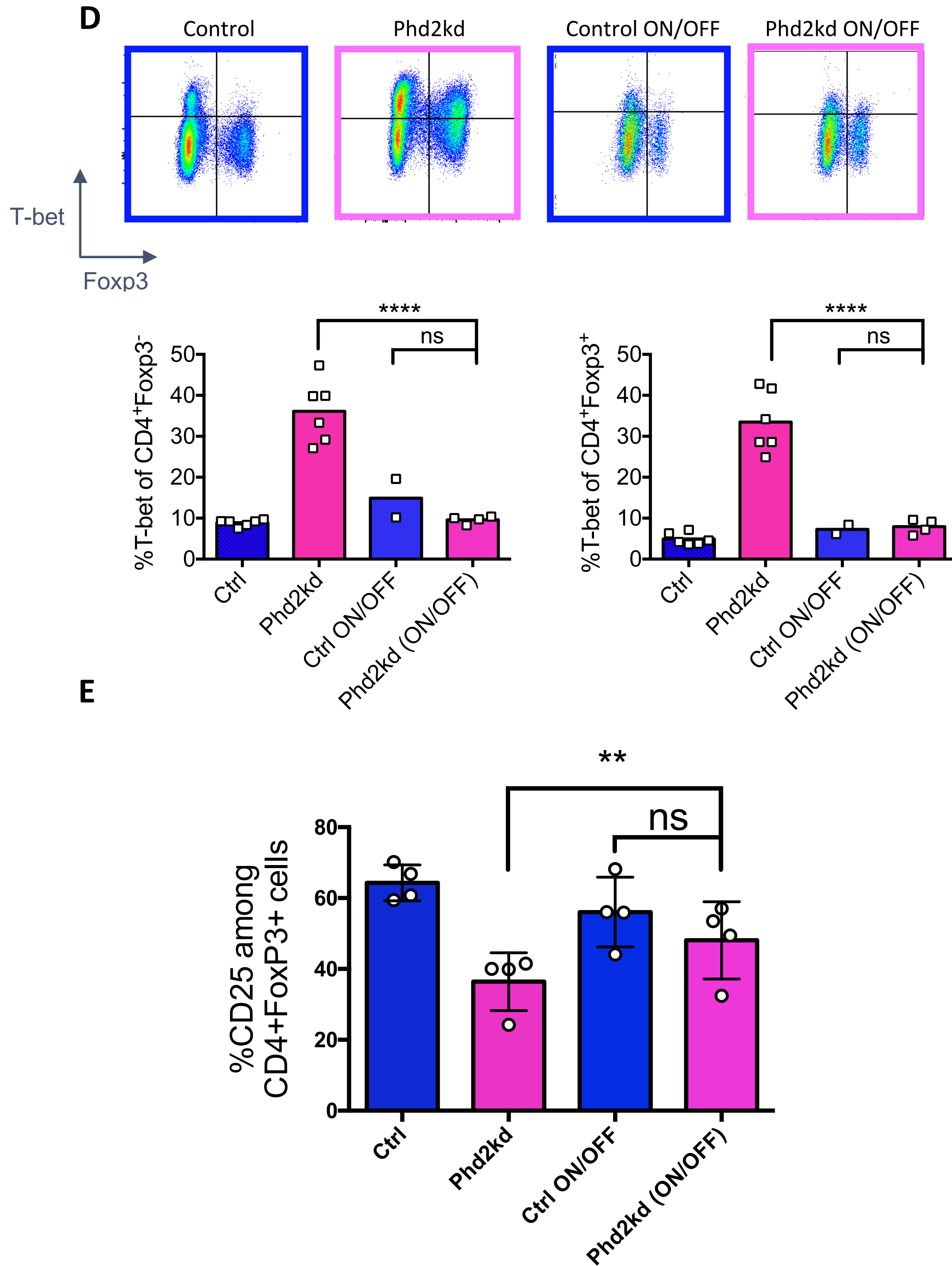


Figure 5.2.8. (cont.) Phd2 knockdown-induced phenotype can be fully reversed upon the removal of doxycycline. Representative dot plots and graphs of expression of **(D)** T-bet and **(E)** CD25 from control and Ph2dkd mice from ON and ON/OFF groups (unpaired *t* test, n= 2-6)(***p*<0.01; *****p*<0.0001.)

5.2.9 Foxp3^{Cre}-Phd2kd mice exhibit similar phenotype to Phd2kd Tregs

To further explore the phenotypic changes that were induced upon Phd2kd silencing, we next asked (1) whether the dysfunction of Phd2kd Tregs was caused by the interaction between other cell populations, and (2) whether the Phd2kd Tregs were one of the major drivers in the overall immune dysregulation. To investigate these questions, Foxp3-restricted conditional *Phd2* knockdown mice (FoxP3-Cre-LSL-CAGGrtTA-Phd2miR, Foxp3^{Cre}-Phd2kd) were used, which were generated by crossing Foxp3-Cre mice with mice in which Phd2-miRNA is only transcribed upon the removal of a LoxP-STOP-LoxP (LSL) sequence. Control^{Foxp3} (Foxp3-Cre-LSL-CAGGrtTA) and Foxp3^{Cre}-Phd2kd mice were then treated with doxycycline before being analysed **[Figure 5.2.9A]**.

In general, Foxp3^{Cre}-Phd2kd mice did not demonstrate dramatic phenotypic changes relative to the global Phd2kd mice. In contrast to global Phd2kd mice, in which 4 weeks of dox treatment was sufficient to induce massive lymphadenopathy, Foxp3^{Cre}-Phd2kd mice demonstrated more subtle changes early on and remained on dox treatment for 12 weeks before more obvious changes were seen. Interestingly, Foxp3^{Cre}-Phd2kd mice after 12 weeks dox treatment demonstrated similar patterns of lymphadenopathy within the LNs, with an overall increase in general immune cell populations, including Tregs **[Figure 5.2.9B].** Nonetheless, no significant differences in the expression of other markers (i.e. T-bet, TNF-α, CD25, CD44/CD62L) were found when compared to control mice.

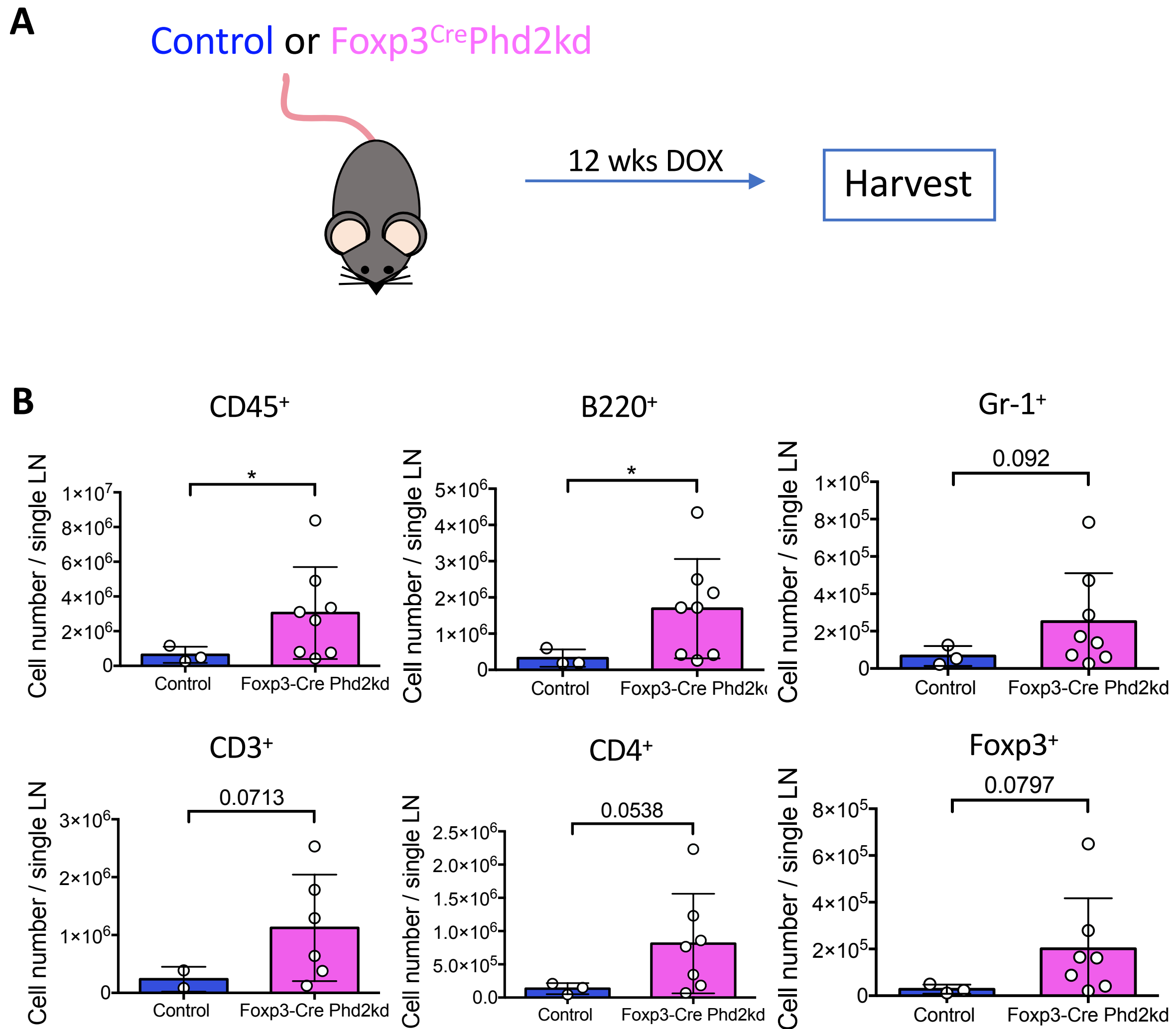


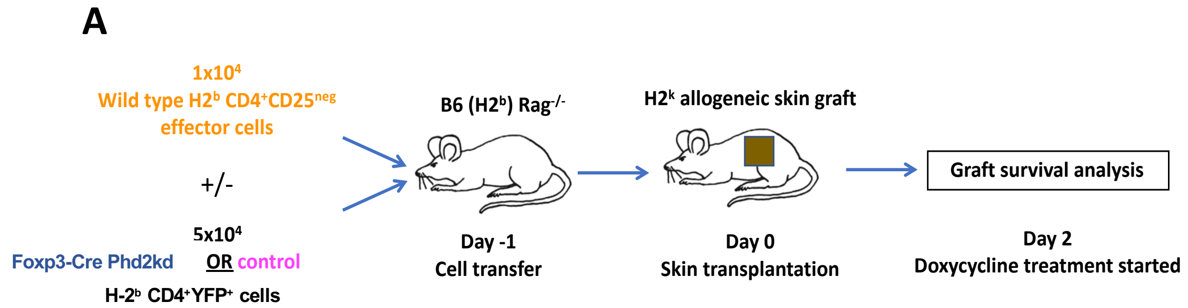
Figure 5.2.9. Foxp3Cre Phd2kd mice exhibit similar phenotype to Phd2kd Tregs. **(A)** Schematic of experimental plan. Control (blue) and (FoxP3-Cre-LSL-CAGGrtTA-Phd2miR, Foxp3^{Cre}-Phd2kd)(pink) were treated with dox (2 mg/mL with 30% sucrose drinking water ad libitum) for 12 weeks and lymph nodes were harvested for flow cytometric analysis. **(B)** Graphs of CD45^{+}, CD3^{+}, B220^{+}, Gr-1^{+}, CD4^{+}, and Foxp3^{+} cell numbers per LN from control and Foxp3^{Cre}-Phd2kd mice (unpaired *t* test, n= 3 for Control$^{CAG/Foxp3}$ mice, and n=8 for Foxp3^{Cre}-Phd2kd group)(**p*<0.05.)

5.2.10 Foxp3^{Cre}Phd2kd Tregs are have impaired ability to prolong allograft survival

Upon observing that Foxp3^{Cre}-Phd2kd mice eventually develop a similar, but limited, inflammatory phenotype, we next sought to determine whether Foxp3^{Cre}-Phd2kd Tregs also had impaired suppressive function. The same adoptive transfer and skin transplantation model as with the global Phd2kd Tregs (as outlined in chapter 5.2.2.) was used, but this time with the transfer of Foxp3^{Cre}Phd2kd Tregs **[Figure 5.2.10A]**. Tregs from control and Foxp3^{Cre}-Phd2kd mice were sorted and isolated with high purity through their expression of yellow fluorescent protein (YFP), which is expressed as part of the Cre transgene. After their skin grafts were monitoring for 100 days after transplantation, C57BL/6 Rag mice receiving Foxp3^{Cre}-Phd2kd Tregs demonstrated a similar trend in rejecting their grafts faster than those receiving control Tregs. However, there was no statistically significant difference between the groups potentially due to the low group sizes **[Figure 5.2.10B]**. A second assay under the same conditions is currently being planned to confirm whether there is a consistent trend.

Overall, the data presented here suggest that the dysfunction found within Tregs upon the silencing of *Phd2* can be induced intrinsically within Treg populations. Moreover, these dysfunctional Tregs are at least partially responsible for the overall immune dysregulation found within knocking down *Phd2*, although the phenotype was less pronounced within Foxp3-restricted silencing relative to global silencing.

A



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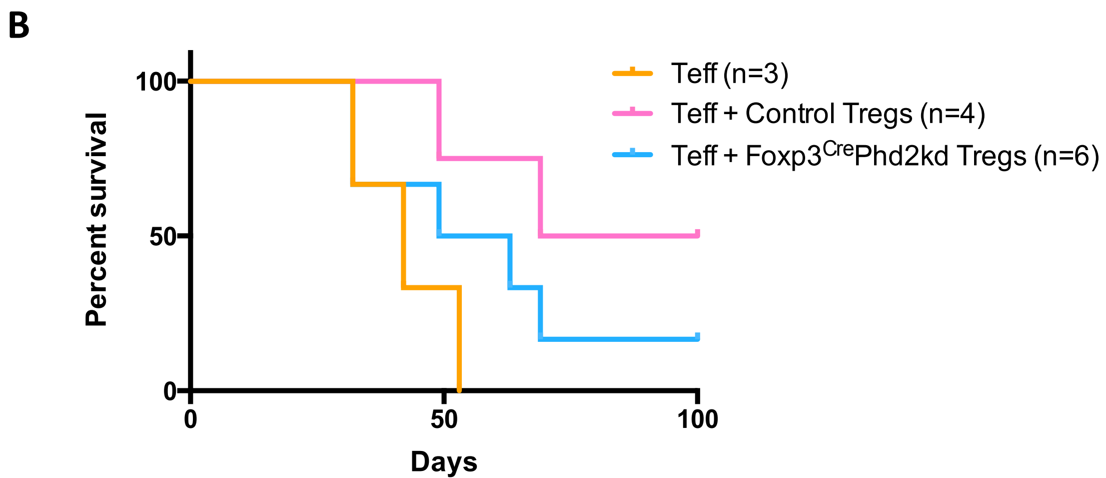

Figure 5.2.10. Foxp3CrePhd2kd Tregs are have impaired ability to prolong allograft survival. (A) Schematic of experimental skin transplant model design. C57BL/6 *Rag1*$^{-/-}$ (H-2^{b}) received WT (H-2^{b}) CD4^{+}CD25^{neg} effector T cells (Teff) with or without CD4^{+}YFP^{+} Tregs from either H-2^{b} control or Foxp3^{Cre}-Phd2kd mice. A day later, mice received allogeneic H-2^{k} skin grafts and were started on doxycycline treatment on subsequent day (2mg/mL in drinking water, ad libitum). **(B)** Graft survival graph of three groups of mice receiving WT Teffs only (n=3, orange), WT Teffs + control Tregs (n=4, pink), and WT Teffs + Foxp3^{Cre}-Phd2kd Tregs (n=6, blue). Survival data were analysed using log-rank test.

