2021

Molecular Determinants of Fetal Tolerance and the Transition to Adult Immunity

Yi Wei Lim

LOYOLA UNIVERSITY CHICAGO

MOLECULAR DETERMINANTS OF FETAL TOLERANCE AND THE TRANSITION TO ADULT IMMUNITY

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

BY

YI WEI LIM

CHICAGO, ILLINOIS

DECEMBER 2021

ACKNOWLEDGEMENTS

I would like first to thank my mentor, Dr. Makio Iwashima, for all the support and guidance he has provided to develop me into an all-rounded scientist. Throughout my training in his lab, Makio has never stopped encouraging and challenging me to push my limits and go beyond my comfort zone to become a better scientist. I will always cherish the deep discussions we had about my project and other immunology topics, as well as all the lessons and training he has provided me in the past six years.

Next, I would like to thank the members of my dissertation committee, Dr. Phong Le, Dr. Jose Guevara-Patino, Dr. Mashkoor Choudhry, and Dr. Erin Lowery, for all their insights and guidance throughout my dissertation project. Their expertise and constructive feedback have kept my project moving forward and allowed me to develop into a better scientist.

I would also like to thank all the past and present members of the Iwashima lab, who became one of my important support systems throughout this journey. They were my sounding board for my ideas, my editors for my writing, and my audiences for all my presentations. They generated a working environment that made research fun and collaborative, and I will cherish these friendships forever.

I am also highly appreciative of all the support from the students, faculty, and staff of the Biochemistry and Molecular Biology program, the Microbiology and Immunology department, and the guidance and support from Pat Simms at the flow cytometry core. I would also like to thank the nurses at Gottlieb Memory Hospital and Loyola University Medical Center for their participation in umbilical cord blood collection.

I want to thank all my family and friends for their endless love and support throughout my journey. Mainly my parents and my sister, always believing in me and supporting all my dreams with their never-ending encouragement, motivation, and positivity.

Lastly, this work would not be possible without the funding support from the NIH/NIAAA T32 training grant AA013527 and the Van Kampen Cardiopulmonary Research Fund.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	Alcoholic liver cirrhosis
AIRE	Autoimmune regulator
AP-1	Activator protein-1
APB	Adult peripheral blood
APC	Antigen presenting cells
ARDS	Acute respiratory syndrome
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
BCG	Bacillus calmette-guerin
BPI	Bactericidal/permeability-increasing protein
C-terminal	Carboxyl terminal
CBT	Umbilical cord blood-derived Foxp3+ T cells
cDC2	Classical dendritic cells 2
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNS	conserved non-coding
CRAC	Calcium release-activated calcium
CSIF	Cytokine synthesis inhibitory factor
cTEC	Cortical thymic epithelial cells
CTL	Cytotoxic T lymphocytes

CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DAG	Diacylglycerol
DC	Dendritic cells
DETC	Dendritic epidermal T cell
DN	Double-negative
DP	Double-positive
E17.5	Embryonic days 17.5
EtOH	Ethanol
FAE	Fetal alcohol exposure
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorders
Foxp3	Forkhead box P3
GALT	gut associated lymphoid tissue
GATA3	GATA-binding protein
gMFI	Geometric mean fluorescence intensity
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
hsp70	Heat shock protein 70
IBD	Inflammatory bowel disease
ICAM-1	Intracellular adhesion molecule 1
Id3	Inhibitor of DNA binding 3
IEL	Intraepithelial lymphocytes

IFN	Interferon
IGV	Integrative Genomics Viewer
IKZF	Ikaros zinc finger
IL	Interleukin
IL-2Rα	IL-2 receptor alpha
IP_3	Inositol-1,4,5-triphosphate
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRAK-M	IL-1R-associated kinase-monocyte
ITAMs	Immunoreceptor tyrosine-based activation motifs
iTreg	*In vitro* induced Tregs
KGF	Keratinocyte growth factor
LAP	Latency-associated peptide
LAT	Linker for activation of T cells
LCMV	Lymphocytic choriomeningitis virus
LPL	Lamina propria lymphocyte
Mbd	Methyl-binding domain
MFI	mean fluorescence intensity
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cells
N-terminal	Amino terminal
NET	Neutrophil extracellular traps
NFAT	Nuclear factor of activated T cells
NICU	Neonatal Intensive Care Unit

NIMA	Non-inherited maternal alloantigens
NK	Natural killer
NKT	Natural Killer T cells
NTx	Neonatal thymectomy
PA	Phosphatidic acid
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid DC
PEth	Phosphatidyl ethanol
PHA	Phytohemagglutinin
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLD	Phospholipase D
pMHC	Peptide on MHC molecule
PP	Peyer patches
PRR	Pattern recognition receptor
PTK	Protein tyrosine kinase
pTreg	Periphery Treg
RAR	Retinoic acid receptor
Reg	Regenerating islet-derived protein
RORγt	Retinoid-related orphan receptor γ-t
RSV	Respiratory syncytial virus
RTE	Recent thymic emigrants
SAGE	Serial analysis of gene expression

SI	Small intestine
SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76kda
SOS	Son of sevenless
SP	Single-positive
sPLA2	Secretory phospholipase A2
T-bet	T-box transcription factor
T9	Transferrin receptor 9
TCR	T cell receptor
Tet	Ten-eleven-translocation
Tfh	Follicular T helper
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TMP	Thymic multipotent precursor
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
TSDR	Treg-specific demethylated regions
tTreg	Thymic Tregs
UCB	Umbilical cord blood
VEO-IBD	Very early onset-inflammatory bowel disease
xGVHD	Xenogeneic graft versus host disease
Zap-70	Zeta-associated protein of 70kda

ABSTRACT

The perinatal immune system is highly tolerogenic and is phenotypically and functionally distinct from the adult immune system. This tolerogenic nature is a double-edged sword for newborns. While it is beneficial to prevent excessive inflammation against the vast array of foreign antigens encountered after birth, it also causes a lack of immune responses to life-threatening infections. My dissertation research aims to investigate the mechanisms by which perinatal T cells contribute to immune tolerance in infants. A deeper understanding of the nature of the perinatal immune system will provide pivotal knowledge to develop safe and effective strategies to protect infants from infection and to establish immune homeostasis with commensal microbes.

Using umbilical cord blood (UCB) T cells as a model to study perinatal immunity, we found that antigen receptor stimulation of T cells in UCB leads to the development of Foxp3+ T cells in both CD4+ and CD8+ T cell subsets. These UCB-derived Foxp3+ T cells are phenotypically and epigenetically distinct from canonical thymus-derived Tregs (tTregs) in adults, but they carry immune regulatory functions *in vitro* and *in vivo*. The development of Foxp3+ T cells requires CD36hi monocytes. Adult blood contains a group of lymphocytes that inhibits monocyte-induced Foxp3+ T cell development, showing how perinatal blood differs from adult blood. Foxp3+ T cell development also requires IL-2. Alcohol, which is known to cause immunological defects, reduces the expression of CD25, a component of the high affinity IL-2 receptor, and blocks Foxp3+ T cell development. The result suggests that immunological

dysfunctions found among infants born from alcoholic mothers may be in part due to the impaired development of these Foxp3+ T cells during their fetal life.

To further elucidate the mechanisms that contribute to perinatal immunological tolerance, we investigated the expression of Helios, another transcription factor known to be expressed by tTregs along with Foxp3. We found that Helios is expressed significantly more frequently by UCB and neonatal peripheral blood T cells than adult T cells. Similar results were observed in mice. The expression frequency decreased rapidly after birth. The data suggested that T cells from fetal/perinatal origin express Helios. Indeed, we found that most gut-associated T cells, which are known to originate from the fetal thymus, express Helios in the fetus and maintained Helios expression throughout adulthood. Additionally, human T cells that matured in mice that received UCB hematopoietic stem cells also express Helios. Gene knockout of Helios in UCB T cells showed a significant increase in expression of multiple effector cytokines, suggesting that one of Helios' functions is to suppress effector cytokine production by activated T cells.

Together, these data demonstrated multiple mechanisms by which T cells can contribute to immune tolerance in neonates. First, the perinatal peripheral environment promotes T cells to differentiate into a unique group of Foxp3+ T cells that carry suppressive functions. Second, perinatal T cells express high levels of Helios, which suppress activated T cells to produce effector cytokines. Together, both intrinsic (Helios) and extrinsic (CD36hi monocytes) mechanisms promote the tolerogenic nature of the perinatal immune system.

CHAPTER ONE: REVIEW OF LITERATURE

T Cell Development

The mature immune cells originate from bone marrow-resident hematopoietic stem cells (HSCs) that differentiate through a highly hierarchical developmental progression. This differentiation process progresses with increasing restricted lineage potential, ultimately leading to the different mature blood cell lineages. The Weissman group was pioneers in identifying two critical progenitor cells: the common myeloid progenitor (CMP), which eventually develops into erythrocyte-megakaryocyte progenitors or granulocyte-monocyte progenitors, and the common lymphoid progenitor (CLP), which ultimately differentiates into the different lymphocyte subsets, including T cells[1,2].

While most of hematopoiesis occurs in the bone marrow, T cell development takes place in the thymus to yield mature, self-tolerant, functional T cells[3,4]. T cell development does not happen cell-autonomously but requires signals from stromal cells, including thymic epithelial cells and mesenchymal fibroblasts[5]. Although the thymus provides the niche that supports T cell development, the thymus-resident T cell progenitor has limited self-renewing potential. Therefore, sustained T cell production requires continual seeding of blood-borne bone marrow-derived progenitors from the periphery[6,7].

The Bleul group identified the most immature precursors, thymic multipotent precursor (TMP), within the early T lineage progenitors in the thymus. These TMPs can give rise to T, B, and dendritic cells on the single-cell level under respective lineage permissive conditions[8]. However, TMPs respond to Notch signals provided by the thymus microenvironment to induce

T cell lineage commitment and rapidly lose their B cell lineage potential. Their finding suggests that bone marrow-derived hematopoietic precursors only commit to T cell lineage after seeding the thymus[9].

αβ T Cell Development

Two lineages of T cells generated in the thymus can be identified by the expression of αβ or γδ T cell receptor (TCR) complexes. Within the αβ T cell lineage, two major subsets were distinguished by surface expression of CD4 and CD8 in the 1980s [10]. T cells undergo a series of differentiation steps defined based on their surface expression of CD4 and CD8 in a spatial-temporal manner in the thymus[11,12]. The most immature subset of thymocyte precursors does not express CD4 and CD8, thus denoted as double-negative (DN). DN is the critical stage that progenitors are committed to either αβ or γδ T cell lineage[13]. During the DN stage, immature thymocytes undergo rearrangement in the TCRβ, TCRγ, and TCRδ genes[14,15]. Successful rearrangement of the TCRβ chain will be complex with a germline-encoded pre-TCRα, forming the pre-TCR complex[16,17]. Signaling via the pre-TCR complex subsequently led to a burst of proliferation, upregulation of CD4 and CD8 co-receptors, initiation of TCRα gene rearrangement, and the silencing of the TCRγ gene[18]. αβ T cell lineage thymocytes then progress to the CD4+ CD8+ double-positive (DP) stage, where they complete TCRα gene rearrangement and subsequently undergo positive and negative selection[19].

