Modeling of human thymic Treg cell development using an artificial thymic organoid system

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Abstract

FOXP3$^+$ Regulatory T (Treg) cells represent a major mechanism of immunological tolerance. Dysregulation and instability of this CD4$^+$ T cell sub-population have been associated with various autoimmune diseases, such as IPEX syndrome, rheumatoid arthritis, and SLE. Their rarity and the lack of a unique set of surface markers together present a significant challenge for human Treg studies. Recently Bifsha et al. demonstrated that a 3D artificial thymic organoid (ATO) culture system supports the differentiation of mature human CD8$^+$ and CD4$^+$ T cells from CD34$^+$ hematopoietic stem and progenitor cells. This system based on OP9 cells transduced with human Delta-like 4 (OP9-hDLL4) creates a thymic-like environment. Thus, knowing that a notable proportion of Treg cells are generated during thymopoiesis, we hypothesize that this ATO system provides a means of differentiating human FOXP3$^+$ Treg cells in vitro. Using this system, we have successfully generated human Treg cells baring classical markers of constitutively elevated FOXP3 and CD25 expression. This phenotype and the cells' ability to repress inflammatory cytokine secretion were maintained up to 21 days post-expansion. Moreover, we have also demonstrated that these cells suppress effector T (Teff) activation and proliferation. Overall, the ATO system presents a first-time opportunity for generating functional human Treg cells in vitro and serves as a platform for the study of human Treg biology and Tregopathies such as IPEX syndrome, as well as potential applications of Treg cell therapy in settings such as allograft tolerance and type I diabetes.

Résumé

Les lymphocytes T régulateurs FOXP3$^+$ (Treg) constituent un des mécanismes majeurs du maintien de la tolérance immunologique. Une instabilité ou une dérégulation de ce sous-type de lymphocytes CD4$^+$ entraine le développement d'une variété de pathologies auto-immunes telles que le syndrome IPEX, la polyarthrite rhumatoïde ou encore le lupus érythémateux disséminé. Cependant, leur rareté et l'absence de marqueurs de surface permettant de les isoler de manière spécifique sont des obstacles significatifs à l'étude de leur fonction chez l'Homme. Récemment, Bifsha et al ont établi un système d'organoïde de thymus artificiel en 3D permettant de différencier des lymphocytes CD4 et CD8 humains matures à partir d'une population de cellules souches hématopoïétiques CD34$^+$. Ce système basé sur des cellules OP9 transduites avec le ligand human Delta-like 4 (OP9-hDLL4) reproduit l'environnement thymique. La majorité des Treg circulant étant d'origine thymique, nous émettons l'hypothèse que ce système d'OTA permettra de supporter le développement de Tregs humains in vitro. Grâce à ce système, nous avons généré avec succès une population de cellules exprimant à la fois FOXP3 et CD25 en grande quantité, deux marqueurs servant à définir classiquement les Tregs humains. Ce phénotype est maintenu jusqu'à 21 jours post-expansion et ces cellules répriment avec succès l'expression de cytokines pro-inflammatoires, une autre caractéristique intrinsèque de cette population. De plus, nous avons démontré que ces cellules suppriment l'activation et la prolifération de lymphocytes T effecteurs in vitro. Ainsi, ce système d'OTA représente une innovation majeure pour générer des Tregs humains in vitro et pourra servir de plateforme pour étudier la biologie des Tregs, le développement de pathologies liées aux Tregs comme le syndrome IPEX et pour des applications potentielles dans le domaine de la thérapie cellulaire comme le traitement du rejet de greffe ou encore du diabète de type 1.

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Contribution of authors

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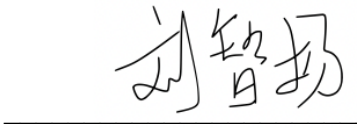

List of abbreviations

Abbreviation	Full name
MHC	Major histocompatibility complex
CD	Cluster of differentiation
Th	T helper
Treg	Regulatory T
TCR	T cell receptor
SLE	Systemic lupus erythematosus
IPEX	Immunodysregulation, polyendocrinopathy enteropathy x-linked
tTreg	Thymic-derived Treg
pTreg	Peripherally-induced Treg
AIRE	Autoimmune regulator
TRA	Tissue restricted antigen
STAT	Signal transducer and activator of transcription
APC	Antigen presenting cell
LAG3	Lymphocyte activation gene 3
FOXP3	Forkhead box P3
IL	Interleukin
mTEC	Medullary thymic epithelial cells
Tconv	Conventional T cells
RTE	Recent thymic emigrants
pDC	Plasmacytoid dendritic cells
TGF-β	Transforming growth factor beta
IKZF	Ikaros family zinc finger
ISP	immature single positive
DP	Double positive
DN	Double negative
HSPC	Hematopoietic stem and progenitor cell
TSP	Thymic seeding progenitor
iTreg	Induced Treg
Teff	Effector T cell
CNS	Conserved non-coding sequence
TSDR	Treg specific demethylated region
DAMP	Danger associated molecular pattern
ILC	Innate lymphoid cell
NK	Natural killer
NF-κB	Kappa-light-chain-enhancer of activated B cells
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
TIGIT	T cell immunoreceptor with Ig and ITIM domains
FcRL3	Fc receptor-like protein 3
DC	Dendritic cell
GARP	Glycoprotein-A repetitions predominant protein
MMT	Matrix metalloprotease
PKC	Protein kinase C
NFAT	Nuclear factor of activated T-cells

AP-1	Activator protein-1
TIP60	Tat-interacting protein 60
ROR	Retinoic acid receptor-related orphan receptor
RUNX1	Runt-related transcription factor 1
AML	Acute myeloid leukemia-1
Bcl-2	B cell lymphoma-2
Ets	Erythroblast transformation specific
HAT	Histone acetyltransferase
TET	Ten-eleven translocation
CBP	Cyclic AMP response element binding protein (CREB)-binding protein
APECED	Autoimmune-polyendocrine-candidiasis-ectodermal dystrophy syndrome
APS-1	Autoimmune polyglandular syndrome type-1
LRBA	Lipopolysaccharide-responsive and beige-like anchor protein
P-TEFb	Positive transcription elongation factor b
NMR	Nuclear magnetic resonance
SP	Single positive
cTEC	Cortical thymic epithelial cell
ADAM	A disintegrin and metalloproteinase
NICD	Notch intracellular domain
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
EDP	Early double positive
SSP	Semi-mature single positive
TSSP	Thymus specific serine protease
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
LTi	Lymphoid tissue inducer
TSLP	Thymic stromal lymphopoietin
DC-LAMP	Dendritic cell lysosomal associated membrane glycoprotein
Nr4a1	Nuclear receptor subfamily 4 group A member 1
Lck	lymphocyte-specific protein tyrosine kinase
ITAM	Immunoreceptor tyrosine-based activation motifs
ZAP70	Zeta-chain-associated protein kinase 70
LAT	Linker of activation of T cells
PLC	Phospholipase C
PIP2	Phosphatidylinositol 4,5-bisphosphate
DAG	Diglyceride
IP3	Inositol triphosphate
PKC	Protein kinase C
MAPK	Mitogen-activated protein kinase
PI3K	Phosphatidylinositol-3 kinase
IEL	Intraepithelial lymphocytes
M-CSF	Macrophage-colony stimulating factor
ATO	Artificial thymic organoid
iPSC	Induced pluripotent stem cell
TdT	Terminal deoxynucleotidyl transferase
CAR	Chimeric antigen receptor

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Introduction

1.1 Immune tolerance toward self-antigens

Constant antigen sampling is the basis of adaptive immunity, and the recognition of friend versus foe is crucial for mounting protective immune responses while preventing auto-reactivity simultaneously. While the innate immune system excels at eliciting a rapid response, adaptive immunity can provide antigen-specific responses that are capable of creating immunological memory[1].

During thymic development, V(D)J recombination of the αβ TCR gene segments has been estimated to be capable of generating over 10^{20} different combinations[2]. This diversity brings versatility but also the chance of causing undesired immune responses, such as activating self-reactive T cells that lead to autoimmune diseases. Several mechanisms have been employed to prevent such occurrences, such as the induction of anergic cells in the periphery, clonal deletion in the thymus and the periphery, presence of co-inhibitory molecules, and secretion of immunomodulatory cytokines[3-6].

$CD4^+$ T cells, also known as helper T cells, are a versatile subset of T cells that play a crucial role in regulating adaptive immune responses by tightly controlling their type, magnitude, and duration. A group of $CD4^+$ T cells, termed regulatory T (Treg) cells, play a central role in maintaining immune homeostasis. Particularly, their importance in promoting self-tolerance as well as preventing tissue damage following pathogen clearance has been well demonstrated in numerous mouse and human studies[7-9]. Different genetic defects affecting Treg differentiation, stability, or function have been associated with organ-specific autoimmunity such as type 1 diabetes (T1D) and systemic autoimmunity such as systemic lupus erythematosus (SLE) and immune dysregulation, polyendocrinopathy enteropathy X-linked (IPEX) syndrome[8].

1.1.1 Central tolerance

Generation of immune tolerance can occur at multiple sites and stages during T cell development. In the thymus, developing T cells are selected for their ability to recognize peptides presented on major histocompatibility (MHC) molecules through the processes of β and positive selection. At these two key steps, survival and further differentiation signals are driven by TCR activation, which indicates successful recognition of peptide-MHC (pMHC) complexes, whereas cells not receiving activation signals are ultimately destined for apoptosis in a process known as "death by neglect[10]." During their development, autoreactive thymocytes are depleted from the effector T (Teff) population via negative selection. Thymocytes that encounter a strong TCR stimulation are destined for apoptosis, in a process known also known as clonal deletion.

The crucial role of the thymus in maintaining self-tolerance was discovered from the observation that thymectomy at day 3 after birth, but not day 7, induced systemic autoimmunity and multiorgan failure in mice[11]. This phenomenon was later associated with the development of thymic Treg (tTreg) cells. When coupled with CD28 co-stimulation and common γ chain cytokine signaling via STAT5, some autoreactive cells are capable of differentiating toward the Treg cell lineage, up-regulating markers such as Forkhead box protein 3 (FOXP3) and CD25 (IL2RA), which are commonly associated with regulatory T cells[12]. $CD4^+FOXP3^+CD25^+$ Treg cells are the most abundant type of regulatory T cell and have thus been the best characterized regulatory T cell population. However, other regulatory/suppressive T cell populations have also been described and characterized, including FOXP3-expressing $CD8^+$ Treg and CD4 CD8 DN Treg cells, as well as $FOXP3^-$ Tr1 cells [13-15].

The unique transcription factor AIRE is specifically expressed in medullary thymic epithelial cells (mTECs), allowing for their expression of tissue-restricted antigens (TRAs) from other cell types and organs[16]. Combined with their elevated level of MHC II expression and surface

expression of co-stimulatory molecules CD80/CD86, they have been long identified as a key factor of thymic Treg development[16]. Due to these characteristics, mTECs have been recognized as a critical factor in driving central tolerance and particularly Treg differentiation. Indeed, multiple studies have demonstrated that the lack of Treg cells with particular specificity rather than impaired Treg activation often drives the development of immunopathologies, again highlighting the importance of self-antigen encounter during thymic selection[17].

1.1.2 Peripheral tolerance

Numerous studies have demonstrated that although TCR reactivity is critical for Treg generation, there is no direct causational effect of high TCR reactivity and Treg differentiation. Therefore, it is possible for autoreactive T cells to maturate as conventional T (Tconv) cells[18-20]. This requires additional mechanisms to prevent autoreactive T cells that "escaped" Treg differentiation/negative selection from triggering autoimmune responses.

Modulation of co-stimulatory signals represents a major mechanism of peripheral tolerance. In the absence of co-stimulation, TCR-stimulated T cells will present an anergic phenotype and are also prevented from adopting an activated phenotype upon future antigen exposure[21]. CD28 provides the most well-described co-stimulatory signal on T cells interacting with CD80/CD86 (B7.1/7.2) on antigen-presenting cells, and this interaction is also the primary costimulation signal provider for naïve T cell activation[5]. Some co-inhibitory molecules have shared ligands with co-stimulatory molecules but bind with higher affinity. This is the case for CTLA-4, which competes with CD28 for CD80/CD86 binding. Co-inhibitory molecules can also target other proteins involved in TCR signaling. Lymphocyte activation gene 3, or LAG3, shares homology with CD4 and out-competes CD4 for MHC class II binding[5]. Yet other co-inhibitory molecules form additional receptor-ligand pairs for signal transduction, such as PDL1-PD1[22]. It is well established

that tTreg cells are key mediators of peripheral tolerance, and Treg cells are well-known for expressing co-inhibitory molecules, including CTLA-4, LAG3, and PD1[5]. In addition to utilizing contact-dependent suppressive mechanisms, Treg cells also secrete immunomodulatory cytokines such as TGF-β and IL-10 to carry out their function in maintaining immune homeostasis.

In addition to tTreg differentiation during thymic development, extra-thymic generation of peripherally induced Treg (pTreg) cells is also vital. It was also demonstrated that recent thymic emigrants (RTEs) have elevated sensitivity toward FOXP3 inducers and reduced sensitivity toward negative regulators of FOXP3 expression[23]. The favouring of pTreg differentiation from RTEs within the naïve $CD4^+$ T cell population further supports the notion that autoreactive T cells can escape negative selection/tTreg induction and require backup mechanisms for self-tolerance. Additionally, while tolerization of the human microbiota could be provided in part by plasmacytoid dendritic cells (pDCs) which are capable of transferring peripheral antigens to the thymus and inducing Treg differentiation, it is clear that this mechanism does not provide full protection against many barrier site commensal microorganisms[24,25]. Consequently, pTreg cells generated in the mesenteric lymph nodes are crucial players in tolerizing food allergens and the microbiota[26,27]. In the periphery, Treg induction is highly dependent on the cytokine TGF-β[24,28]. Particularly, the mesenteric lymph node contains elevated levels of TGF-β generated by stromal cells, and the SMAD2/3 complex downstream of TGF-β signaling is a well-known inducer of FOXP3 expression[29]. The disruption of this tolerogenic environment disrupts the balance between Th17 and Treg cells and plays a key role in inflammatory colitis disease progression[30].

Based on these discoveries, a protocol for *in vitro* Treg differentiation has been described. In mice, naïve $CD4^+$ T cells can up-regulate FOXP3 in response to TCR stimulation, co-stimulation, TGF-β, and IL-2. This *in vitro* generated population, termed induced Treg (iTreg)

cells, are capable of suppressing Teff expansion *in vivo*, and are far more unstable than either tTreg and pTreg cells[31].

1.2 FOXP3$^+$ Regulatory T (Treg) cells

Not only did day 3 thymectomy experiments shed light on our understanding of thymic development and central tolerance, but they also ultimately led to the discovery of an immunoregulatory CD25$^+$ population[32]. This subset of cells has a relatively delayed emergence during thymic development compared to conventional T cells, and thus caused the contrasting effects of thymectomy at various time points after birth[17]. Their unique phenotype and function drew interest from different immunological fields such as autoimmunity and transplant immunology and opened the door for cancer immunology studies.

1.2.1 Phenotype of Treg cells

Initial studies discovered that the suppressive T cell population is enriched in CD4$^+$ CD5^{hi} cells[32]. Identification of Treg cells eventually narrowed down to a CD25$^+$ population comprising 5-10% of CD4$^+$CD8$^-$ thymocytes and peripheral CD4$^+$ T cells, and less than 1% of peripheral CD8$^+$ T cells in mice[32]. Specifically, the transfer of CD25-depleted T cells into athymic nude mice resulted in multiorgan autoimmunity, which highly resembles the previously described "scurfy" mouse model that spontaneously develops systemic autoimmunity. However, reconstitution and co-transfer of CD4$^+$ CD25$^+$ T cells with CD25-depleted T cells were able to rescue the autoimmune phenotype[33]. A few years later, the mutation causing the scurfy phenotype in mice was mapped to the *foxp3* locus. Soon after, mutations in the human *FOXP3* gene were identified as the cause of IPEX syndrome, a systemic autoimmune disease that shared characteristics with the Treg cell

depletion in mice[34]. FOXP3 was then established as the master transcription factor for Treg cells in both mice and humans due to its central role in tTreg cell development as well as pTreg cell induction and phenotype maintenance, particularly in sustaining constitutive upregulation of CD25, and repression of TCR-mediated inflammatory cytokine secretion and proliferation[35,36]. Moreover, Treg cells also repress CD127 (IL-7Rα) expression and express elevated levels of activation markers such as CTLA4, ICOS, and GITR at resting states in both humans and mice, whereas LAG3 is preferentially expressed on the surface of activated Treg cells[37-39]. In humans, the Treg cell population is also subdivided based on their expression of FOXP3 and CD25 into naïve/resting Treg ($FOXP3^{low}$ $CD45RA^{+}$), effector Treg ($FOXP3^{+}$ $CD45RA^{-}$), as well as a $FOXP3^{low}$ $CD45RO^{-}$ population that contains unstable Treg cells[40]. Due to the downregulation of CD127 and up-regulation of CD25 in activated T cells, the isolation and characterization of $FOXP3^{low}$ Treg cells have been severely limited.

In both humans and mice, thymic-derived Treg cells are also characterized by their expression of Helios (IKZF2), a transcription factor that is expressed in post-β-selection thymocytes, namely immature single positive (ISP) cells ($CD3^{-}$ $CD4^{+}$ $CD8^{-}$) and double positive (DP) cells ($CD3^{lo/+}$ $CD4^{+}$ $CD8^{+}$)[41-43]. Work by Thornton *et al.* showed that Helios expression is gradually lost in most murine thymocytes at the single positive stage but is maintained in Treg cells found in the thymus[42]. However, the characterization of Helios expression in human thymocytes only covered the double negative (DN) to DP stages[41]. Helios expression is generally thought to be tTreg-specific in mice, as most described *in vitro* TGF-β induced Treg (iTreg) and pTreg cells in tolerance models do not express Helios[44].

Moreover, transcriptomic analysis of murine $Helios^{+}$ and $Helios^{-}$ Treg cells revealed that over 1000 genes are differentially expressed, with $Helios^{-}$ Treg cells containing increased

similarities to conventional T cells[26]. Particularly, the chromatin structure regulator Satb1, whose ectopic expression in Treg cells causes reprogramming toward Teff lineages, is only fully repressed in Helios$^+$ Treg cells[26]. Additionally, demethylation of the conserved non-coding sequence (CNS) 2 of the FOXP3 gene, also known as the Treg specific demethylated region, or TSDR, is less complete in pTreg cells relative to tTreg cells[26]. The TSDR methylation status strongly correlates with stable FOXP3 expression and thus Treg lineage stability by acting as a demethylation-dependent transcriptional enhancer[45]. Furthermore, Helios$^+$ and Helios$^-$ Treg cells showed similar lineage stability when transferred into wild-type recipients. However, when transferred into lymphoreplete recipients, Helios$^-$ Treg cells more readily lose FOXP3 expression[26].

Comparatively, Helios$^+$ and Helios$^-$ Treg cells are similarly suppressive against naïve T in murine adoptive transfer models. However, Helios$^+$, but not Helios$^-$ Treg cells have the capacity to suppress inflammatory cytokine secretion of previously activated Teff cells[26]. Aside from stability and functional differences, human Helios$^+$ but not Helios$^-$ Treg cells have also been identified as co-expressors of TIGIT and FcRL3, two markers unmodulated by TCR-induced activation[46]. Importantly, this discovery allowed for the specific isolation and functional analysis of human tTreg cells. Collectively, these discoveries identified phenotypic and functional differences, as well as markers to distinguish between tTreg and pTreg cells.