5.3 Discussion

In this chapter, the effects of *Phd2* silencing *in vivo* on the immune phenotype were revealed, highlighting a prominent role for the HIF/Phd2 pathway in immune homeostasis. Moreover, the results presented here suggest that the immune dysregulation exhibited by Phd2kd mice may be driven by an impairment of Tregs.

The silencing of *Phd2* had evident effects on the immune system. However, because *Phd2* inhibition was systemic in our model, it was necessary to determine whether the overall phenotype can be driven by its effects on the immune system alone. In this regard, our group had previously conducted experiments to clarify this point. In these experiments, congenic recipient mice (CD45.1) received bone marrow transplantation (BMT) from either Phd2kd or control mice (CD45.2) [472]. After 8-weeks engraftment following 10-weeks dox treatment, only mice that received BMT from Phd2kd mice developed a similar phenotype (enlargement of LNs and multilineage expansion) as observed in Phd2kd mice. This demonstrated that the phenotype of Phd2kd mice was largely driven by intrinsic silencing effects on the immune system.

While previous studies have demonstrated the consequences of *Phd2* silencing using Cre-mediated recombination in adult mice, citing increased angiogenesis and erythrocytosis [547-549], these did not report changes in physical appearances or immune phenotype similar to those observed within our model. Thus, to address concerns of whether our observations were specific to our particular model, our group have also analysed samples from mice with Cre-recombinase

mediated *Phd2* inactivation (RosaERTCre;Phd2fl/fl), finding similar trends of immune dysregulation in both models [472].

Given the similarities in phenotype to other pathologies associated with *Foxp3* disruption, we were surprised to find an expansion of highly activated ($CD62L^{lo}CD44^{+}$) $Foxp3^{+}$ Treg populations as reflected in both cell number and proportion in Phd2kd mice **[Figure 5.2.3]**. However, when their function was assessed *in vivo*, Phd2kd Tregs had completely lost their ability to control graft rejection **[Figure 5.2.2]**. Our in depth immunophenotyping of transcription factors and cytokines further reveal a unique Treg population that arises upon the silencing of *Phd2* **[Figure 5.2.4]**. Our findings are especially relevant in regard to a recent study by Lee et al., in which the authors observed an inflammatory phenotype in mice with Treg-specific VHL deficiency (*Vhl*$^{fl/fl}$*Foxp3*^{Cre}) accompanied by loss of suppression in Tregs but no increase in Treg population [550]. Moreover, they reported significant HIF-1α-dependent conversion of Tregs to IFN-γ producing-Th1 effector cells in *Vhl*$^{fl/fl}$*Foxp3*^{Cre} mice. While we observed an upregulation in expression of Th1-type molecules such as TNF-α and T-bet in Phd2kd Tregs, we importantly did not observe an increase in IFN-γ production or loss of Foxp3 expression in Treg populations in either Phd2kd or $Foxp3^{Cre}$-Phd2kd mice. It is thus possible that the differences in phenotype observed in disrupting different parts of this pathway may be attributed to other components upstream of VHL responsible for HIF inhibition. Furthermore, *Phd2* silencing did not upregulate IL-17 or ROR-γt expression in either $Foxp3^{neg}$ or $Foxp3^{+}$ populations in our model, despite previous reports suggesting that HIF-1α expression can enhance Th17 skewing and impede Treg differentiation [465, 466]. It may be possible that the $Foxp3^{+}$ Treg populations in Phd2kd mice may eventually convert to IFN-γ or IL-

17-producing effector cells over time given longer duration of dox treatment. Overall, further investigation on the specific pathways activated as a result of the inhibition of Phd2 will elucidate its specific effects on Tregs.

One of the most intriguing phenotypic changes found in Foxp3^{+} Tregs in Phd2kd mice was a significant downregulation in CD25 expression **[Figure 5.2.5]**, leading to an expansion of CD25^{neg}Foxp3^{+} Tregs that are not commonly found within the LNs [551]. Interestingly, CD25^{neg}/CD25^{low}Foxp3^{+} Treg populations have been found to be upregulated in patients with autoimmune pathologies such as SLE [552-556]. While previous studies have established an indispensable role for CD25 signalling in the differentiation and induction of Tregs, its function in mature Tregs is not as clear [557]. Congenital disruption of IL-2R signalling in Foxp3-expressing cells leads to *Scurfy*-like phenotype [530, 557]. However, mice with these deficiencies are still able to generate Foxp3^{+} Tregs in the thymus and periphery, albeit with lower frequency and impaired suppressive potency. While loss of CD25 expression inhibits Treg-mediated suppression through IL-2 deprivation, CD25^{neg} Tregs may also have intrinsically impaired function through disruption of STAT5 signalling [557]. Interestingly, in the context of autoimmunity, Tregs from CD25 KO mice were reported to take on an activated/effector phenotype (CD62L^{neg}CD44^{+}) with loss of suppressive function, similar to what was observed within our Phd2kd model. Moreover, post-thymic CD25 deletion in Tregs have revealed that CD25 expression is necessary for long-term survival of Tregs and their suppressive function, but not lineage stability [558]. Collectively, these reports suggest that CD25 expression is indispensable for Treg suppressive function and may be a marker for activated Tregs within an autoimmune environment.

The significant expansion of $CD25^{neg}$ Tregs in Phd2kd mice also begs the question regarding the source of this cell population. Whether they are originally $Foxp3^{+}$ Tregs that lost CD25 expression or are Tregs induced from Tconvs that gained Foxp3 expression is unclear within our current model, in part due to a lack of means to distinguish thymic (tTregs) or peripheral Tregs (pTregs). However, the former reasoning may be more likely given our TSDR analysis that demonstrates that $Foxp3^{+}$ T cells from Phd2kd mice have high demethylation levels regardless of CD25 expression **[Figure 5.2.6]**. Relative to tTregs that consistently demonstrate nearly complete demethylation, some studies have shown that pTregs have more variable degrees of demethylation [559], although this has also been refuted by other reports [560]. Thus, it may be beneficial for future studies to explore whether the $CD25^{neg}$ Tregs can gain or regain CD25 expression and suppressive function. Interestingly, a study by Zelaney et al. suggested that $CD25^{neg}$ Tregs are a population of committed regulatory cells that regain $CD25^{+}$ expression upon homeostatic expansion and/or activation [561]. While the results within this thesis demonstrated that the removal of dox can reverse the immune dysregulated phenotype and replenish $CD25^{+}Foxp3^{+}$ Treg populations **[Figure 5.2.8]**, the question of whether they are Tregs that regained CD25 expression or newly expanded/induced Tregs was not definitively determined. Therefore, future studies using Phd2kd models with fluorescently labelled $Foxp3^{+}$ cells may be able to help to answer some of these questions regarding the origin and suppressive function of $CD25^{neg}$ Treg populations.

The $Foxp3^{Cre}$-Phd2kd model provided valuable insight regarding the inflammatory phenotype observed upon *Phd2* silencing. Firstly, although the phenotype induced in $Foxp3^{Cre}$-Phd2kd mice was weaker in comparison **[Figure 5.2.9]**, the induction of similar inflammation and loss of

suppressive function within Tregs **[Figure 5.2.10]** corroborated our findings that the immune dysregulated phenotype in global Phd2kd mice is at least partly due to effects on Tregs. Moreover, the lack of significant change in expression of other markers such as T-bet, TNF-α, CD44, CD62L, and CD25 within Tregs suggests that global Phd2kd Tregs adopted the observed phenotype indirectly, rather than through a Treg-intrinsic Phd2-mediated mechanism. Nonetheless, the phenotype observed in Foxp3^{Cre}-Phd2kd mice may be less pronounced due to limitations within our current model, in which a mixture of heterozygote males and females was used. Because *Foxp3* is located on the X chromosome, random inactivation of one allele in heterozygotic females will generate approximately half Tregs expressing the WT allele and the other half being Phd2 silenced. Future studies will compare our findings using homozygotic models or restricting experiments to male mice.

Taken together, this chapter provides evidence that Phd2 plays a critical role in Treg function and homeostasis, in which its impairment may induce immune dysregulation. The focus of subsequent studies in the next chapter will be to identify the Phd2 targets that drive this inflammatory phenotype and exploit this pathway to determine if Treg function and fitness can be enhanced.

6 Modulation of Treg Phenotype and Function Under Chronic Hypoxia and HIF-2α Inhibition

6.1 Introduction

An important factor to consider within the context of Treg cell therapy is the stability of cell products *in vivo*. Tregs can lose suppressive function or even convert to pathogenic effector T cells under certain conditions, such as inflammation [560, 562, 563]. As PHD2 is inactivated under hypoxic conditions, such as those found in allograft microenvironment, this suggests that oxidative tension may be another factor that destabilises Treg function. Thus, identifying the specific components that drive Treg dysfunction upon PHD2 inhibition may be therapeutically promising within the context of transplantation and other immune pathologies.

While PHD2 may interact with other proteins [564], it principally targets HIF-1α and HIF-2α for VHL-mediated proteasomal degradation. Although reports investigating the role of HIF-2α on Tregs are sparse, recent studies have highlighted the effects of HIF-1α in Treg biology. In particular, HIF-1α has been shown to modulate the balance between Th17 and Treg differentiation [464-466]. Nonetheless, our own data **[Figure 5.2.4]** demonstrated no significant increases in Th17-associated markers or cytokines upon the silencing of *Phd2*, suggesting that

this is not the principal cause of the immune dysregulation within our model **[Figure 5.2.1]**. Moreover, our adoptive cell transfer and Foxp3^{Cre}-Phd2kd data further demonstrate that *Phd2* silencing has a direct effect on differentiated Foxp3-expressing Tregs in dysregulating their function **[Figure 5.2.10]**. With regards to the role of HIF-1α on Treg function specifically, previous reports have been largely incongruent. While some have reported that HIF-1α can drive Treg instability and loss of suppressive function [467, 565], others have found that HIF-1α expression enhances suppression and is necessary for optimal function *in vivo* [468, 469]. Therefore, it is evident that further research is necessary to clarify the roles that HIF-α molecules play in Treg biology. These insights may permit the manipulation of this pathway to promote stability and function of Tregs under hypoxic environments.

In this chapter, we therefore aimed to build on our previous findings and prior literature to identify the components that drive an immune dysregulated phenotype upon silencing *Phd2*. Moreover, we sought to determine whether these models mimic physiological or pathological conditions. Finally, we investigated whether our insights within this pathway can be translated for therapeutic use in the form of pharmacological inhibition.

Hypothesis: The phenotypic changes observed in Phd2kd mice are driven by HIF-α, which can be controlled to modulate Treg function

Aim: To explore the effects of HIF-α inhibition and chronic hypoxia on Treg biology and function

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Figure 6.2.1 in this chapter has been reproduced/adapted with permission from Yamamoto et al. [472] and is entirely my own work.

6.2 Results

6.2.1 *Hif2a* inhibition reverses *Phd2* knockdown-induced immune dysregulated phenotype

To first assess whether the immune dysregulated phenotype observed within Phd2kd mice was indeed driven by HIF signalling, we used *Phd2* and *Hif* double-KD mice developed by our collaborators at the Hypoxia Biology Group. Due to mice possessing ColA1 alleles, two different targeting sequences can be expressed, thereby allowing the generation of mice in which *Phd2* was targeted with either *Hif1a* (CAGGrtTA-Phd2miR/Hif1amiR, P2H1kd) or *Hif2a* (CAGGrtTA-Phd2miR/Hif2amiR, P2H2kd).

Along with Control^{CAG} and Phd2kd mice, P2H1kd and P2H2kd mice were administered the same course of dox treatment (2mg/mL in drinking water). Throughout the treatment period, while P2H1kd mice had weight loss similar to Phd2kd mice, P2H2kd mice had comparable mean weight changes to control mice **[Figure 6.2.1A]**. Moreover, upon harvesting peripheral lymph nodes after 4 weeks dox treatment, concomitant silencing of *Hif1a* partially ameliorated *Phd2* knockdown-induced lymphadenopathy, as reflected in LN size and weight **[Figure 6.2.1B]**. Nonetheless, concomitant *Hif2a* knockdown had comparable measurements to control mice. This trend was consistent when measuring the absolute counts of CD45$^+$, CD3$^+$, B220$^+$, and Gr-1$^+$ immune cell populations within the peripheral LNs, in which *Hif2a* silencing reversed leukocyte expansion observed in Phd2kd mice **[Figure 6.2.1C]**. Taken together, this demonstrated that the immunological effects induced by the silencing of *Phd2* was primarily *Hif2a* isoform dependent.

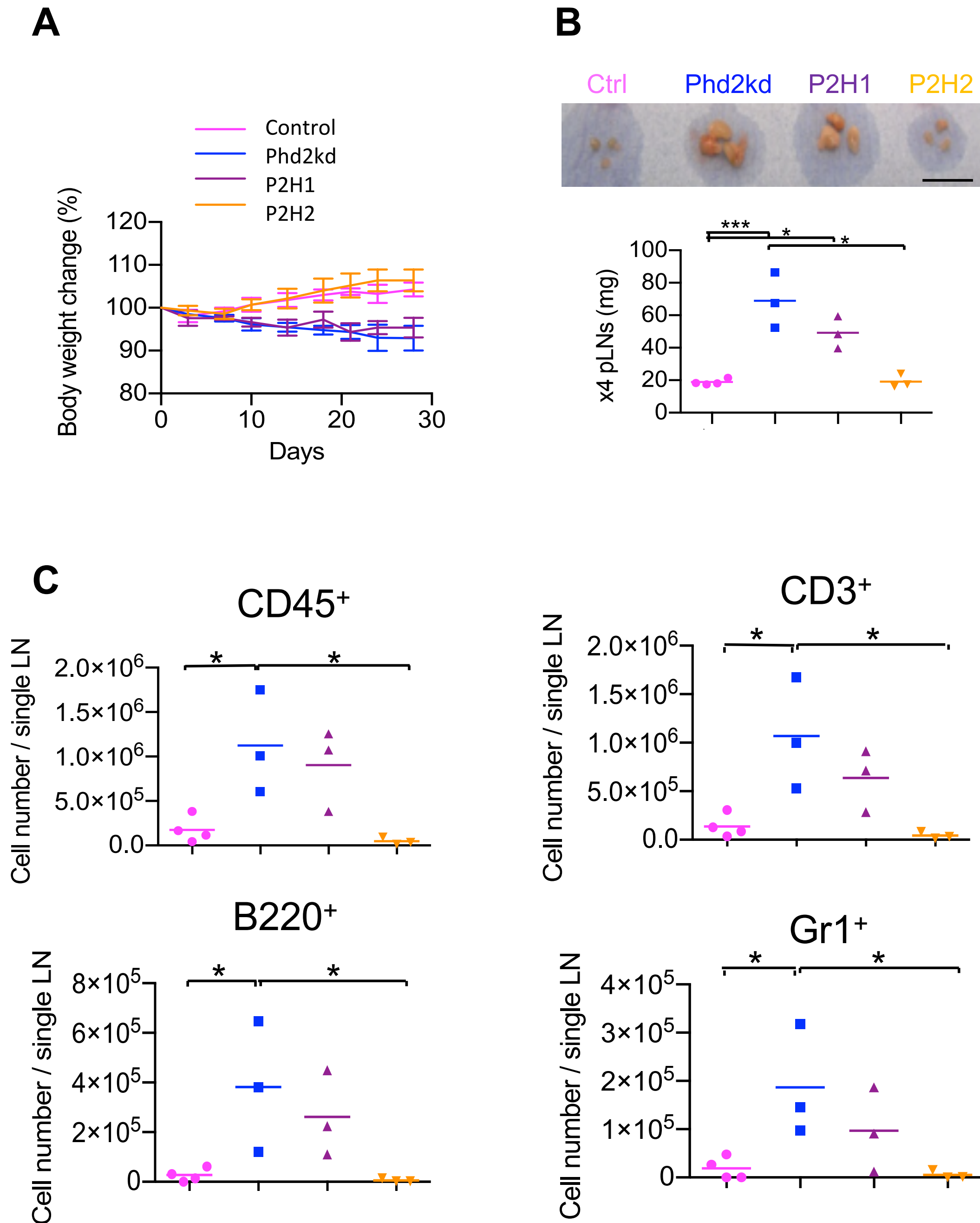


Figure 6.2.1. Hif2a inhibition reverses Phd2 knockdown-induced immune dysregulated phenotype. Control, Phd2kd, P2H1 (PHD2 HIF-1α double knockdown, CAGGrtTA-Phd2miR/Hif1amiR), and P2H2 (PHD2 HIF-2α double knockdown, CAGGrtTA-Phd2miR/Hif2amiR) mice were treated with dox (2 mg/mL with 30% sucrose drinking water ad libitum) for 4 weeks. **(A)** Mean percentage of body weight changes relative to d0 (day dox treatment was started) was recorded for the duration of doxycycline treatment (repeated-measures ANOVA with Tukey's post-hoc test, n=3-7.) **(B)** Representative image and tissue weights (mg) of LN taken from mice after 4 weeks dox (one-way ANOVA with Tukey's post hoc test, n=3-7)(*p<0.05, **p<0.01, ***p<0.001.) **(C)** Graphs of $CD45^+$, $CD3^+$, $B220^+$, and Gr-1^+ cell numbers per pLN (one-way ANOVA with Tukey's post hoc test, n=3-7))(*p<0.05).