Both positive and negative selection process involves the engagement of TCR with a major histocompatibility complex (MHC) molecule with a self-peptide. The goal of positive selection is to select thymocytes that express TCR that recognize self-MHC molecules. Positive selection occurs in the thymus cortex mediated by cortical thymic epithelial cells (cTECs) that express MHC class I or MHC class II with a self-peptide. A peptide-MHC complex that induces

weak TCR signaling promotes thymocyte survival during positive selection and does not induce TCR-mediated apoptosis[20–22]. After positive selection, DP thymocyte that recognizes MHC class I in the context of self-peptide further differentiate into CD4-CD8+ single-positive (SP) T cells, while those that recognize self-peptide in the context of MHC class II differentiate into CD4+CD8- SP T cells[23](Figure 1).

Conversely, thymocytes that express TCR that binds self-peptide bound to MHC too strongly undergo activation-induced apoptosis[24]. This process is known as negative selection, where the goal is to remove T cells that will recognize self[5]. Negative selection occurs in the medulla and is mediated by medullary thymic epithelial cells (mTECs) and dendritic cells (DC). Positively selected thymocytes migrate from the thymic cortex to the medulla via CCR7-mediated attraction by mTECs[25,26]. In the thymic medulla, mTECs express numerous tissue-restricted self-antigens promiscuously through the autoimmune regulator protein(AIRE)[27–3034,35]. mTECs can directly and indirectly, in cooperation with thymic dendritic cells, present these tissue-specific self-antigens to eliminate self-reactive T cells and promote the generation of regulatory T cells (Tregs)[31–33]. AIRE-expressing mTECs mediates Treg generation when thymocytes bind MHC-self peptides at high-avidity[34,35]. The generation of thymic Tregs (tTreg) aids in controlling self-reactive T cells that have escaped the negative selection in the thymus into the periphery. Subsequently, the Foxp3+CD25+ thymocytes can be found mainly in the medullary region of the thymus[36,37]. Therefore, mTECs play an essential role in establishing self-tolerance in T cells through both negative selection and generation of tTregs.

After the completion of both positive and negative selection, SP thymocytes undergo final functional maturation that involves the upregulation of cell surface markers CD62L, sphingosine-1-phosphate receptor 1 ($S1P_1$), and chemokine receptor CCR7[25,38]. These markers

facilitate the emigration of SP thymocytes from the thymus into the circulation known as recent thymic emigrants. Once in the periphery, these recent thymic emigrants continue their post-thymic education with progressive maturation of both surface phenotype and immune function ultimately into fully mature naïve CD4+ and CD8+ T cells[39,40].

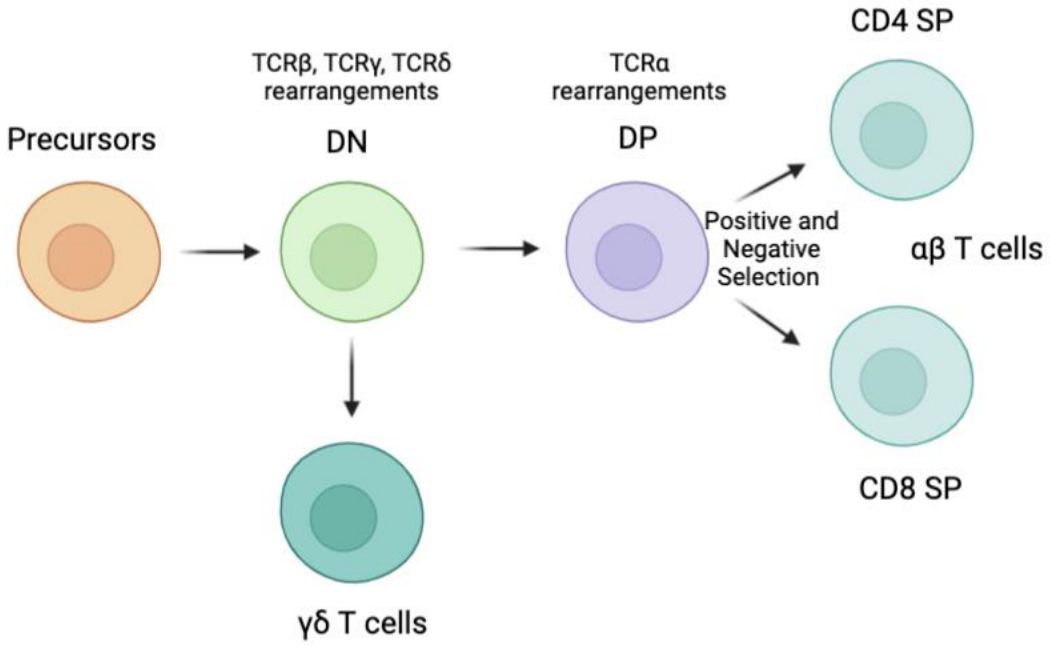

Figure 1: αβ and γδ T Cell Development in the Thymus. The diagram depicts simplified stages of αβ and γδ T cell thymopoiesis from thymocyte precursors to CD4-CD8- double-negative (DN), CD4+CD8+ double-positive (DP), and CD4+ or CD8+ single-positive (SP) stage.

γδ T Cell Development

γδ and αβ T cells arise from a common progenitor cell in the thymus where they commit to either αβ or γδ T cell lineage in the DN stage[13]. Progenitors that productively rearrange TCRγ and TCRδ chain will express γδ TCR on the cell surface and undergo a burst of proliferation. However, the majority of them avoid progression through the SP stage and egress to the periphery with a CD4-CD8- DN phenotype (Figure 1). Molecular events leading to αβ or γδ T cell lineage decision is not resolved, but several factors that can contribute to γδ T cell lineage commitment has been identified. For instance, the expression of transcription factor SOX13

promotes γδ T cell development while opposing αβ T cell development[41]. Expression of inhibitor of DNA binding 3 (Id3) also regulates the adoption of the γδ T cell fate in the thymus[42]. TCR signal strength is also implicated in determining lineage choice where DN cells receiving a stronger TCR signal adopts a γδ T cell fate while DN cells receiving weaker TCR signals committing to the αβ lineage[43].

Early genetic work of γδ TCR rearrangement during fetal, neonatal and adult thymocyte development revealed an organized and sequential rearrangement of specific γ and δ gene segments in developing γδ T cells during ontogeny[44,45]. These studies showed that upon thymic egress, most γδ T cells localized to non-lymphoid peripheral tissues, and the tissue localization segregates with the surface expression of specific TCR Vγ chains[46,47]. This ordered rearrangement resulted in timed production of defined γδ T cell population with specific combinations of Vγ and Vδ chain, appearing in waves during development and populate different tissues in the adult animal.

The first wave of thymocytes bearing γδ TCR encoded by Vγ5 and Vδ1 gene segments appear early (E14.5) in embryonic life and populates the skin epidermis[48]. Subsequently, Vγ6+ γδ T cells are generated and localize to tissues such as the uterine, tongue, and lungs[49,50]. Next, Vγ7+ γδ T cells that mainly populate the gut mucosal tissue are generated, even though their development timeline is still under debate. Vγ7 chain can be detected in the thymus as early as E13, with the highest expression between E17-E19. However, Vγ7 chain is detected in the fetal gut and liver at E11, before developing thymic lobes are colonized by T cell progenitors[51]. Lastly, Vγ1+ and Vγ4+ expressing γδ T cells are generated and found in the periphery (blood, spleen and lymph nodes)[44,52,53]. In summary, γδ T cells with different Vγ chain usage appeared in waves during ontogeny and localized to different anatomical sites in mice (Figure 2).

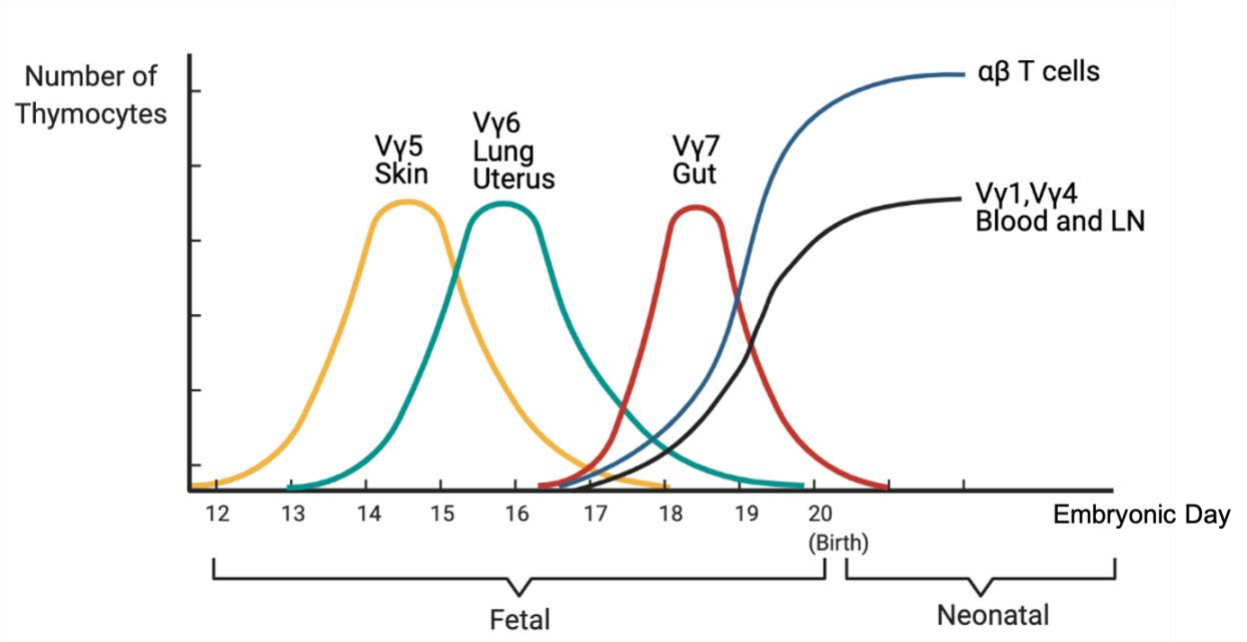

Figure 2: Successive Waves of γδ T Cells of Different Vγ Chain Usages Across Development in the Thymus. Diagram depicts the developmental timing of specific Vγ chain expression in the thymus and their corresponding anatomical localization after thymic egression. Figure adapted from Ribot *et al.*[54] using Tonegawa nomenclature.