1.2.2 Active mechanisms of Treg cell-mediated suppression

1.2.2.1 TGF-β secretion

Treg cells harness several "active" and "counteractive" mechanisms to carry out their suppressive function. As their major active mechanism, Treg cells secrete immunomodulatory cytokines such as TGF-β, IL-10, and IL-35 to suppress aberrant immune responses[47]. In contrast, counteractive mechanisms allow Treg cells to sequester activation stimuli such as pMHC, co-stimulatory

molecules, and inflammatory cytokines[47]. While initial reports claim that TGF-β secretion is crucial for Treg function, others have reached opposite conclusions[48,49]. Later, it was discovered that Treg cells are the only lymphocyte population that expresses Glycoprotein-A repetitions predominant protein (GARP), which binds to latent TGF-β and facilitates its transport to the cell surface[47]. The latent domain of TGF-β possesses an integrin-binding domain, which is utilized by epithelial cells to sequester and activate TGF-β via the metalloprotease MMP14[50]. This additional layer of regulation may explain the contrasting conclusion drawn from different experimental models. Overall, TGF-β secretion is one mechanism used by Treg cells in specific contexts but does not appear to be a major Treg suppression mechanism.

1.2.2.2 IL-10 secretion

Another factor secreted by Treg cells is IL-10, a multifaceted cytokine with differential effects on different immune cell populations. In APCs, IL-10 signaling reduces phagocytic activity and co-stimulatory molecule expression, thus decreasing antigen presentation. In T cells, IL-10 signaling diminishes T cell proliferation and TCR-mediated inflammatory cytokine secretion. However, it acts as an activating signal for NK and B cells and also triggers B cell isotype switching[47]. While mice carrying Treg-specific impairment of IL-10 production failed to control inflammation at barrier sites such as the intestines and lungs, these mice do not develop systemic autoimmunity, indicating that Treg cell suppressive function is not fully dependent on IL-10 secretion either[51].

1.2.3 Counteractive mechanisms of Treg cell-mediated suppression

1.2.3.1 Expression of co-inhibitory molecules

Aside from active mechanisms, Treg cells also compete with Teff cells for critical activation and proliferation signals. One counteractive mechanism is competition between Treg and Teff cells for

contact-dependent signals required for Teff activation, such as the famed co-inhibitory molecule CTLA4 (also known as CD152). Through its higher affinity for CD80/CD86 (also known as B7.1/B7.2), CTLA4 outcompetes CD28 and thus blocks Teff activation and induces anergy of antigen-specific Teff cells. Additionally, Treg cells exploit the high-affinity binding of CTLA4 with its ligands to capture, endocytose, and degrade CD80/CD86 molecules of DC origin[47].

1.2.3.2 pMHC sequestration

More recently, it was also discovered that during antigen presentation, Treg cells can uptake dendritic cell (DC) membrane containing MHC II and co-stimulatory molecules via a trogocytosis-like mechanism[52]. This discovery unveiled how Treg cells can suppress only naïve Teff cells in an antigen-specific manner *in vivo* while preserving the APC's ability to activate other non-self-antigen-specific Teff cells. Together, Treg cells deplete local pro-inflammatory soluble factors and membrane-bound T cell activating signals for suppressor function.

1.2.3.3 IL-2 deprivation

Being the first characteristic by which Treg cells were identified, their elevated level of CD25 expression facilitates another counteractive mechanism by allowing them to preferentially uptake IL-2 in comparison to steady state Teff cells expressing only the intermediate affinity receptor complex comprised of CD122 (IL-2Rβ) and CD132 (IL-2Rγ, also known as common γ chain)[47]. Their unmatched level of CD25 expression also provides a competitive advantage over other CD25-expressing populations such as activated Teff cells, B cells, ILCs, and NK cells[47]. In combination with FOXP3-mediated repression of cell-intrinsic IL-2 secretion, prioritized IL-2

consumption by Treg cells depletes the local microenvironment of IL-2 and thus limits Teff proliferation[53].

1.2.3.4 Conversion of extracellular ATP to adenosine

Treg cells also use ectonucleotidases CD39 and CD73 to convert ATP and ADP to AMP and then AMP to adenosine, respectively[54]. Extracellular ATP acts as danger-associated molecular pattern (DAMP) and creates a pro-inflammatory environment, whereas adenosine drives immune cell activity toward an anti-inflammatory state[55]. Moreover, the adenosine A_{2A} receptors sensing the level of extracellular adenosine also trigger differential downstream effects in Teff and Treg cells; namely, A_{2A} signaling results in decreased kappa-light-chain-enhancer of activated B cells (NF-κB) activation in Teff cells, whereas in Treg cells A_{2A} receptor triggers Treg expansion and enhanced suppressor activity[56,57].

1.3 Treg dysregulation in diseases

It is generally believed that developmental, homeostatic and/or functional deficits in $FOXP3^+$ Treg cells predisposes to the onset of autoimmune and chronic inflammatory diseases[9,39,58-60]. By definition, autoimmune diseases caused by mutations in the *FOXP3* gene are classified as IPEX syndrome, the hallmark human autoimmune disease provoked by defective Treg cell development and/or function[60,61]. The disease is highly heterogenic, where different mutations cause distinctive Treg phenotypes. In addition to mutations in the *FOXP3*, CD25, CTLA-4, and LRBA mutations are also known to cause IPEX-like "Tregopathies[60]." In the absence of LRBA, CTLA-4 is more prone to lysosomal degradation, and as such, LRBA-mutant Treg cells are impaired in their ability to suppress Teff proliferation[62,63].

In addition to IPEX and IPEX-like syndromes, the predominant role of malfunctioning Treg cells has also been demonstrated in Autoimmune Polyglandular Syndrome Type 1, or APS-1, which is caused by mutations in the *AIRE* gene[64]. Failure of tissue-restricted antigen (TRA) expression in the thymus results in incomplete self-tolerance in forms of both negative selection and Treg induction, and thus induces autoreactivity toward multiple different organs, especially of the endocrine system. Lastly, "conventional" chronic inflammatory and autoimmune diseases are also characterized by defective Treg cell development, survival, or function.

1.3.1 IPEX syndrome

The IPEX syndrome is an X-linked recessive autoimmune disease with a rare incidence of approximately 1 in 1.6 million. Due to mutations in the FOXP3 protein, Treg cell function is either partially or completely abrogated, depending on the type and location of mutations[61]. Clinical manifestations usually involve autoimmune enteropathy resulting in chronic diarrhea, type I diabetes and/or thyroiditis (enteropathy), and eczematous dermatitis[65]. Other organs can also be affected, commonly accompanied by elevated lymphocyte infiltration and/or antibody production[66]. Without treatment, IPEX patients rarely survive past the first two years of life, often as a result of sepsis, due to barrier site destruction caused by chronic inflammation[61]. However, the current standard treatment with systemic immunosuppressants has many side effects. The only curative therapy so far is allogeneic hematopoietic stem and progenitor cell (HSPC) transplantation [61].

Over 60 IPEX-causing FOXP3 mutations have been described and each has vastly dissimilar effects on FOXP3 expression, Treg phenotype and function[61,66,67]. Aside from nonsense and frameshift mutations, different missense mutations can cause defective suppressive capacity and/or failure to repress inflammatory cytokine secretion. While IPEX-causing mutations can be

found cross the entire coding sequence of the FOXP3 gene, the majority of these mutations occur within the C-terminal forkhead domain, which is required for DNA binding as well as certain protein-protein interactions (discussed below). Within the forkhead domain, mutations have very heterogeneous effects on Treg function. Based on NMR data, defective DNA binding was suggested to cause the R397W mutant phenotype, where there is a loss of transcriptional repression and Teff suppression[68,69]. Whereas other mutations such as A384T only affect FOXP3 regulation of certain gene subsets, resulting in WT-like inflammatory cytokine repression, but failing to suppress Teff cell proliferation[70,71]. This mutant was later shown also to decrease FOXP3-TIP60 interactions, which is a stabilizing factor for FOXP3:NFAT binding and cooperative transcriptional regulation[71,72]. R347H, another mutation found in the forkhead domain, has relatively normal Teff suppression and represses inflammatory cytokine secretion, but failed to up-regulate CD25, suggesting decreased Treg stability in this mutant[73,74]. Given the variable phenotypes and clinical manifestations of different IPEX-causing FOXP3 mutations, it is important to further characterize and understand how different IPEX-causing FOXP3 mutations drive Treg dysfunction. Not only will it help the prognosis and treatment of IPEX syndrome, but it will also in a broad sense help us understand how Treg dysregulation may occur in other autoimmune diseases and autoinflammatory disorders.

1.3.2 APS-1

APS-1, also known as APECED, is caused by mutations in the autoimmune regulator (*AIRE*) gene. Although extra-thymic AIRE expression was reported, it is still generally agreed that most of its impact lies in its expression in medullary thymic epithelial cells (mTECs)[16]. As the key mediator of negative selection, the atypical transcription factor AIRE has no identified DNA binding motifs but can induce promiscuous gene expression encoding for almost 4000 tissue-restricted antigens

(TRAs)[64,75]. It functions by targeting genes with a repressed chromatin state, recruiting a repressor complex, but using it to activate gene transcription[76]. Additionally, AIRE was also shown to modulate RNA elongation by releasing stalled RNA polymerases through its interaction with P-TEFb[64]. Due to aberrant negative selection and Treg induction, APS-1 is characterized by organ-specific autoimmunity, commonly targeting cells of hormone-secreting organs such as parathyroid chief cells and pancreatic β-islet cells[64].

1.3.3 Other chronic inflammatory and autoimmune diseases in humans

Treg cell dysregulation or dysfunction is also implicated in a much broader spectrum of immunological diseases and conditions, such as rheumatoid arthritis, SLE, atherosclerosis, multiple sclerosis, type I diabetes, immunological degenerative diseases, etc[29,39]. Diminished Treg cell numbers and reduced Treg:Teff cell ratio in peripheral blood have been reported in RA and SLE in numerous studies[9,58,59]. Additionally, Teff cells from RA patients have also shown reduced susceptibility toward Treg-mediated suppression has also been reported in *in vitro* settings, suggesting alternative or parallel mechanisms for breach of tolerance[77]. In addition to suppressing Teff cells, Treg cells have also been shown to suppress antibody production as well as affinity maturation—two key events for humoral autoimmune diseases[59]. Due to their immunomodulatory nature, enhancing Treg cell activity, survival, stability, and localization have been used to alleviate a wide range of autoimmune/autoinflammatory disorders [78,79].

1.4 Function and binding partners of FOXP3

Being the established master transcription factor of Treg cells, FOXP3 has been extensively studied since its identification. Early experiments involving the cloning of FOXP3 fragments

identified its functional domains as well as a number of binding partners[80]. Many of these interactions have been shown to be disrupted by mutations that cause IPEX syndrome, the rare disease where the FOXP3 gene is mutated. These natural FOXP3 mutants aided in the initial characterization of FOXP3 functional domains and defining interacting proteins of FOXP3.

The FOXP3 protein acts as a homodimeric transcription factor composed of 3 main functional domains: (1) the N-terminal repressor domain (PRR), (2) the zinc finger leucine-zipper domain required for dimerization, and (3) the C-terminal forkhead domain responsible for DNA binding[81]. However, all three functional domains are involved in protein-protein interactions, with over 360 direct and indirect binding partners[82]. Unsurprisingly, FOXP3 is involved in regulating approximately 700 genes to create the distinctive phenotype of Treg cells[81].

1.4.1 Functional domains of the FOXP3 protein

The N-terminal domain of FOXP3 is required for maintaining the anergic phenotype of Treg cells. Due to FOXP3-dependent repression of nuclear factor of activated T cells (NFAT):AP-1-mediated transcription, TCR signaling in Treg cells does not induce proliferation and secretion of inflammatory cytokines such as IL-2 and IFN-γ, as in Teff cells[68,80]. Also, the N-terminal of FOXP3 is also involved in the sequestration of AP-1, which often acts synergistically with NFAT due to their overlapping transcription targets [68]. Moreover, the FOXP3 N-terminal domain is involved in repressing RORα-dependent transcription, as it is a key mediator of Th17 polarization. This key function is critical for maintaining a proper Treg/Th17 balance, which is disrupted in many autoimmune and autoinflammatory disorders[83]. Lastly, this domain also binds to the chromatin modifiers such as the histone lysine acetyltransferase TIP60, which, together with P300, provides the vital role of stabilizing the FOXP3 protein level by inhibiting ubiquitination and its subsequent proteasomal degradation[71].

The zinc finger leucine zipper domain is required FOXP3 dimerization and thus DNA binding. Specifically, mutation/deletion of the leucine zipper results in loss of transcriptional repression by FOXP3 and subsequently loss of Treg function. Moreover, FOXP3 isoforms lacking this leucine zipper have been proposed to act as a dominant negative form of FOXP3 involved in the regulation of other functional isoforms[68,71] .

The C-terminal forkhead domain is responsible for direct DNA binding and is also the domain where most IPEX-causing FOXP3 mutations lie. Aside from DNA binding, this domain is also shown to interact with NFAT and NF-κB, and loss of FOXP3-NFAT interactions via the forkhead domain results in loss of IL-2 repression[68]. While most IPEX mutations in the forkhead domain affect transcriptional repression by FOXP3, other mutations result in defective suppression of Teff cells, while leaving transcriptional repression intact[68]. Overall, each functional domain of FOXP3 is utilized for certain protein-protein interactions that are critical for its function.

1.4.2 NFAT

The transcription factor NFAT is a key modulator of T cell activation through cooperative interactions with AP-1. In response to productive T cell activation, NFAT:AP-1 drives the expression of genes such as *IL-2*, *IL-4*, *CD25*, and *CTLA4*, whereas TCR activation in the absence of co-stimulatory signals drives the expression of a set of NFAT-dependent, AP-1-independent genes responsible for inducing T cell anergy[84,85]. While FOXP3 drives the expression of certain genes such as *CD25* and *CTLA4*, pro-inflammatory cytokine secretion is repressed. It was discovered that NFAT:FOXP3 complexes occupy the same binding site as NFAT:AP-1 in the *il2* promoter, thus prompting the hypothesis that FOXP3 competes with AP-1 for cooperative NFAT binding as one mechanism of carrying out its suppressive function[84].

1.4.3 NF-κB

While NF-κB regulation of the FOXP3 locus is extensively characterized, far less work is done on the direct interactions between FOXP3 and NF-κB family proteins. While initial studies have reported FOXP3 interactions with RelA, an NF-κB subunit, inhibits NF-κB-dependent transcription, some subsequent studies failed to recreate similar results[86,87]. More specifically, it was initially discovered that FOXP3 directly blocks NF-κB mediated transcription downstream of PKCθ, IκB kinase β (IKKβ), or TNF-α stimulation while leaving its DNA-binding activity intact. However, all evidence was confined to either overexpression or reporter assays. To reconcile such conflicting results, it was also hypothesized that FOXP3-NF-κB interactions are indirect, potentially through NFAT or Runx1[87].

1.4.4 Runx1

Runx1, also known as acute myeloid leukemia 1 (AML-1), is a key regulator of T cell development at β-selection and positive selection. Perhaps more importantly, this transcription factor is required for $CD4^+$ T cell development and homeostasis in the periphery as a negative regulator of pro-apoptotic Bcl-2 family proteins[87,88]. An inter-domain region of FOXP3 was identified to facilitate FOXP3-Runx1 interactions, and ablation of such interactions results in ineffective IL-2 repression and failed suppression of Teff cells. While Runx1 acts as a transcription activator with other transcription factors such as Ets-1 and NF-κB, upon FOXP3 binding it acts as a transcriptional repressor and results in differential regulation of their common target genes[89].

1.4.5 HATs: TIP60 & P300

Another key aspect of Treg cells is their unique epigenetic landscape, particularly in the CNS2 region which contains the major TSDR (see below). However, FOXP3 interactions with histone

modifiers have additional roles as well. FOXP3 is known to interact with two histone acetyltransferases (HATs) TIP60 and P300. Both of which are described to induce FOXP3 acetylation[90]. While FOXP3 acetylation by P300 has been shown to prevent ubiquitination and subsequent proteasomal degradation of the FOXP3 protein, the role of TIP60-dependent acetylation of FOXP3 is less clear. However, Treg-specific deletion of TIP60 resulted in severe systemic autoimmunity, with disease kinetics similar to that of scurfy mice[90]. Later it was shown that P300 binding to TIP60 results in the acetylation of TIP60, which leads to the binding of TIP60 to FOXP3 and subsequent acetylation of FOXP3. Defects in FOXP3:TIP60 interactions results in impairment of Treg suppressive function, but not repression of pro-inflammatory cytokine secretion[72]. Additionally, a recent study also demonstrated that FOXP3 acetylation by P300 downstream of IFN-β signaling significantly enhances the induction of the Treg-specific transcriptional program[91]. Together, these findings highlight the important role of TIP60 acting both as a scaffold and a post-translational modifier of FOXP3 to promote its stability and function.

1.5 Regulation of FOXP3 expression

Expression of the FOXP3 protein is tightly controlled, particularly through its promoter region and four highly conserved CNS regions (Figure 1)[29]. The promoter region of FOXP3 contains binding sites for NFAT and c-Rel (NF-κB family protein), implicating the role of TCR signaling in driving FOXP3 expression. Additionally, the FOXP3 promoter region also contains binding sites for AP-1, Foxo proteins, GATA3, SMAD3, as well as the H3K4 histone methyltransferase MLL4[29,92]. So far, SMAD3 is the only transcription factor found to bind to the CNS1 region, and thus, it has been shown that this region is pivotal for TGF-β-dependent pTreg induction[93]. The methylation status of the CNS2 region of FOXP3, more commonly known as the TSDR, is perhaps one of the most

distinctive features of thymic Treg cells[26]. This region also contains binding sites for many factors involved in the transcriptional regulation of FOXP3: STAT5, STAT3, Runx1, Ets-1, and even FOXP3 itself[29,94,95]. While CNS3 is not required for maintaining FOXP3 expression on differentiated Tregs, deletion of CNS3 results in defective tTreg generation, particularly in thymocytes receiving lower TCR stimulation[96]. Through its c-Rel binding, CNS3 was suggested to act as a pioneering element for tTreg induction through TCR-dependent mechanisms[93]. Additionally, a newly discovered control region, CNS0, is recognized by the pioneering factor and epigenetic modifier Satb1. The expression of Satb1 in thymocytes peaks at the DP and CD4SP stages, suggesting its role in priming Treg differentiation from newly positively selected thymocytes[97].

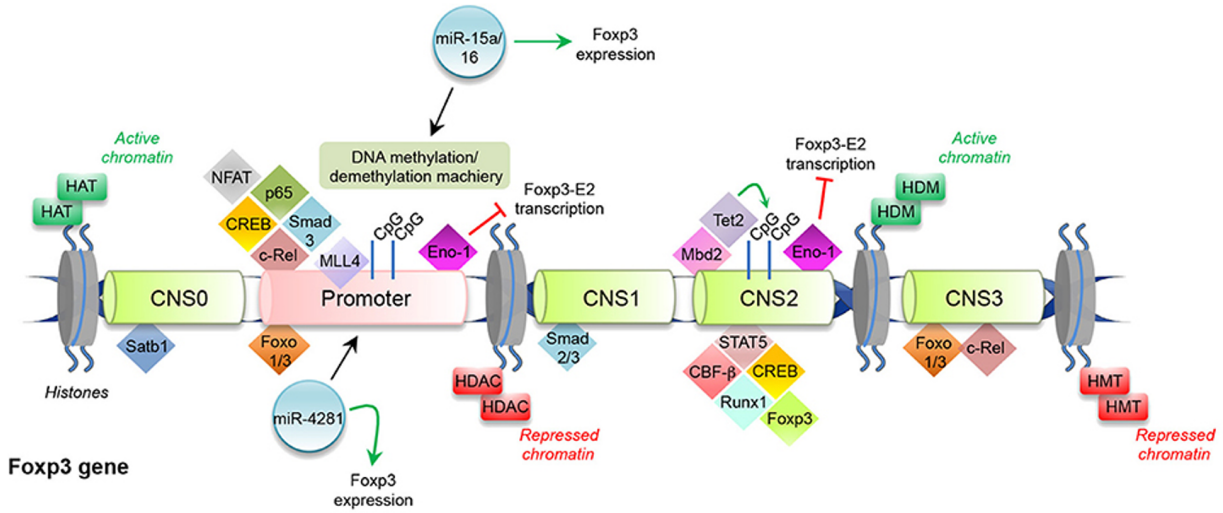

Figure 1: The FOXP3 locus is regulated by multiple different signaling pathways. FOXP3 transcription is regulated at its promoter region and at its 4 CNS regions. Specifically, CNS0 it's a pioneering region required for initial expression, but not maintenance of FOXP3. The CNS1 region is regulated by SMAD2/3 complex downstream of TGF-β. CNS2, also known as TSDR, is regulated by epigenetic modifications via Tet2, STAT5 downstream of IL-2 signaling, as well as the FOXP3 protein itself. CNS3 is regulated by c-Rel downstream of TCR signaling and the FOXP3-stabilizing Foxo family proteins, which is also regulated by TCR signaling via subcellular localization. Figure adopted *from Colamatteo A, Carbone F, Bruzzaniti S, et al. 2020*[29].