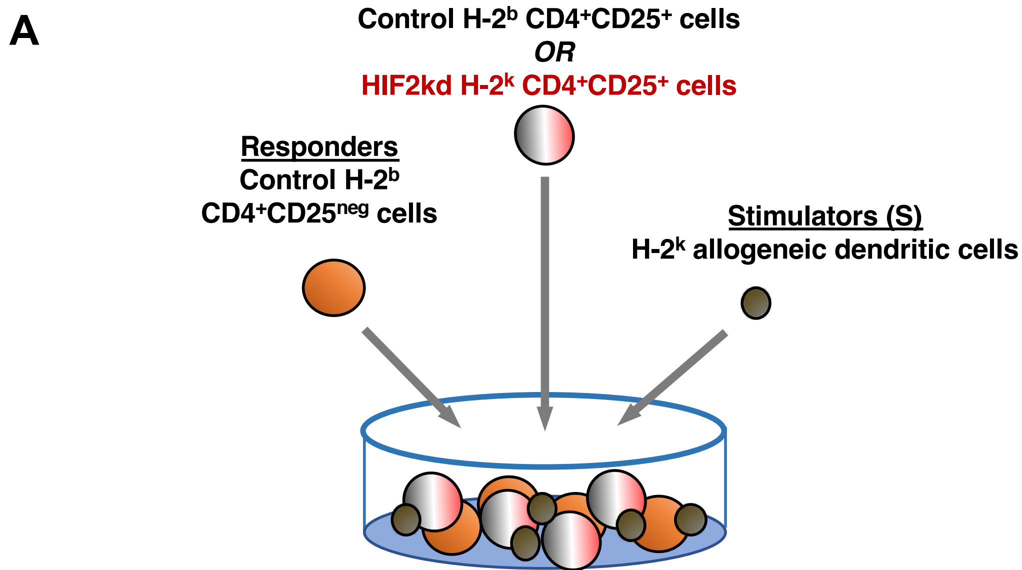
6.2.2 *Hif2a* knockdown Tregs demonstrate greater suppression *in vitro*

Previous studies have investigated effects of *Hif1a* inhibition in modulating Treg biology. Nonetheless, the role that *Hif2a* plays in Treg suppressive function has not yet been closely examined. Our previous data indicated that the inflammatory phenotype observed within Phd2kd mice was in large part due to a dysfunction in Tregs **[Figure 5.2.2]**. Thus, based on the finding that concurrent *Hif2a* inhibition completely ameliorated immune dysregulation, we hypothesised that *Hif2a* have detrimental effects on Treg function.

While HIF-α isoforms are expected to be readily destabilised under normoxia, we were first curious to establish whether *Hif2a* inhibition had any effects on Tregs under normoxic conditions. To investigate this, *Hif2a* single knockdown (HIF2akd) mice were used. Control^{CAG} and HIF2akd mice were treated for 4 weeks with dox (2mg/mL in drinking water) before $CD4^{+}CD25^{+}$ Tregs from spleens were isolated for *in vitro* functional analyses. Syngeneic WT $CD4^{+}CD25^{neg}$ were cocultured with CBA alloDCs (generated in the presence of GM-CSF and IL-4) and Control^{CAG} or HIF2akd Tregs at various Teff:Treg ratios (1:1, 1:2, 1:4, and 1:8) **[Figure 6.2.2A]**. Doxycycline (2mg/mL) was incorporated in culture medium to ensure persistent *Hif2a* knockdown throughout the course of the incubation period.

Here, HIF2akd Tregs demonstrated enhanced suppression of Teff proliferation over control Tregs **[Figure 6.2.2B]**. Thus, taken together, these data suggest that *Phd2* silencing increases *Hif2a*, which drives inflammatory response and has a role in dysregulating Treg function. Moreover, *Hif2a* inhibition may in turn enhance Treg suppressive function even in an oxygenated environment.

A



B

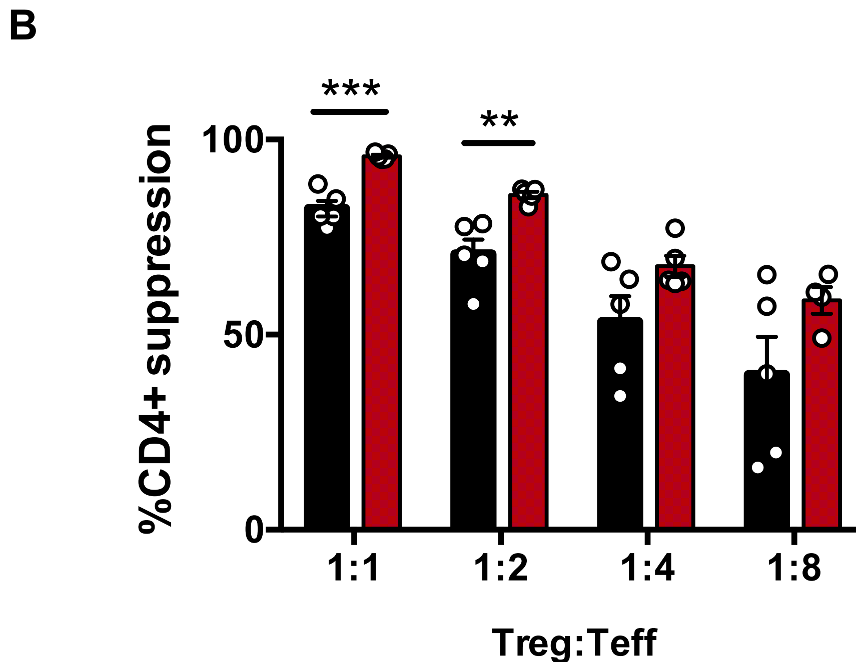
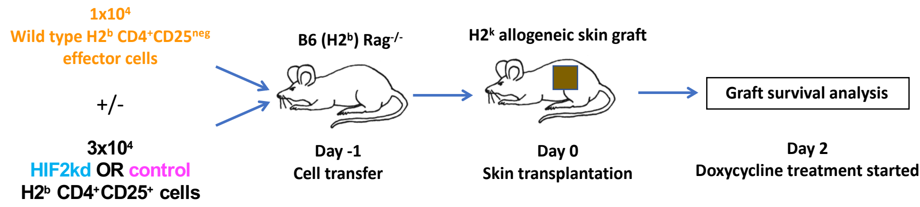


Figure 6.2.2. Hif2a knockdown Tregs demonstrate greater suppression *in vitro*. (A) Schematic of *in vitro* suppression assay design. Splenocyte-derived CD4^{+}CD25^{+} Tregs were isolated from splenocytes of control or HIF2akd (CAGGrtTA-Hif2amiR) CBA mice, which were pre-treated for 4 weeks of doxycycline (2 mg/mL with 30% sucrose drinking water ad libitum). Tregs were then cultured with control CD4^{+}CD25^{neg} Teff responders stimulated with CBA allogeneic DCs at various Treg:Teff ratios (1:1, 1:2, 1:4, and 1:8) and incubated for 4 days. Dox was added to culture medium in all assays. **(B)** Graph shows responder proliferation rate, which was measured by [(3)H]thymidine uptake. Data shown as division index values, in which proliferation is normalised to the proliferation of responders with beads alone (unpaired *t* test)(**p<0.01; ***p<0.001; ns=not significant.)

6.2.3 *Hif2a* knockdown Tregs do not prolong skin allograft survival

Given their enhanced suppression *in vitro*, their functionality *in vivo* was assessed using an adoptive cell transfer and skin transplantation model. C57BL/6 Rag (H-2^b) mice received $CD4^+CD25^+$ Tregs isolated from $Control^{CAG}$ or HIF2akd mice and C57BL/6 WT $CD4^+CD25^{neg}$ Teffs. On the subsequent day, mice were transplanted with allogeneic CBA (H-2^k) skin grafts and monitored for 100 days post-transplantation. Dox treatment was initiated the day after transplantation **[Figure 6.2.3A]**. In contrast to the findings from the *in vitro* assays, HIF2akd Tregs demonstrated significant functional impairment and did not prolong allograft survival **[Figure 6.2.3B]**. While repeat experiments with the same conditions will be conducted in future studies to confirm these findings, the discrepancy between the *in vitro* and *in vivo* functional assays suggest that there is high complexity within the HIF/PHD pathway that may require further investigation.

A



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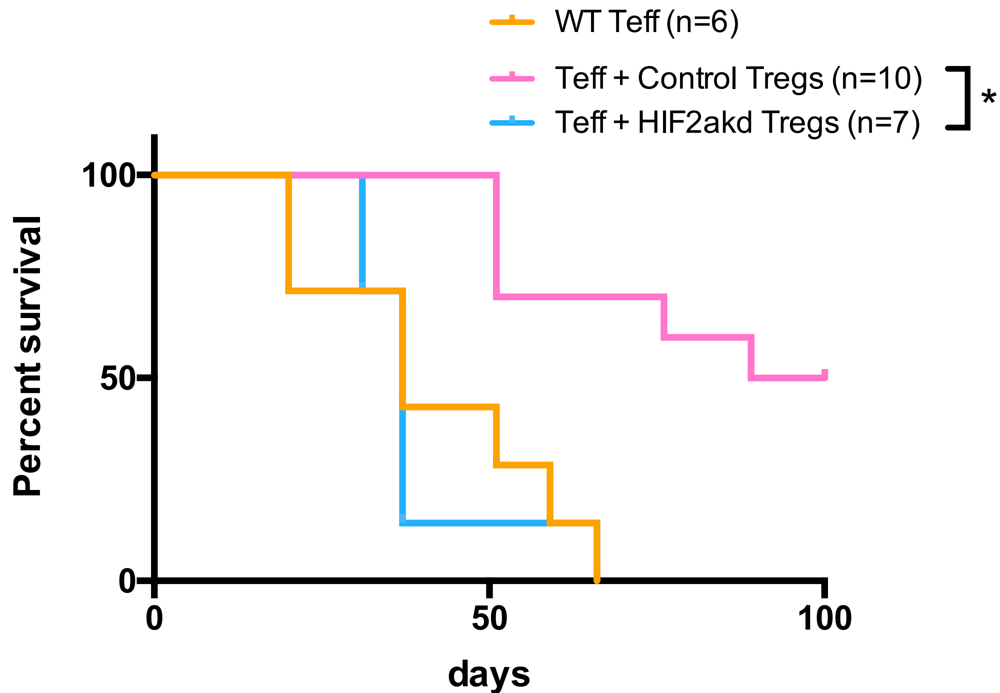


Figure 6.2.3. ***Hif2a*** **knockdown Tregs do not prolong skin allograft survival. (A)** Schematic of experimental skin transplant model design. B6 *Rag1*$^{-/-}$ (H-2^b) received WT (H-2^b) CD4$^+$CD25^{neg} effector T cells (Teff) with or without CD4$^+$CD25$^+$ Tregs from either C57BL/6 control or HIF2akd mice. A day later, mice received allogeneic CBA (H-2^k) skin grafts and were subsequently started on doxycycline treatment (2mg/mL in drinking water, ad libitum). **(B)** Graft survival graph of three groups of mice receiving WT Teffs only (n=6, orange), WT Teffs + control Tregs (n=10, pink), and WT Teffs + IL-33-Tregs (n=7, blue). Survival data were analysed using log-rank test (*p<0.05).

6.2.4 Mice exposed to chronic hypoxic conditions exhibit similar immune dysregulated phenotype demonstrated by Phd2kd mice

Given the dramatic changes observed in immune phenotype and Treg function from the genetic manipulation of the HIF/PHD2 pathway, we sought to investigate whether these findings were translatable to physiological conditions. We first asked whether the immunological effects of *Phd2* inhibition can also be observed in mice exposed under chronic hypoxic conditions, in which HIF signalling pathways would be expected to be activated. To explore these questions, C57BL/6 WT mice were maintained within hypoxic chambers (10% O_2) for 6 weeks before harvesting for phenotypic analysis **[Figure 6.2.4A]**.

As expected, control mice kept under hypoxic conditions demonstrated an increase in haematocrit levels compared to those kept under normoxia **[Figure 6.2.4B]**. Interestingly, mice exposed to hypoxia also exhibited weight loss **[Figure 6.2.4C]**. Moreover, similar to the phenotype observed within Phd2kd mice, there was significant lymphadenopathy **[Figure 6.2.4D]**, correlating with increases in absolute cell numbers of major immune cell populations, including Foxp3^{+} Tregs **[Figure 6.2.4E]**.

In a separate study, activation/memory markers (CD62L and CD44) were also analysed, in which the trends were again consistent with those observed between control and Phd2kd mice. While many of the parameters did not demonstrate statistical significance, perhaps due to low group sizes (n=3), there was an overall shift away from naïve (CD62L^{+}CD44^{neg}) towards effector populations (CD62L^{neg}CD44^{+}) in CD4^{+} populations of hypoxic mice **[Figure 6.2.4F]**. Significant shifts in other markers of interest (i.e. CD25 and Th1 markers; data not shown) were not

observed. Overall, mice exposed to chronic hypoxic conditions demonstrated an inflammatory phenotype that partially exhibited some of the effects seen with genetic *Phd2* inhibition.