αβ T Cell Subsets

Within the adaptive arm of the immune system, αβ T cells can elicit immune responses, maintain immune homeostasis, and establish immune memory. Peripheral αβ T cells subset (also commonly referred to as conventional T cells) can be classified by surface expression of CD4 or CD8 coreceptors, representing T helper and cytotoxic T lymphocytes, respectively. CD4+ T cells recognize antigen bound to MHC class II, while CD8+ T cells recognize antigen bound to MHC class I[55]. CD4+ and CD8+ T cells can be further distinguished into different subsets based on their function, unique defining transcription factor, and cytokine profile discussed in the following sections.

CD4+ T Cell Subsets

CD4+ T cells play a central role in immune protection mainly by producing cytokines to help B cells make antibodies, induce macrophages to increase microbicidal activity, or chemokines to recruit immune cells to the site of infection, etc. Upon activation, naïve CD4+ T cells can differentiate into different T helper (Th) subsets such as Th1, Th2, Th17, and Tregs depending on the cytokine milieu of the microenvironment produced by antigen presenting cells (APCs) or other neighboring cells (Figure 3).

T helper type 1 (Th1). Th1 cells were first discovered along with Th2 cells by the Coffman group back in 1986, where they observed that CD4+ T cells is divided into two distinct populations with different cytokine profiles[56]. Cytokines interleukin-12 (IL-12) and interferon-γ (IFN-γ) initiates the signaling cascade to generate Th1 cells[57,58]. These cytokines promote the expression of the master transcription factor for Th1 differentiation, T-box transcription factor (T-bet), and suppress the development of other Th subsets[59,60]. Th1 produces cytokines such as IFN-γ, IL-2, and tumor necrosis factor (TNF) which mainly functions to aid in eliminating intracellular bacteria and viruses by other immune cells type such as macrophages[61–63].

T helper type 2 (Th2). CD4+ T cells activated in the presence of IL-4 and IL-2 give rise to Th2 cells[64]. Th1 cytokine IFN-γ can inhibit Th2 responses[65]. Th2 cells express the master transcription factor GATA-binding protein 3 (GATA3) and produce IL-4, IL-5, IL-10, and IL-13[56,66,67]. The expression of GATA3 leads to the production of IL-4, IL-5 and inhibits Th1 differentiation[66,68,69]. Th2 cytokines mainly play a role in the elimination of extracellular pathogens as well as mediating airway hypersensitivity by acting on B cells, mast cells, and eosinophils[70–74].

T helper type 17 (Th17). For the longest time, Th1 and Th2 comprise the main effector population of CD4+ T cells differentiated from naïve CD4+ T cells in the periphery. In 2003, a third major CD4+ effector T cell population differentiated from naïve CD4+ T cells was identified[75]. These cells were designated as Th17 and characterized by their production of IL-17A, IL-17F, and IL-22. Th17 mediate immune responses against extracellular bacteria and fungi. They are also involved in various autoimmune disorders such as rheumatoid arthritis, psoriasis, and Crohn's disease[76–80]. While Th1 and Th2 cytokines inhibit Th17 differentiation, IL-6, TGF-β, IL-21, and IL-23 promote Th17 differentiation from activated CD4+ T cells[76,81]. IL-6 and low concentrations of TGF-β induce the Th17 master transcription factor retinoid-related orphan receptor γ-t (RORγt)[82]. IL-21 and IL-23, on the other hand, promote stabilization and maintenance of Th17 cells[83,84].

Regulatory T cells (Tregs). Th1, Th2, and Th17 mainly generate inflammatory responses to fight pathogens. On the other hand, Tregs are specialized for immune suppression by preventing aberrant or excessive inflammation and promoting self-tolerance.

Gershon and Kondo started the concept of suppressor T cells in 1970 when they found that T cells can also dampen immune responses[85]. Active research of these suppressor T cells and markers to identify them went on for several years but quickly collapsed in the mid-1980s. In parallel with the study of suppressor T cells above, different researchers investigated how breaching self-tolerance can lead to autoimmune disease development. This approach eventually led to the finding of T cells and thymocytes with autoimmune-suppressive activity[86]. Nishizuke and Sakakura demonstrated in 1969 that neonatal thymectomy (NTx) three days after birth resulted in autoimmune destruction of the ovaries[87]. Others further showed that thymectomy in conjunction with sublethal radiation resulted in autoimmune thyroiditis and type 1 diabetes

development in adult rats[88,89]. Subsequently, they discovered that the transfer of syngeneic T cells protected rats from developing diabetes and thyroiditis induced by adult thymectomy and sublethal irradiation[90,91]. These results suggested that the thymus produces a subset of T cells with suppressive function to prevent T cell-mediated autoimmune disease, prompting the investigation to identify specific markers expressed by these suppressive T cells.

In 1995, Sakaguchi *et al*. discovered CD4+ T cells that carry suppressive functions express the IL-2 receptor α chain, CD25[92]. The depletion of CD4+CD25+ T cells led to autoimmune disease development and heightened immune response when foreign antigens were introduced. Reconstitution of CD4+CD25+ T cells prevented autoimmune disease development and normalized the response against foreign antigens[92]. The frequency of CD4+CD25+ T cells in the periphery correlated well with the NTx model findings. CD4+CD25+ T cells can be detected in the periphery of neonates from around three days after birth[93,94]. NTx at day 3 eliminates the presence of these cells in the periphery and causes autoimmune disease. Subsequently, the transfer of syngeneic CD4+CD25+ immediately prevents autoimmune disease[93]. These results demonstrated that the thymus produces CD4+CD25+ T cells with suppressive function, later known as Tregs.

The master transcription factor for Tregs, forkhead box P3 (Foxp3), was initially discovered in 2001. A single *Foxp3* mutation in the X-chromosome is the disease-causative gene in Scurfy mice, where they develop severe autoimmunity spontaneously[95]. Mutation of the human Foxp3 (the ortholog of murine Foxp3) causes a similar human disease called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome)[96,97]. The autoimmune phenotype observed in Scurfy mice and IPEX patients closely resembled mice deficient in CD4+CD25+ Tregs, giving rise to the hypothesis that Foxp3 is crucial for Treg

development and function. By 2003, several studies supported the role of Foxp3 in the generation of Tregs and their suppressive function. Peripheral CD4+CD25+ T cells and CD4+CD8-CD25+ thymocytes express *Foxp3* mRNA. Overexpression of Foxp3 in CD4+CD25- T cells converted them into phenotypically and functionally Treg-like cells *in vitro* and *in vivo*[98–100].

While Foxp3 expression in mice is limited to Tregs, human T cells can transiently upregulate Foxp3 expression upon TCR activation[101]. This transient Foxp3 induction after TCR activation may be an inhibitory feedback mechanism to prevent hyperactivation of T cells as they are hyporesponsive and have reduced cytokine production such as IFN-γ, TNF, and IL-10. However, these activated effector T cells that transiently upregulate Foxp3 are not Tregs as they do not carry suppressive function and still produce higher levels of IFN-γ, TNF-α, and less IL-10 compared to Tregs upon PHA restimulation[102].

While Tregs can be generated from the thymus (tTregs), Tregs can also be generated in the periphery (pTregs) or *in vitro* (iTregs) from naïve CD4+ T cells upon TCR activation. As discussed previously, tTregs are generated in the thymus when thymocytes bind MHC-self peptides at high-avidity to maintain self-tolerance[34,35]. On the other hand, naïve CD4+ T cells in the periphery can differentiate into pTregs when stimulated with cognate antigen in the presence of TGF-β, retinoic acid, and IL-2[103,104]. TGF-β signaling induces the expression of Foxp3 by CD4+ T cells in the presence of IL-2 and inhibits Th17 differentiation through the actions of transcription factors, Smad3 and STAT5[105–108]. IL-2 signaling is required for TGF-β to convert naïve CD4+CD25- T cells into CD4+CD25+Foxp3+ Tregs[105].

Since the development mechanism for pTregs and tTregs is different, it is proposed that the development of tTregs is essential to maintain self-tolerance and prevent autoimmunity. In

contrast, pTregs are generated in the periphery to control immune response against environmental challenges. Several reports demonstrated that pTregs accumulate at tissue sites constantly exposed to foreign antigens such as the intestine and the placenta[109–111]. Furthermore, tissue severely damaged by inflammatory effector responses against pathogens subsequently produces anti-inflammatory cytokines such as TGF-β to induce Tregs specific for foreign antigens[112,113].

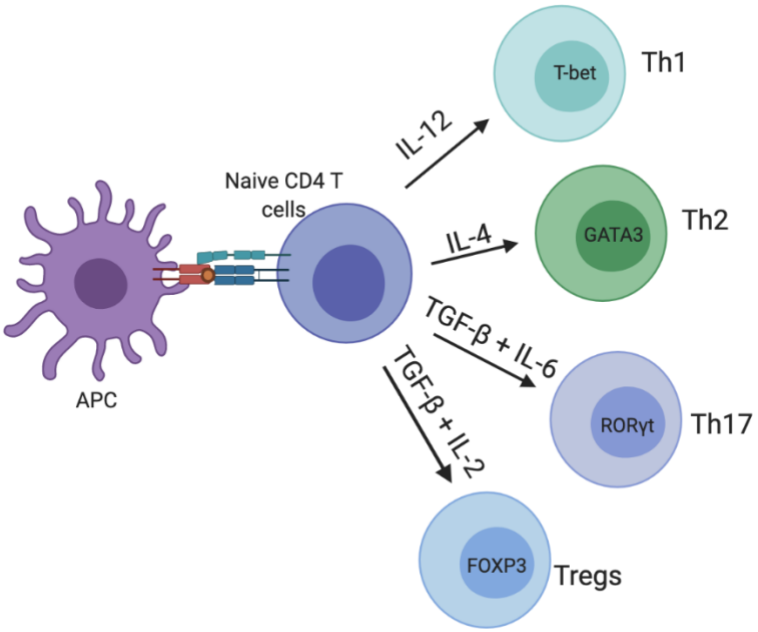

Figure 3:Naïve CD4+ T Cell Differentiation into the Different T Helper or Regulatory Subsets. Diagram depicting the differentiation of stimulated naïve CD4+ T cells by APC into different Th subsets depending on the cytokine milieu. The master transcription factor is denoted within each Th and Treg subset.