1.5.1 Epigenetic regulation at the TSDR

Demethylation of CpG sequences within the TSDR region plays a nonredundant role in maintaining constitutive FOXP3 expression, as Treg cells carrying a methylated TSDR do not exhibit a stable Treg cell phenotype[98]. During thymocyte development, the TSDR region is methylated at double positive and single positive stages, and gradual demethylation starts in FOXP3$^+$ CD4SP cells, continuing until the cells reach a fully mature Treg state[29,99]. More intriguingly, studies have shown that TSDR demethylation and FOXP3 expression are independent, yet complementary processes both triggered downstream of TCR signaling in the thymus[98]. As Tet proteins are required for DNA demethylation through base excision repair, consistent expression of Tet2, as well as Tet3, is maintained in Treg cells in an IL-2-dependent manner, and failure of

their expression leads to loss of Treg phenotype and FOXP3 expression [100-102]. Additionally, the histone acetyltransferase P300 and its analogue CBP also interact with TSDR and play an important role in maintaining Treg stability, as their combined deletion results in reduced Treg lineage stability in inflammatory environments partly through their regulation of GAG3 expression. GATA3 has long been identified to cooperate with FOXP3 in Treg cells, promoting FOXP3 expression levels and migration toward sites of inflammation[82,103]. In addition, Ets-1 binding to TSDR was demonstrated to be methylation-status sensitive *ex vivo*, while those of CREB and c-Rel were not[45]. However, the specific composition of such methylation status-sensitive Ets-1-containing transcription complex remains to be characterized. Finally, using a dCas9-Tet1 system, it was demonstrated in the past year that maintaining TSDR demethylation alone in the absence of Treg-inducing cytokines is capable of inducing FOXP3 expression[104]. Despite this approach failing to generate cells with a functional Treg phenotype, their findings further highlight the importance of TSDR methylation status for FOXP3 expression.

1.5.2 STAT5 and common γ cytokines

Due to their high expression of CD25 and requirement of IL-2 for a healthy Treg population, the STAT5 signaling in Treg cells has been extensively studied. The transcription factor STAT5, downstream of IL-2, IL-7, IL-9, and IL-15, was shown to bind to both the FOXP3 promoter and TSDR region[53,74]. Additional studies have also shown that STAT5 binding occurs predominantly in the TSDR region and is required for the stable expression of FOXP3. Furthermore, lineage stability of TSDR-deficient Treg cells can be improved in the presence of elevated IL-2, a phenomenon by which the authors suggested the possibility of direct FOXP3 promoter:TSDR interactions[95]. Through upregulation of CD25 by FOXP3 and stabilization of FOXP3 expression

by STAT5, IL-2 signaling has been implicated as an unreplaceable mechanism for maintaining a stable Treg phenotype[53].

In addition to Satb1 binding, the newly discovered CNS0 region of FOXP3 was also reported to include STAT5 binding sites[105]. During tTreg differentiation, Treg progenitor cells can be separated into $FOXP3^{-}$ $CD25^{+}$ and $FOXP3^{low}$ $CD25^{+}$ progenitors[106]. The latter population was overrepresented in CNS0-deficient mice, while mature newly generated $CD73^{-}$ $FOXP3^{+}$ Treg cell were significantly reduced. However, despite being required for initial upregulation and stabilization of FOXP3 expression, CNS0 is dispensable after Treg maturation, suggesting a role of CNS0 in maintaining FOXP3 expression before TSDR demethylation is sufficient to confer FOXP3 stability[105].

Lastly, through its involvement in the translational profile of Treg cells, IL-2 regulates the translation of transcriptional activators of the FOXP3 locus. Specifically, IL-2 signaling drives upregulation of the eukaryotic translation initiation factor eIF4E in Treg cells, which results in increased translation of certain gene subsets, including those driving Treg proliferation as well as Foxo3[107]. However, cell cycle progression is a destabilizer of FOXP3 expression, Foxo3 is a known stabilizer and inducer of FOXP3 expression[107-109]. These data suggest the possibility of additional roles of IL-2 in regulating of Treg lineage stability and homeostasis. Together, through direct binding of STAT5 to the FOXP3 locus and other indirect mechanisms, IL-2 signaling plays irreplaceable roles in the induction as well as stable maintenance of FOXP3 expression.

1.5.3 TGF-β and SMAD proteins

The cytokine TGF-β has a wide range of functions, modulating proliferation, differentiation, and embryonic development[110,111]. Depending on the cell type and timing during development, TGF-β can often exert different or even opposite effects[110]. Such is also true for its involvement in the

immune system, as it plays a critical role in the induction of both pTregs and Th17 cells[112]. TGF-β downstream signaling is a complex network revolved around SMAD proteins, with the capacity of modulating transcription levels as well as epigenetic modifications. The target genes and the effect of targeting, be it activation or repression, are highly dependent on SMAD partners, resulting in the often context-specific effect of TGF-β signaling[110]. At the *FOXP3* locus, SMAD2/3 downstream of TGF-β signaling binds to both the promoter region and CNS1. The role of TGF-β signaling in pTreg and iTreg generation was mostly contributed by the CNS1 region, as its deletion resulted in severely lowered pTreg numbers in mesenteric lymph nodes and iTreg numbers following *in vitro* induction assays[50]. While initial reports claim that removing TGF-β signaling has no effect on tTreg development, several experiments suggested the opposite[113-115]. Particularly, TGF-β signaling blockade results in the loss of Treg populations 3-5 days after birth, when the first Treg cells should emerge. However, Treg cells appear after 7 days and have heightened responsiveness toward IL-2, resulting in their increased expansion. Mechanistically, both death by neglect and negative selection triggers apoptosis, and the sensing of which drives uptake of apoptotic cells and induction of TGF-β secretion by APCs[115-117]. TGF-β then promotes survival of both APCs and thymocytes, favouring Treg induction over negative selection[113].

1.5.4 TCR and co-stimulation

As aforementioned, TSDR deletion results in unstable FOXP3 expression and Treg function[26]. Specifically, it has been shown that cell cycle progression reduces the stability of FOXP3 transcription but is counteracted by stabilization through interactions between the promoter region and TSDR[118,119]. Through 3C capture, it was demonstrated that binding of NFAT to both regions of the FOXP3 locus triggers promoter:enhancer looping in Tregs, which was enhanced by stimulation of the TCR signaling pathway[119]. This interaction was significantly higher than any

other regional interactions at the FOXP3 locus and is barely present in Teff cells[119]. Additionally, in developing T cells, TCR signaling occurs before FOXP3 expression and a persistent TCR signal is required for Treg cell differentiation[87,120]. Moreover, due to the self-specific nature of their TCRs, most Treg cells experience regular but infrequent antigen exposure in the periphery[87]. It has been hypothesized that recurring TCR stimulation via self-antigen recognition may be vital for the maintenance of Treg cell phenotype[87]. Specifically, the PI3K:Akt axis downstream of TCR and co-stimulatory signals induces nuclear localization of NF-κB family transcription factors, particularly c-Rel[121,122]. c-Rel has identified binding sites in the CNS3 region of FOXP3, and c-Rel deficient mice was shown to have reduced tTreg and pTreg levels[29,93]. The Foxo proteins Foxo1 and Foxo3 bind to the FOXP3 promoter and act as transcriptional activators. However, their phosphorylation via the PI3K:Akt axis results in their nuclear export[29]. As such, both TCR and CD28 co-stimulatory signaling must be optimized for the induction and maintenance of stable FOXP3 expression.

1.6 Thymocyte development and tTreg differentiation

The thymus acts as the site of development for T cells, where they partake in key selection processes required for their maturation before entering into circulation and carrying out their role in the adaptive immune system (Figure 2)[123,124]. HSPCs, being highly enriched in CD34 expressors, migrate into the thymus, and commit toward the T cell lineage. While the previously coined term "thymic seeding progenitors (TSPs)" suggest that these cells are primed for T cell differentiation, single cell analysis on human postnatal uncommitted thymocytes identified that this population exhibit a gene expression profile highly similar to HSPCs in the bone marrow[125]. Cells at the first stage, termed double negative (DN) stage due to the lack of CD4 and CD8 expression, includes a

heterogenous population of primed as well as committed T cell precursors[123,124]. Upon successful V(D)J recombination of the TCRβ chain, the latter up-regulates both CD4 and CD8, entering into the double positive (DP) stage. VJ recombination of the TCRα chain then takes place, readying the cells for positive selection and subsequent transition into single positive (SP) stages. Upon successful positive selection, developing thymocytes are also subjected to negative selection and Treg induction to prevent autoreactive thymocytes from developing into Teff cells. In the thymus, CD25 expression is not induced upon TCR signaling during β-selection nor positive selection, and as such, CD25 expression in DP and SP thymocytes is considered to be associated with Treg cells or their precursors, the latter being reported to exist in as early as pre-DP thymocytes[106,126]. In addition to $CD25^{+}$ Treg precursors, another $FOXP3^{low}$ Treg precursor population was also identified. TCR sequencing has shown that the two Treg cell precursor populations have dissimilar TCR repertoires and differential protection against experimental models of autoimmunity[127] (Figure 3).

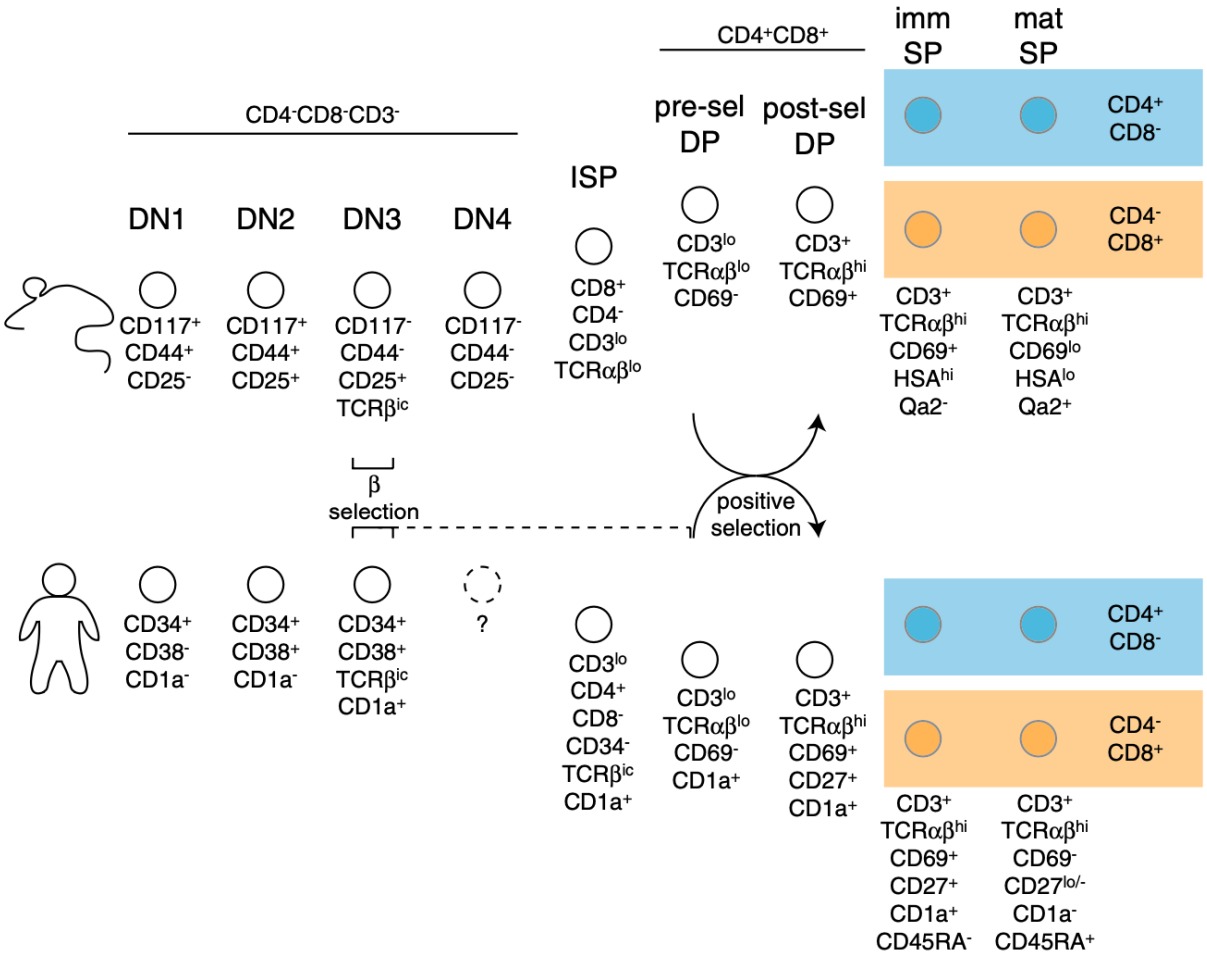

Figure 2: Developmental stages of human and mouse thymocytes. Thymic selection is broadly separated into CD4 CD8 DN, CD4 CD8 DP, and CD4 or CD8 SP stages. In mice, DN cells are further divided into 4 stages based the expression of CD117, CD44, and CD25, whereas 3 DN stages are established for human thymocytes based on the expression of CD34, CD38, and CD1a (top and bottom, respectively). An alternative nomenclature uses CD7 and CD5, rather than CD34 and CD38. Both nomenclatures converge at the $CD1a^+$ population, which is the final DN stage. After β-selection, a transitional ISP stage ($CD4^+$ $CD3^-$) was observed in both mice and human thymocytes. Similarly, a $CD3^-$ DP stage is also shared. Upon positive selection, CD3, αβTCR, as well as CD69 is up-regulated. In humans, CD27 is also up-regulated after positive selection. After DP-SP transition, the SP cells can also be further divided. In mice, the two SP populations are separated based on CD69, HSA, and Qa2 expression, whereas in humans, the distinction is made based on the expression of CD69, CD45RA, CD1a, and CD27. Adopted from *Halkias J, Melichar HJ, Taylor KT, Robey EA. 2014*[124].

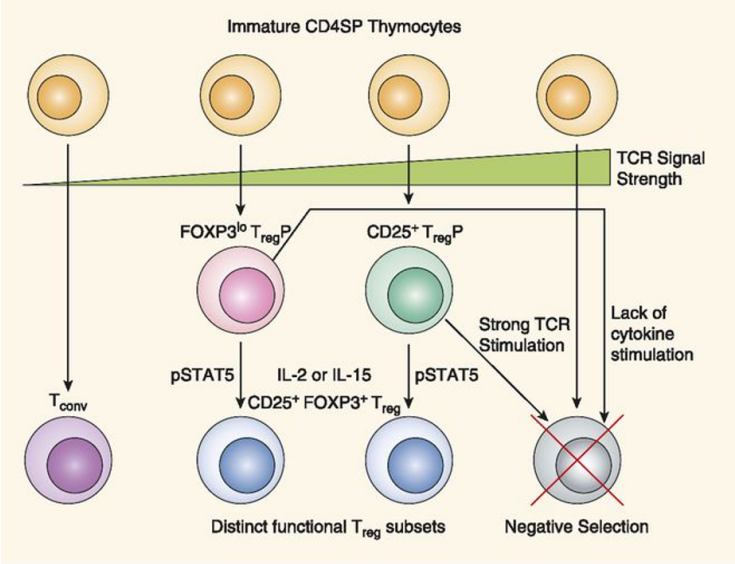

Figure 3: Current model on thymic Treg development. During thymic development, the strength or absence of TCR signaling dictates cell fate. Thymocytes not receiving TCR signaling are destined for death by neglect, whereas thymocytes receiving weak TCR signaling are destined to become Teff/Tconv cells. Thymocytes receiving higher thresholds of signaling become Treg cells through different intermediates, namely, the FOXP3^{low} intermediate and the CD25^{+} intermediate. Thymocytes receiving further elevated TCR stimulation are destined for negative selection due to their high reactivity. Adopted from *Owen DL, Sjaastad LE, Farrar MA. 2019*[106].

1.6.1 Stages of thymic selection

Each of the broad categorizations of DN, DP, and SP can be further subdivided. In mice, DN cells are divided into four stages based on their expression of CD44 and CD25, while in humans, such division is less clear[128]. One classification involves the HSPC enrichment marker CD34 and the ectoenzyme CD38, while the other is based on CD7 and CD5 expression, and both nomenclatures converge at the upregulation of CD1a, which marks T cell lineage commitment and the start of V(D)J recombination[41,123,124,129,130]. In humans thymocyte development, CD7 acts as a marker of Notch signaling[125]. Upon successful rearrangement of the TCRβ chain or the TCRγ and δ chains marked by signaling through the pre-TCR or γδTCR, respectively, developing T cells encounter their first lineage differentiation choice. TCR stimulation also halts ongoing recombination at the

other β, γ, and δ chains to prevent the generation of bispecific T cells in the phenomenon known as allelic exclusion. Successful β-chain rearrangement promotes the αβ branch of T cell development in the event termed β-selection, whereas successful γδTCR signaling promotes differentiation of γδ T cells[131]. Successful β-selection triggers the largest amplification event throughout thymocyte development, as TCR rearrangement strictly prevents proliferation[132]. This takes full advantage of a rearranged and productive TCRβ chain to maximize the efficiency of successful αβ T cell development. The DN-DP progression is also marked by transitional immature single positive (ISP) and early double positive (EDP) stages. In humans thymocyte development, ISP cells are characterized by CD4 expression and without expressing CD8 and CD3, whereas in mice, CD8 is up-regulated before CD4 during the ISP stage. At the EDP stage, cell surface expression of the other co-receptor (CD8 in humans and CD4 in mice) and low levels of αβTCR are up-regulated sequentially[123,124,133]. Upon positive selection at the DP stage, i.e., successful pMHC engagement by the fully rearranged αβTCR, surface expression of CD3 and αβTCR is elevated, accompanied by the expression of CD69 in mice and both CD69 and CD27 in humans[124].

The second major lineage commitment event occurs post-positive selection in the form of CD4 versus CD8 lineage commitment. Two classical models exist for this event, one suggesting random co-receptor selection and the elimination of T cells that have chosen the wrong co-receptor (i.e., the stochastic-selective model) and the other suggesting TCR signaling pattern directly downregulating the expression of the unnecessary co-receptor (i.e., the instructive model)[134]. Due to the finding that, compared to CD8, CD4 has a much stronger interaction with Lck, a key mediator of TCR signaling, it was understood that stronger, but even more so longer duration of TCR signaling strongly favours CD4SP differentiation[135]. The best model thus far, namely the kinetic signaling model, was proposed due to discovering that regardless of MHC specificity, all

thymocytes transiently downregulate CD8 expression[135]. Thus, the presence/continuation of TCR signaling at this $CD4^+$ $CD8^{low}$ stage drives CD4SP lineage commitment, whereas disruption of TCR signaling results in sensitization of common γ cytokines such as IL-7, which are known to promote silencing of the CD4 locus, and hence CD8SP differentiation[136]. The transcription factor ThPOK plays a central role in promoting CD4SP lineage commitment, and its transcription is promoted by strong TCR signaling, further demonstrating the importance of TCR signaling as the basis of CD4 versus CD8 cell fates[137,138]. On the other hand, Runx3 is required for CD8 T cell development, although its selective induction mostly occurs after CD8 lineage commitment[137,139]. Further classification of SP thymocytes separates semimature single positive cells (SSP) and fully mature SP cells based on their expression of CD45RA/RO and CD69, where fully mature SP cells express CD45RA but not CD69. Additional human thymocyte maturation markers include CD27 and CD1a, the latter being expressed starting at the pro-T cell (designated DN2 in certain literature), marking T-lineage commitment, and downregulated at the last step of maturation[124].