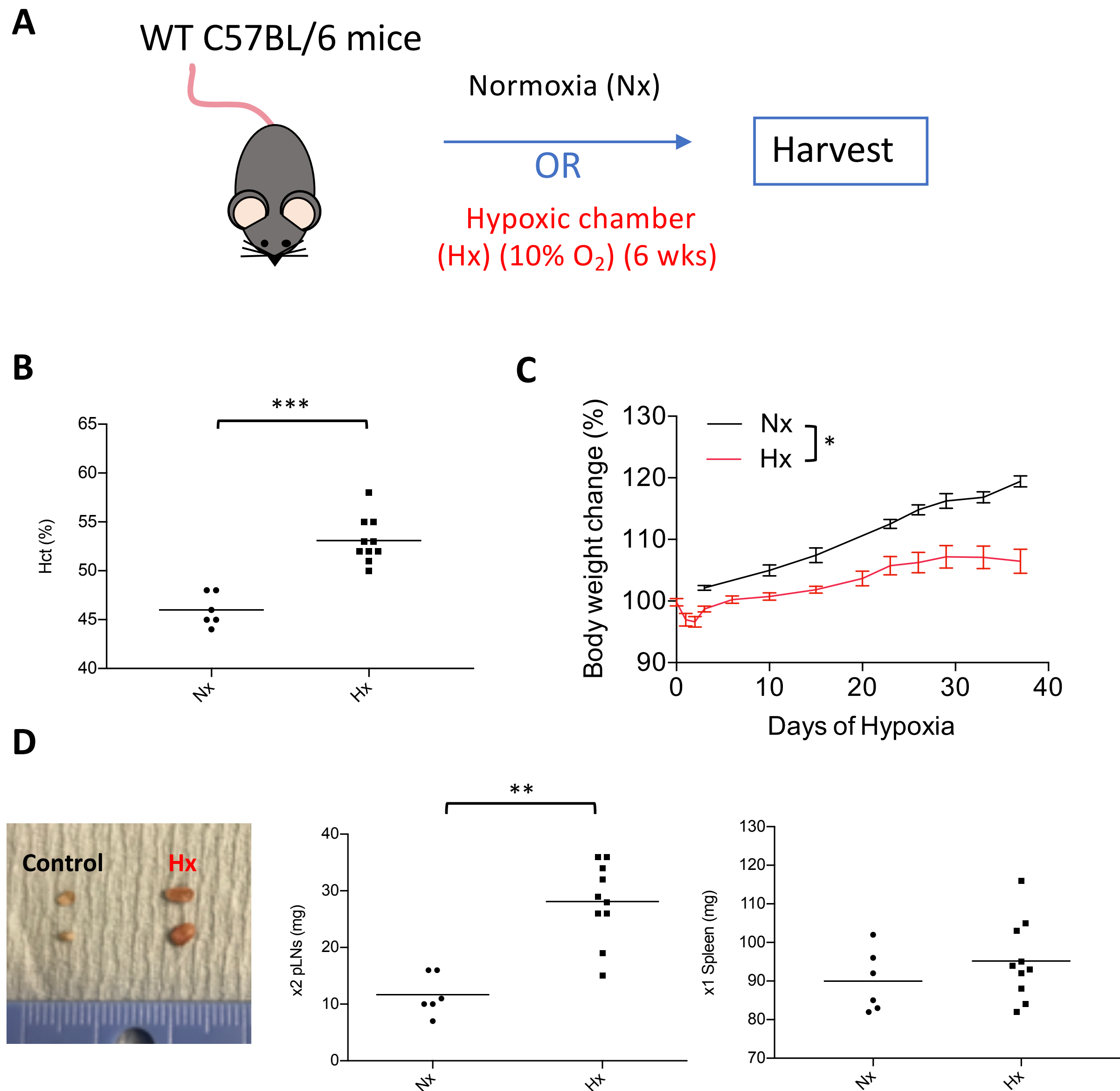


Figure 6.2.4. Mice exposed to chronic hypoxic conditions exhibit similar immune dysregulated phenotype demonstrated by *Phd2kd* mice. (A) Schematic of experimental design. WT C57BL/6 mice were maintained in either normoxic cages (Nx) or hypoxic 10% O_2 (Hx) cages for 6 weeks. **(B)** Mean percentage of body weight changes relative to d0 (day put into chambers) was recorded for the duration of the 6 weeks. (repeated-measures ANOVA with Tukey's post-hoc test, n=6-10) **(C)** Hematocrit levels within peripheral blood and **(D)** tissue images and weights (mg) of pLNs (2 per mouse) and spleens were recorded at the end of treatment. (unpaired *t* test, n= 6-10)(***p*<0.01; ****p*<0.001.)

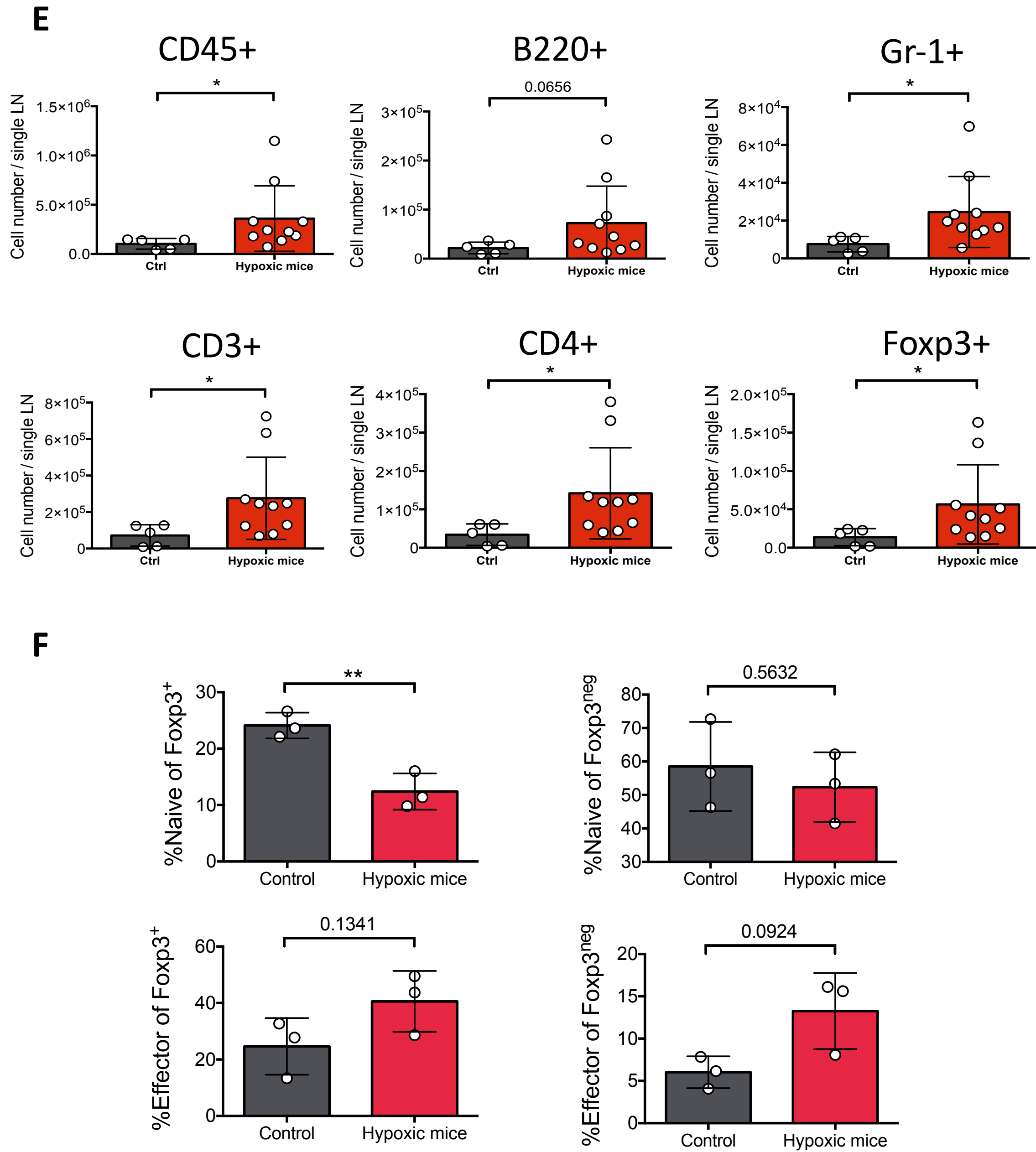


Figure 6.2.4. (cont.) Mice exposed to chronic hypoxic conditions exhibit similar immune dysregulated phenotype demonstrated by *Phd2kd* mice. (E) Graphs showing flowcytometric data of cell numbers per pLN of $CD45^+$, $B220^+$, $Gr\text{-}1^+$, $CD3^+$, $CD4^+$, and $Foxp3^+$ populations and **(F)** proportion of naïve ($CD62L^+CD44^{neg}$) and effector ($CD62L^{neg}CD44^+$) cells within $Foxp3^+$ and $Foxp3^{neg}$ populations (unpaired *t* test, n=3-10)(*p<0.05, **p<0.01.)

6.2.5 Hypoxia induced-inflammation can be corrected by *Hif2a* inhibition

The use of hypoxic chambers to induce chronic hypoxic conditions enables the exploration of the effects of inhibition of HIF in a physiological setting. Given the reversal of immune dysregulation in Phd2kd mice upon the concomitant inhibition of *Hif2a* **[Figure 6.2.1]**, we next asked whether it can also ameliorate hypoxia-driven inflammatory effects. To examine this, experiments were conducted with mice that carried a tamoxifen-inducible Cre recombinase-expressing transgene (*Rosa26Cre*^{ERT2}) that were homozygous for floxed *Hif2a* alleles (RosaERTCre *Hif2a*$^{fl/fl}$, HIF2aKO). One week after tamoxifen treatment, Control^{Cre} (RosaERTCre) or HIF2aKO were maintained in hypoxic chambers (10% O_2) for 4 weeks before harvesting for phenotypic analysis **[Figure 6.2.5A]**.

Consistent with previous findings, *Hif2a* silencing was found to abrogate the development of lymphadenopathy, as reflected in size and weight of peripheral LNs **[Figure 6.2.5B]**. Although not significant, leukocyte expansion in the LNs was also minimised in HIF2aKO mice, as reflected in absolute cell numbers of CD45$^+$, CD3$^+$, B220$^+$, and Gr-1$^+$ immune cell populations **[Figure 6.2.5C]**. The same trend was again observed upon examining the activation/memory markers (CD62L and CD44), in which the shift in frequency from naïve (CD62L$^+$CD44^{neg}) to effector (CD62L^{neg}CD44$^+$) was lessened in HIF2aKO mice **[Figure 6.2.5D].** Taken together, this suggest that chronic hypoxia induces inflammatory responses that may be driven primarily by HIF-2α.

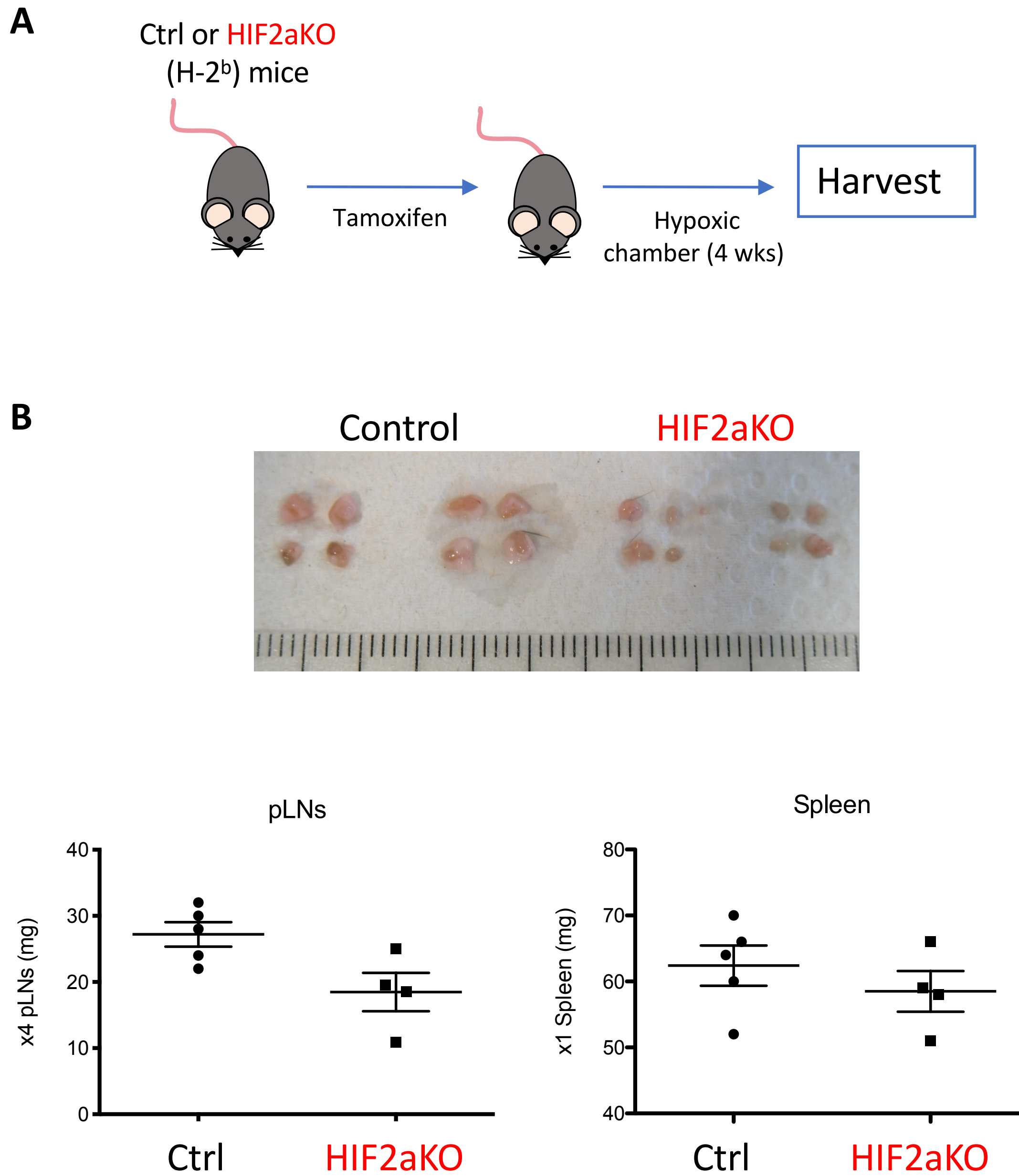


Figure 6.2.5. Hypoxia induced-inflammation can be corrected by *Hif2a* inhibition. **(A)** Schematic of experimental design. C57BL/6 control or HIF2aKO (*Rosa*ERT*Cre* *Hif2a*$^{fl/fl}$) mice were administered tamoxifen treatment a week before being maintained in hypoxic 10% O_2 (Hx) cages for 4 weeks. **(B)** Tissue images and weights (mg) of pLNs (2 per mouse) and spleens were recorded at the end of treatment.

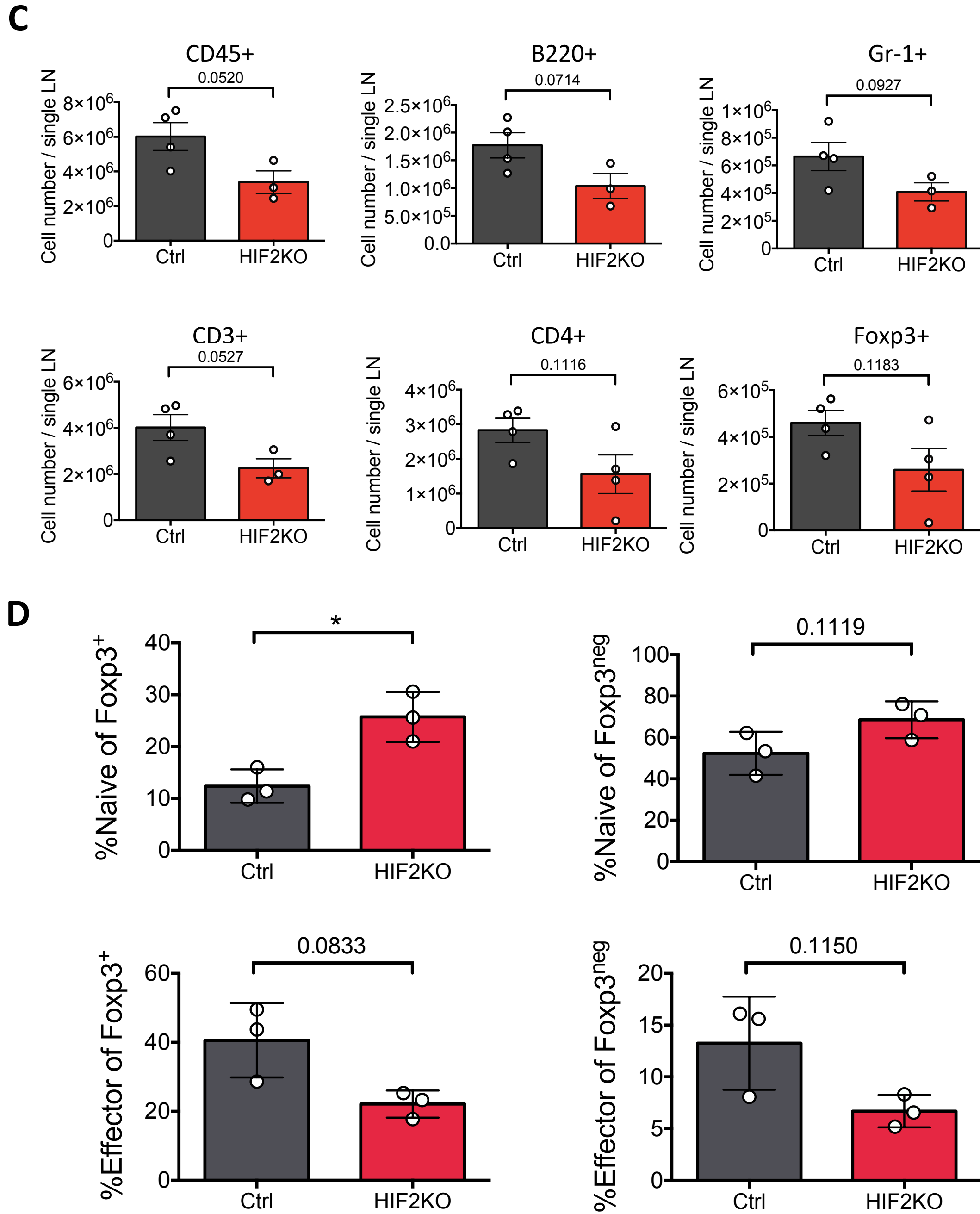


Figure 6.2.5. Hypoxia induced-inflammation can be corrected by *Hif2a* inhibition. **(C)** Graphs showing flowcytometric data of cell numbers per pLN of $CD45^+$, $B220^+$, $Gr\text{-}1^+$, $CD3^+$, $CD4^+$, and $Foxp3^+$ populations and **(D)** proportion of naïve ($CD62L^+CD44^{neg}$) and effector ($CD62L^{neg}CD44^+$) cells within $Foxp3^+$ and $Foxp3^{neg}$ populations (unpaired *t* test, n=3-10)(**p<0.01, ***p<0.001)

6.2.6 HIF-2α inhibition in human Tregs enhances suppressive potency *in vitro*

In the previous mouse experiments, we established an important role for *Hif2a* in modulating Treg function. We next asked whether these findings are relevant to human Tregs. To investigate whether HIF-2α inhibition can enhance Treg function, experiments were performed using a clinically relevant HIF-2α antagonist (PTN2385), which allosterically inhibits dimerization of HIF-2α with HIF-1β [566]. PTN2385 is currently being tested in clinical trials for the treatment of renal cancer.