Mechanisms of regulatory and suppressive functions by Tregs. Tregs themselves have to be TCR-activated in the presence of IL-2 to be suppressive[114]. Once activated, Tregs can suppress conventional T cells independently of an antigen, a phenomenon known as the bystander effect[115]. Tregs possess regulatory and suppressive functions mediated via several mechanisms, some of which include: 1) Tregs can inhibit TCR-induced proliferation by

inhibiting IL-2 production by conventional T cells in a cell-contact dependent manner in the absence of APCs. 2)Tregs can modulate the function of APCs through the expression of coinhibitory molecule cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). CTLA-4 is constitutively expressed by both murine and human Tregs[116–118]. CTLA-4 expression by Tregs suppresses the proliferation of conventional T cells in the presence of APCs by downregulating costimulatory molecules CD80/86 on APCs[119]. Blockade of CTLA-4 both *in vitro* and *in vivo* abrogated suppression of conventional T cell proliferation and protective effects of Tregs in murine colitis models[116,117,120]. 3) Tregs can produce immunosuppressive cytokines, including TGF-β and IL-10. Tregs can produce high amounts of soluble or membrane-bound TGF-β, and blocking TGF-β is shown to abrogate suppression of T cell proliferation by Tregs partially[121,122]. Immunoregulatory effects of IL-10 production by Tregs have been demonstrated in several inflammatory disease models [123–125]. 4) Tregs may also suppress by expressing hallmark effector T cell transcription factors such as T-bet. Treg subset has been shown to upregulate Th1 transcription factor T-bet in the presence of IFN-γ, subsequently promoted the expression of chemokine receptor CXCR3 to promote T-bet+ Tregs accumulation at Th1 inflammatory sites[126]. Therefore, it is well appreciated that Tregs can suppress immune responses to maintain homeostasis and tolerance using many different mechanisms.

Epigenetic regulation of Foxp3. Tregs are characterized by Foxp3 expression, which has been shown to mediate its differentiation, maintenance, and function[99,100,127,128]. Because Foxp3 plays such a critical role in Treg biology, extensive studies have been conducted to understand the molecular mechanisms that govern and regulate the induction of this transcription factor. In addition to the promoter, three conserved non-coding DNA sequence (CNS) intronic enhancer element at the *Foxp3* gene locus has been identified[129]. These enhancer regions are designated as

CNS 1, 2, and 3 and have been shown to contribute differentially to tTreg and pTreg differentiation. CNS1 region is shown to be essential for iTreg/pTreg development while CNS2 is crucial for the maintenance of Foxp3 expression and CNS3 controls *de novo* Foxp3 expression and tTreg differentiation (Figure 4)[129].

CNS1 enhancer region contains binding sites for transcription factors such as the nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), retinoic acid receptor (RAR), and Smads (Figure 4)[130,131]. Smad2/3 binding to CNS1 is responsible for TGF-β mediated induction of Foxp3 in pTregs[132]. The deletion of CNS1 led to abrogation of Foxp3 induction in naïve T cells and pTreg differentiation but did not affect tTreg differentiation in the thymus[129]. Deletion of CNS1 is similar to the phenotype of TGF-β1-and Smad2/3-deficient mice, where tTreg levels were relatively normal, but pTregs were significantly reduced in numbers[132,133]. CNS1-deficient mice demonstrate mucosal inflammation and abortion of fetuses, highlighting the importance of pTregs in maintaining mucosal homeostasis and in pregnancy[111,134].

The CNS2 enhancer region contains Stat5, NFAT, RUNX1/Cbfβ, CREB, and Foxp3 binding sites (Figure 4)[135]. CNS2 region is highly enriched with CpG sites that can be epigenetically regulated through methylation. This region is also known as one of the Treg-specific demethylated regions (TSDRs). Methylation of CpG islands is generally accepted as an epigenetic mechanism to repress gene transcription[136]. The fully demethylated CpG sites on CNS2 contribute to the stable expression of *Foxp3* by tTregs [137,138]. Foxp3 protein binds to demethylated CNS2 and enhances its expression via a positive feedback loop[129]. The CNS2 CpG islands are highly methylated in both Foxp3- effector T cells and *in vitro* generated Tregs (iTregs), leading to a non-permissible chromatin configuration at the CNS2 enhancer region[135,138,139]. Consequently, Foxp3 expression in iTregs is highly unstable and is lost during

restimulation in the absence of TGF-β[135]. Additionally, this region's complete methylation prevents abnormal Foxp3 induction in non-Tregs such as Th1, Th2, Th17, CD8 T cells, and NK cells[140–142].

The commitment of a stable Treg lineage is established early during tTreg development, where the TSDR demethylation occurs continuously throughout its tTreg maturation process. The most immature $CD24^{hi}$ subset among thymic Foxp3+ Tregs started with a substantially methylated TSDR, and demethylation frequency increases as they mature, identified as $CD24^{lo}$ population[143]. The CpG demethylation of CNS2 in Tregs were controlled by the ten-eleven-translocation (Tet) family of the demethylation factor[144]. Downregulation of Tet2 prevented TSDR demethylation in tTregs[145]. Methyl-binding domain (Mbd) proteins-deficient tTregs had a marked impairment of Tet2 binding at the TSDR region, leading to a decrease in TSDR demethylation and Foxp3 expression in tTregs[146].

The CNS3 enhancer region contains binding sites for c-Rel, an NFkB family member, and plays a vital role in tTregs and pTregs differentiation (Figure 4). c-Rel directly binds to CNS3 and promotes *Foxp3* transcription by the formation of a Foxp3-specific enhanceosome[147,148]. CNS3-deficient mice demonstrate a significant decrease in the frequency of tTregs, as well as impairment in TGF-β-mediated Foxp3 induction in peripheral naïve T cells[129]. c-Rel deficient mice exhibit a similar reduction in Foxp3 induction as CNS3-deficient mice[129].

Kitagawa *et al.* recently identified a region approximately 8-kb upstream of the transcriptional start site (TSS) of *Foxp3* that is also important for Foxp3 expression regulation. This region is defined as CNS0 (8.5kb upstream of TSS) and contains binding sites for Satb1 (Figure 4)[149]. Satb1 is a global genome organizer that induces transcriptional and epigenetic regulation via forming a novel nuclear architecture[150]. The binding of Satb1 to CNS0 is predicted

to be the pioneering element that is required for subsequent activities of the other CNS elements for Treg lineage commitment. T cell-specific Satb1 deficiency impaired Treg-specific super-enhancer activation and decreased expression of Treg signature genes[151]. Genetic ablation of Satb1 leads to autoimmunity due to impaired tTreg development[152]. Although Satb1 deletion impairs tTreg development, it enhances the development of pTregs in the periphery in mice[149]. These data suggest that Satb1 has differential effects on tTreg and pTreg cell development.

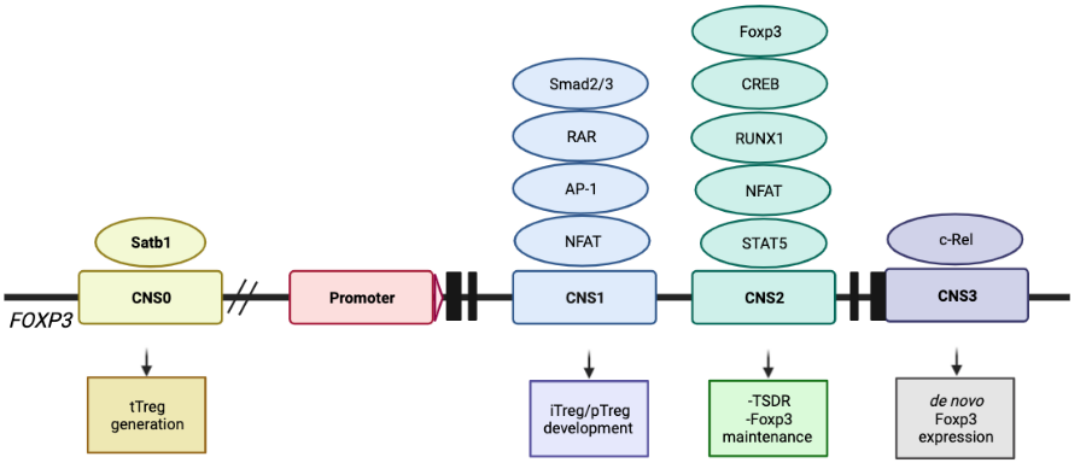

Figure 4: Schematic Diagram of Transcriptional Regulation of the *Foxp3* Locus. Regulatory regions within *Foxp3* locus, including the promoter, CNS0, CNS1, CNS2, and CNS3 with the binding of transcription factors and the function of each regulatory region were depicted. Figure adapted from Lee *et al.* [153].

CD8αβ+ T Cell Subsets

Upon recognizing its cognate antigen in the context of MHC class I, naïve CD8+ T cells undergo clonal expansion and subsequent effector differentiation to generate cytotoxic T lymphocytes (CTL) in the presence of IFN-α/β or IL-12[154–156]. CD8+ T cells specialize in the eradication of acute viral or intracellular bacterial pathogens and malignant cells. Like Th1 cells, CD8+ T cells can generate robust amounts of IFN-γ and TNF[157,158]. In addition to the release of proinflammatory cytokines, CD8+ CTL mediates target cell killing by granule exocytosis pathway or FasL/Fas pathway[159]. CTL can release secretory granules containing perforin and

granzyme protein to lyse neighboring cells directly[160]. Perforin deficient mice cannot clear lymphocytic choriomeningitis virus (LCMV) *in vivo* and have lower efficiency in eliminating tumor cells[161–163].

While research mainly focuses on CD4+ Tregs, descriptions of CD8+ Tregs are scarce. In humans, thymic CD8+ Tregs is described to express markers and function similar to their CD4+ tTreg counterpart[164]. CD8+ Tregs have been implicated in inflammatory disorders, including inflammatory bowel disease (IBD), type 1 diabetes, and multiple sclerosis[165–167]. CD8+ Tregs have been shown to inhibit CD4+ Th1 and CD4 follicular T helper (Tfh) cells[168,169].

CD8αα+ T Cells

An important component of the intestinal immune system is the intraepithelial lymphocytes (IEL), which directly contacts enterocytes and serve as the frontline T cells in the gut. Within the mouse IEL fraction, there is a sizeable unconventional subset of αβ T cells that express CD8αα molecules instead of co-receptors CD4 or CD8αβ[170]. The frequency of these IEL varies among species where 10% of mouse small intestine IEL consists of these CD8αα+ αβ T cells but is undetectable in human intestines[171,172]. CD8αα+ T cells have been detected in the mouse small intestine lamina propria lymphocyte (LPL) fraction although this study did not differentiate if they are αβ or γδ T cells[173]. Unlike conventional CD8αβ+ αβ T cells that have high TCR repertoire diversity, analysis of both human and mouse IEL repertoire diversity revealed that TCRα and β chain are oligoclonal in the gut[174,175]. Furthermore, CD8αα+ αβ T cell also undergoes self-antigen-dependent agonist selection process in the thymus[176,177].