1.6.2 Notch signaling

Notch signaling is a simple yet highly conserved system used to regulate cell fate and differentiation of numerous cell types in events such as vasculature, neuron arborization, as well as HSPC differentiation[140]. The core Notch signaling pathway is rather simple: both Notch proteins and their ligands, Delta and Jagged protein families, are transmembrane proteins, and binding receptor:ligand binding triggers two sequential Notch cleavage events mediated by ADAM metalloproteinases and the γ-secretase[140]. The cleaved Notch intracellular domain (NICD) is then liberated and translocates into the nucleus, where it interacts with CSL, also known as RBP-J, to regulate downstream transcription[140,141]. The indispensable nature of Notch signaling for T cell lineage commitment was demonstrated through the work of two different groups simultaneously.

Radtke *et al.* identified that Notch deletion caused a severe reduction in thymocyte numbers as well as disruption of the thymic architecture. Specifically, blockage occurs at the very first stage of murine T cell development: DN1, a stage where cells are still uncommitted. Alternatively, Notch deletion causes thymocytes to adopt the B cell lineage[142]. Concurrently, Pui *et al.* discovered that ectopic expression of a constitutively active form of Notch1 resulted in T cell development in the bone marrow at the expense of B cell lymphopoiesis[143]. Subsequential discoveries demonstrated that TEC-specific DLL4 expression is required for normal T cell development *in vivo*, once again with development blocked at the DN1 stage if TECs are DLL4-deficient[144].

1.6.3 Spatial-temporal regulation of thymocyte development

Based on histological analysis, the thymus can be broadly divided into two regions, the cortex and the medullary. Both regions contain functionally instinct types of epithelial cells, namely cTECs and mTECs[145]. Due to their unique expression of thymoproteasomes as well as specialized proteases cathepsin L and TSSP, cTECs express a distinct set of self-peptides supporting low-affinity TCR:pMHC interactions to promote positive selection of both MHC class I- and class II-restricted thymocytes[146-148]. On the other hand, mature mTECs are characterized by their upregulation of AIRE and CD80, with a subset of high CD86 expressors[149,150]. While the complete set of TEC differentiation signals is yet under investigation, it was found that cTECs and mTECs share a common progenitor and that the maturation of both cTECs and mTECs requires lymphocyte-derived signals, as thymocyte development blockade results in a loss of thymic structure[148,151]. For example, RANK signaling in mTECs is required for their upregulation of AIRE and MHC II, and the ligand RANKL is secreted by LTi cells, γδT cells, SP thymocytes, as well as NKT cells[148,152].

Although thymocyte movement within the cortex or medulla is random, migration between thymic regions is tightly controlled by numerous signals. Predominantly, this process is regulated by chemokines, while other important cues include ligand availability, as in the case of DLL4 and IL-7[153-156]. HSPCs enter the thymus at the cortico-medullary junction, and migrate toward the cortex, due to CCL25 and CXCL12 production by cTECs and expression of their receptors, CCR9 and CXCR4, on DN thymocytes. Upon positive selection, migration of DN thymocytes away from the cortico-medullary junction into the subcapsular region is then mediated by CCRL1. However the exact mechanism remains to be elucidated[148]. Following DP-SP transition, thymocytes up-regulate CCR7. Both mTECs and medullary fibroblasts produce its ligands CCL19 and CCL21[148]. SSP thymocytes were also reported to express CCR4, and its ligands CCL17 and CCL22 are expressed by thymic DCs and components of the Hassall's corpuscles, respectively, suggesting their role in promoting Treg generation and negative selection[157-159]. Expression of the GPCR S1PR1 (or S1P1) is required for thymocyte egress. The activation marker CD69 was shown to interact with S1PR1, resulting in the internalization and degradation of the latter[160]. Consequently, it was hypothesized that the discontinuation of TCR signaling results in S1PR1 upregulation, marking the end of their maturation and selection process[161,162].

1.6.4 Contribution of various thymic populations toward tTreg induction

Thymic development is orchestrated through crosstalk between many different cell types, including cTECs, mTECs, DCs, and even thymocyte-thymocyte interactions[148,151,163,164]. As previously mentioned, mTECs and thymic DCs, in particular, are of high importance for tTreg development, particularly due to the TRA expression on mTECs and the ability of thymic DCs to mediate self-antigen:MHC transfer from mTECs via trogocytosis[158,159,165-168]. Surprisingly, approximately half of TRA-specific Treg cells are induced through antigen presentation by thymic

DCs[169]. Upon terminal differentiation, mTECs can also form special thymic bodies such as the Hassall's corpuscles, which are associated with human tTreg induction[157-159].

Specifically, thymic stromal lymphopoietin (TSLP) production was detected within Hassall's corpuscles, and associated with activated $CD11c^+$ thymic DCs[158]. TSLP is a common γ-like cytokine mostly secreted by epithelial cells that has well-described roles in mediating Th2 responses, including allergic inflammation[170]. Its receptor is composed of CD127 and TSLPR, which together triggers STAT5 as well as weak STAT3 signaling. Its unique property lies within the upregulation of MHC-II and CD86 without inducing inflammatory cytokine secretion, making it suitable for promoting Treg differentiation and Th2 responses[170].In addition to $CD25^+$CD4SP cells being co-localized with activated DCs ($DC\text{-}LAMP^+$) in Hassall's corpuscles, isolated human $CD11c^+$ conventional DCs and $CD123^+$ plasmacytoid DCs pre-treated with TSLP are capable of inducing $CD25^+$ $FOXP3^+$ regulatory cells from CD4SP thymocytes, but not peripheral naïve $CD4^+$ T cells, *in vitro*[158,159]. This process is dependent on TCR stimulation and CD28 co-stimulation signals as well as IL-2 availability, as antibody blocking of HLA-DR, CD80 and CD86, or IL-2 results in severely decreased Treg numbers[158].

B cells also contribute toward tTreg development, as they were able to promote the generation of $CD25^+$ Treg precursors but not subsequent Treg maturation[106]. Additionally, genetic perturbations resulting in B cell defects, such as AID or CD40L deficiency, also resulted in decreased Treg proportions[106]. Lastly, a more recent study identified B cell licensing via CD40:CD40L (CD154) interactions also induced AIRE expression on thymic B cells, suggesting an additional TRA source other than mTECs[171].

1.7 Role of TCR and co-stimulatory signals during Treg development

Despite all cell-intrinsic and cell-extrinsic factors that influence thymic development, the whole process is still unarguably centered around TCR signaling. Not only does its signaling affect commitment to different T cell lineages during thymocyte development, but it also biases the future adaptation of CD4SP thymocytes toward different helper fates[172]. Multiple readouts have been used for assessing the strength of TCR:pMHC interactions. The surface marker CD5 was identified as a negative regulator of TCR signaling, and its expression level is directly correlated to the activation strength in developing thymocytes[173]. Furthermore, naïve $CD5^{hi}$ cells have been found to experience increased downstream TCR-induced phosphorylation events compared to $CD5^{low}$ cells[174]. Nur77, also known as Nr4a1, is another commonly used readout for TCR signaling due to its induction by antigen-specific stimulation but not cytokine-mediated stimulation in T cells and B cells[175,176]. Much of our knowledge on the intricacies of TCR signaling influencing thymic development was established using these two markers in combination with TCR transgenic models.

1.7.1 Overview of TCR stimulation and CD28 co-stimulation signaling

The αβTCR and δγTCR themselves lack signal transduction motifs but are associated with CD3 complexes[177]. The αβTCR:CD3 complex is usually comprised of an αβTCR, a CD3εγ, and a CD3εδ heterodimer, as well as a CD3ζ homodimer[177,178]. CD4 and CD8 co-receptors are associated with the kinase Lck through their intracellular domains, and co-localize with the TCR:CD3 complex upon TCR:pMHC engagement, due to their recognition of MHC-II and MHC-I, respectively[177]. This triggers Lck-mediated phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3 molecules, recruiting another protein tyrosine kinase, Zap70, which subsequently recruits the adaptor protein LAT. Through direct binding or additional adaptor proteins, LAT recruits effector signaling molecules such as phospholipase C γ (PLCγ) and VAV1,

resulting in the complex referred to as the LAT signalosome[178]. PLCγ next cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diglyceride (DAG) and inositol triphosphate (IP3), activating protein kinase C θ (PKCθ) and ER-calcium release, respectively[178]. VAV1 promotes the mitogen-activated protein kinase (MAPK) signaling pathway, whereas PKCθ is responsible for triggering NF-κB-mediated transcription, and Ca^{++} release activates calcineurin to allow for NFAT nuclear translocation[178]. Additionally, the co-stimulatory molecule CD28 plays a vital role in activating PLCγ activation, as it is constantly associated with phosphatidylinositol-3 kinase (PI3K), a regulator of PLCγ activation[177,179]. PI3K activation also inhibits a negative regulator in GSK3β, further enhancing downstream signaling[179]. Lastly, CD28 can also act as docking sites for LAT-associated proteins, allowing it also to transduce signals that activate AP-1 and NF-κB-dependent transcription[179].

1.7.2 Unique aspects of TCR signaling in thymocytes

Although a unique, altered peptide pool is used for positive selection, the vastly different TCR signaling response of developing versus peripheral T cells was found to be mainly attributed thymocyte-intrinsic properties[10]. Unlike peripheral T cells, TCR stimulation alone in the absence of co-stimulation is able to trigger downstream signaling DP thymocytes, conferring more sensitivity, allowing for positive selection with very low levels of surface αβTCR expression[178]. More surprisingly, aberrant stimulation in CD28 co-stimulation can drive DN-DP transition in the absence of V(D)J rearrangement, bypassing β-selection altogether[180]. In addition to broad sensitivity differences, new data suggest that the proper combination of signal intensity and duration is required for positive selection[10]. The pro-apoptotic Bcl-2 family protein Bim is required for negative selection. However, its deletion could rescue self-reactive T cells only if deletion

occurred in the medulla[10]. Due to this observation, the authors proposed the sustained signal model, where negative feedback from strong sustained signals will promote higher levels of negative feedback, particularly MAPK phosphatases, resulting in a death by neglect-like phenomenon. In contrast sustained lower levels of activation are capable of supporting positive selection[10]. Indeed, low but sustained Erk (classical MAPK) phosphorylation is key to driving positive selection, whereas its robust albeit short-lived activation results in cell death[181,182].

More recently, a few TCR signaling regulators have been found to primarily affect T cell development, but not peripheral T cell function, of which the protein Themis drew particular interest. Its deletion does not affect earlier stages of T cell development but blocks positive selection and thus DP-SP transition[183]. In addition, the few peripheral T cells in Themis deficient mice almost exclusively exhibit a memory-like phenotype[184]. While it was agreed that Themis exerts its effect primarily through the regulation of phosphatase Shp1, whether Themis functions as a positive or negative regulator is still under heavy debate. As they witnessed higher TCR signaling as characterized by Ca^{++} influx and Erk phosphorylation, one group suggested that Themis acts as a negative regulator of TCR signaling strength by restricting Shp1 access toward TCR signaling effectors[184]. On the other hand, the alternative proposition was that Themis act as a positive regulator by blocking Shp1:substrate interactions and catalytic cysteine oxidation, due to the evidence of Shp1 enzymatic activity inhibition[185]. Forming a unified theory may be difficult, as both death by neglect and negative selection result in apoptosis[183]. Nevertheless, Themis is a clear example of TCR signal tuning in developing thymocytes in addition to the more well-known and finely characterized modulators such as CD5.

1.7.3 Impact of TCR signaling strength on tTreg development

The instructive role of TCR signaling during multiple stages of thymocyte development is evidently clear, as in events such as αβ versus γδ and CD4SP versus CD8SP lineage commitment[131,186-188]. Unsurprisingly, it also has a significant impact on agonist selection. For NKT cells, CD8αα^{+} intraepithelial lymphocytes (IELs) cells, and particularly Treg cells, their differentiation relies on recognizing their cognate antigen[189]. A study using a Nur77 reporter mouse model demonstrated that both iNKT cells and Treg cells experienced higher levels of stimulation relative to conventional T cells[176]. CD25^{+} FOXP3^{-} Treg precursors were also found to respond to stimulation with higher Nur77 transcription, even slightly stronger than differentiated Treg cells[176]. Lastly, it was shown that mice carrying a Treg-associated transgenic TCR has significantly reduced Treg cell frequencies. The authors also discovered that Treg cell frequency is inversely correlated to clonal frequency[20]. Such intra-clonal competition for strong, persistent TCR:pMHC interactions further supports notion that elevated TCR signaling plays an instructive role during thymic Treg development.

1.8 Limitations of human Treg studies

While their importance is well recognized, the study of Treg cells—human Treg cells in particular—presented with several major obstacles. Firstly, human Treg cells lack a unique set of markers necessary for their identification and isolation, as classical markers like CD25 and FOXP3 are also transiently expressed on conventional T cells post-activation[190]. Indeed, this limited set of markers failed to capture the complexity of *in vivo* human Treg cells, despite their regular usage in human studies[191]. Secondly, unlike their mice counterparts, for the longest time, functional human Treg cells cannot be induced from naïve CD4^{+} T cells from peripheral blood[191]. Although

very recently, a publication claimed that they could generate functional human iTreg cells using solely TCR stimulation supplemented with TGF-β and IL-2[91]. Consequently, human Treg studies, IPEX syndrome studies, in particular, relied on artificially overexpressing FOXP3 in conventional T cells using retroviral vectors[71,192,193]. While these artificial Treg cells demonstrate some properties of *bona fide* Treg cells, including immunosuppression and a generally anergic phenotype, they lack several key characteristics such as a demethylated TSDR, which is capable of supporting the stable expression of the endogenous copy of FOXP3[73]. Furthermore, un-physiologically high levels of FOXP3 may mask the defectiveness in certain FOXP3 mutations, and as these cells lack sustained endogenous FOXP3 expression, they are not suitable for studies on FOXP3 induction nor maintenance of the Treg phenotype. Lastly, all patient studies have been done on peripheral T cells[66]. Whether certain defects in the FOXP3 protein affect imprinting during Treg development or cause skewing of the Treg cell TCR repertoire remains unknown. Together, the limitations of existing approaches urge the need for new methods to study and manipulate human Treg cells.

1.9 *In vitro* models of thymocyte development

In addition to *in vivo* mouse models, recapitulation of thymic development *in vitro* has always brought up high interest. Developed by Jenkinson *et al.*, the fetal thymic organ culture was the first *in vitro* system capable of supporting complete T cell development[194]. Over a decade later, Schmitt *et al.* developed a monolayer system where T cell development is supported independently of isolated thymic stromal cells[195]. In the past 5 years, two different 3D organoid systems were described, supporting T cell differentiation with significantly higher efficiency[196]. Since their establishment, these *in vitro* systems have been utilized for the furtherance of our understanding

of multiple aspects of T cell development, including but not limited to the impact of growth factors and peptides, thymocyte-stromal cell interaction, TCR signaling strength, generation of tolerance, as well as thymocyte migration[124,197,198].

1.9.1 Thymic organ cultures

Fetal thymic organ culture (FTOC) systems utilize 2-deoxyguanosine treatment of fetal thymi[199]. Such treatment exhibits strong toxicity toward developing thymocytes, while leaving stromal cells intact. While whole lobe cultures maintain the extracellular matrix and thymic lobe structure, reaggregation cultures require additional steps of thymus disaggregation and stromal cell purification[194]. The purified stromal cells are then mixed with thymocyte progenitors for reseeding onto polycarbonate cell culture inserts for optimal oxygen and nutrient exchange at the air-liquid interface[194]. These approaches played a key role in the mapping and ordering of developmental stages, particularly because neither immortalized nor primary thymic stromal cell lines maintain *in vivo* characteristics in *in vitro* monolayer systems[123,194]. Aside from supporting murine thymocyte development, mouse FTOCs can also support human T cell development in a hybrid FTOC system until the SP stages[123]. Moreover, it is a common practice to remove part of the thymus during cardiovascular surgery for congenital diseases to allow for better heart and blood vessel access[200,201]. Therefore, it is also possible to create human FTOCs as well as a different system for the study of human thymic development. An alternative approach was to use thymic slices, which preserve the thymic architecture while allowing high-resolution microscopy techniques for thymocyte tracking[124,202,203]. Together, these approaches are exceptional, physiological methods for the *in vitro* study of thymocyte development.

1.9.2 Monolayer cultures

Although organ cultures can suffice the need for many studies on thymic development, its scalability is still an issue. Around the same time that Notch signaling was shown to be indispensable for T cell differentiation, an *in vitro* monolayer B cell differentiation system was described[142-144,204]. Afterward, it was also demonstrated to support NK cell differentiation[205]. This system uses a murine bone marrow stromal cell line derived from osteopetrotic mice, which lacks functional macrophage-colony stimulating factor (M-CSF) secretion, thus favouring lymphoid over myeloid cell differentiation[206,207]. The same group subsequently demonstrated that OP9 stromal cells transduced with either DLL1 or DLL4 can support T cell differentiation up to SP stages, with predominantly CD8SP cells [208,209]. Like their B cell differentiation system counterparts, exogenous FLT3L and IL-7 are required for progenitor survival, proliferation, and differentiation[204,210]. The OP9 cells are also secretors of SCF, IL-7, and to a much lesser extent FLT3L[204]. The cooperative signaling of these ligands is sufficient in promoting T cell differentiation from both human and murine $CD34^+$ HSPCs.

1.9.3 3D artificial thymic organoids

The more recently described 3D artificial organoid (ATO) systems have significant advantages over the organoid system in accelerated differentiation, higher frequency of selection, and greater cellularity[196,201,209,211,212]. Similar to reaggregated thymic organ cultures, the ATO cultures are seeded onto cell culture inserts in a stromal cell-HSPC mix[194,196,201]. However, in addition to the exogenous cytokines provided in OP9-DLL4 monolayer cultures, ATO cultures are also supplemented with ascorbic acid, which has been shown to accelerate T cell maturation presumably through its ability to activate TET proteins for increased epigenetic modifications[196,201,213,214]. Moreover, using TCR transgenic iPSCs, Montel-Hagen *et al*. have

observed allelic exclusion, thus demonstrating that there is true positive selection taking place in the organoid system[215]. Although both murine MHC class I and II can support human T cell positive selection, CD4SP cell frequency is still much lower in ATO systems compared to the periphery[201,215,216]. Moreover, despite demonstrating positive selection, there are no characterizations or proof of negative selection nor Treg cell induction in the ATO system to date.

1.10 Rationale, hypothesis, and experimental aims

Despite their well-established importance in disease and homeostasis, human T_{reg} studies have been limited, partly due to a lack of cell type rarity and the lack of *in vitro* differentiation methods from $CD4^+$ naïve T cells. Alternatively, we propose to explore the feasibility of utilizing ATO systems to support thymic Treg cell development from $CD34^+$ HSPCs. This will provide a more physiological means of generating human Treg cells *in vitro*. Such advancements can provide a basis for various human Treg studies to further our understanding of Treg development, survival, and lineage stability. More importantly, once established, this system can be used for investigating monogenic diseases affecting the Treg population, such as IPEX syndrome. From a clinical standpoint, our study can also lead to Treg cell therapies in the form of autologous Treg cell transplants for correcting genetic diseases and controlling other autoimmune, autoinflammatory, or allergic diseases by harnessing the suppressive power of Treg cells.