Ex vivo-expanded human Tregs were first generated from three separate peripheral blood donors (K1, K7, K9) using a well-defined 2-week expansion protocol in the presence of anti-CD3/anti-CD28 beads and recombinant human IL-2 **[Figure 6.2.6A]**. Expanded Tregs were cultured with anti-CD3/anti-CD28 beads and autologous peripheral blood mononuclear cells (PBMCs) at various Treg:PBMC concentrations. DMSO (control, 0.02%, black) or PT2385 (20 μM, red) were added to suppression cultures and incubated for approximately 72 hours until phenotypic analysis. Percent suppression of $CD4^+$ and $CD8^+$ proliferation was calculated based on normalisation to bead-stimulated PBMC only conditions for DMSO and PT2385-treated cultures, respectively.

Consistent with the mouse data, Treg cultures with HIF-2α antagonist treatment exhibited significantly enhanced suppressive potency of $CD4^+$ and $CD8^+$ PBMC proliferation relative to DMSO control Tregs in all three donors **[Figure 6.2.6B]**. Overall, this also correlated with downregulated activation markers (CD25 and HLA-DR) within $CD4^+$ and $CD8^+$ PBMC populations

in HIF-2α-inhibited cultures versus DMSO cultures, further supporting increased suppressive potency of HIF-2α inhibited Tregs **[Figure 6.2.6C]**. In conclusion, this data further demonstrates that HIF-2α play a role in modulating Treg biology and that HIF-2α inhibition could provide a clinically-relevant strategy for the enhancement of Treg suppressive function.

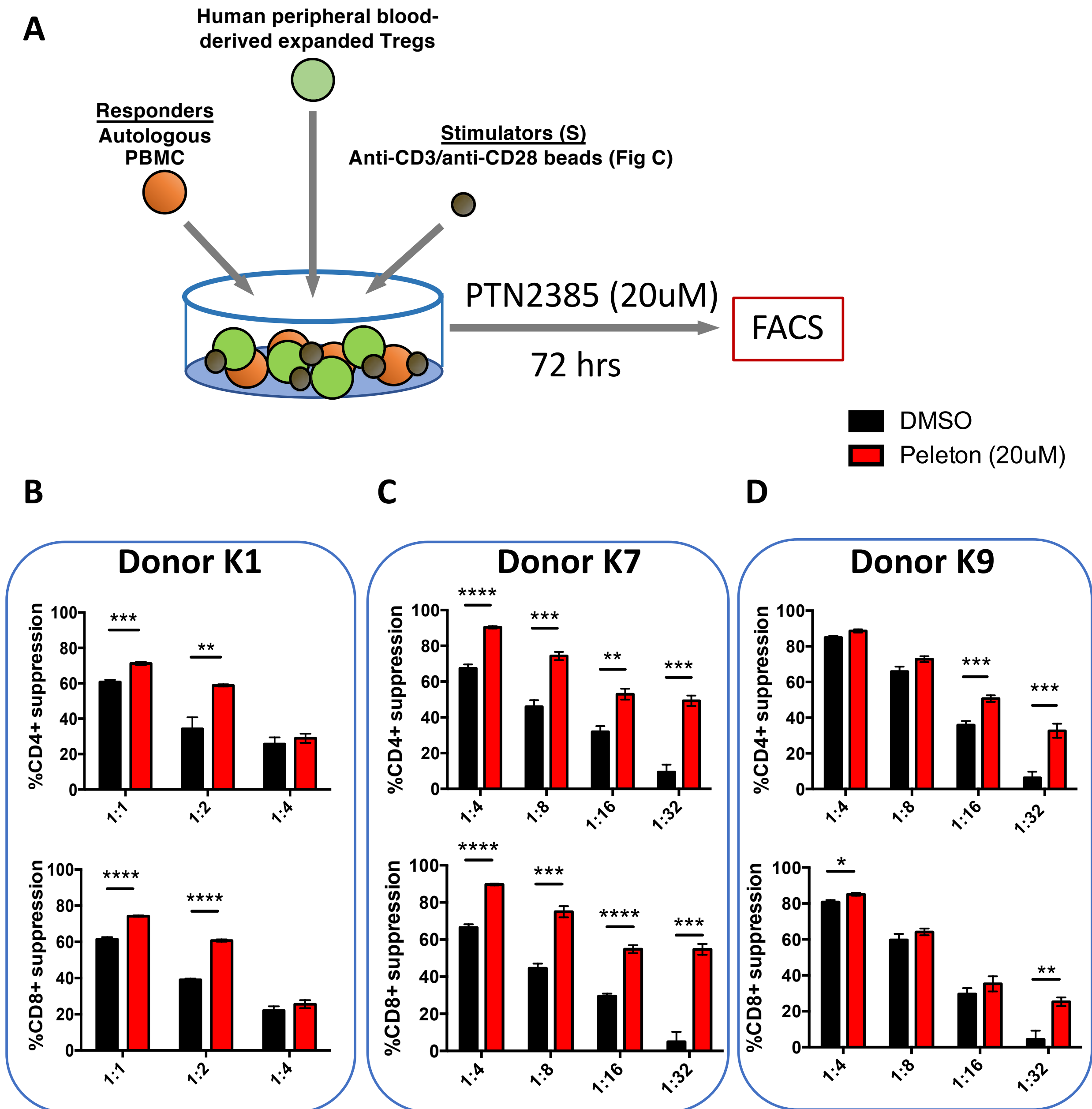
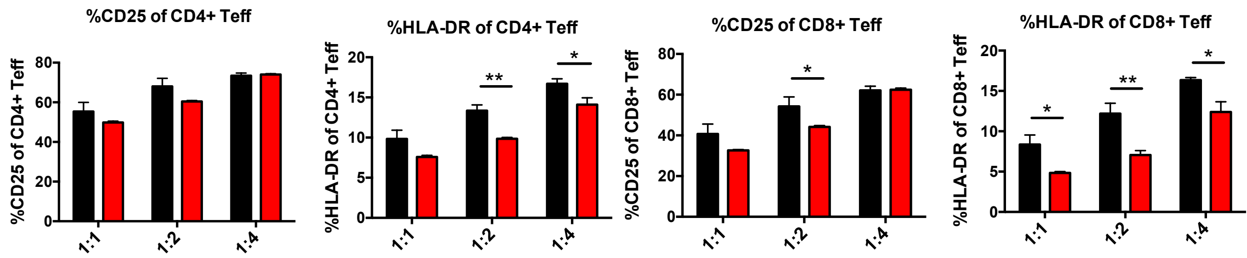


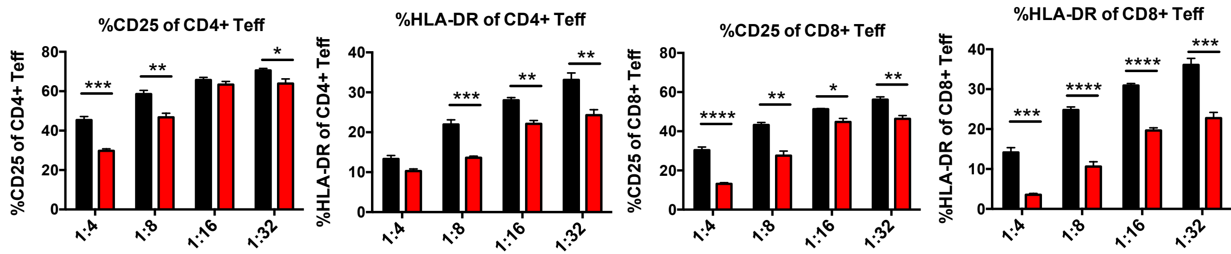
Figure 6.2.6. HIF-2α inhibition in human Tregs enhance suppressive potency *in vitro*. (A) Schematic of *in vitro* suppression assay design. Human $CD4^{+}CD25^{+}CD127^{lo}$ Tregs were flow sorted and expanded *in vitro*. Expanded Tregs were then added at various ratios to a culture of anti-CD3/anti-CD28 stimulated violet proliferation dye (VPD)-stained PBMCs (responders) with DMSO (control, 0.02%) HIF-2α antagonist (PTN2385, 20uM) and incubated for 72 hours before being harvested for flow cytometric analysis. **(B-D)** Data represented as percentage suppression of $CD4^{+}$ or $CD8^{+}$ responders, in which control or PTN2385 groups were respectively normalised to proliferation of responders with beads and DMSO or PTN2385 alone. This was repeated for 3 separate donors (K1, K7, K9) (unpaired *t* test)(*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.)

E **Donor K1**

DMSO
Peleton (20uM)



F **Donor K7**



G **Donor K9**

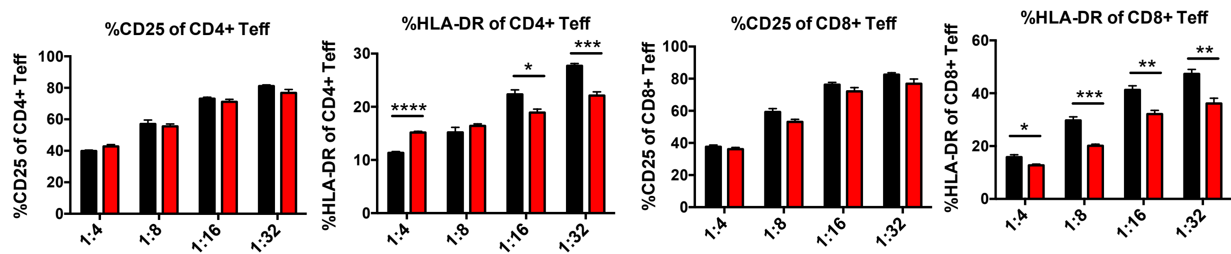


Figure 6.2.6. (cont.) HIF-2α inhibition in human Tregs enhance suppressive potency *in vitro*. (E-G) Graphs representing activation marker (CD25 and HLA-DR) expression of $CD4^+$ or $CD8^+$ responders, separated by donor (unpaired *t* test)(*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.)

6.3 Discussion

The data presented in this chapter confirm that immune dysregulation caused by the silencing of *Phd2* is induced by increased HIF signalling. Specifically, our double knockdown models targeting *Phd2* concurrently with *Hif1a* or *Hif2a* strongly suggest that the latter is the principal driver of the phenotype observed with Phd2 silencing**.** Moreover, these data demonstrate that the phenotype caused by *Phd2* inhibition is akin to immune responses induced under chronic hypoxic conditions, in which *Hif2a* silencing similarly reverses this effect**.** Taken together, along with data showing that HIF-2α inhibition may also increase suppressive potency of Tregs *in vitro*, the findings in this chapter reveal a previously unreported role that the HIF-2α/Phd2 pathway plays in modulating Treg biology.

While PHD2 mainly targets HIF molecules and other proteins involved in VHL-mediated degradation, it has been shown to also interact with other proteins [564]. Thus, it was first necessary to confirm that the Phd2kd phenotype acted through HIF signalling pathways. Given previous literature that emphasised the role of HIF-1α in modulating Treg function, we were surprised to find that HIF-2α was the dominant driver of this phenotype **[Figure 6.2.1.]**. Nonetheless, although not statistically significant relative to Phd2kd mice, the data also suggest that HIF-1α inhibition may have a modest effect in ameliorating the inflammatory phenotype. Moreover, while PHD2 is considered to be the predominant enzyme involved in HIF-α degradation, it is important to consider the activity of the other HIF-regulating molecules in future studies. For example, certain HIF-regulators such as FIH have been shown to inhibit HIF-1α at higher efficiency than HIF-2α [567]. Additionally, other regulators have been demonstrated

to inhibit HIF-1α, but their effects on HIF-2α have not yet been elucidated [568-570]. Taken together, it is possible that under *Phd2* inhibition, there are additional mechanisms present to inhibit HIF-1α, leaving HIF-2α as the prominent signalling molecule. Developing methods that reliably detect HIF molecule levels and their activity within these experiments will be important to clarify these questions.

From the current study, it is unclear the mechanisms by which HIF-2α and Foxp3 signalling pathways converge, and whether there is a direct or indirect interaction of the two molecules. Thus, to investigate the functional interplay between these pathways, we have recently conducted single-cell RNA sequencing analysis on lymph node-derived CD4$^+$ T cells from control, Phd2kd, and P2H2kd mice after 4 weeks of doxycycline treatment. While the analysis is still preliminary, there are distinct differences in gene expression between control and Phd2kd CD4$^+$ T cells. Notably, using unsupervised clustering analysis to characterise groups of cells based on similarities of their transcriptomes, we identified unique *Foxp3*-expressing islands which are present in Phd2kd samples but not in control or P2H2kd samples **[Appendix 3]**. Deeper analysis within these datasets may allow the identification of certain genes of interest.

Many of the previous studies have investigated the HIF/PHD pathways primarily from a binary perspective, in which normoxia has generally been equated to complete HIF inhibition. However, our *in vitro* suppression assays suggest the possibility that there is a degree of HIF-2α signalling present even under oxygenated conditions. Unexpectedly, there was a significant increase in Treg suppression upon the inhibition of HIF-2α, implying that HIF-2α silencing is incomplete under normoxic conditions **[Figure 6.2.2. and Figure 6.2.6.]**. In essence, while we cannot determine the

magnitude of activity, HIF-2α signalling may still be active at low levels and have roles in enhancing Treg function even in normoxia.