CD8αα+ αβ T cells carry immunoregulatory functions in the gut. Using TCRαβ specific for lymphocytic choriomeningitis virus (LCMV)-derived peptide gp33 transgenic mice, self-antigen stimulation led to a decrease in IL-2, IFN-γ, and IL-10 production by CD8αα+ αβ IELs.

Furthermore, in the presence of LCMV infection in the intestinal mucosa of these mice, CD8αα+ αβ IELs do not exhibit cytotoxic activity albeit being activated[178]. Additionally, CD8αα+ αβ IELs can prevent CD4+CD45RB^{hi} mediated colitis in severe combined immunodeficiency (SCID) mice in an IL-10 dependent manner[179]. These data highlight how CD8αα+ αβ T cells differ phenotypically and functionally from conventional CD8αβ+ αβ T cells.

γδ T Cells

γδ T cells were discovered in the mid-1980s, but their biological functions and antigens they recognize are still poorly understood[180–182]. This T cell lineage blurs the traditional boundaries between the innate and adaptive immune systems. γδ T cells exert an innate-like rapid immune response by recognizing a broad spectrum of molecules, including non-peptide antigens in a classical MHC or MHC-like molecule dependent[183–186] and independent manner[187,188]. Unlike conventional αβ T cells, which reside primarily in secondary lymphoid organs, γδ T cells are enriched in mucosal sites of the body such as the skin[189], intestinal epithelium[47,190,191], lung, tongue, and uterus[50]. γδ T cells express a CD3-associated heterodimeric T cell receptor (TCR) molecule on their surface, but their diversity of Vγ/Vδ chains appears to be limited and dictated by their anatomical localization (Figure 2)[46,47].

The biology of γδ T cells has been extensively studied in mouse models. They are among the first T cells to develop in the murine thymus. Their differentiation and effector functions are developmentally pre-programmed and occur at distinct waves during development. In mice, γδ thymocytes were detected in the thymus as early as gestation day 14.5 (E14.5), where their frequency and numbers outnumbered that of αβ thymocytes[192]. The frequency of γδ thymocytes reaches its peak at E16.5 then gradually decreases through the first postnatal week until it reaches adult levels of about 0.3-0.5% around ten days after birth[192]. This early emergence of γδ

T cells is also reflected in the periphery, where they are highly represented in fetal and neonates of many different species. γδ T cells can be detected as early as 6-9 gestational weeks in the human liver and primitive gut[193].

γδ T Cells in the Gut

Most of γδ T cells in the intestine IEL fraction express CD8αα homodimer but not CD8β chain[170]. γδ IEL is implicated to be of fetal origin as the transfer of fetal liver from E15-16 can generate γδ T cells in the IEL compartment[173]. Vγ7 chain can be detected in the thymus at E13, and in the fetal gut and liver as early as E11, before T cell progenitors colonize the developing thymic lobes[51].

The developmental route of IEL γδ T cells has been the subject of controversy for many years as several studies showing γδ IEL from thymus-dependent and thymus-independent origin. In athymic mice, γδ T cells are still present in the IEL compartment, albeit at a lower number than euthymic mice[173]. γδ T cells development and maintenance in the gut are independent of the microbiota, as the cell numbers isolated from germ-free and conventionally raised mice were similar[194,195].

The importance of γδ IEL in maintaining gut tissue homeostasis is evident by the reduction of epithelial cell proliferation in both the small intestine and colon in TCRδ$^{-/-}$ mice[196]. These γδ IEL express keratinocyte growth factor (KGF), a potent intestinal epithelial cell mitogen, and are important to preserve the integrity of damaged epithelial surfaces in Dextran Sulfate Sodium (DSS)-induced colitis model system[197]. Large numbers of γδ T cells localized at DSS-induced epithelial cell damage sites and TCRδ$^{-/-}$ mice demonstrated more DSS-induced mucosal injury and delayed tissue repair after the termination of DSS treatment[197]. TCRδ$^{-/-}$ mice also have increased gut permeability attributed to a decrease in intestinal tight junctional

complexes[198]. This perturbation correlates with increased susceptibility to the development of spontaneous colitis in aged TCRδ$^{-/-}$ mice around eight months of age[199]. The lack of γδ T cells is also correlated with increased levels of IFN-γ in the intestinal epithelium in different inflammatory bowel disease animal models[200]. The transfer of γδ IEL ameliorated colitis with decreased IFN-γ and increased TGFβ1 production[199]. In patients with celiac disease, individuals on a gluten-free diet and not experiencing active celiac disease have a higher frequency of CD8+ γδ IEL that express inhibitory NK receptor NKG2A and intracellular TGFβ1. Crosslinking of NKG2A or γδ TCR of these CD8+ γδ IEL led to secretion of TGFβ1 *in vitro,* suggesting the regulatory potential of CD8+ γδ IEL in celiac disease patients[201].

In addition to maintaining intestinal homeostasis, γδ IEL may carry effector roles against pathogens. In the presence of intestinal microbiota, γδ IEL can produce antimicrobial protein regenerating islet-derived (Reg) protein family, C-type lectins, including RegIIIγ, which kills Gram-positive bacteria[194,202]. Gene expression profile analysis of γδ IEL shows that they constitutively express effector function genes such as Granzyme A, B, and RANTES even without the presence of an infection[203]. Indirectly, IEL γδ T cells can protect against pathogens by promoting intestinal epithelial barrier function to restrict epithelial transmigration of pathogens such as *Toxoplasma* and *Salmonella typhimurium*[198].

γδ T Cells and Tolerance

The role of γδ T cells in tolerance induction was initiated when McMenamin *et al.* demonstrated that OVA exposure in the respiratory mucosa activates splenic CD8+ γδ T cells that specifically suppressed OVA-specific IgE antibody response in rats and mice[204,205]. The depletion of γδ T cells *in vivo* and *in vitro* abolished the induction and maintenance of orally induced-systemic tolerance against OVA, reflected in the production of anti-OVA antibody and

OVA-specific T cell proliferative responses[206]. Mechanistically, γδ T cells inhibit the priming of CTL precursors, IL-2 production and inhibit humoral responses[207].

To further investigate how γδ T cells can contribute to the establishment of oral tolerance, Kapp *et al.* isolated and generated γδ T cell clones from small intestine IEL fraction. These γδ T cell clones express IL-10 and transforming growth factor-β1 mRNA and can potently inhibit the generation of CTL response *in vitro*[208]. Locke *et al.* demonstrated that CD8αα+ γδ IEL is required for self-tolerance using a NOD mouse model of spontaneous type 1 diabetes. Transfer of CD8αα γδ IEL prevented the development of diabetes in a 3-day old neonatal thymectomy (NTX)-NOD mouse model similar to CD4+CD25+ Tregs transfer. The induction of CD4+CD25+ Tregs via oral insulin in euthymic mice also requires CD8αα+ γδ IEL in the gut to prevent diabetes in NOD mice[209].

γδ T Cell Cytokine Production

Depending on the source and conditions of activation, γδ T cells can produce a variety of cytokines including IFN-γ, TNF-α, IL-4, IL-5, IL-6, and IL-10[210–212]. Many studies generated γδ T cell clones to study their function and cytokine production due to the low frequency of γδ T cell. CD4+ and CD4-CD8- γδ T cell clones isolated from TCRα$^{-/-}$ mice conformed to the typical Th2 phenotype: high levels of IL-4, IL-5 and IL-10 and undetectable levels of IFN-γ, or to the typical Th1 phenotype, displaying high levels of IFN-γ, respectively. These Th1 and Th2 associated γδ T cell clones could generate appropriate Th1 and Th2 associated IgG production when cultured with naïve B cells[212]. Primary γδ T cell clones from noninflamed human skin tissue produce IFN-γ and TNF after 24-hour stimulation with PMA and ionomycin[213]. γδ T cell clones established from the small IEL fraction are shown to produce large amounts of IL-10 and little to no IL-2, IL-,4, and IFN-γ[214]. On the other hand, γδ T cell clones established similarly

from the spleen produces high levels of type 1 cytokines such as IFN-γ and TNF[215]. Freshly isolated lamina propria γδ T cells produce IL-10 and IL-17 after PMA ionomycin activation, but not those isolated from the spleen, payer's patches, mesenteric lymph nodes, and peripheral blood[216]. These data suggest that the cytokine profile of γδ T cells varies depending on their cellular localization.

T Cell Signaling and Activation

T cell activation initiates when the TCR binds to its cognate antigen presented by the MHC molecule (pMHC). This TCR/pMHC engagement subsequently led to the phosphorylation of tyrosine residues on the immunoreceptor tyrosine-based activation motifs (ITAMs) on the CD3 complex by Src family kinases, Lck[217]. CD4 and CD8 are both associated with Lck and acts as co-receptors to augment antigen receptor responses[218].

The phosphorylated tyrosine molecules on CD3 ITAMs led to the recruitment of a protein tyrosine kinase (PTK) known as the zeta-associated protein of 70kDA (Zap-70). Zap-70 binds to these phosphor-tyrosine molecules via their SH2 domain[219,220]. Upon ITAMs binding, Zap70 is released from its autoinhibitory conformation. This exposes its regulatory phosphorylation sites to be phosphorylated by Lck or Zap70 itself (via transphosphorylation)[221,222]. Activated Zap70 can now further activate downstream substrates, including linker for activation of T cells (LAT) and SH2 domain-containing leukocyte phosphoprotein of 76kDA (SLP-76)[223,224].

LAT and SLP-76 served as essential adapters for subsequent signaling factors to bind and activate multiple signaling pathways. LAT deficient of critical tyrosine residues inhibits T cell activation[224]. The loss of LAT or SLP-76 results in a near-complete loss of TCR signal transduction[225,226]. Phosphorylation of tyrosine residues on LAT led to the recruitment of

downstream signaling molecules such as Grb2 family proteins, SLP-76, and phospholipase C-γ1[227,228]. Grb2 family of proteins can bind LAT via their SH2 domain while binding other downstream proteins via their SH3 domains.