Hypothesis: Knowing that a large fraction of T_{reg} cells is generated during thymic development and that ATOs can support thymocyte maturation and selection, we hypothesize that ATOs provide a thymic-like environment capable of supporting human $FOXP3^+$ T_{reg} cell differentiation from thymocytes *in vitro* and that provisioning of key signals such as STAT5 cytokines may promote

Treg cell development in the ATO system. The project aims to modify these ATO systems to support *bona fide* T_{reg} development and to characterize stages and instructive signals of human thymic T_{reg} differentiation.

Experimental aims: This project has 2 aims -

Aim 1: Establish an ATO system capable of supporting Treg cell development from $CD34^{+}$ HSPCs.

Aim 2: Assess functionality and phenotypic stability of ATO-derived Treg cells.

Materials and methods

2.1 Isolation of human CD34$^+$ HSPCs

Total mononuclear cells were purified using Ficoll Paque density gradient media from fresh umbilical cord blood, following MACS isolation of CD34$^+$ cells (Miltenyi, cat: 130-046-702) or FACS isolation of CD34$^+$ CD3$^-$ cells using BD FACSArea Fusion 1. All samples were collected from patients after obtaining informed consent and all processes are approved by institutional research ethics board of McGill University Health Centre and CHU Ste Justine.

2.2 Generation and maintenance of ATO cultures

OP9-hDLL4 were obtained as a gift from Dr. Elie Haddad. After trypsin harvest, OP9-hDLL4 cells and HSPCs are counted using Trypan Blue and are mixed at a 1:23 HSPC:feeder ratio, with 2500-7500 HSPCs seeded per ATO. The OP9:HSPC mix is pelleted and adjusted to 24000 cells /µL (or 1000 HSPCs /µL) and seeded onto polycarbonate (Nunc, cat: 137060) or hydrophilic polytetrafluoroethylene (PTFE) (Millicell, cat: PICM0RG50) cell culture inserts. The cell culture insert is then transferred using tweezers into a 6-well plate containing 1 mL of differentiation media. The differentiation media consists of MEM-alpha (Gibco, 12571063), supplemented with 20% FBS, 1% Penicillin-Streptomycin, 800 µM of L-phospho-ascorbate (Sigma Aldrich, cat: A8960-5G), 2.5-10 ng/mL recombinant human IL-7 (Peprotech, cat: 300-19) and 5-10 ng/mL recombinant human FLT3L (Peprotech, cat: 200-07) as specified per experiment. Recombinant IL-2 (gift from NIH) is supplemented at 1000 U/mL and recombinant human TSLP (Biolegend, cat: 582404) is supplemented at 50 ng/mL where stated. Media is exchanged every 3 to 4 days.

At the time of harvest, 1 mL of PBS is added forcefully at the top of the insert to dislodge the organoid and further mixed 2-5 times to detach hematopoietic cells. OP9 feeder cells remain attached to the cell culture insert. Typical yields consist of approximately 500,000 – 2,000,000

live cells/ATO. PBS containing hematopoietic cells are then transferred to a 5 mL round bottom polystyrene tube (FACS tube) for staining.

2.3 Staining and flow cytometric analysis

Cells harvested from ATO cultures are stained with antibodies/dyes listed below. Extracellular antibodies are stained for 20 minutes. When staining for intracellular markers, the cells are fixed for 30 minutes and permeabilized with the eBioscience FOXP3/Transcription Factor Staining Buffer Kit (ThermoFisher, cat: 00-5523-00). Intracellular markers are stained for 45 minutes. All staining steps are incubated at 4˚ C away from light. Antibodies were stained at manufacturer's recommended concentrations or via antibody titration. After staining, samples were run through BD FACSCanto II or BD LSRFortessa X-20. Results were analyzed with FlowJo version 10.4.

Table 1: List of antibodies and dyes used in flow cytometry

Antibody	Clone	Company & Catalog #
CD3-PE	UCHT1	Biolegend, 300441
CD3-BV785	OKT3	Biolegend, 317330
CD3-BV785	UCHT1	Invitrogen, 16-0038-81
CD4-FITC	RPA-T4	BD Pharmingen, 555346
CD4-AF700	RPA-T4	Biolegend, 300526
CD8-APC	RPA-T8	BD Pharmingen, 555369
CD8-PE-Cy7	RPA-T8	Biolegend, 301012
CD8-PerCP	RPA-T8	Biolegend, 301030
CD34-FITC	581	Biolegend, 343504
CD34-Pacific blue	581	Biolegend, 343512
CD7-PE	M-T701	BD Pharmingen, 555361
CD7-BUV395	M-T701	BD Pharmingen, 563845
CD1a-APC	HI149	BD Pharmingen, 559775
CD5-AlexaFluor 700	L17F12	Biolegend, 364026
CD117-PerCP	104D2	BD Pharmingen, 562687
CD56-APC	HCD56	Biolegend, 318318
CD56-PE-Cy7	HCD56	Biolegend, 318310
αβTCR	IP26	Biolegend, 306708
HLA-DR-Super Bright 436	LN3	Invitrogen, 62-9956-42

CD86-BV605	IT2.2	Biolegend, 305429
CD19-PerCP	HIB19	Biolegend, 302228
CD11c-PE	B-ly6	BD Pharmingen, 555392
CD25-BV605	BC96	Biolegend, 302632
CD127-PE-Texas Red	HIL-7R-M21	BD Pharmingen, 562397
FOXP3-PE	236A/E7	BD Pharmingen, 560852
FOXP3-R718	259D/C7	BD Pharmingen, 566935
Helios-Pacific Blue	22F6	Biolegend, 137220
Ki-67	B56	BD Pharmingen, 564071
IL-2	MQ1-17H12	Biolegend, 500350
7-AAD	–	BD Biosciences, 559925
Annexin V-FITC	–	Biolegend, 640906
eBioscience Fixable Viability Dye eFluor 506	–	Invitrogen, 65-0866-14
eBioscience Fixable Viability Dye eFluor 780	–	Invitrogen, 65-0865-14

2.4 Stimulation and expansion of ATO-derived thymocytes

Viable, $CD4^+$ $CD8^+$ $CD56^-$ cells were FACS purified at week 4 from ATOs cultured on PC inserts with 10 ng/mL of IL-7 and FLT3L. After sorting, cells are then plated into U-bottom 96-well plates at 10000 cells/well and stimulated with 0.5-8 µL/mL of αCD3/αCD28 tetramers (Stem cell, cat: 10971) or 1 – 100 ng/mL of αCD3 (OKT3) as specified. Cultures were maintained in MEM alpha supplemented with 1000 U/mL of IL-2 and 10 ng/mL of IL-7 for 11 days, with media change every 2 days. At the end of culture, cells were harvested for immunophenotyping.

2.5 Expansion of ATO-derived Treg cells

ATOs cultured on PTFE inserts treated with 50ng/mL of TSLP and 10 ng/mL of IL-7 and FLT3L were harvested at week4 and stained for surface expression of CD4, CD8, and CD25. Allogenic feeder cells derived from total PBMCs were irradiated with 20Gys of irradiation, washed 3 times with PBS, and plated at 20000 cells/well in U-bottom 96-well plates in MEM alpha media

supplemented with 1000 U/mL of IL-2 and 10 ng/mL of IL-7. Viable, $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ cells were sorted directly into the prepared wells and stimulated with αCD3 (OKT3) at 30 ng/mL. Culture media is exchanged every 2 days and the culture is re-stimulated with αCD3 at 30 ng/mL at day 11 and split into two different wells. At day 21, cultures were harvested. One well of each condition (expanded from $CD25^+$ and $CD25^-$ CD4SP cells) was directly phenotyped. Other culture was activated with 25 ng/mL of PMA (Sigma-Aldrich, cat: 524400) and 1 mg/mL of ionomycin (Sigma-Aldrich, cat: I0634) and treated with BD GolgiStop (BD Pharmingen, 554724) at a 1:1000 dilution for 3 hours at 37˚C. After the incubation period, cells were stained and assessed for cytokine secretion.

2.6 Statistical analysis

Statistical analysis was done with GraphPad Prism 9.0 software. Results were expressed as means with error bars representing standard deviation.

Results

3.1 Validation of human T cell differentiation in the ATO system

To utilize ATOs to generate and study Treg differentiation, we first validated T cell differentiation in this system using various thymocyte markers. MACS-purified $CD34^+$ HSPCs and OP9-hDLL4 cells were mixed and seeded onto polycarbonate cell culture inserts and supplemented with the cocktail of IL-7 (2.5 ng/mL), FLT3L (5 ng/mL), and phospho-ascorbate (800μM) as previously described (see Figure 4 for schematic)[201]. The OP9-hDLL4 cell line is derived from osteopetrotic mice lacking functional M-CSF expression and is transduced with the human Delta-like 4 (hDLL4) to support human T cell development. After 3 weeks of culture, we have found that most cells in the culture have lost expression of the stem cell marker CD34 and up-regulated CD45 surface expression (Figure 5A). Additionally, as early as week 3, > 80% of the cells gained the expression of CD7, an early DN stage marker. CD1a expression is gradually up-regulated in these $CD7^+$ cells, which marks commitment toward the T cell lineage[123]. CD3 expression can also be detected as early as week 3 but increased over 10-fold between week 3 and week 7 (Figure 5B-C). While the surface CD3 expression is associated with the pre-TCR and the TCR, the former is expressed at very low levels, resulting in CD3 levels only being detectable post positive selection[186]. The co-receptors CD4 and CD8 are up-regulated prior to elevated CD3 expression, and thus we were able to detect a significant portion of cells expressing the co-receptors before up-regulating CD3 (Figure 5). While we could detect thymocytes of all developmental stages up to the final CD4SP and CD8SP stages in accordance with previous publications, neither CD25 nor FOXP3 expression was detected in any of the populations (data not shown).

Additionally, knowing that Treg cell development has slower kinetics compared to Teff cells based on the day 3 thymectomy experiments, we hypothesized that our assessment timings

might be too early. However, as the thymocytes viability drops drastically after 7 weeks, we were not able to maintain the culture for a further extended time. We next asked the question of whether the dosage of exogenous cytokines limited the speed of differentiation. Indeed, we found that increasing the concentration of IL-7 and FLT3L accelerated the differentiation and the emergence of $CD3^+$ cells (Figure 5A, 6A). Thus, in all subsequent experiments, we used elevated concentrations of IL-7 (2.5 ng/mL to 10 ng/mL) and FLT3L (5 ng/mL to 10 ng/mL).

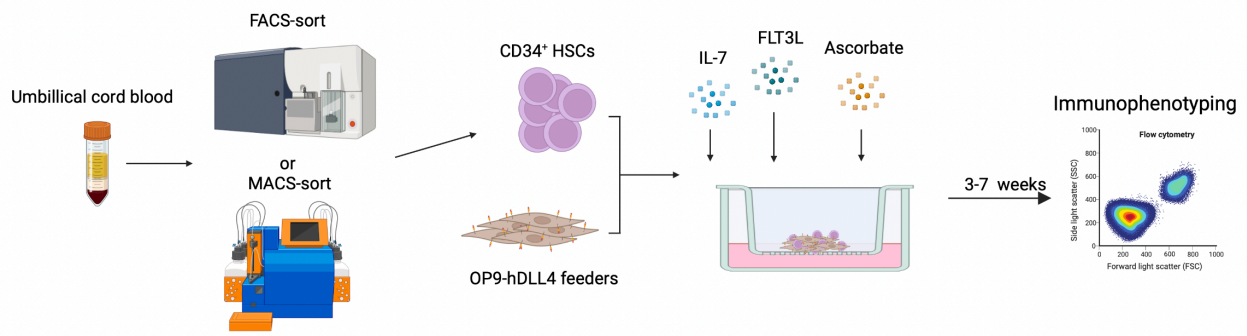

Figure 4: Experimental setup of ATO-based T cell differentiation. $CD34^+$ cells were FACS- or MACS-purified from umbilical cord blood and co-cultured with OP9 cells transduced with human Delta-like 4 (hDLL4). The cell mix was then seeded onto cell culture inserts and supplemented with IL-7, FLT3L, and phospho-ascorbate. The culture was maintained for 3–7 weeks and immunophenotyped thereafter.

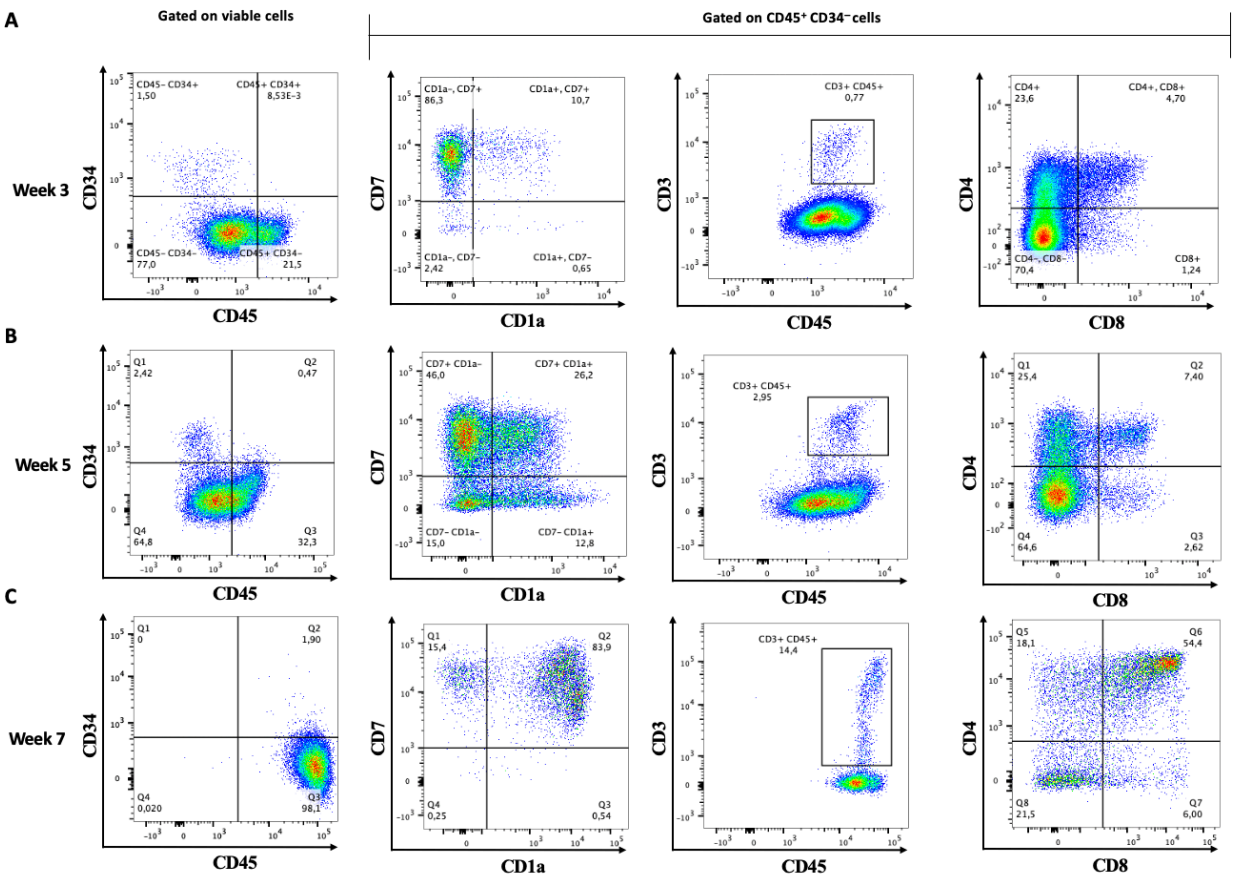

Figure 5: Validation of standardized human T cell differentiation in the ATO system. To assess T cell differentiation in ATO systems, multiple ATOs were seeded in parallel and harvested at various timepoints to assess CD34, CD45, CD7, CD1a, CD3, CD4, and CD8 expression. Cultures were seeded on polycarbonate inserts and maintained in the presence of IL-7 (2.5 ng/mL), FLT3L (5 ng/mL), and phospho-ascorbate (800µM). $CD34^+$ cells represent HSPCs, and T cell precursors were identified as $CD7^+$ $CD1a^+$ cells. Positively selected cells were determined by elevated CD3 expression. $CD3^-$ $CD4^+$ cells were classified as ISP cells, whereas $CD3^-$ $CD4^+$ $CD8^+$ cells represent early DP cells. **(A)** Pseudocolour plots of cells harvested 3 weeks after differentiation. Left most plot gated on total viable cells; right three graphs gated on $CD34^-$ $CD45^+$ cells. **(B)** Flow plots of cultures harvested 5 weeks after differentiation. **(C)** Cultures were harvested at week 7. Different fluorophores were used for CD45, CD3, and CD4 to include the staining of additional markers. N = 1.

3.2 Effect of IL-2 on T cell differentiation and Treg development

Having identified that the standard ATO system does not support Treg development, we set to test the effect of various cytokines implicated in Treg development in the ATO system. Since STAT5 signaling is vital for Treg development and differentiation, particularly due to its regulation of the FOXP3 locus at CNS0 and CNS2/TSDR, and that CD25 is expressed on mature Treg cells and a subset of Treg precursors, IL-2 became our first candidate. In our initial experiments, 1000 U/mL of rhIL-2 was included in the cytokine cocktail, in addition to 10 ng/mL of IL-7 and FLT3L. This concentration was selected as it is used in *in vitro* human Treg expansion cultures[217]. However, with IL-2 supplementation, up-regulation of CD3 was strongly hindered (Figure 6). Moreover, we found that CD1a expression was significantly lower in the presence of IL-2 and that the population was enriched in $CD117^+$ cells, indicating that most of the population was not primed toward the T cell lineage. This blockade was observed from the first harvest at week 3, and was not relieved even at the latest timepoint, which is week 5 (data not shown). Together, these results indicate that IL-2 blocks early-stage T cell differentiation.

In humans, CD117 expression is detected on NK cell precursors[218]. Although IL-15 is indispensable for NK cell differentiation *in vivo*, NK cell *in vitro* differentiation can be supported by either IL-2, IL-7, and IL-15[219,220]. Previous literature shows that early STAT5 signaling is a potent inducer of NK differentiation from HSPCs. A CD56 staining was incorporated, and we confirmed the presence of $CD56^+$ cells in ATOs with this setup (data not shown).

To overcome this issue of developmental blockade by IL-2, we next supplemented the culture with IL-2 two weeks after the initial seeding to allow the differentiating HSPCs to bypass the early-stage blockade. From seeding till week 3, cultures were maintained in media with IL-7, FLT3L, and phospho-ascorbate, whereas IL-2 was added into the cocktail from week 3, and cultures were harvested at weeks 4 and 5. With this approach, we obtained $CD3^+$ T cells from IL-

2 treated ATOs, which allowed us to assess whether IL-2 can induce/enrich Treg or Treg precursor populations in the ATO. FOXP3 expression was detected on a low frequency (~1%) of $CD3^+$ T cells in cultures supplemented with IL-2, but not in controls that did not receive exogenous IL-2 throughout the entire differentiation process (Figure 7A&B). However, $CD3^+$ $FOXP3^+$ cells did not up-regulate CD25 and were exclusively CD4 CD8 DP thymocytes (Figure 7B&C). While this approach might have generated $FOXP3^{low}$ $CD25^-$ Treg precursors, mature Treg cells were not generated at any of the assessed time points, as the culture was again mostly composed of $CD117^+$ NK or NK precursors by week 5 (data not shown). Having observed that thymocyte differentiation in ATOs is not a synchronized process, we found that it was difficult to supplement IL-2 without severely adversely affecting the outcomes of the culture.