Additionally, especially under physiological and pathological conditions, it may be speculated that different gradients of oxidative stress can induce different HIF-dependent immune responses. Preliminary *in vivo* studies that were conducted to assess the function of HIF-2α further illustrate this complexity within the system. In contrast to the *in vitro* studies **[Figure 6.2.2]**, Tregs from HIF-2α mice that were adoptively transferred with WT Teffs into C57BL/6 Rag mice with a skin allograft had impaired capacity to prolong skin allografts relative to control Tregs **[Figure 6.2.3.]**. There may be several possibilities for this discrepancy in function *in vivo* versus *in vitro*. One explanation may be that a degree of HIF-2α signalling is indispensable for certain Treg processes *in vivo*. For example, HIF-2α may play a similar role as HIF-1α, in which a recent study by Miska et al. suggests that HIF-1α-deficient Tregs may have impaired migratory capabilities [565]. Additionally, it is possible that HIF-2α may be necessary for maintaining stability or adapting to certain environments with fluctuating and strenuous levels of oxidative stress, such as in graft microenvironments. Finally, there is limited information in the current literature regarding the regulation of HIF-1α and HIF-2α isoforms relative to each other. While there does not appear to be competition between the two isoforms for DNA-binding sites as they generally bind to different sequences [571], it is conceivable that there may be competition for co-activators such as HIF-1β or p300 that are necessary for their transcriptional activity [392]. Moreover, although the two HIF isoforms preferentially induce different genes, studies have demonstrated that they may compensate for each other when one isoform is deficient. For

example, while HIF-1α is demonstrated to be the predominant inducer of glycolytic enzymes, HIF-2α has been shown to induce glycolytic genes when HIF-1α is not present [570, 572]. Other studies have also demonstrated that HIF-1α in some contexts may also induce genes such as matrix metalloproteinases (MMP), which are typically induced by HIF-2α [573]. It is thus possible that inhibition of HIF-2α may allow for increased HIF-1α signalling, either due to increased availability of co-activators or as a compensatory mechanism. Future studies will therefore aim to further elucidate the effects of HIF-2α inhibition on Treg activity *in vivo*.

Nonetheless, we believe that our findings using pharmacological HIF-2α inhibition is important in revealing the possibility of enhancing the suppressive potency of human Tregs through the modulation of this pathway **[Figure 6.2.6.]**. Peloton (PT2385) is a first-in-class HIF-2α antagonist that is currently in clinical trials for the treatment of clear cell renal cell carcinoma (ccRCC) [566]. *VHL* inactivation is common in ccRCC and thus lead to upregulated activation of HIF-2α signalling, which may induce increased expression of genes involved in tumour growth and metastasis, such as *VEGF* [574]. Based on titration experiments, we selected a dose of HIF-2α antagonist (20 μM) that did not itself significantly affect PBMC proliferation, activation, (i.e. CD25 and HLA-DR) or toxicity. Thus, we believe that the decrease in proliferation and activation of PBMCs upon the incorporation of PT2385 was reflective of increased Treg-mediated suppression. Yet there are limitations in the design of the current study due to PT2385 being directly incorporated within heterogenous cultures. While this model may be reflective of how PT2385 can be used in the context of *in vivo* administration, future experiments will be necessary to determine the direct effects of PT2385 on Tregs alone and whether Tregs need stimulation or signalling from PBMCs

to enhance their suppression under these conditions. Importantly, oral administration of PT2385 has been well-tolerated without significant adverse events in ccRCC patients within clinical trials [566]. In the context of transplantation, this invites the possibility of *in vivo* HIF-2α inhibition to enhance suppressive function of endogenous Tregs to control rejection. Alternatively, the *in vitro* data also demonstrates potential for HIF-2α inhibitors to be used within *ex vivo*-Treg expansion protocols. Nonetheless, the function of HIF-2α in Tregs *in vivo* will need to be further elucidated to clarify the feasibility of these strategies.

Although there have been previous studies that have investigated the effects of systemic chronic hypoxia in mice using hypoxic chambers from a haematological standpoint, its effects on the immune system have not yet been closely examined. Here, the findings in this chapter demonstrate that our previous findings from the genetic manipulation of the HIF/PHD2 pathway is physiologically consistent with effects of chronic hypoxia **[Figure 6.2.4]**. Additionally, the use of hypoxic chambers allows for further versatility in experiments to observe effects of hypoxia concurrently with genetic or pharmacological manipulation of HIF/PHD. Similar to *Phd2* inactivation, chronic hypoxia induces a similar immune dysregulated phenotype with lymphadenopathy, which can be reversed with *Hif2a* inhibition **[Figure 6.2.5]**. Nonetheless, further investigation of the activity and regulation of both HIF isoforms under different hypoxic conditions will be beneficial. In particular, previous studies have highlighted the dynamic interplay between the HIF isoforms that reveal a profound complexity in adapting to different levels of hypoxia. For example, studies have shown in some cell lines that HIF-1α is the dominant isoform to become activated under shorter durations (2-24 hrs) of extreme hypoxia (<0.1% O_2). In contrast, HIF-2α appears to remain active for longer durations (48-72 hrs) under

predominantly mild hypoxic conditions (<5% O_2) and may be the major isoform activated to deal with chronic hypoxia [402, 570, 575]. Moreover, similar to the Phd2kd model, the activity of the various HIF-regulators will need to be further elucidated. Certain regulators of HIF are oxygen independent and some have been demonstrated to regulate HIF-1α preferentially over HIF-2α. Notably, the hypoxia associated factor (HAF) and heat-shock protein 70/carboxyl terminus of Hsp70-interacting protein (Hsp70/CHIP) complex has been shown to inhibit HIF-1α but not HIF-2α [570, 576-578]. Taken together, the data present promising avenues of investigation into HIF and hypoxia-driven immune responses from a physiologically-relevant standpoint, where future studies will seek to develop better understanding of the interworking of this system.

In conclusion, the findings within this thesis provide insight into a previously unreported role of the HIF/PHD2 pathway in modulating Treg function and immune response. Due to the novelty of the data and the complexity of the intricate HIF/PHD system, many questions remain to be explored. Nonetheless, our present and future findings may have potentially far-reaching implications that are not only relevant in transplantation, but also in a variety of immune-related pathologies.

7 Discussion

7.1 Chapter introduction

The pleiotropic nature of both the IL-33/ST2 [309, 333, 361, 372, 579-582] and HIF/PHD pathways [396, 442, 537-539, 570] has been well-established in recent literature, revealing the importance of the two pathways in directing the immune system. In this thesis, the importance of their regulatory properties, and in particular, their role in modulating Treg function are highlighted. While their identity as “double-edged swords” within the immune system brings challenges in harnessing their regulatory properties [300, 309, 333, 372, 467, 537, 538, 540, 582, 583], the findings presented within this thesis demonstrate that elucidating the inner workings of these pathways serve not only to identify their tolerogenic functions, but to also mitigate or reverse their potential inflammatory properties.

7.2 Summary of experimental results

7.2.1 Chapter 3

In Chapter 3, the primary aim was to explore the role of IL-33 within the general immune cell environment, with a particular focus on its effect on Treg biology. Thus, to capture a broad understanding of the effects of IL-33 in immune cells, splenocytes from control (PBS-treated) versus IL-33-treated CBA mice were compared using multiplexed gene expression analysis. Here, IL-33-treated mice exhibited an overall downregulation of integral T cell-associated genes **[Figure 3.2.1]**, correlated with upregulation of Treg-associated genes **[Figure 3.2.2]**.

To determine whether this was also reflected at the protein level, control and IL-33-treated mice were analysed by flow cytometry, in which IL-33 treatment induced significant expansion of $ST2^{+}$ Tregs within the spleen, LNs, and peripheral blood **[Figure 3.2.3]**. Importantly, IL-33 treatment significantly upregulated ST2 expression and promoted an effector phenotype ($CD62L^{neg}CD44^{+}$) within Treg populations, but not $Foxp3^{neg}$ populations **[Figure 3.2.4]**. IL-33-Tregs also exhibited upregulated Treg-associated markers, such as CD25, CTLA-4, ICOS, PD-1, and CD39/CD73, which were commonly co-expressed with ST2 **[Figure 3.2.6]**. To assess Foxp3 and ST2 dynamics *in vivo* after IL-33 treatment, peripheral blood from control and IL-33-treated mice were sampled over multiple time points during and after cessation of treatment. Here, Foxp3 and ST2 expression levels remained significantly upregulated above physiological levels even up to two weeks after treatment **[Figure 3.2.5]**. The capacity of IL-33 treatment to increase the Treg:Teff ratio led us to explore whether this can be further accentuated with rapamycin, due to its effects in hindering

Teff activation while enhancing Treg survival. Indeed, the combination of IL-33 and rapamycin treatment significantly upregulated Foxp3 and ST2 over IL-33 treatment alone **[Figure 3.2.7]**

Finally, to establish whether Treg populations could be similarly expanded with IL-33 treatment *in vitro*, $CD4^+$, $CD4^+CD25^{neg}$, and $CD4^+CD25^+$ cells were stimulated with tolerogenic DCs and IL-33. While Foxp3 and ST2 expression were upregulated within $CD4^+$ and $CD4^+CD25^+$ cultures with the addition of IL-33, Tregs were not significantly induced within $CD4^+CD25^{neg}$ cultures **[Figure 3.2.8]**. Significant expansion of $ST2^+$ Tregs was also observed with immunogenic DCs and IL-33 **[Figure 3.2.9]**.

Taken together, data from Chapter 3 demonstrated that IL-33 preferentially expands a Treg population with high expression of ST2 and other suppressive markers both *in vivo* and *in vitro.* Thus, the findings in this chapter indicate IL-33 may exhibit a regulatory function through the expansion of a specific populations of Tregs that could potentially be harnessed for therapy.

7.2.2 Chapter 4

After establishing the capacity of IL-33 to expand a specific population of Tregs with high expression of ST2, we next aimed to assess the functionality of IL-33-Tregs and determine whether they may have therapeutic potential. First, the suppressive potency of Tregs from PBS (control) or IL-33-treated CBA mice were compared in an *in vitro* suppression assay. Despite their upregulation of many markers central to Treg-dependent mechanisms of suppression, IL-33-Tregs did not exhibit greater suppression *in vitro* against $CD4^+$ Teffs in either bead or allogeneic

DC-stimulated conditions **[Figure 4.2.1]**. Nonetheless, to determine whether IL-33-Tregs demonstrated any functional advantages *in vivo*, control and IL-33-Tregs were compared in an adoptive transfer and fully MHC-mismatched skin transplant model. In contrast to *in vitro* assays, IL-33-Tregs was better able to prolong skin allografts than control Tregs **[Figure 4.2.2]**.

To develop a better understanding of the mechanisms underlying the functional advantages of IL-33-Tregs, control and IL-33-Tregs were compared through multiplexed quantitative transcriptomic analysis. Consistent with the immunophenotyping data, some of the most differentially upregulated genes within IL-33-Tregs were those associated with Treg suppressive function **[Figure 4.2.3]**. Interestingly, IL-33-Tregs also significantly upregulated genes associated with graft-homing **[Figure 4.3.4]**. This was also confirmed at the protein level, suggesting that IL-33-Tregs had better capacity to migrate to allografts *in vivo*, a feature that was not reflected in the *in vitro* assays.

To determine whether these findings were consistent with other mouse strains, the effects of IL-33 treatment on C57BL/6 mice were also assessed. While flow cytometric analysis revealed that IL-33 treatment demonstrated a similar effect in phenotype in C57BL/6 mice **[Figure 4.2.5]**, C57BL/6 IL-33-Tregs did not show better capacity to prolong CBA skin allografts than control Tregs **[Figure 4.2.6]**. Transcriptomic analysis revealed that IL-33 treatment did not induce a significant shift in gene expression of C57BL/6 mice, relative to what was observed within CBA mice **[Figure 4.2.7]**. Comparison of splenocytes from control C57BL/6 and CBA mice through transcriptomic analysis, revealed that C57BL/6 mice may have an intrinsically more Th1-biased phenotype, which may explain their differences in responsiveness to IL-33 **[Figure 4.2.8]**.

In conclusion, IL-33-Tregs demonstrated functional advantages *in vivo* through their graft-homing phenotype and thus may have therapeutic potential within the context of transplantation.

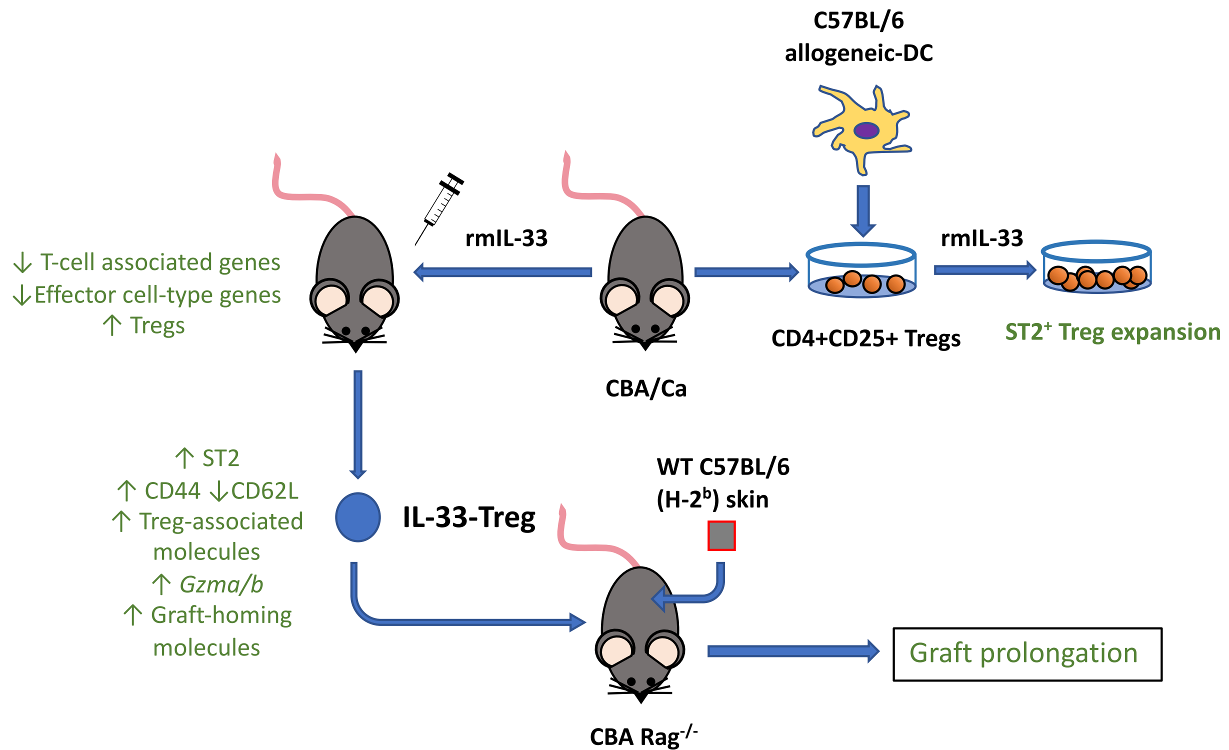


Figure 7.2.2. Summary schematic representing major findings from chapters 3 and 4.

7.2.3 Chapter 5

In Chapter 5, we investigated the modulation of another molecular pathway that we hypothesised may give insight into Treg plasticity, which is another area of consideration for Treg-based therapy. To this end, we explored the modulation of the HIF/PHD pathway by using global conditional *Phd2* knockdown (Phd2kd) and Foxp3-restricted *Phd2* knockdown (Foxp3^{Cre}-Phd2kd) mice models.

Firstly, the phenotypic effects of dox-inducible global *Phd2* silencing on the immune system were analysed. Phd2kd mice after 4 weeks of dox treatment exhibited an autoimmune-like phenotype with lymphoproliferation, including significant upregulation of $Foxp3^+$ populations **[Figure 5.2.1]**. Nonetheless, when assessed *in vivo* in adoptive cell transfer and skin transplant models, Phd2kd Tregs had significantly impaired ability to prolong allograft survival relative to control Tregs **[Figure 5.2.2]**. Flow cytometric analysis revealed that Phdk2d Tregs adopted an effector phenotype ($CD62L^{low}CD44^+$) **[Figure 5.2.3]** and significantly upregulated Th1-type markers **[Figure 5.2.4]**. Interestingly, Phd2kd $Foxp3^+$ Tregs significantly downregulated expression of CD25 **[Figure 5.2.5]** but remained strongly demethylated at the TSDR **[Figure 5.2.6]**.