One well-known target in the Grb2 complex is the son of sevenless (SOS) that subsequently led to Ras activation and its downstream signaling pathway[229,230]. SLP-76 can bind both Grb2 and PLCγ, serving as an important functional link of the RAS and calcium signaling pathway[223]. The recruitment and activation of PLCγ1 catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG)[231]. IP_3 leads to calcium release from intracellular calcium stores in the endoplasmic reticulum via the IP_3 receptor. This deprivation of intracellular calcium storage leads to the opening of cytoplasmic calcium release-activated calcium (CRAC) channels leading to the influx of calcium from the extracellular environment[232,233]. Calcium regulates calcineurin, which dephosphorylates and induces the nuclear localization of NFAT transcription complexes from the cytosol, ultimately leading to NFAT-dependent gene transcription such as IL-2[234,235]. The increase of DAG leads to activation of its downstream target RasGRP, a Ras activator, that subsequently mediates the activation of the Ras signaling pathway downstream[236]. DAG also activates protein kinase C-θ, which eventually leads to the activation of the NF-kB pathway (Figure 5)[237].

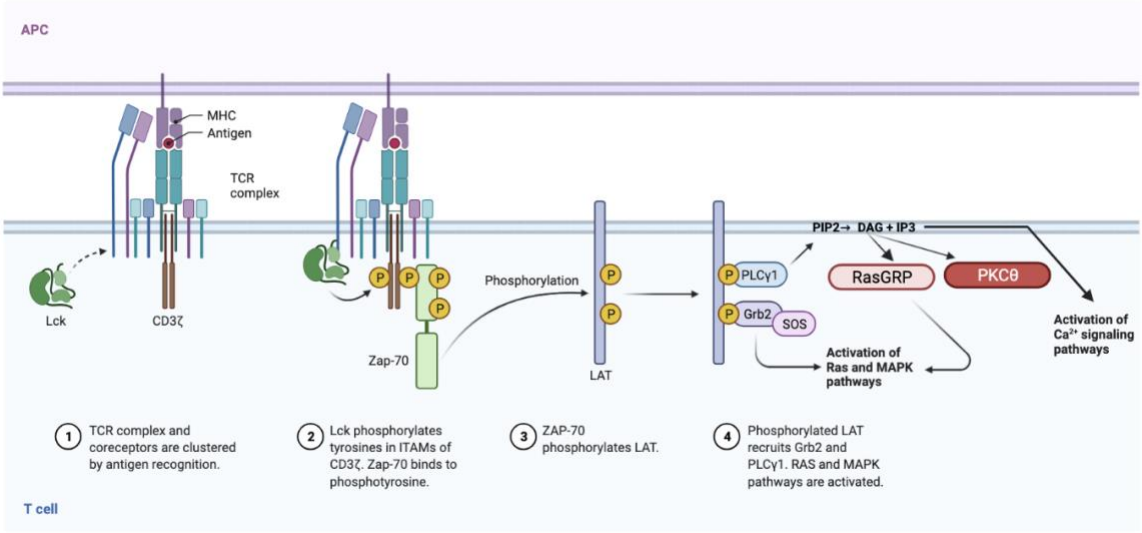

Figure 5: Simplified Schematics of the TCR Signaling Pathway. Image adapted from Biorender.

Immune Tolerance

Our immune system is developed to generate protective immune responses against foreign antigens and tolerate self-antigens to preserve the integrity of our tissue. Tolerance can be defined as a state of unresponsiveness against a specific antigen by a fully competent immune system that is still capable of eliciting protective responses against foreign antigens.

Ray Owen first demonstrated the concept of immunological tolerance in 1945. He observed that nonidentical, dizygotic twin cattle shared red blood cells that persist into adult life[238]. It was known that mixing red blood cells during regular blood transfusions would trigger a severe immunological reaction. Still, the lack of immune response against dizygotic red blood cells in these calves suggested that immune tolerance is generated in response to foreign cells acquired before birth. Furthermore, the majority of these dizygotic cattle twins are entirely tolerant of each other's skin allograft[239,240]. In 1953, Billingham, Brent, and Medawar demonstrated that injection of allogeneic tissue into mice *in utero* led to tolerance against subsequent skin allografts from the same allogeneic strain in adulthood. They showed that the

establishment of tolerance is dependent on a critical time window when alloantigens are exposed. Mice that were injected with foreign cells after birth demonstrated a lack of tolerance against subsequent skin allografts[241]. These seminal studies laid the groundwork for the concept of acquired immunological tolerance, for which Burnet and Medawar were awarded the Nobel Prize in Physiology or Medicine in 1960.

Immune tolerance can be divided into central and peripheral tolerance. Central tolerance occurs when newly generated T and B cells test their receptors to recognize self-antigens in the thymus or bone marrow, respectively. Strongly autoreactive T and B cells are censored by clonal deletion or receptor editing mechanism[24,242]. Autoreactive T cells developing in the thymus can also undergo "clonal diversion" where T cells expressing self-reactive TCR differentiate into tTregs[35]. tTregs can then exit into the periphery to suppress other autoreactive T cells to maintain self-tolerance. Therefore, autoreactive T cells can get deleted through the negative selection that eliminates self-reactive clones from the repertoire (clonal deletion) or gets imprinted into self-reactive clones with suppressive and regulatory functions (clonal diversion).

While central tolerance mechanisms are efficient, they cannot eliminate all self-reactive lymphocytes. Autoreactive lymphocytes that escaped these mechanisms and entered the periphery can encounter new self-antigens in secondary lymphoid organs such as lymph nodes and the spleen. Therefore, the tolerance state of the immune system is further maintained by multiple peripheral tolerance mechanisms. One of the mechanisms is clonal anergy. It was first described by Nossal, who discovered the presence of mature, autoreactive B cells in the circulation that failed to respond to antigen stimulation[243]. This phenomenon is observed in T cells activated in the absence of costimulatory signals (e.g., CD28 co-stimulation) or presence of

coinhibitory signals (e.g., CTLA-4 mediated inhibition), resulting in their unresponsiveness to subsequent stimulation[244].

Another mechanism of peripheral tolerance is the presence of tolerogenic antigen-presenting cells (APCs). DCs can modulate T cell response based on the costimulatory or coinhibitory signals they provide. DCs can promote tolerogenic response by delivering coinhibitory signals or not provide adequate costimulatory signals for T cell activation and proliferation. Healthy cells do not activate resting DCs, whereas direct stress, virally induced cytokines, and necrotic signals can activate them[245]. Tolerogenic DCs are not confined to a single DC subset but may be generated by incomplete maturation or induced in the presence of anti-inflammatory cytokines such as IL-10 and TGF-β[246]. Furthermore, repetitive stimulation of human naïve T cells with immature DCs can convert naïve T cells into IL-10 producing regulatory T cell subset, Tr1 cells, that do not express high levels of CD25 or canonical Treg transcription factor, Foxp3[247].

Of note, in addition to tTregs and pTregs, other Foxp3 negative regulatory T cell populations have been characterized to contribute to peripheral tolerance. A population of CD4+CD25-Foxp3- T cells was detected in the mesenteric lymph node and spleen when oral tolerance is induced and mediates suppression via TGF-β dependent mechanism. These cells express latency-associated peptide (LAP) on their surface and suppress T cell-mediated colitis and autoimmune encephalomyelitis[248,249]. Another subtype of peripherally induced Treg is the IL-10 producing type 1 regulatory T (Tr1) cells that are Foxp3 negative[250]. Foxp3 is not required for Tr1 induction or function since suppressive Tr1 cells can be generated and isolated from peripheral blood of IPEX patients, a disease due to Foxp3 mutations[251]. IL-10 producing Tr1

cells have been demonstrated in various immune-mediated diseases such as diabetes and celiac disease[252,253].

In summary, immune tolerance can be achieved by multiple mechanisms (both central and peripherally) to prevent excessive inflammation and autoimmune disease development.

Perinatal Immunity

According to the World Health Organization, the perinatal period (comprises both fetal and neonatal stages) is defined as the period before and a short period after birth. In humans, it starts during gestational week 22 up to 1 week after birth[254]. Using conserved key stages of neurodevelopment during fetal brain formation, the perinatal period in mice starts from embryonic day 9 (E9) and up to 7 days after birth[255,256] (Figure 6).

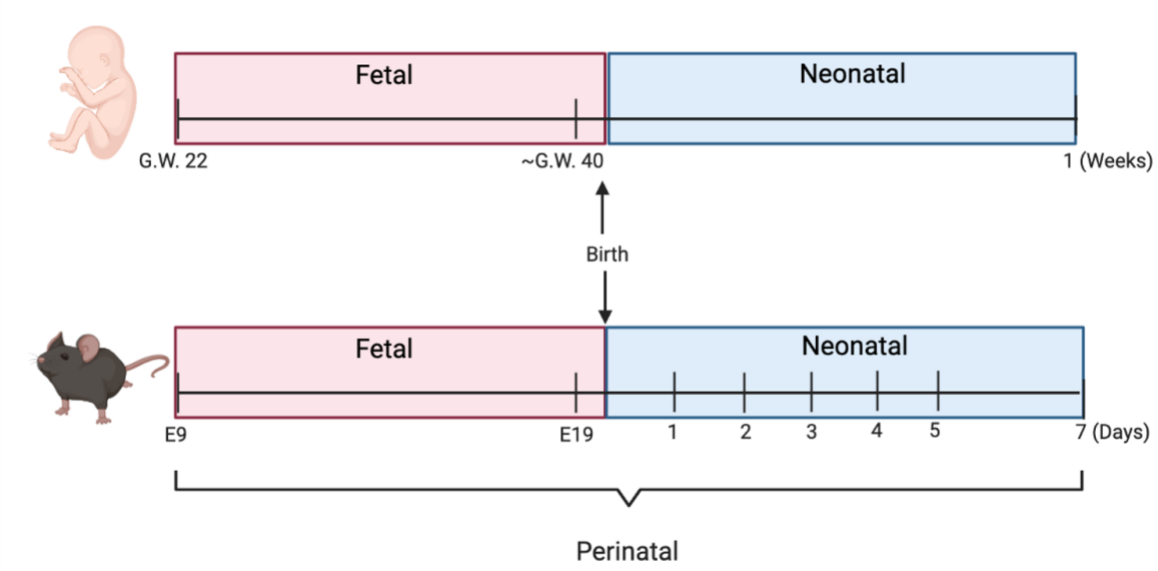

Figure 6: Perinatal Period in Human and Mice.