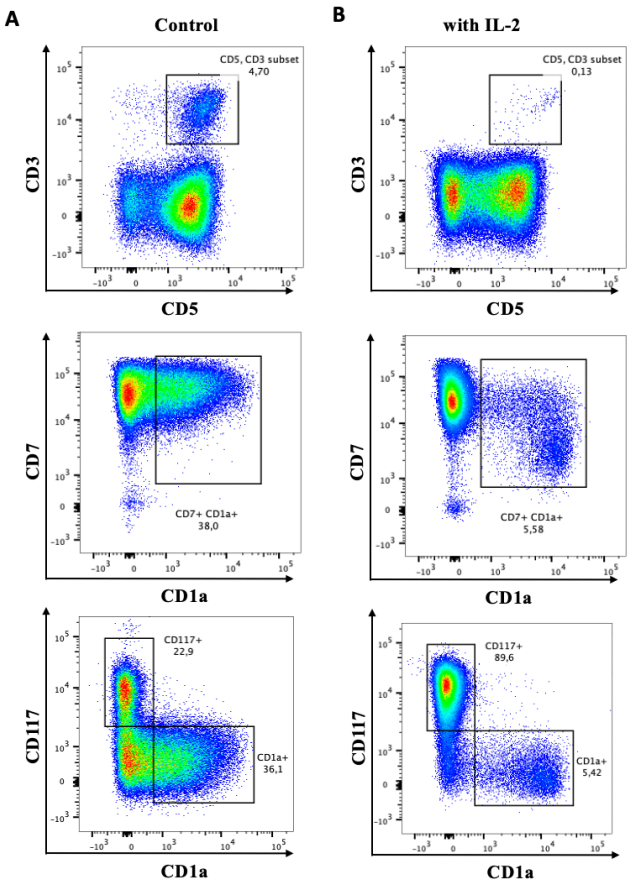

Figure 6: Early administration of IL-2 blocks T cell differentiation. ATOs were seeded onto polycarbonate inserts were supplemented with IL-2 (1000 U/mL) in addition to IL-7 (10 ng/mL) and FLT3L (10 ng/mL) and phospho-ascorbate (800µM) and harvested after 3 weeks of culturing. Flow plots were pre-gated on viable, $CD45^{+}$ cells. ***(A)*** Flow plots of CD3, CD5, CD7, CD1a, and CD117 in the control group without IL-2. ***(B)*** Flow plots of ATO cultures supplemented with IL-2. N = 1.

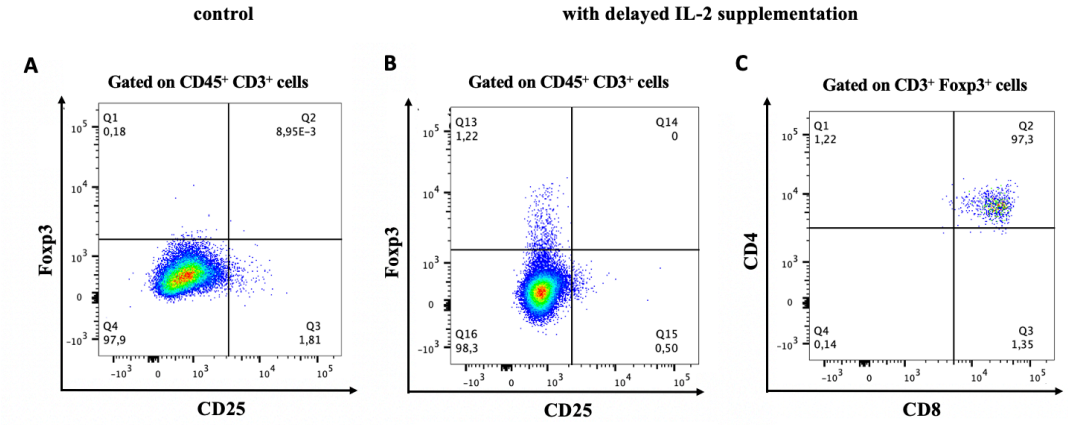

Figure 7: Delayed supplementation of IL-2 induces FOXP3 expression. ATO cultures seeded on polycarbonate inserts were supplemented with IL-7 (10 ng/mL) and FLT3L (10 ng/mL) plus phospho-ascorbate (800µM) throughout the entire duration of differentiation. IL-2 (1000 U/mL) was supplemented after 21 days, and the cultures were harvested at week 4. ***(A&B)*** Both flow plots are pre-gated on viable, $CD45^+$ $CD3^+$ cells. ***(A)*** Expression of FOXP3 and CD25 in the control group without IL-2 supplementation. ***(B)*** Flow plot of cultures with delayed IL-2 supplementation. ***(C)*** CD4 and CD8 expression on $FOXP3^+$ cells in part B. Pre-gated on $CD45^+$ $CD3^+$ $FOXP3^+$ cells. N = 1.

3.3 Physical properties of ATOs alter differentiation kinetics

Additionally, two different types of cell culture inserts have been described to support T cell differentiation in separate ATO systems. We assessed whether physical properties of the insert might affect thymocyte differentiation efficiency and kinetics, which is critical for us to capture mature Treg cells should they arise[196,201]. In addition to PC inserts used in studies utilizing FTOC systems, PTFE inserts were also used to support T cell development in an MS-5 cell line-based ATO system. The MS-5 cell line, which is also long described to support murine hematopoietic cell growth *in vitro*, was transduced with human Delta-like 1 to support T cell development by Seet *et al*[196,221].

While OP9-based ATOs on PTFE inserts also supports T cell development, significantly less cellularity was observed compared to the OP9-based ATOs seeded onto PC inserts. This could be caused by less efficient feeder cell attachment on PTFE inserts[222]. Additionally, we established

that the differentiation kinetics is vastly dissimilar, despite the same culturing conditions in terms of seeding number and cytokine concentration. As early as week 3, over 90% of the population gained CD1a expression on PTFE-based ATOs, while CD1a expression was detected in less than half of the population in PC-based ATOs, marking the T cell lineage-committed cells. Moreover, positively selected ($CD3^+$) cells were mostly CD4 or CD8 SP cells on PTFE-based organoids, while on PC-based organoids, $CD3^+$ cells are mostly comprised of CD4 CD8 DP cells that have not yet differentiated toward CD4 or CD8 SP lineages (Figure 8). Additionally, the DP population in ATOs seeded on PTFE inserts has reduced expression of CD8 relative to the CD8 SP cells, suggesting that they are at the transitional phase between DP and SP stages (Figure 8C)[223]. Moreover, at week 4, the culture lacks $CD3^-$ $CD4^+$ $CD8^{+/-}$cells, which correspond to the ISP and early DP ($CD3^-$ $CD4^+$ $CD8^+$) populations, respectively. These two transitional populations have undergone productive TCRβ chain rearrangement, but not TCRα chain rearrangement. In contrast, the populations that have not undergone β-selection (DN) and those that are positively selected ($CD3^+$, SP or DP) are both present at each timepoint. Together, these two observations demonstrated that there is a blockade of continuous T cell development and differentiation in the ATOs seeded onto PTFE inserts.

Since the $CD3^+$ cells in ATOs seeded onto PTFE inserts are composed of predominantly CD4 or CD8 SP cells at harvest, we next verified that these cells are indeed newly differentiated T cells. To confirm that these SP T cells on PTFE insert ATOs are not contaminating peripheral T cells that were initially seeded into the culture, we stained for CD1a, and found that these cells are CD1a expressors, indicating that they are still thymocytes in development[124]. However, as an extra precautionary measure, FACS-sorted $CD34^+$ $CD3^-$ HSPCs were used in all future experiments, including repeats, to exclude the possibility that these $CD3^+$ cells originated from contaminating

cells of MACS purification. Together, these results indicate that OP9 feeder cells can support T cell differentiation on PTFE inserts at an accelerated pace compared to PC inserts. However, this came at the expense of reduced cellularity and blockade of continuous β and positive selection beyond the first wave of differentiation.

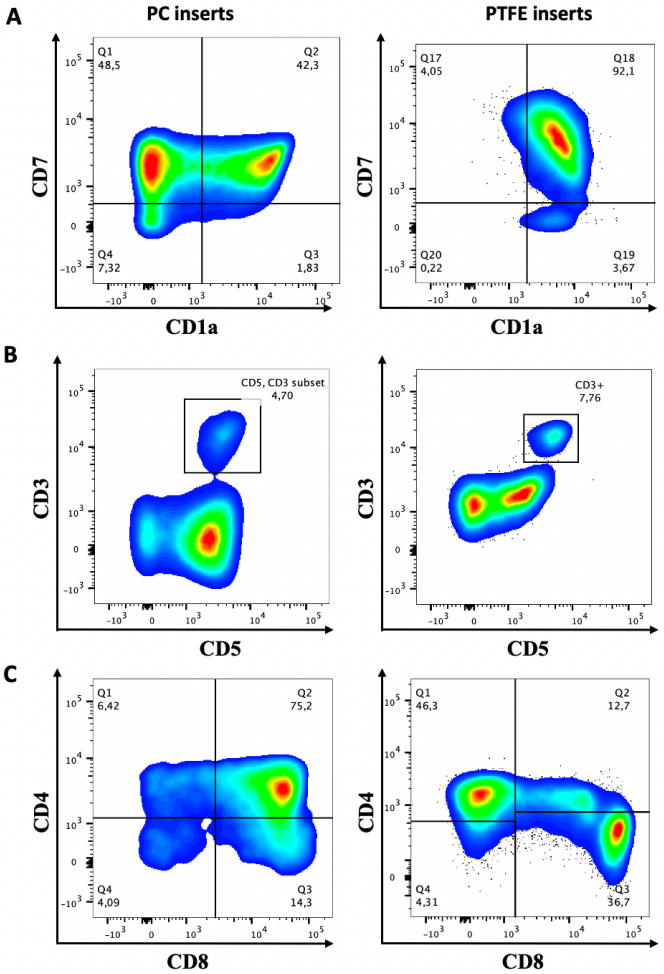

Figure 8: Physical properties of ATO systems drive altered developmental kinetics. ATOs were seeded onto polycarbonate (PC), and PTFE inserts at 7500 HSPCs/ATO and 1:23 ratio of HSPCs:OP9 feeder cells. Both cultures were maintained in media with IL-7 (10 ng/mL) and FLT3L (10 ng/mL), plus phospho-ascorbate (800µM) and harvested after 3 weeks of culturing. ***(A&B)*** Flow plots showing CD7, CD5, CD1a, and CD3 expression on viable $CD34^{-}$ $CD45^{+}$ cells. ***(C)*** CD4 and CD8 expression of $CD45^{+}$ $CD3^{+}$ cells. N = 5.

3.4 TSLP enriches for a CD4SP FOXP3$^+$ CD25$^+$ population

Having confirmed that PTFE inserts can support T cell development on OP9 cell line-based ATOs, we then raised the question whether cytokines other than IL-2 may enhance Treg induction in the ATO system. Like many common γ cytokines such as IL-2, IL-7, and IL-15, the common γ-like cytokine TSLP is capable of activating STAT5 signaling, and it is long established to directly signal in T cells in the periphery[74,224]. Additionally, it is known to condition different thymic DC populations to support Treg development[158,159,170]. Therefore, we investigated whether TSLP supplementation could promote Treg development in ATOs. The cultures were seeded onto PTFE inserts and supplemented with IL-7 (10 ng/mL), FLT3L (10 ng/mL), phospho-ascorbate (800μM), and TSLP (50 ng/mL) for 4 weeks before harvesting. The addition of TSLP did not affect early-stage T cell differentiation, as similar frequencies of precursors and CD3$^+$ thymocytes were detected (Figure 9A). Remarkably, it enriched for a CD4SP FOXP3$^+$ CD25$^+$ population after 4 weeks of culture. The expression levels of both proteins were exceptionally high, forming a distinct FOXP3$^+$ CD25$^+$ population, in contrast to the low FOXP3 expression level detected in ATO cultures supplemented with IL-2. Furthermore, this population is also amongst the higher expressors of Helios, the transcription factor associated with developing thymocytes and maintained in thymic Treg cells, but not Teff cells (Figure 9B-D). In our experiments, we witnessed a 10-fold increase in Treg cell frequencies, and a final count of approximately 200 Treg cells per ATO (Figure 9E).

However, it remains unclear whether the effect of TSLP is cell-intrinsic or cell-extrinsic. Since TSLP can promote HLA-DR and CD86 expression on professional APCs such as dendritic cells, we hypothesized that there might be other hematopoietic lineages that developed from CD34$^+$ HSPCs and, in turn, provided the necessary signals required for thymic Treg differentiation[158,159]. To investigate how TSLP induced Treg differentiation in ATOs, we

immunophenotyped the cultures for HLA-DR and CD86 expression, binding partners of the TCR complex and the co-stimulatory molecule CD28, respectively. We also characterized other lymphoid populations using basic markers for B cells (CD19), conventional DCs (CD11c), and NK cells (CD56), which can be differentiated from HSPCs using similar OP9 monolayer setups[204,205,225,226]. While mature human T cells up-regulate MHC-II in response to TCR stimuli and thymocyte-thymocyte positive and negative selection via MHC class I or MHC-like molecules have been described, we did not observe any HLA-DR expression on $CD3^+$ developing thymocytes in the ATO culture (Figure 10A)[164,227-229]. Thus, it is likely that other lymphoid populations mediated the selection of CD4SP thymocytes. Indeed, we observed both DCs and B cells in the culture with both HLA-DR and CD86 expression in TSLP-treated conditions (Figure 10B). These results are in line with previous studies showing that TSLP-activated DCs were able to induce Treg differentiation from CD4SP thymocytes. However, there may be a thymocyte-intrinsic role of Treg induction via STAT5. Unlike previous studies where TSLP treatment of DCs occurred prior to CD4SP thymocyte-DC co-culture, developing thymocytes are also subject to TLSP signaling in our setup. Further TSLPR staining is required, although the rapid internalization of TSLPR upon signaling may pose additional challenges for interpreting of whether TSLP directly influences Treg differentiation. In conclusion, we found that TSLP was able to promote the development of mature $FOXP3^+$ $CD25^+$ Treg cells in the ATO system, a first-time demonstration of *in vitro* Treg differentiation from $CD34^+$ HSPCs (working model depicted in Figure 11).

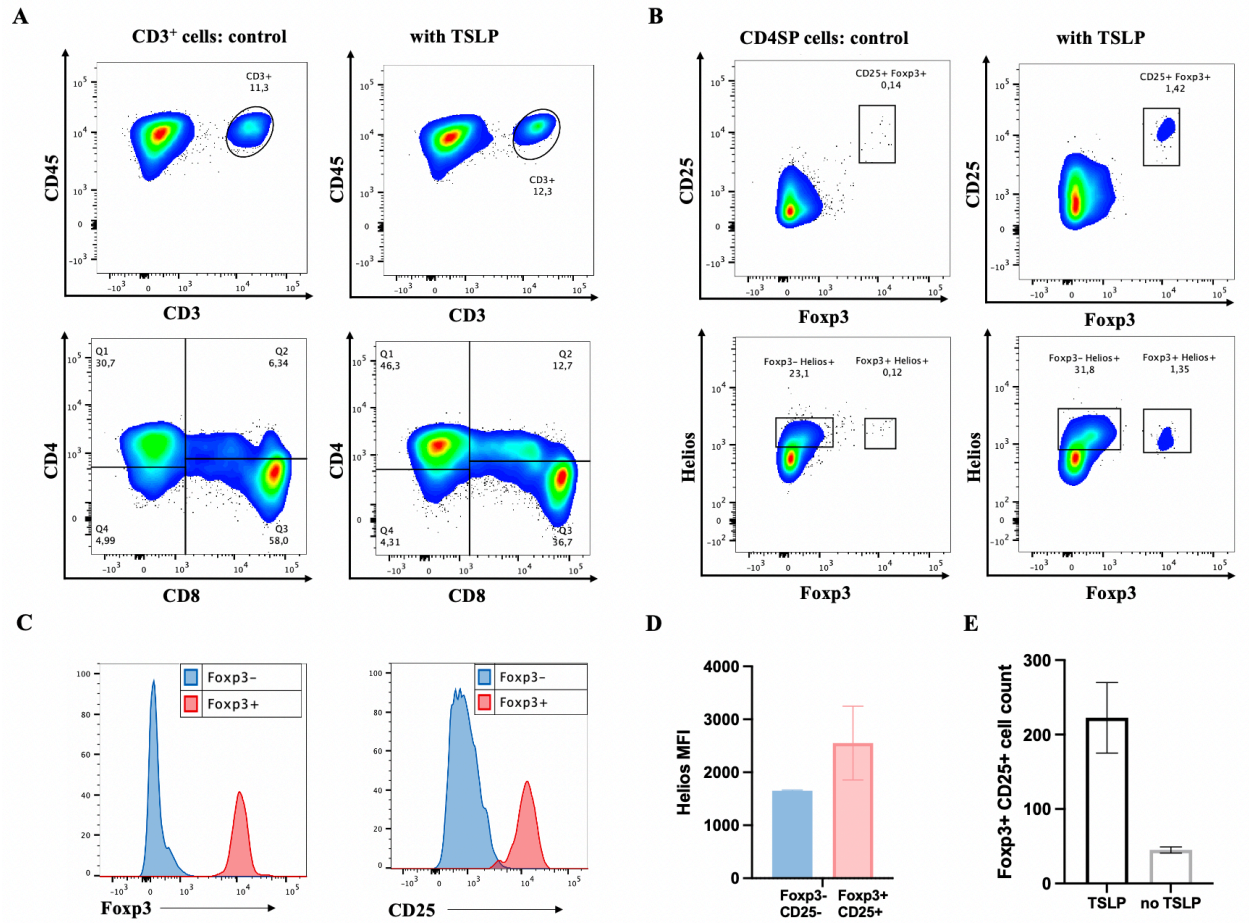

Figure 9: TSLP promotes the generation of a FOXP3$^+$ CD25$^+$ CD4SP population. ATOs cultured on PTFE inserts were supplemented with TSLP (50 ng/mL), in addition to IL-7 (10 ng/mL) and FLT3L (10 ng/mL), plus phospho-ascorbate (800µM). Cultures were harvested after 4 weeks of culturing. ***(A)*** CD3, CD4, and CD8 expression with TSLP supplementation compared to no TSLP control. ***(B)*** FOXP3, CD25, and Helios expression on CD3$^+$ CD4SP cells. ***(C)*** Histogram of FOXP3 and CD25 expression levels. Gated on FOXP3$^+$ CD25$^+$ cells in part B (top). ***(D)*** MFI of Helios between FOXP3$^+$ and FOXP3$^-$ populations. ***(E)*** Absolute count of FOXP3$^+$ CD25$^+$ cells generated per ATO. N = 2.

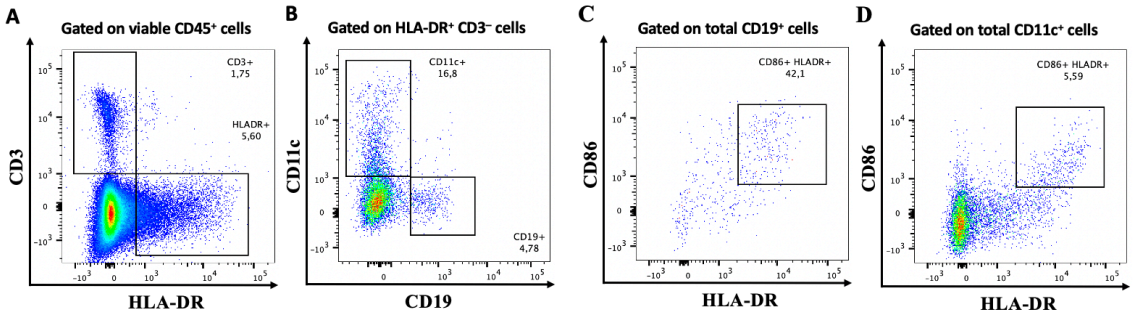

Figure 10: Activated professional APCs are detectable in ATO systems. Cells were harvested after 4 weeks of culturing from ATOs supplemented with IL-7 (10 ng/mL) and FLT3L (10 ng/mL), phospho-ascorbate (800µM), and TSLP (50 ng/mL). ***(A)*** CD3 and HLA-DR expression on viable $CD45^+$ cells. ***(B)*** CD11c and CD19 expression on $HLADR^+$ $CD3^+$ cells. ***(C-D)*** CD86 and HLA-DR expression on total $CD19^+$ and total $CD11c^+$ cells, respectively.