The phenotypic changes induced by the silencing of *Phd2* followed a time-dependent response, Here, many of the immune cell changes were apparent within the first two weeks of dox treatment **[Figure 5.2.7]**, which further emphasised that PHD2 most likely plays fundamental roles in modulating immune homeostasis. Moreover, upon the removal of dox treatment, the inflammatory phenotype in Phd2kd mice could be eventually reversed, indicating that this was not a permanent change in immune phenotype **[Figure 5.2.8]**.

To investigate whether there was a Treg-intrinsic mechanism that caused Treg dysregulation and whether the inflammatory phenotype developed due to their dysregulation, Tregs from mice with Foxp3-specific knockdown of *Phd2* ($Foxp3^{Cre}$-Phd2kd mice) were additionally examined. Here, $Foxp3^{Cre}$-Phd2kd mice exhibited similar features of inflammation and lymphoproliferation, albeit with a weaker phenotype **[Figure 5.2.9]**. $Foxp3^{Cre}$-Phd2kd Tregs similarly demonstrated impaired suppressive function when assessed in an adoptive cell transfer and skin

transplantation model **[Figure 5.2.10]**. Taken together, the silencing of *Phd2* in mice induced immune dysregulation that may be driven by an impairment of Treg populations.

7.2.4 Chapter 6

Building off our findings from Chapter 5, we next aimed to identify the molecules responsible for immune dysregulation in Phdk2d mice. To determine whether this was HIF-driven, dox-inducible double knockdown models silenced with *Phd2kd* and *Hif1a* (P2H1kd) or *Hif2a* (P2H2kd) were used. Interestingly, while dox-treated P2H1kd mice still exhibited signs of inflammation comparable to Phd2kd mice, P2H2kd mice did not develop these features **[Figure 6.2.1]**. This suggested that HIF-2α signalling had a principal role in driving inflammation in Phd2kd mice. Thus, to examine whether HIF-2α signalling had a role in modulating Treg function, a global dox-inducible *Hif2a* knockdown (HIF2akd) model was also used. HIF2akd Tregs were assessed in an *in vitro* suppression assay, which revealed that they have greater suppressive capacity over control Tregs **[Figure 6.2.2]**. Surprisingly, however, this was not replicated in the preliminary skin transplant experiment **[Figure 6.2.3]**, suggesting high complexity and fine tuning of HIF/PHD pathway *in vivo*.

We were next curious to investigate whether these findings could be replicated with hypoxia treatment. Mice maintained in hypoxic chambers (10% O_2) for 6 weeks demonstrated weight loss, increased haematocrit levels, and lymphoproliferation **[Figure 6.2.4]**. Furthermore, when *Hif2a* knockout (HIF2aKO) mice were maintained under chronic hypoxia, they did not develop

inflammatory features, consistent with our findings that HIF-2α is mechanistically responsible for these effects **[Figure 6.2.5]**.

Finally, to explore whether these findings were translatable to humans, the effects of a clinically-relevant HIF-2α antagonist on human Tregs were assessed. Tregs cultured with a HIF-2α antagonist in an *in vitro* suppression assay exhibited significantly enhanced suppressive potency of $CD4^+$ and $CD8^+$ PBMC proliferation **[Figure 6.2.6]**.

In summary, this chapter provides evidence that *Phd2* silencing induces an inflammatory phenotype driven principally by HIF-2α. Additionally, these data demonstrate that our findings may be both physiologically and clinically relevant within the context of transplantation and other immune pathologies.

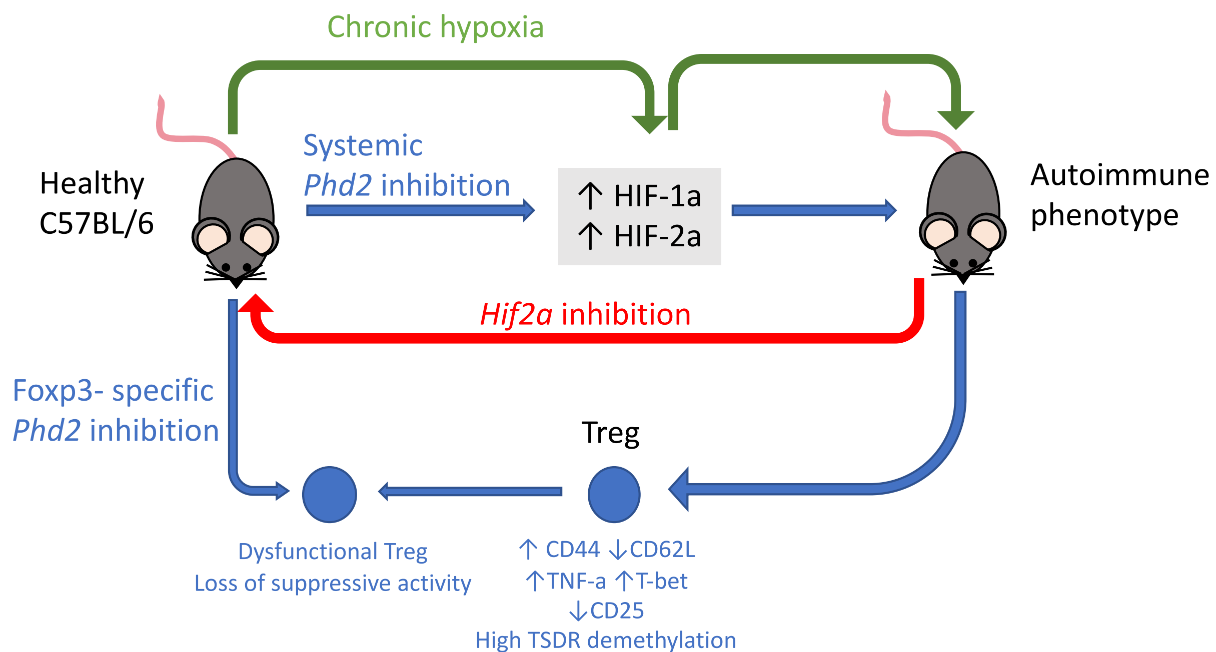


Figure 7.2.4. Summary schematic representing major findings from chapters 5 and 6.

7.3 General Discussion

Through the help of preclinical models within the past two decades, the therapeutic potential for Tregs has become evident, paving the way for Phase I/II clinical trials for the treatment of transplantation, GVHD, and autoimmune diseases [187]. While the initial clinical trials for assessing the feasibility of Treg cell therapies have been promising, various concerns will still need to be addressed for safe and effective implementation. The data presented in this thesis serve two main contributions for furthering Treg and transplantation research. Firstly, the in-depth investigations into these molecular pathways reveal currently underappreciated heterogeneity within Treg biology that are worthy of further investigation for advancement within the field. Secondly, these findings demonstrate the potential for the manipulation of these specific molecular pathways in enhancing Treg cell therapies and beyond.

7.3.1 Deeper insights into Treg diversity and plasticity

Our exploration within these pathways have unveiled many interesting characteristics of Treg biology. One theme that was consistently highlighted within our study were the features of heterogeneity present within Treg populations alone. While the discovery of Foxp3 as a marker for Tregs has become a catalyst for the study of Treg biology, it is evident that a greater emphasis on elucidating the various populations present within Treg populations and the factors that

govern their activity are necessary to fully optimise their therapeutic use. In this regard, these findings may contribute to the growing knowledge of research within this field and are important considerations for therapeutic application.

The unique population of $ST2^+$ Tregs examined highlights the diversity present within Treg populations. Here, we found that IL-33 expands a unique and highly activated Treg subset characterised with upregulated expression of its own receptor, ST2, and other Treg-associated suppressive markers. Interestingly, transcriptomic analysis of IL-33-Tregs revealed that they exhibit a distinct signature, similar to tissue-resident Tregs, such as visceral adipose tissue (VAT) Tregs [485, 487]. Tissue-resident Tregs have recently been recognised as specialised Tregs that have important roles in maintaining organ homeostasis [584]. While ST2 is highly expressed within tissue-resident Tregs and especially within visceral adipose tissue (VAT) Tregs, it is barely detectable within the blood and lymphoid tissues at physiological levels. Within our study, however, IL-33 treatment significantly expanded $ST2^+$ Tregs within lymphoid tissues (spleens and LNs) and blood, in which $ST2^+$ Tregs stably persisted at least two weeks after treatment was ceased. Moreover, IL-33-Tregs (which highly expressed ST2) had superior capacity in prolonging allograft survival. Therefore, while $ST2^+$ Tregs have been associated with tissue-resident Tregs, their enhanced function and stability in other organs suggest that they may also have other functions within the body. Although the source of $ST2^+$ Tregs and their “optimal” or natural function within the immune system will need to be further elucidated, this distinct population underscores the diversity within Tregs as a whole.

Tied in within the theme of heterogeneity of Tregs is the matter of plasticity of Treg phenotype and function, which was highlighted within our investigation of the HIF/PHD pathway. Although Tregs are considered to be a predominantly stable cell lineage, it has become increasingly clear from recent literature that certain environmental factors can disable their suppressive function or even cause them to lose *Foxp3* expression and convert to proinflammatory T cells [391]. Interestingly, the data presented in this thesis reveal that oxygen tension and the activation of HIF signalling pathways may be one of the environmental stimuli that disrupts Treg function *in vivo*. Namely, we demonstrate that *Phd2* silencing in mice induces an autoimmune-like phenotype, correlating with significant expansion of Tregs with impaired suppressive function likely driven by HIF-2α. Phd2kd Tregs were characterised by a highly activated phenotype (CD62L^{low}CD44^{+}) and elevation of Th1-type markers (T-bet and TNF-α but not IFN-γ). Yet, perhaps the most intriguing shift observed within Phd2kd Tregs was a significantly downregulated expression of CD25. Upregulation of CD25^{neg} Treg populations has been observed in a selection of patients with autoimmune pathologies [552-556]. While CD25 expression is known to be critical for Treg function and survival [254, 558], it is not clear within our model or other autoimmune pathologies whether CD25 has a role in Treg stability. Interestingly, regardless of the absence of CD25 expression, Phd2kd Tregs maintained high demethylation of TSDR, which has been considered to be a reliable measure of Treg stability [585, 586]. Here, our results indicate that this measure may be applicable to the maintenance of Foxp3 expression but not necessarily suppressive function, which we believe is an important distinction. Another question that follows is whether these Tregs can regain CD25 expression or if they have terminally differentiated into cells that have lost suppressive function. Likewise, it is unclear whether

CD25^{neg} Tregs have a specialised role within the immune system or whether they are a consequence of pathological immune dysregulation. One study suggested that CD4^{+}CD25^{neg}Foxp3^{+} cells may constitute a reservoir of cells that can regain CD25 after activation [561]. This may be consistent with our findings, in which CD25 expression returns within Phd2kd Tregs after the withdrawal of doxycycline, although it is possible that these cells are a newly expanded population of CD25-expressing Tregs. In summary, these investigations shine a light on relevant questions regarding the plasticity present within Foxp3^{+} Tregs, in which CD25 expression may play an important role.

Taken together, our explorations within these two pathways have highlighted features within Treg biology that have been often overlooked in previous literature and could guide further avenues of research within the field.

7.3.2 Manipulation of the IL-33/ST2 and HIF/PHD pathways for improving cell therapies

The findings in this thesis show that the manipulation of molecular pathways may open up possibilities for the refinement of Treg cell-based therapies and provide solutions to concerns surrounding current treatment strategies in transplantation and beyond.

Currently, most of the ongoing or completed clinical trials have revolved around the use of *ex vivo* polyclonally-expanded Tregs [187]. While current expansion protocols for polyclonal Tregs have been deemed safe and reliable for use in clinical trials, one of the practical challenges that

still requires consideration has been the generation of the high number of polyclonal Tregs required for therapy [187, 255-257]. Additionally, there are concerns that repetitive stimulation with currently established protocols may yield effector T cell contamination [504-506]. Along with a degree of patient-to-patient variability in the quality and quantity of expanded cell product, strategies to either expand a greater number of Tregs and/or to enhance their suppressive potency without sacrificing stability are in demand. There is therefore an opportunity to improve these protocols with the use of IL-33. The addition of IL-33 treatment can significantly expand Treg cell numbers both *in vivo* and *in vitro*, with IL-33-Tregs exhibiting a greater potential in protecting allograft survival. From a practical standpoint, IL-33 can be conveniently incorporated into currently established protocols for the expansion of Tregs *ex vivo*. Moreover, our *in vitro* suppression assays with human Tregs treated with HIF-2α antagonist, suggest that the HIF-2α pathway can be similarly exploited. While the effect of HIF-2α inhibition on Tregs *in vivo* will need to be further clarified, our *in vitro* data reveal the potential for a HIF-2α antagonist to be employed in *in vitro* expansion protocols.

A separate concern for Treg cell therapy has been the theoretical risk of global immunosuppression, especially with the use of polyclonal Tregs [507]. Currently, many preclinical and clinical studies have attempted to overcome this issue with the expansion of donor-specific Tregs, which may limit unwanted off-target effects. Nonetheless, the generation of sufficient numbers of donor-specific Tregs may be limited through current methods due to the limited number of donor-specific Tregs that can be reliably expanded [587]. Furthermore, there have been numerous recent animal studies focusing on the generation of antigen-specific Tregs using genes encoding chimeric antigen receptors (CARs) [588]. However, their practicality, safety, and

overall effectiveness *in vivo* is still under investigation. An alternative strategy to prevent nonspecific immunosuppression of Tregs may be to restrict the anatomical location of their suppressive activity. The graft-homing attributes of IL-33-expanded Tregs are therefore attractive for their potential to regulate alloreactive responses locally. As described previously, animal studies have demonstrated that Treg homing to the allograft is critical for graft survival in Treg cell-based therapies [266]. Thus, IL-33-Tregs, which express many of the chemokine receptor molecules essential for graft homing, may confer enhanced specificity over polyclonal Tregs due to their potential to preferentially migrate to graft microenvironments [266, 516, 517]. Moreover, their enhanced expression of ST2 compared with Teffs may allow for selective activation and expansion of Tregs with IL-33 treatment **[Figure 3.2.3]**. Recent studies have also demonstrated that $ST2^+$ Tregs may be effective in the treatment of other pathologies. For example, due to the prevalence of $ST2^+$ Tregs within adipose tissues, much research has focused on their role in tissue metabolism, in which they have been reported to be involved in the maintenance of insulin sensitivity and glucose tolerance, as well as dampening inflammation associated with obesity [589, 590]. Other studies have recently investigated the potential of mouse and human $ST2^+$ Tregs to promote tissue repair through their enhanced production of amphiregulin (AREG) [360].

As highlighted within our investigations of the HIF/PHD pathway, Treg phenotypic and functional stability *in vivo* is another potential concern for Treg-cell based therapies. Tregs frequently migrate to hypoxic regions [591], which may induce transient or sustained stress. In particular, Tregs that are recruited to inflammatory regions may experience prolonged periods of relative hypoxia [592]. Based on our findings, it is possible that Tregs may lose suppressive function within

hypoxic regions. Modulation of the HIF/PHD pathway within Tregs in hypoxic environments may thus have potential in maintaining or elevating their suppressive function and mitigating plasticity. As described earlier, one feasible strategy may be through HIF-2α antagonism, of which some molecules are currently in clinical trials [566]. While oral administration of a HIF-2α antagonist has been well tolerated in patients with clear cell renal carcinoma, developing methods for local administration may be a viable strategy for targeted inhibition. Moreover, treatments based around the manipulation of HIF/PHD pathway within Tregs may provide potential therapeutic strategies in autoimmune diseases, in which both Treg plasticity and elevated HIF signalling are potential drivers of disease progression [538]. Taken together, elucidating the environmental stimuli, such as oxygen tension, which affect Treg stability, may provide valuable opportunities for therapeutic intervention.

7.4 Future directions

These findings pave the way for future avenues of research in transplantation and beyond. In particular, future work within these pathways should focus on experiments that elucidate underlying mechanisms and their applicability to human biology.