The perinatal immune system is phenotypically and functionally distinct from the adult immune system, which is highly tolerant. During development, the fetus is programmed to exist in a semi-allogeneic environment *in utero*. Postnatally, newborns must rapidly develop a functional system capable of tolerating non-harmful commensal microbes and antigens while

fighting off harmful pathogens. Therefore, the primary goal of the perinatal immune system is to attain tolerance, with reduced alloantigen recognition and poor responses against foreign antigens[238,241,257–259]. This tolerogenic propensity is important for preventing excessive inflammation when neonates are first exposed to benign antigens such as commensal microbes, maternal and food antigens. However, increased tolerance renders newborns highly susceptible to life-threatening infections, contributing to 40% of the 3 million annual worldwide neonatal deaths[260]. Many of these deaths are attributed to vaccine-preventable illnesses, but neonatal immunity's tolerant nature reduces vaccine efficacies such as measles, malaria, and polio in infants[261,262]. Transplacentally transferred maternal antibodies can provide some early protection against pathogens in neonates. Maternal antibodies against measles, rubella, and varicella can be detected in infants up to 4 months after birth[263]. However, this passive protection mechanism is short-lived and decays when the child is about six months of age.

Tolerance Establishment

In 1953, Billingham, Brent, and Medawar demonstrated tolerance against non-self can be established if introduced within a critical time window[241]. Failure to establish tolerance during this critical time window is reflected in the development of allergic disease or inflammation in adulthood.

In humans, the development of tolerance likely begins *in utero*. Maternal exposures to the farming environment increase Treg abundance and suppressive function in UCB [264,265]. Production of allergy-associated Th2 cytokines IL-5 and IL-13 are also lower in neonates from farming mothers[265]. Childhood exposure to the farming environment is correlated with the protection against allergic disease development in adult life[266,267]. Exposure of children younger than one year to stables and farm milk consumption is associated with lower frequencies of

asthma, hay fever, and atopic sensitization compared to those aged 1-5 years[266]. Subjects that lived on a farm during the first five years of life had the lowest prevalence of allergic rhinitis even among the oldest age group (61-75 years), suggesting the lifelong protective effect of childhood farm living[267].

The establishment of tolerance *in utero* is also essential to tolerate maternal antigens when the fetuses are developing in a semi-allogeneic host. Several studies have observed that hematopoietic cells of maternal origin can be found in the developing fetus. Maternal DNA can be detected in both the cellular and plasma fractions of UCB[268]. Further, maternal cells have also been detected to cross the placenta and reside in fetal lymph nodes[257]. This micro-chimerism can persist into adulthood where human leukocyte antigen (HLA)-disparate maternal cells have been detected in healthy adults[269].

Fetal exposure to non-inherited maternal alloantigens (NIMAs) induces the development of Tregs that suppress anti-maternal immunity and persist into adulthood. These fetal T cells proliferate substantially in mixed leukocyte reactions with another non-related adult APCs but have lower proliferation against maternal APCs. These differences were abrogated when fetal Tregs are depleted from the culture, demonstrating that fetal Tregs suppress proliferation against maternal alloantigen in an antigen-specific manner[257]. Tolerance towards NIMAs was observed in organ transplant recipients with higher graft survival if they received organs expressing maternal HLA antigens rather than paternal HLA antigens not inherited by the recipient[270,271]. Interestingly, newborns exclusively breastfed have a higher frequency of Tregs, reduced NIMA-induced T cell proliferation, and inflammatory cytokine production than those who were formula-fed. These data indicated that exposure of newborns to maternal cells through breastfeeding could also increase Treg frequency and promote tolerance against NIMA[272]. These

studies suggest a human equivalent of murine actively acquired tolerance demonstrated by Billingham, Brent, and Medawar back in 1953.

Tolerance against skin commensal microbe is established during the first to the second week of life in mice. Adult colonization of the skin commensal microbe (*S. epidermis*) led to subsequent skin inflammation, while at seven days after birth, colonization led to tolerance. This phenomenon coincides with the rapid influx of Tregs to the skin between six and thirteen days after birth. Blocking Treg migration to the skin abrogates tolerance establishment against *S. epidermis* in neonates[273].

In summary, establishing tolerance against harmless foreign antigens needs to occur within a critical development time window to prevent inflammation and atopic disease development.

Perinatal Immune System is Functional

Experiments demonstrating qualitative and quantitative differences in fetal and adult immune response demonstrated that the impaired newborn immune responses persist up to 18 months after birth[274,275]. The perinatal immune system is initially thought to be different from the adult immune system because it is inert, functionally impaired, or compromised due to limited antigen experience leading to insufficient immune response. It is now clear that the perinatal immune system is highly active but biased towards achieving a state of tolerance due to its developmental requirement.

In neonatal mice, it has been shown that protective CTL responses can be generated by murine retrovirus[276]. Furthermore, neonatal T cells can be activated *in vivo* if the antigen is presented by professional APC isolated from adult mice[277]. These data suggest that neonatal T cells are not intrinsically tolerant biased but became tolerant when non-costimulatory neonatal

APCs activate them. Lastly, depending on the immunization method, murine neonatal T cells can generate Th1 or Th2 T cell responses[278].

In humans, fetal thymocytes respond to PHA activation as early as 14 gestational weeks, providing evidence of immune competence in the fetus[279]. The human fetus can mount T cell response to some pathogens but result in antigen-specific tolerance towards others. For instance, fetuses exposed to mumps virus *in utero* developed persistent cellular immune responses as they developed delayed hypersensitivity towards inactivated virus skin test[280]. Neonatal T cells can elicit a Th1 type response like adult levels in certain vaccinations or maternal infections. Bacillus Calmette-Guerin (BCG) vaccinations at birth trigger a Th1 memory response of a similar magnitude compared to when it is given later in life[281]. CD4+ T cells from infants vaccinated with BCG at birth produced similar concentrations of IFN-γ as compared to immune adults[282]. UCB from helminth infected or mycobacterial-sensitized mothers produced more antigen-specific cytokines (IL-5, IL-10, and IFN-γ) than those from uninfected or non-sensitized mothers[283]. Infants exposed to malaria *in utero* have elevated frequencies of CD4+ effector memory T cells. Higher frequency of their CD4+ and CD8+ T cells express IFN-γ and TNF in response to malaria antigens[284]. These findings suggest that fetuses and neonates can generate functional and protective pathogen-specific T cell responses like adults under certain infections and vaccination.

In contrast, T cells from babies with congenital toxoplasmosis failed to proliferate and produce IFN-γ and IL-2 when cultured with toxoplasma lysate antigens compared to T cells from infected adults. This lack of response by these neonatal T cells is antigen-specific because they achieve similar or greater response to concanavalin A activation or mixed leukocyte reaction as adult T cells[285]. These data demonstrate that perinatal T cells can generate an immune response

depending on the pathogen. It is still unclear why specific pathogens can elicit a response while others do not.

Perinatal versus Adult Immune System

While the perinatal immune system is functional, some critical differences between the perinatal and adult immune systems contribute to the tolerant biased response by fetuses and newborns.

Innate Immunity

Several studies indicated that antigen-specific priming in the fetus could occur from around 20-22 gestation weeks[286,287]. However, circulating monocytes at all gestational ages express reduced levels of MHC class II molecules compared to adults, leading to impaired antigen-presenting activity[288]. DCs are professional APCs required for the initiation of an immune response. In their immature form, DC capture, process, and presents antigens on MHC-peptide complexes at their surfaces upon activation[289]. While APB and UCB DCs have comparable immature phenotypes, the upregulation of surface HLA-DR and CD86 costimulatory molecules was significantly diminished in UCB DCs upon LPS activation[290]. The surface expression of CD80, CD86, and HLA-DR did not reach until adult levels around 6-9 months after birth for monocytes and plasmacytoid DC (pDC)[291,292].

In addition to antigen presentation, APCs can modulate the immune response generated to be inflammatory or anti-inflammatory by cytokines they produced after activation[293]. CD8α+ DC promotes Th1 responses *in vivo,* while CD8α- DC promotes a Th2 type profile. This phenomenon was due to the high levels of IL-12 production by CD8α+ DCs[294]. The induction of Th1 CD4+ T cells by UCB DCs was significantly lower compared to their adult counterparts[290]. The lower Th1 induction is due to a decrease in IL-12 and IFNα/β production by UCB DCs[290,292].

LPS stimulation of UCB mononuclear cells demonstrates hyperproduction of IL-6, IL-8, and IL-10 while IL-12, IFN-γ, and TNF were significantly lower. This hypoproduction of IL-12, IFN-γ, and TNF persist up to the first six months after birth compared to adults[291]. Additionally, while human fetal classical dendritic cells 2 (cDC2) are capable of migrating to lymph nodes, respond to toll-like receptor ligation and induce adult CD4+ T cell proliferation like adult cDC2s, they limit the ability of adult CD4+ T cells to produce inflammatory cytokines through the upregulation of arginase-2 in a coculture set up[295].

Another component of the innate immune system is natural killer (NK) cells. NK cell functions are tightly regulated in the presence of activating and inhibitory receptors. For instance, CD94/NKG2A is an inhibitory receptor, while CD94/NKG2C is an activating receptor capable of binding the same human leukocyte antigen-E (HLA-E) molecule[296]. Neonatal NK cells express higher levels of the inhibitory receptors CD94/NKG2A and lower capacity to lyse cognate target cells without undergoing differentiation compared to adult NK cells[297,298]. Furthermore, UCB NK cells also have diminished degranulation ability and IFN-γ production[299,300].

γδ T cell compartment. Another difference between the perinatal and adult immune systems is the γδ T cell compartment. γδ T cells are highly represented in young animals in many animal species and are disproportionately crucial for immune protection[48,301]. γδ T cells are essential for effective primary response against *E. vermiformis* and *Cryptosporidium parvum* in younger mice but not as prominent when adult mice were infected[302,303]. γδ T cells deficient weanlings showed a delayed acquisition of immune resistance to *E. vermiformis* and *Cryptosporidium parvum* compared to their wild-type counterparts. The requirement for γδ T cell for immune protection is no longer observed when adult mice were challenged[302,303].

The γδ V gene usage has been reported to differ between human neonates and adults as well. UCB γδ T cells predominantly express Vδ1 while adult PBMC predominantly expresses Vδ2 [304]. Overrepresentation of Vγ9Vδ2+ γδ T cells in adults was previously thought to reflect an antigen-specific selection process resulting from postnatal exposure to pathogens. Surprisingly, it is found that Vγ9Vδ2+ are the predominant circulating γδ T cell subset in the second-trimester fetus, and its frequency decreases with gestation age. These fetal circulating Vγ9Vδ2+ γδ T cells were phosphoantigen reactive and showed a preprogrammed effector potential with the capability to produce Granzyme A, Granzyme K, and IFN-γ[305]. These data suggest that different γδ T cell subsets can become predominant at different developmental stages and may carry important functions that remain to be elucidated.