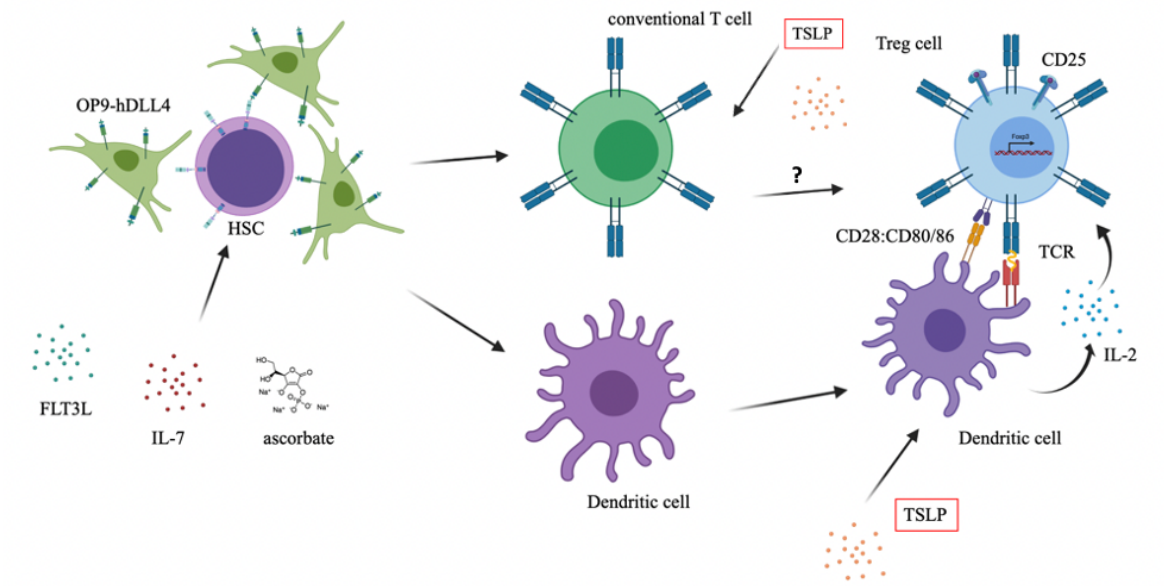

Figure 11: Summary of a working model of Treg induction in ATO systems. The majority of HSPCs differentiate into T cells, but other hematopoietic cells are also present in the culture. TSLP primes existing APCs, particularly DCs, in the culture. DCs activate, and up-regulate MHC II and co-stimulatory molecules for T_{reg} differentiation. TSLP may also act directly on T cells to promote T_{reg} differentiation via STAT5 signaling.

3.5 Differential response to TCR stimulation by ATO-derived thymocytes

In addition to triggering positive and negative selection, TCR signaling strength also acts as a central modulator of thymocyte lineage fates. Particularly, strong TCR stimulation has been associated with agonist selection, which leads to the development of Treg cells, iNKT cells, MAIT cells, and CD8αα T cells[176,230]. With this premise, we next evaluated the effect of differential doses of TCR stimulation on ATO-derived thymocytes, with or without CD28 co-stimulation. While CD69, CD27, and CD45RA/RO expression have been described to differentiate between different SP populations, namely ISP, immature SP (in some literature referred to semimature), and mature SP cells, in ATO-derived thymocytes, these markers do not effectively separate $CD3^+$ and $CD3^-$ SP cells[124]. As such, TCR stimulation assays were completed with total CD4SP cells sorted from PC-based ATOs at weeks 4-5, where > 95% of $CD4^+$ cells do not express CD3, confirmed by

staining done in parallel with FACS sorting. After an 11-day stimulation period in the presence of IL-7 and IL-2, cells are harvested for immunophenotyping. Stimulating with αCD3/αCD28 tetramers at concentrations optimized for peripheral T cells (8 µL/mL), we found the culture to be comprised of mostly $CD56^+$ cells with variable expression of CD3, and a near-complete loss of CD4 (Figure 12A). At significantly reduced levels of polyclonal stimulation (0.5 µL/mL of tetramers), there were still equivalent frequencies of $CD3^+$ $CD56^-$ and $CD3^+$ $CD56^+$ cells. Intriguingly, we did observe that at this stimulation intensity, the $CD3^+$ $CD56^-$ population contains a fraction of CD4SP cells, all of which are nearly exclusively expressors of CD25 and FOXP3 (Figure 12B). While the induction of $CD56^+$ $CD3^+$ cells was unexpected, the literature also highlighted that, like Treg cells, NKT cells require a higher TCR stimulation threshold for differentiation[231]. These data hinted that differential activation thresholds may instruct ATO-derived thymocytes toward different T cell lineages.

To further explore the above phenomena, we investigated the effect of αCD3 (OKT3) stimulation alone on ATO-derived thymocytes, since co-stimulation enhances TCR signaling intensity. Expectedly, αCD3 alone can still trigger survival and proliferation of sorted thymocytes, and fewer $CD56^+$ cells were induced (Figure 13A). Moreover, amongst the $CD56^-$ $CD3^+$ population, CD4SP, and CD8SP cells exist in similar frequencies. Additionally, at 1 ng/mL of αCD3, we were also able to detect a $CD25^-$ $CD3^+$ CD4SP population (Figure 13B). These results are in accordance with previous literature stating that co-stimulation is dispensable and even detrimental for positive selection, and that CD25 expression is triggered by higher levels of TCR stimulation, as in the case of $FOXP3^-$ $CD25^+$ Treg precursors[106,232,233]. In conclusion, these findings demonstrate that TCR and STAT5 signalling can cooperatively induce the stable expression of CD25 and FOXP3 from CD4 SP thymocytes. However, it is yet premature to

confidently conclude that the combination of optimal TCR stimulation and STAT5 signaling is sufficient to induce Treg cell differentiation from developing thymocytes.

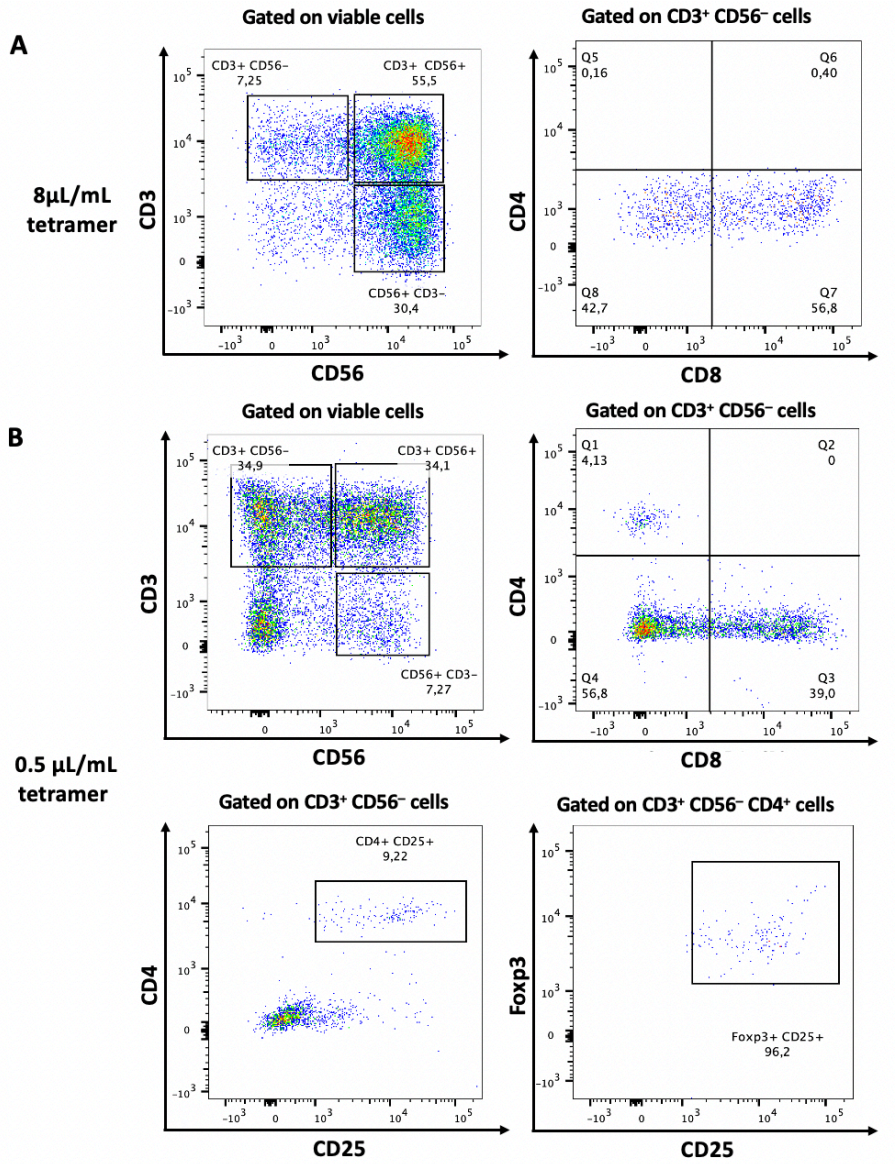

Figure 12: ATO-derived thymocytes are highly sensitive toward polyclonal TCR stimulation. Total CD56$^-$ CD4SP (CD3$^{+/-}$) were sorted from ATOs cultured on PC inserts for 4 weeks and expanded for 11 days using αCD3/αCD28 tetramers in the presence of IL-2 (1000 U/mL) and IL-7 (10 ng/mL). ***(A)*** Flow plot of cultures stimulated with 8 µL/mL of tetramers. Gated on viable cells (left) or CD3$^+$ CD56$^-$ cells (right). ***(B)*** Flow plot of cultures stimulated with 0.5 µL/mL of tetramers. Gated on viable cells (top left), CD3$^+$ CD56$^-$ cells (top right and bottom left), or on CD3$^+$ CD56$^+$ CD4SP cells (bottom right). N = 2.

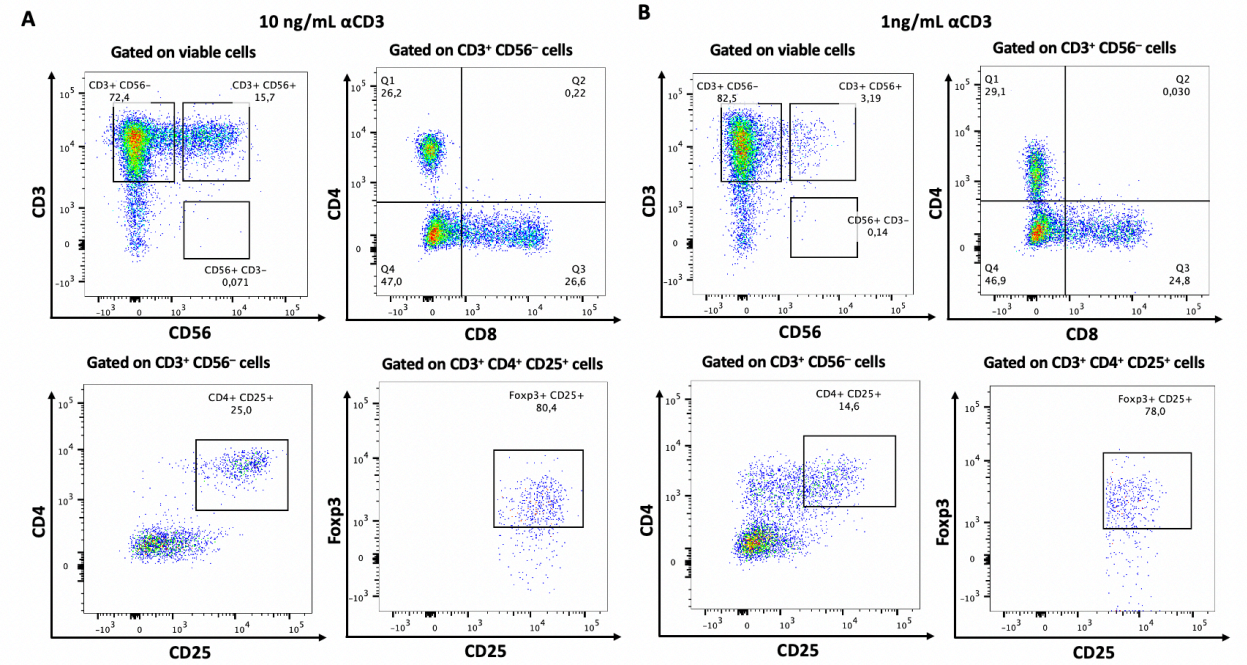

Figure 13: Polyclonal TCR stimulation of ATO-derived thymocytes can alter their lineage choices. Total CD56$^-$ CD4SP (CD3$^{+/-}$) were sorted from ATOs cultured on PC inserts for 4 weeks and expanded for 11 days using αCD3 (OKT3) alone without co-stimulation in the presence of IL-2 (1000 U/mL) and IL-7 (10 ng/mL). Flow plots on top show in order CD3 versus CD56 expression on total viable cells (top left) and expression of CD4 and CD8 on CD3$^+$ CD56$^-$ cells (top right). Bottom flow plots show CD25 and FOXP3 expression in CD3$^+$ CD56$^-$ CD4SP cells. ***(A)*** Flow plots of cultures stimulated with 10 ng/mL of αCD3. ***(B)*** Flow plot of cultures stimulated with 1 ng/mL of αCD3. N = 1.

3.6 Phenotypic and functional analysis of expanded, ATO-derived Treg cells

To further characterize the ATO-derived FOXP3$^+$ CD25$^+$ cells and prove their identity as authentic Treg cells, further assessments are necessary, which requires a significantly larger quantity of cells. Particularly, these cells need to be expanded for functional tests such as suppression assays and cytokine secretion assessments. To increase our final yield of expanded Treg cells, total CD25$^+$ cells, rather than CD25^{hi} cells, were sorted from PTFE-based ATOs. The cells were activated in the presence of feeders and αCD3 in the presence of IL-7 and IL-2 for 21 days, with a restimulation at day 11. The cultures were then either directly immunophenotyped or restimulated for cytokine

secretion assays. Since $CD3^-$ CD4SP cells are virtually absent in PTFE inserts at the time of harvest, or 4 weeks after seeding, CD4SP $CD25^-$ cells were expanded in parallel as a control (see Figure 14A for schematic).

After 21 days, a $FOXP3^{hi}$ population was maintained in cultures expanded from CD4SP $CD25^+$ cells, but not CD4SP $CD25^-$ cells. Furthermore, this population was identified as the highest expressors of CD25 and lacked expression of CD127. More importantly, cytokine secretion assays revealed that the $FOXP3^{hi}$ population repressed IL-2 secretion, whereas IL-2 secretion was readily detectable in $FOXP3^-$ cells (Figure 14B). Additionally, we also found that $FOXP3^{hi}$ cells actively repressed TCR-induced cell cycle progression, based on their low levels of Ki-67 expression (Figure 14C). Lastly, given the lenient gate of CD25 and higher proliferation of Teff cells *in vitro*, $FOXP3^{hi}$ cells comprise only 10% of the total CD4SP $CD25^+$ expansion culture. Compared to Teff cells expanded from CD4SP $CD25^-$ cells, Teff cells that are effectively co-cultured with ATO-derived Treg cells at a 1:10 ratio showed lower expression of Ki-67, suggesting that the ATO-derived Treg cells are suppressive (Figure 14D).

While this set of assays needs further testing, such as a more accurate measurement of suppression with proliferation dye labeling and assessment of TSDR methylation status, these data do indicate that our optimized ATO system is capable of supporting the differentiation of stable and functional human Treg cells from $CD34^+$ HSPCs, providing the first-time opportunity for studying Treg development *in vitro*, and providing a novel tool for human Treg cell manipulations for both research and clinical applications in Treg cell therapy.

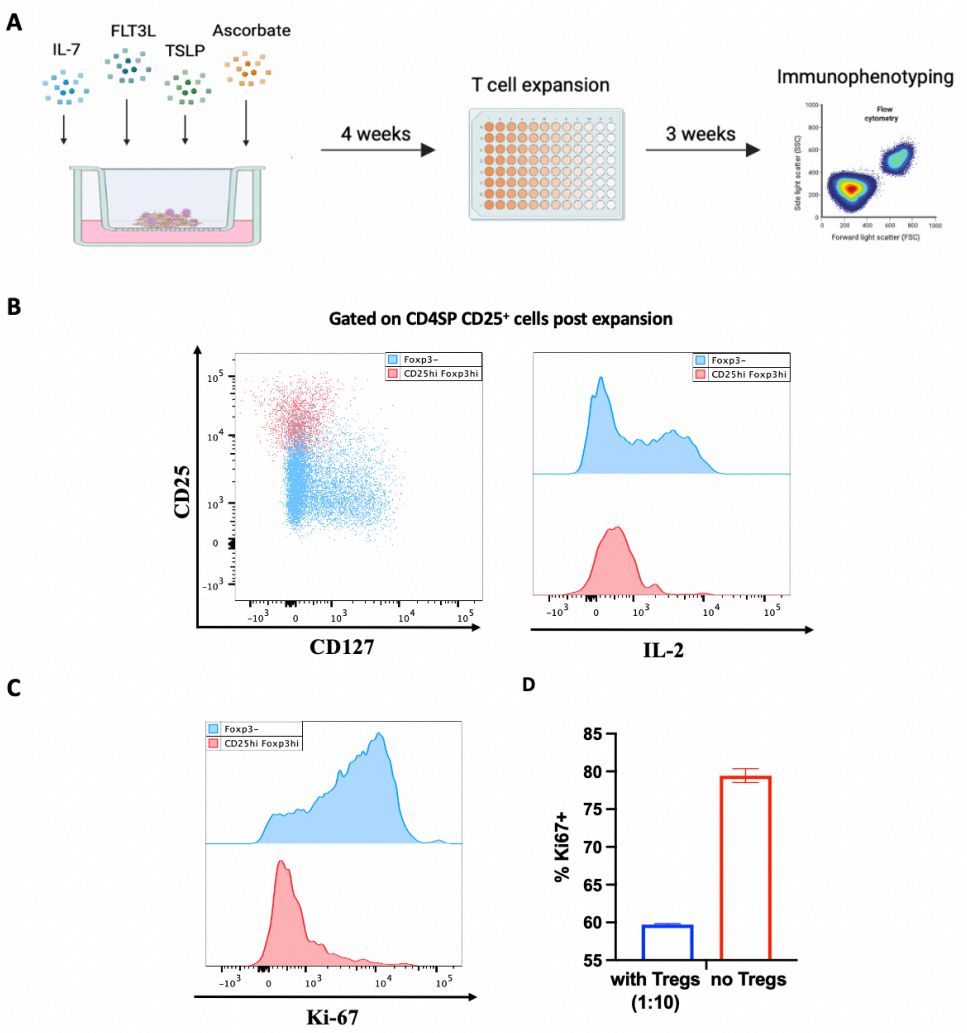

Figure 14: CD4SP CD25$^+$ cells from ATOs contain functional Treg cells that stably maintain their phenotype. From ATO cultures supplemented with TSLP, CD4SP CD25$^+$ and CD4SP CD25$^-$ cells were sorted and stimulated with αCD3 (OKT3) and co-cultured with irradiated feeders derived from allogeneic PBMCs in the presence of IL-2 (1000 U/mL) and IL-7 (10 ng/mL). Cells were restimulated on day 11 and harvested on day 21 for direct immunophenotyping and cytokine assays using PMA/ionomycin stimulation and Golgi transport inhibitor. ***(A)*** Schematic of the experimental layout. ***(B)*** Expression of CD25 and CD127 of FOXP3^{hi} and FOXP3$^-$ cells (left) and IL-2 secretion (right). ***(C)*** Ki-67 expression of FOXP3^{hi} and FOXP3$^-$ cells. ***(D)*** Frequency of Ki-67$^+$ cells of the total Teff population (FOXP3$^-$) in CD25$^+$ cell-depleted expansion cultures and co-cultures of 1 Treg: 10 Teff cells. N = 1. Error bars represent experimental duplicates.