A major aim for future studies must focus on further clarifying the mechanisms underlying IL-33-Treg-mediated suppression of alloresponse *in vivo*. While IL-33-Tregs demonstrated enhanced graft-protective function with increased graft-homing potential, our assays are limited by an inability to track the precise kinetics of Treg migration and activity at sites of alloresponse. Moreover, our transcriptomic and immunophenotyping analysis revealed that IL-33-Tregs upregulated many markers associated with Treg-mediated suppression (i.e. CTLA-4, CD39/CD73, and Granzyme A and B), but their preferred mechanisms of suppression is yet to be determined within our model. These questions may be further investigated with the use of various transgenic models in order to identify the important mechanisms involved in their protective function. It may thus be beneficial in future work to repeat skin transplantation experiments with the aim of harvesting skin grafts and draining LNs at distinct time points for histological examination, which may provide further insight into the exact mechanisms of suppression. Additionally, this may also indicate whether other features of $ST2^+$ Tregs, such as enhanced wound healing, play a role in their graft-protective functions.

In the case of the HIF/PHD pathway, elucidating the link between Foxp3 and HIF-2α will be a priority. Firstly, it is necessary to understand whether a direct interaction exists between Foxp3

and HIF-2α. As previous studies have demonstrated that there is physical interaction between HIF-1α and Foxp3 using chromatin immunoprecipitation sequencing (ChIP-Seq) [537], similar methods may be applied here. Moreover, as described earlier, we have conducted preliminary single-cell RNA sequencing analysis to attempt to identify other genes of interest that may have prominent roles within the pathway. Here, we compared $CD4^+$ cells from control, Phd2kd, and P2H2kd mice after 4 weeks of dox treatment or 4 weeks of dox followed by 1 week off in order to capture gene expression changes that are rapidly reversed **[Appendix 3]**. Additionally, we aim to do sequencing of samples from $Foxp3^{Cre}$-Phd2kd mice. By cross-analysing samples from all of these data sets, we hope to identify genes of interest to target further investigations. Finally, molecular analysis of cells from mice under chronic hypoxia will be conducted to further elucidate the mechanisms underlying the observed immune changes.

A major focus of future research will be to attempt to translate our findings to human cells. In particular, there has been minimal literature elucidating the role of the IL-33/ST2 axis in human Tregs. This may be in part due to the low proportion of $ST2^+$ Tregs found within human tissue in the quiescent state [360]. Additionally, the lack of commercially available monoclonal antibodies for the reliable detection of human ST2 may have also impeded translation to human cell lines. Nonetheless, recent reports suggest that the pathway may indeed play a similar role within human Treg biology. Liu et al. indicate that recombinant IL-33 can enhance expansion of human Tregs, which maintain suppressive potency and upregulate secretion of IL-13 [386]. Moreover, findings from other studies demonstrate the accumulation of highly activated human $ST2^+$ Tregs in peripheral blood and tumour lesions of colorectal cancer patients [385]. In a similar manner, it may be helpful to analyse clinical samples and biopsies from transplantation patients to

determine if there is increased ST2 expression within the graft microenvironment. Within our investigations in mice, the use of *in vivo* models was critical to evaluate the full effects of IL-33/ST2. Future studies will also aim to assess the effects of this pathway in Tregs using established humanised mouse models [270, 593].

Within our investigation of the HIF/PHD2 pathway, we demonstrated the translatability of our findings in mice to human cells, in which HIF-2α inhibition increased the suppressive function of Tregs in both species. This will guide future experiments in using HIF-2α antagonism in other *in vitro* cultures, such as human Treg expansion, or *in vivo* treatment within humanised mouse models. A number of clinical samples may also yield promising findings. For example, changes in Treg phenotype may be interesting to measure in patients treated with oral HIF-2α antagonists within clinical trials. In terms of our findings from the chronic hypoxia model, it may be fruitful to analyse clinical samples from people experiencing mild hypoxia, such as extreme mountain climbers, to observe whether they exhibit similar evidence of inflammation at high altitudes.

Finally, while studies directly linking the IL-33/ST2 and HIF/PHD2 pathways have been limited thus far, there may be potential to modulate these pathways for combination therapy in order to enhance Treg function. Similar to how IL-33 and rapamycin treatment together demonstrated synergistic effects on Treg expansion **[Figure 3.2.7]**, simultaneous manipulation of these pathways may warrant further investigation in future studies. As the two pathways have demonstrated potential for use in both *in vivo* and *ex vivo* strategies, there may be a number of ways that they can be incorporated together. For example, IL-33 and PT2385 stimulation may be used together to enhance Treg function *ex vivo* for adoptive cell therapies.

7.5 Concluding remarks

Modern immunosuppression has revolutionised the field of transplantation but remains limited by serious off-target effects. Treg cell-based therapies may hold the key to overcoming this challenge. Unlike pharmacological immunosuppression, Treg cell therapy provides physiological control and the potential for specificity. This study expands on our understanding of Treg biology through identifying specific molecular pathways that may be manipulated for enhanced Treg function. The potential for Treg modulation with small molecules or biologics may therefore prove highly valuable for addressing a number of immune-mediated pathologies.

Publications

Publications

Kawai K, Uchiyama M, Hester J, & Issa F. *IL-33 drives the production of regulatory T cells with enhanced in vivo suppressive activity in transplantation*. AJT, 2020. [In review][*].

Yamamoto A, Macklin P, Hester J, Kawai K, et al. *Systemic silencing of HIF hydroxylase PHD2 causes reversible immunoregulatory dysfunction*. JCI, 2019; 130 (9): 3640-3656.

Kawai K, Uchiyama M, Hester J, Wood K.J. & Issa F et al. *Regulatory T cells for Tolerance. Human Immunology*, 2018; 79(5):294-303.

[*]Portions of the IL-33 project have been submitted for publication and currently undergoing review.

Abstracts

Kawai K, et al. (2020) *The HIF/PHD2 pathway in regulatory T cell biology* and *Modulation of IL-33/ST2 pathway in regulatory T cells for transplantation tolerance*. Two speaker sessions presented at the European Society of Transplantation (Copenhagen, Denmark).

Kawai K, et al. (2019) *The HIF/PHD2 pathway in regulatory T cell biology*. Speaker/Poster session presented at the Oxford Immunology Symposium (Oxford, UK).

Kawai K, et al. (2019). *Modulation of IL-33/ST2 pathway in regulatory T cells for transplantation tolerance.* Speaker session presented at British Transplantation Congress (Harrogate, UK).

Kawai K, et al. (2018). *Modulation of IL-33/ST2 pathway in regulatory T cells for transplantation tolerance.* Poster session presented at the European Congress of Immunology (Amsterdam, Netherlands).

Kawai K, et al. (2018). *Modulation of IL-33/ST2 pathway in regulatory T cells for transplantation tolerance.* Poster session presented at the Oxford Immunology Symposium (Oxford, UK).

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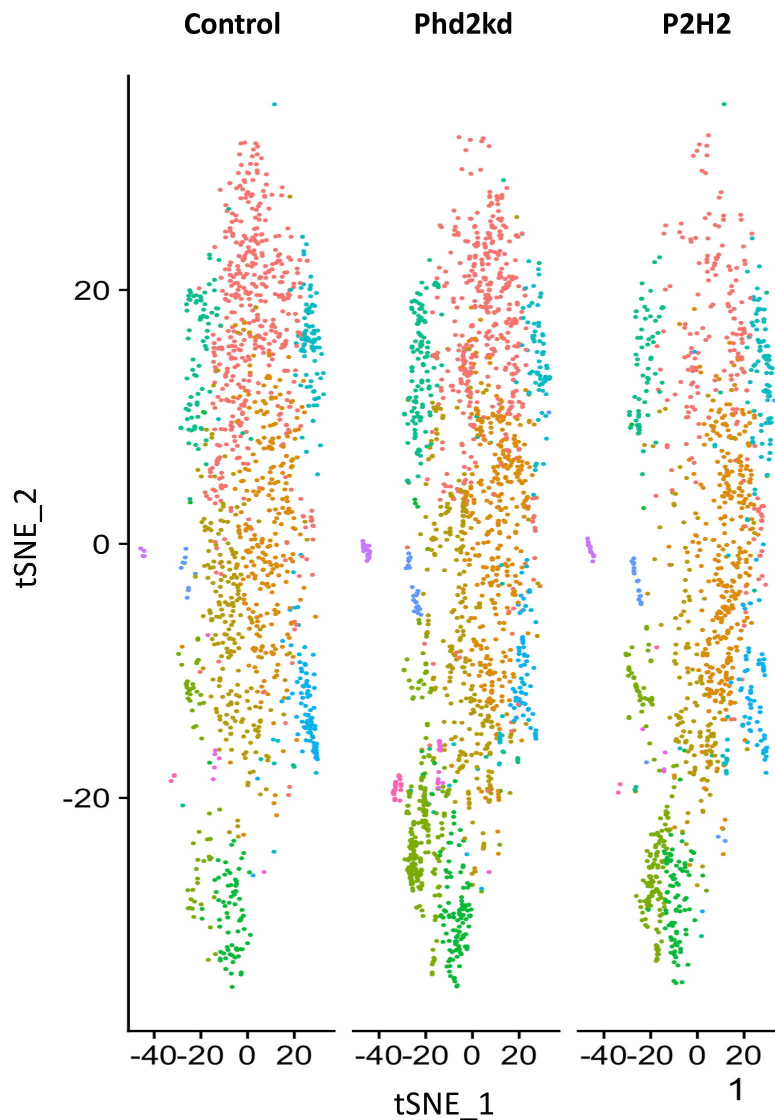
Appendices

	probe.ID	Log2 fold change	BY.p.value
Il7r-mRNA	NM_008372.3:1020	-1.04	0.000909
S100a8-mRNA	NM_013650.2:227	2.11	0.019
Lck-mRNA	NM_010693.2:1180	-1.26	0.019
Il18r1-mRNA	NM_001161842.1:620	-1.06	0.024
Jak1-mRNA	NM_146145.2:4080	-0.73	0.024
Ccl9-mRNA	NM_011338.2:1125	1.63	0.0287
Bcl10-mRNA	NM_009740.1:1168	-0.504	0.0325
Mpo-mRNA	NM_010824.2:1648	4.69	0.037
Lcn2-mRNA	NM_008491.1:190	2.44	0.0391
Apoe-mRNA	NM_009696.3:129	-0.795	0.0431
Egr3-mRNA	NM_018781.2:518	-1.21	0.0431
Mrc1-mRNA	NM_008625.1:3992	1.06	0.0431
Vim-mRNA	NM_011701.4:34	0.873	0.0431
C3-mRNA	NM_009778.2:285	3.35	0.0431
Ikbke-mRNA	NM_019777.3:618	-1.07	0.0431
Thy1-mRNA	NM_009382.3:425	-1.05	0.0431
Cd7-mRNA	NM_009854.1:234	-1.31	0.0431
Ncr1-mRNA	NM_010746.3:391	-1.29	0.0431
Il2rb-mRNA	NM_008368.3:2365	-0.807	0.0431
Cd247-mRNA	NM_001113391.2:215	-1.39	0.0431
Chil3-mRNA	NM_009892.2:823	5.51	0.0431
Cd6-mRNA	NM_001037801.2:1315	-1.18	0.0431
Itk-mRNA	NM_010583.3:404	-1.58	0.0431
Zap70-mRNA	NM_009539.2:1030	-0.894	0.0431
Lgals3-mRNA	NM_001145953.1:665	1.16	0.0431
Pparg-mRNA	NM_011146.1:1060	3.33	0.0431
Xbp1-mRNA	NM_013842.2:825	1.21	0.0431
Ccr7-mRNA	NM_007719.2:755	-1.44	0.0431
Cd3e-mRNA	NM_007648.4:380	-1.23	0.0481
Cd55-mRNA	NM_010016.2:1058	-0.787	0.0481
C1qbp-mRNA	NM_007573.2:630	0.548	0.0481
Nfatc3-mRNA	NM_010901.2:2260	-0.502	0.0481
Camp-mRNA	NM_009921.2:355	1.96	0.0481
Ccl24-mRNA	NM_019577.4:335	8.89	0.0481
Cd81-mRNA	NM_133655.2:575	0.243	0.0481

Appendix 1. Significantly differentiated genes between splenocytes of PBS and IL-33-treated mice. Statistically significant (adjusted *p* value <0.05) differentially regulated genes between splenocytes of PBS (n=3) and IL-33-treated (n=3) mice, as described previously, are shown with respective Log2 fold change and probe ID. Adjusted *p* value calculated with control of False Discovery Rate (FDR) using Benjamini-Yekutieli method.

	probe.ID	Log2 fold change	BY.p.value
Tcf7-mRNA	NM_009331.3:1810	-1.3	0.00472
Il1rl1-mRNA	NM_001025602.2:815	2.38	0.00472
Ifitm2-mRNA	NM_030694.1:87	2.7	0.00472
Gzmb-mRNA	NM_013542.2:1020	5.47	0.00581
Ccr2-mRNA	NM_009915.2:2965	1.8	0.00581
Stat1-mRNA	NM_009283.3:1590	-1.15	0.0073
Ccr7-mRNA	NM_007719.2:755	-1.71	0.00758
Card11-mRNA	NM_175362.2:545	-0.739	0.00893
Gzma-mRNA	NM_010370.2:188	4.33	0.00893
Icos-mRNA	NM_017480.1:142	1.56	0.00893
Ccr9-mRNA	NM_009913.6:820	1.32	0.00933
Stat6-mRNA	NM_009284.2:3465	-0.515	0.0103
Batf-mRNA	NM_016767.2:750	1.52	0.0114
Lgals3-mRNA	NM_001145953.1:665	1.68	0.0114
Ccr4-mRNA	NM_009916.2:394	1.78	0.0123
Klrg1-mRNA	NM_016970.1:825	1.94	0.0123
Ltb-mRNA	NM_008518.2:163	-1.22	0.0154
Jun-mRNA	NM_010591.2:2212	-1.49	0.0154
Cd96-mRNA	NM_032465.2:34	-1.13	0.0154
Tigit-mRNA	NM_001146325.1:730	1.67	0.0206
Smad4-mRNA	NM_008540.2:2885	-0.716	0.0206
Lag3-mRNA	NM_008479.1:1700	2.77	0.0222
Itgb1-mRNA	NM_010578.1:1855	0.953	0.0223
H2-K1-mRNA	NM_001001892.2:1370	-0.604	0.0223
Itga6-mRNA	NM_008397.3:910	-0.591	0.0223
Sell-mRNA	NM_001164059.1:664	-1.14	0.0233
Vim-mRNA	NM_011701.4:34	1.09	0.0397
Lta-mRNA	NM_010735.1:1115	-1.5	0.0397
Gata3-mRNA	NM_008091.3:1943	1.11	0.04
Il6ra-mRNA	NM_010559.2:2825	-1.32	0.0441
Hif1a-mRNA	NM_010431.2:1294	0.921	0.0441
Cd27-mRNA	NM_001033126.2:235	-0.873	0.0441
Gpr183-mRNA	NM_183031.2:238	1.09	0.0441
Fos-mRNA	NM_010234.2:1330	-0.415	0.0441
Jak1-mRNA	NM_146145.2:4080	-0.613	0.0441
Psma2-mRNA	NM_008944.2:136	0.376	0.0441
Cd200r1-mRNA	NM_021325.3:1090	1.21	0.0494

Appendix 2. Significantly differentiated genes between control and IL-33-Tregs. Statistically significant (adjusted *p* value <0.05) differentially regulated genes between CD4^{+}CD25^{+} Tregs of PBS (n=3) and IL-33-treated (n=4) mice, as described previously, are shown with respective Log2 fold change and probe ID. Adjusted *p* value calculated with control of False Discovery Rate (FDR) using Benjamini-Yekutieli method.



Appendix 3. Single-cell sequencing analysis of Control^{CAG}, Phd2kd, and P2H2 CD4+ T cells. t-distributed Stochastic Neighbour Embedding (tSNE) plots CD4^{+} T cells isolated from Control^{CAG}, Phd2kd, and P2H2 mice after 4 weeks of dox treatment (2mg/mL in drinking water).