Discussion

FOXP3$^+$ Treg cells play a non-redundant role in establishing both central and peripheral tolerance, and a dysfunctional/dysregulated Treg population is implicated in numerous autoimmune diseases. These include inborn errors of immunity that predominantly affect Treg cells' function and stability, such as IPEX syndrome and other IPEX-like diseases caused by mutations involved in IL-2 signaling (e.g., CD25 deficiency), CTLA4 expression/function (e.g., LRBA deficiency), and IL-10 signaling (e.g., CD210 deficiency), etc[67]. Other inborn errors of immunity such as APS-1 cause the formation of an incomplete set of Treg cells required for establishing self-tolerance. While several approaches for Treg cell therapies are currently under investigation, these approaches are not physiological, as they mostly rely on ectopic expression of FOXP3, which lacks many layers of regulation, as the endogenous FOXP3 still exhibits an effector-like landscape, particularly at CNS2/TSDR[73,234,235]. These FOXP3-transduced cells are less stable than *ex vivo* Treg cells and are thus less ideal for cell therapies. In an experimental autoimmune arthritis model, murine Treg cells can lose FOXP3 expression and differentiate into Th17 cells that are capable of secreting IL-17, exacerbating disease progression[236]. Therefore, Treg cell stability is key for progressing toward Treg cell therapy, and methods for generating stable human Treg populations *in vitro* are yet lacking.

Protocols for *in vitro* T cell differentiation from HSPCs using monolayers have been described for over two decades, and T cell development in both monolayer and subsequent ATO systems has been shown to mirror the developmental stages observed *in vivo*[158,159,195]. However, due to the efficiency of T cell generation, traditional monolayers are not suitable for T cell therapy. These newly described ATO systems are much more efficient at generating mature T cells (~3% versus ~18%), and the differentiation shows accelerated differentiation kinetics compared to

monolayer systems[196,237]. These advancements enable to use *in vitro* T cell development as a platform for future Teff and Treg cell therapies. However, there are no illustrations of *in vitro* thymic Treg cell differentiation[158,159,195]. Even then, the described Treg differentiation protocols require *ex vivo* CD4SP thymocytes and DCs, which are not easily accessible for therapeutic applications nor Treg developmental studies. Given that the Treg population is essential for maintaining immune homeostasis, their development and function require extensive investigation. Specifically, it is far more common to develop autoimmunity due to a lack of Treg cells with a particular specificity than impairment of their activation and/or downstream function[17]. Given that the TCR plays a key instructive role during T cell development as well as function, identifying a defined set of factors that induce Treg development will reveal how the distinct Treg TCR repertoire is formed, in turn shedding light on new risk factors associated with both organ-specific and systemic autoimmune diseases.

In this study, we have found that with our cytokine cocktail, which includes the cytokine TSLP in addition to our optimized concentrations of IL-7 and FLT3L, ATO systems can support human $FOXP3^+$ $CD25^+$ Treg development from $CD34^+$ HSPCs. We further demonstrated that these ATO-derived Treg cells could be expanded while maintaining a stable Treg phenotype ($FOXP3^+$ $CD25^+$ $CD127^-$). Additionally, these expanded Treg cells retain functionality, as demonstrated by repression of inflammatory cytokine secretion and anergic phenotype in response to TCR stimulation, as well as suppression of Teff cell-cycling in Treg:Teff co-cultures. Furthermore, we also investigated the effect of differential TCR stimulation on developing thymocytes, searching for a defined set of signals sufficient for driving Treg differentiation. We have found that thymocytes are biased toward different cell fates depending on the strength of stimulation. A properly titrated TCR stimulation with IL-2 supplementation induced sustained

expression of FOXP3 as well as CD25 from total ATO-derived CD4 SP thymocytes. These findings progress toward defining and quantifying Treg-inducing signals. In combination with humanized mouse models, this system also acts as a platform for studying how different signals and/or genetic defects affecting their development may alter their function and stability in the periphery. Importantly, the described Treg-generating ATO system will allow for further understanding of Treg cell biology, and the development of more physiological Treg cell therapies against different autoimmune/autoinflammatory diseases, particularly IPEX syndrome and APS-1.

4.1 Progression toward defining drivers of thymic Treg differentiation

While optimized stimulations did result in a population acquiring sustained FOXP3 and CD25 expression, in the initial experiments on thymocyte polyclonal stimulation, we have identified a significant fraction of $CD56^+$ cells that have lost cell surface expression of CD3, as well as a $CD56^+$ $CD3^+$ population. Although thymic NK cells have been described by one group to derive independently from T cell precursors and Notch signaling, others have shown the opposite, as illustrated by TCRγ expression in a low frequency of $CD56^+$ NK cells[220,238-240]. Thus, given that both IL-7 and IL-2 were supplemented at doses much higher than physiological levels to promote proliferation and survival, it is possible that thymocytes destined for negative selection were rescued, and redirected toward an NK phenotype. However, additional NK markers such as the NKG2D and CD94, as well as cytokine secretion, should be assessed to fully demonstrate this phenomenon[241,242]. More importantly, VDJ rearrangement status of these $CD56^+$ cells from ATO-derived $CD4^+$ thymocytes must be assessed. Taken together, these results demonstrate that these ATO-derived thymocytes are significantly more sensitive toward TCR stimulation relative to T cells in peripheral blood.

At lowered stimulation doses than those inducing mostly $CD3^-$ $CD56^+$ cells, a $CD3^+$ $CD56^+$ population increased in frequency, and is mostly comprised of either CD8SP or CD4 CD8 DN cells. This population could represent a population directed toward a iNKT or NKT-like differentiation program, but this hypothesis needs to be backed up by staining for PLZF, an NKT-specific transcription factor[243]. As previously described, NKT cells, like Treg cells, require high levels of TCR stimulation. However, the authors did not report similarities and differences in TCR signaling requirements for iNKT versus Treg differentiation[176]. In the same study, the authors did, however, suggest that since Treg cells continuously receive TCR signaling in the periphery, whereas iNKT cells do not, TCR stimulation frequency may play a role in Treg versus iNKT differentiation requirements[176]. Therefore, in the near future, the effect of re-stimulations will also be assessed. Additionally, as soluble and immobilized αCD3 has differential impacts on CD3/TCR complex endocytosis, it is necessary to determine whether induction of $FOXP3^+$ $CD25^+$ cells could be more efficient with certain activation methods compared to others[244,245]. This may also influence other thymocyte fates, since signaling duration, as well as signaling intensity, has been implicated in positive and negative selection, αβ T cell versus γδ T cell lineage commitment, and CD4SP versus CD8SP lineage commitment, as well as the agonist selection of NKT cells versus Treg cells[134,137,188,246,247]. At even lower doses of stimulation, achieved by αCD3 alone without co-stimulation, the $CD56^+$ $CD3^-$ population is near non-existent, and the ratio of $CD56^+$ $CD3^+$ cells to $CD56^-$ $CD3^+$ cells decrease with αCD3 concentration. These results are in line with the observation that co-stimulation is not required and even detrimental for thymic development of Teff cells[232]. With varying concentrations of αCD3, we have also observed a direct correlation between CD25 expression and αCD3 concentration. This is also in accordance with previous studies that have identified $FOXP3^{low}$ $CD25^+$ Treg precursors that are induced by strong TCR

signaling. In the two-step induction model, signaling through STAT5 triggered by IL-2 binding converts this $CD25^+$ precursor population into a mature Treg cell population[106].

Together, these observations bring initiatives for further investigation of the impact of TCR signaling strength and duration on lineage specification of developing human thymocytes, which is now made feasible with the ATO system.

4.2 Antigen specificity and MHC-I and -II expressors in the ATO system

Based on previous literature, we know that OP9 cells do not express MHC II[248]. Therefore, the only MHC-II expressing cells are the identified human HLA-DR expressors derived from $CD34^+$ HSPCs. Additionally, past *in vivo* experiments studying human thymocyte development in humanized mouse models reported that most T cells developed in these mice react toward human rather than murine MHC class II[249,250]. In these experiments, intense reactivity was observed when T cells were co-cultured with allogenic DCs, but not autologous DCs, which is a sign of central tolerance[250]. Our preliminary data have also identified that T cells can develop normally from ATO cultures comprising HSPCs from mixed donors. In the future, we do intend to investigate whether Treg cells from these mixed-donor ATOs are capable of suppressing the reactivity of T cells from single-donor ATOs toward hematopoietic cells derived from mixed-donor ATOs (suppress T cells from ATO of donor X from reacting toward cells derived from ATO of donors X&Y) via bystander activation[251]. These experiments will open the route for *in vivo* investigations on ATO-derived Treg cells in GvHD and other transplant settings, and future therapeutic opportunities[252-254].

Induction of Treg cells with unknown specificity is still a considerable limitation for either *in vivo* studies utilizing disease models in humanized mice or future clinical applications. Therefore, in line with our existing investigation on the impact of TCR signaling strength on Treg cell induction, we wish to test the feasibility of inducing antigen-specific Treg cells. Since direct

peptide loading triggered both positive and negative selection, it is more than likely that specific peptides are also capable of driving Treg development when provided with all other essential signals, such as STAT5[255]. Additionally, since APCs can present many different peptides simultaneously, to ensure Treg cells of the desired specificity are induced, we can incorporate the use of peptide-MHC multimers for Treg induction from immature thymocytes[256,257]. Further tuning may also be done with modulators increasing FOXP3 expression or stability, such as TGF-β or P300, or with modulators of TCR signaling strength, such as αCD28 co-stimulation or cAMP[91,113,258,259]. The latter acts through PKA, which phosphorylates Csk, a negative regulator of Lck, and dampens all TCR downstream signals[259]. These manipulations will aid in developing a protocol for generating Treg cells independent of gene editing, which is faced with its own issues of having off-target effects[260-262].

An alternative approach is to seed HSPCs differentiated from CAR-T or TCR transgenic iPSCs in ATOs. While the CAR-Teff cells can cause dangerous side effects such as cytokine release syndrome caused by hyperinflammation, extremely potent CAR-Treg cells are also prone to inducing undesired immunosuppression[263]. However, since TCR transgenic iPSCs rely on pMHC recognition, experimental or clinical applications are also limited due to the abundance of human MHC class II isoforms. Previous studies have already demonstrated that mature T cells can be generated from ATOs seeded with HSPCs derived from TCR transgenic iPSCs[215,264,265]. Additionally, iPSCs can also be generated from mature T cells, and re-differentiated back into T cells[266]. While this approach may seem inefficient initially, it does provide practically unlimited expansion potential of a T cell clone of interest and ensures antigen recognition once reinfused into the host. Combined with our Treg differentiation protocol, these two approaches could quickly generate Treg cells capable of suppressing antigen-specific autoimmunity, such as type I diabetes.

In conclusion, induction of antigen-specific Treg cells will open the door for Treg cell therapy against any autoimmune diseases with a known antigen.

4.3 Incorporation of TRA expressors in ATO systems

While ATO systems support T cell differentiation and create a diverse TCR repertoire, the system still lacks expression of TRAs, due to the absence of mTECs[212,215]. It has been shown numerously that TEC maturation requires interaction with developing thymocytes[151-153,163,267]. Particularly, mTECs require RANKL secreted by positively selected thymocytes to maturate into AIRE expressing mature mTECs[152]. Slightly over a decade ago, an *in vitro* murine TEC differentiation procedure was described to generate TEC progenitors from iPSC cells[268]. The progenitor cells express various TEC markers, such as FoxN1, AIRE and DLL1. When co-transferred into Nude mice with DN thymocytes, these TEC progenitors can support mature CD8SP T cell differentiation[268]. More recently, a more efficient differentiation protocol utilizing Foxn1 transduction was described which allowed the detection CCL25 and DLL4 expression with a roughly 10-fold efficiency over untransduced iPSC differentiation[269]. While these experiments were done with murine iPSCs, the same RANK-dependent upregulation of AIRE in mTECs is conserved in humans, suggesting the possibility of inducing human TEC cells to be included in a further modified ATO system[270]. In addition to providing TRAs, incorporating TECs will also act as an additional source of MHC-II, which could further increase CD4SP frequency, which is generally lower than CD8SP frequency in ATOs, unlike *in vivo* ratios, where CD4SP cells are of higher frequency. Moreover, additional co-stimulation sources may also increase Treg induction's efficiency. Successful incorporation of mTECs will provide a system that more closely resembles the thymus.

APS1, the disease where a non-functional AIRE causes defective Treg induction, negative selection, and consequently organ-specific autoimmunity, is also a good candidate for ATO applications. Due to the predominant, if not exclusive, function of AIRE in mTECs, disease correction does not require direct genetic manipulation of the developing thymocytes. Therefore, a model for APS1 correction only requires the reinfusion of unmodified thymocytes, whereas the genetically modified TEC cells do not need to be transferred back. Compared to modification on reinfusing cells, this is much safer for clinical purposes being risk-free of unintended DNA breaks, given that major TRAs were being expressed and presented for negative selection and/or Treg induction.

Overall, incorporating TRA-expressing mTECs in ATO systems will further create an *in vit*ro system capable of supporting T cell differentiation with a TCR repertoire and antigen specificity resembling that of T cells generated in the thymus.

4.4 Studying IPEX syndrome with ATO-based Treg differentiation protocol

The ATO system provides abundant opportunities for human Treg cell therapy, and it is also an exceptional platform for studying Treg developmental defects in combination with genomic engineering. As aforementioned, most IPEX studies have relied on retroviral transduction and ectopic expression of FOXP3 on Teff cells, which does not recapitulate all Treg physiological properties, especially events that regulate the initial up-regulation of FOXP3 and Treg lineage commitment[71-73]. Using either CRISPR-Cas9 or Cpf1 (also known as Cas12a), HSPCs can be modified to implement known IPEX-causing FOXP3 mutations, such as A384T, R397W, or C424Y[66].

Recently, multiple advancements have been made to increase HSPC editing efficiency, as it is much more challenging to edit quiescent cells. These advancements include a study using

transient p53 inhibition and cell cycle progression promotion which increased HSPC editing efficiency approximately 1.5 times compared to standard protocols[271]. Due to the low frequency of Treg generation in our current ATO system, reaching a high HSPC editing efficiency is critical. Since it is impossible to select for edited HSPCs before culturing ATOs, as FOXP3 will not be expressed, a FOXP3 reporter will have to be included for the identification of edited Treg cells once they develop. While this approach creates a longer FOXP3 mRNA transcript, which may affect FOXP3 mRNA stability, it provides a possibility of sorting $FOXP3^+$ cells for additional assays and purer starting culture for expansions. To further increase editing efficiency, enrichment methods can also be incorporated. Several enrichment methods have been described based on the observation that the limiting step of CRISPR editing is the import of all necessary elements into the cells. Namely, the introduction of a secondary unlinked edit, knock in or knock out, will enrich cells that carry the desired edit. Specifically, co-deletion of UMPS, an enzyme involved in uracil synthesis, via CRISPR-Cas9 confers resistance toward 5-fluoroorotic acid and exogenous uracil dependency. In contrast, editing of the ATP1A1 gene encoding for the Na^+/K^+ pump can confer ouabain resistance[272,273]. Both enrichment approaches could increase the frequency of edited cells in starting ATO cultures.

Alternatively, if enrichment processes still do not provide the desired frequency of edited HSPCs for ATO seeding, iPSCs can also be used for ATO-based IPEX studies. Since these are cell lines, it is possible to expand edited iPSCs clonally, and thus removing the necessity for a FOXP3 reporter. Moreover, it will also allow the use of nickases, rather than inducing double-strand breaks, making it a safer approach[260,261]. However, the serious issue is that iPSC-derived thymocytes do not express TdT, the specialized non-templated DNA polymerase responsible for adding random nucleotides at V(D)J junctions[215]. This fundamental difference may create biases

in our future IPEX studies. However, it is possible to create iPSC cell lines from IPEX patients and use these cells to study Treg development with IPEX-causing FOXP3 mutations. These cell lines could be maintained and expanded indefinitely, and, in a way, resolve the issue of rare patient samples. Unfortunately, available IPEX patient samples do not exist for every mutation, and such approaches would likely be limited to the more common mutations, such as A384T[66]. These alternative approaches will be explored if necessary.

Currently, we have adopted the approach of CRISPR editing HSPCs and knocking in the mutation and a reporter, without deleting any regulatory elements such as introns and UTRs, since it will allow us to identify whether specific IPEX-causing FOXP3 mutations will cause a bias in the TCR repertoire of Treg cells. Other than development, the CRISPR-edited mutant Treg cells will allow for more physiological characterization of IPEX-causing mutations, including suppression and cytokine secretion, which could be previously measured, and also FOXP3 and Treg functional stability, which was not possible with earlier approaches. Particularly, Treg cells can co-express other CD4 helper T cell master transcription factors, such as Tbet, GATA3, and RORγT, in response to polarizing cytokines[274]. This special mechanism acts to aid their function in part by up-regulating chemokine receptors to home toward sites of inflammation. However, prolonged inflammation can act as destabilizing factors and cause these "functionally adapted" Treg cells to lose FOXP3 expression and become inflammatory ex-Treg cells[78,274]. While, in healthy individuals, the destabilizing signal is usually held in balance, IPEX Treg cells, due to defective protein-protein interactions, may be more prone to becoming dysregulated cell-intrinsically, on top of being in a more inflammatory environment caused by impaired Treg suppressive function, thus creating a feed-forward loop resulting in disease exacerbation.

In addition to *in vitro* stability examinations using Th polarizing cytokines, *in vivo* models could also be used. Several MHC-II transgenic humanized mouse models exist for different autoimmune diseases, particularly those with a defined antigen[275]. Since breached tolerance of tissue-restricted antigens is one of the common clinical manifestations of IPEX syndrome, available humanized mouse models of T1D and thyroiditis will be of particular interest for the analysis of both ATO-derived WT Tregs and IPEX-mutant Treg cells[276,277]. Overall, this first-time description of human Treg cell differentiation from HSPCs using the ATO system provides us with opportunities for more physiological characterization of the biology of IPEX-inducing FOXP3 mutations.

4.5 Conclusion

In summary, in this study, we have found that our cytokine cocktail of TSLP, IL-7, and FLT3L generates an ATO system capable of supporting functional human $FOXP3^+$ $CD25^+$ Treg cell differentiation from $CD34^+$ HSPCs. Additionally, we demonstrated that an adequately titrated dose of TCR and STAT5 signaling alone could drive sustained CD25 and FOXP3 expression from CD4SP thymocytes, albeit the functionality of the latter population remains to be elucidated. With these discoveries, we will continue our search for a defined set of signals sufficient to drive thymic Treg development, which will help us reach our ultimate goal of generating Treg cells of any given specificity. Successful generation of Treg cells with the desired specificity will have countless applications for Treg cell therapies in controlling autoimmune diseases, alleviating allograft rejections, and resolving allergic reactions. Together, these data indicate that the ATO system is a novel tool that brings human Treg cell studies to an unprecedentedly close distance from being widely applicable in disease treatment. ATO-derived Treg cells will be further tested for their functionality and stability *in vitro* and *in vivo* using various humanized mouse models, including

GvHD and T1D. In combination with gene editing, this system will also support our study on IPEX syndrome and the implications of FOXP3 mutations on Treg development and function.

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