In humans, γδ T cell clones from UCB and pre-term peripheral blood produce higher levels of IL-4, IL-5, IL-10, and lower levels of proinflammatory cytokine IFN-γ compared to adult γδ T cell clones upon antigen receptor stimulation. On the other hand, IFN-γ production by neonatal γδ T cell clones achieved similar levels to adult γδ T cell clones when the clones were stimulated with PMA and ionomycin[306]. These data suggest that neonatal γδ T cell clones can produce adult levels of effector cytokines depending on the type of stimulation. Gibbons *et al.* further dissected if the differences in cytokine production between neonates and adult γδ T cells is due to the proportion of γδ T cells with different TCR v gene usage. They found that adult Vγ9Vδ2+ and Vδ1+ clones produce none or very low levels of IL-10 in comparison to neonatal Vγ9Vδ2+ and Vδ1+ clones, suggesting some age-dependent change in function regardless of V gene usage[306].

In summary, while perinatal and adult γδ T cells carry specific differences, γδ T cells are well positioned before and during birth to contribute to both immuno-protection and immuno-regulation properties.

Adaptive Immunity

The perinatal immune system is generally considered to produce a Th2 biased response upon antigen stimulation. In murine studies, neonatal T cells produce higher levels of IL-4 in response to TCR stimulation compared to adult T cells[307]. This group subsequently demonstrated that this Th2 biased response rapidly declines five days after birth to levels similar to adult T cells[308]. In contrast, human perinatal T cells do not predispose to a Th2 response but a more defective Th1 response. UCB naïve T cells produce significantly lower levels of IFN-γ in comparison to adult naïve T cells under neutral conditions[309]. UCB T cells also secrete higher levels of anti-inflammatory cytokine IL-10 upon TCR stimulation under neutral conditions while their adult counterparts do not[310]. However, under Th1/Th2 polarizing conditions or in a TCR-independent manner, UCB and murine T cells can elicit Th1 and Th2 cytokine production similar to or higher than adult-levels[309,311].

Studies in both humans and mice demonstrated a deficiency in CD8+ T cell functionality and magnitude of T cell responses during the perinatal stage. In mice, neonatal CD8+ T cells are deficient in their *in vivo* response against influenza, herpes simplex virus, and respiratory syncytial virus (RSV)[312–314]. This defect is also observed in humans, where infants with fatal RSV and influenza virus show a near absence of CD8+ CTL response[315]. Neonatal CD8+ T cells produce significantly less granzyme B, IFN-γ, and IL-2 than adult CD8+ T cells[316]. Additionally, neonatal CD8+ T cells failed to differentiate into memory T cells but preferentially gave rise to short-lived effector T cells[317]. CD8+ T cells by neonates proliferate and differentiate more

rapidly and demonstrate increased death compared to their adult counterparts following infection[318]. These data highlight intrinsic cell differences in the division and differentiation program by neonatal CD8+ T cells. CD8+ T cells in neonates displayed a distinctive transcription and chromatin landscape, enriched with gene expression signatures characteristic of innate immunity[316].

Increase propensity for Treg development by perinatal T cells. The contribution of perinatal tolerance by Tregs has been shown in humans where maternal alloantigen can promote Treg development and subsequent tolerance that persists into adulthood[257,270,271]. Treg-mediated peripheral tolerance is required during fetal development as the initiation of autoimmunity in IPEX coincides with the emergence of T cells in the second trimester of fetal development[319]. Tregs comprise a significantly greater percentage of total peripheral CD4+ T cells in fetuses (at 25th gestational weeks) than healthy infants and adults (~15% vs. ~5%)[320]. tTregs were detected as early as the 13th gestational week along with the first mature T cells, while extrathymic CD4+CD25+ Tregs were present from the 14th gestational week onwards in human[321,322].

Naïve T cells that are isolated from fetal lymphoid organs preferentially differentiate into functional Tregs upon antigen stimulation are more responsive to allogeneic stimulation than adults, where they proliferate and adopt a Treg fate[257,323]. This enhanced propensity could be due to the elevated expression of various TGF-β family members in fetal lymph nodes compared to adult lymph nodes[257]. However, a higher frequency of naïve T cells isolated from fetal lymphoid organs differentiate into Tregs and express higher Foxp3 than adults upon antigen receptor stimulation[324]. The addition of exogenous TGF-β further enhanced Treg differentiation in both fetal and adult T cells populations. Still, a higher frequency of fetal T cells differentiates into Treg in all conditions[324]. This enhanced Treg differentiation may partly be due to having a higher

sensitivity to TGF-β as indicated by higher levels of SMAD2/3 phosphorylation and the increased expression of Lin28b, a repressor of microRNAs that target TGF- β signaling mediators by fetal naïve T cells [324].

UCB naïve CD4+ T cells also differentiate into Foxp3+ Tregs at a higher frequency than adult naïve CD4+ T cells after antigen receptor stimulation[325]. The Foxp3+ Treg induction is further increased in UCB but not APB naïve T cells in the presence of progesterone. This hormone is implicated in dampening immune responses against fetal and maternal antigens[326]. Furthermore, gene microarray analysis between fetal and adult naïve CD4+ T cells demonstrate substantial differences[323,327]. Fetal naïve T cells share a partial transcriptome and epigenome like adult tTregs, further suggesting that fetal naïve T cells are more poised for tolerance[327].

In mice, the increased propensity of perinatal CD4+ T cells to differentiate into Foxp3+ T cells upon TCR stimulation is also observed. Wang *et al.* demonstrated a higher frequency of sorted CD4+ Foxp3-/GFP- from neonatal thymus and spleen converted into Foxp3+/GFP+ Treg cells upon TCR stimulation than adults without the addition of exogenous TGF-β. They demonstrated that this intrinsic default of Treg generation by neonatal T cells inversely correlates with age, decreasing to adult levels by two weeks after birth[328].

Together these studies suggest both cell-intrinsic and extrinsic mechanisms promote Treg generation from perinatal T cells to establish tolerance.

Soluble Factors

Soluble plasma factors appeared to carry immunosuppressive effects in infants. The blood plasma of newborns contains significantly higher levels of adenosine compared to adults. Adenosine is shown to inhibit toll-like receptor-mediated TNF production by newborn monocytes while preserving IL-6 cytokine production, a cytokine with anti-inflammatory and

Th2 polarizing properties[329,330]. Thus, high adenosine levels may further promote an overall anti-inflammatory milieu in neonates.

Relative to adult plasma, neonatal plasma demonstrated a gestational-age-dependent inadequacy in multiple anti-microbial proteins and peptides. The release of bactericidal/permeability-increasing protein (BPI) by neonatal polymorphonuclear leukocytes is significantly lower than adults[331]. Anti-microbial proteins and peptides such as BPI levels, calprotectin, LL37, and secretory phospholipase A2 (sPLA2) of full-term newborns are also significantly lower than their maternal counterparts[332].

Thymus differences

During gestation in the mouse, the thymic rudiment is first colonized by hematopoietic precursors originating from the fetal liver or the aorta-gonad-mesonephros around E12-14[333–336]. T cells leaving the thymus during the first two weeks of life appeared to be derived from the first wave of lymphoid precursors that seeded the fetal thymus around E10-12[337]. The thymus is colonized by a second wave of precursor cells sometime after 12 days of fetal life, subsequently giving rise to the second generation of thymocytes two weeks after birth[337]. These suggest that the peripheral T cell pool can arise from different waves of lymphoid precursors seeding the thymus during ontogeny, which could be another potential mechanism contributing to perinatal tolerance.

Peripheral CD4+ T cells derived from fetal versus adult thymic precursors are functionally and phenotypically different. Adkin *et al.* generated thymic chimera by implanting fetal thymic lobes in adult host mice and found that fetal-derived T cells produce higher levels of both IFN-γ and IL-4 than adult-derived T cells in an antigen-specific manner[338]. In contrast, antigen response of CD4+ T cells comparing intact neonatal and adult mice demonstrate the

typical neonatal response where they are Th2 biased and produce fewer IFN-γ compared to adult[338]. These data suggest that the combination of fetal precursors and fetal thymic stroma are necessary to produce cells with fetal-like properties[339].

There are differences in cTECs and mTECs proportions between the first week of postnatal life versus the adult thymus. Quantification of cytokeratins, K5 expressed by mTECs, and K8 expressed by cTECs allowed the cTECs/mTECs ratio analysis. There is a predominance of cTEC in neonates in contrast to mTEC predominance in adults[340]. Histologic analysis revealed that the neonatal thymus has minimal medullary areas and unique vascular architectures than the adult thymus. This observation is consistent with immature thymocyte predominance in neonates[340]. Transcriptional diversity is also observed within mTEC and cTEC isolated from the fetus (from different gestational days), newborns, adults, and aged mice[341]. Age-dependent thymic microenvironment changes in the peptide repertoire, antigen processing, and presentation are thought to influence the negative selection and Treg development[342,343].

Furthermore, it is suggested that negative selection in neonates is inefficient[344,345]. Thymic selection threshold is higher in neonates and young versus adult mice, where thymocytes bearing TCRs with low affinity for self-peptide are not efficiently selected into the neonatal T cell repertoire[346]. Alternately, thymocytes with high reactivity against self-peptide MHC will be positively selected, leading to a skewed TCR repertoire in neonates with higher self-reactivity. Increased self-reactivity subthreshold has been linked to higher binding affinity and promiscuity against multiple foreign antigens[347–349]. The strength of TCR signaling during thymic selection is correlated with the cell surface expression of CD5[350]. Both neonatal conventional T cells and tTregs had significantly higher CD5 expression than their adult counterparts in mice[346]. Thus, increasing the thymic selection threshold in neonatal T cell development may endow the limited

TCR repertoire with a more remarkable ability to respond to multiple foreign antigens. Additionally, NTx one to four days after birth led to maintenance of TCR repertoire from early neonatal life that was usually deleted in the adult thymus. These thymectomized mice contain autoreactive T cell clones in the periphery that can expand, ultimately leading to autoimmune disease development[344].

Differences in Progenitor Cells

Previous work has suggested that the ontogeny of the immune system does not progress linearly from fetal to adulthood but rather stratified into layers of distinct immune cell development arising from different waves of hematopoietic stem cells[351]. This concept has been applied to observing different murine γδ T cells lineages and B cells that differ in their ontogeny and arise in succession[352,353]. Accumulating evidence proposes that neonatal T cells are a distinct T cell population with unique functional properties suited for their perinatal life requirements. It is hypothesized that they arise from a different pool of progenitor cells from adult T cells.

Fetal and adult thymocyte precursors can give rise to mature T cells that differ in life span and proliferative capacity in the periphery. Intra-thymic injection of fetal thymocyte precursors generated a different ratio of CD4+ to CD8+ T cells compared to those derived from adult thymocyte precursors[354,355]. Adkins *et al.* demonstrated that E14 and E17 fetal CD4-CD8- thymocyte produce similar outcomes with low CD4:CD8 mature T cell ratio. In contrast, E20 (approximately at birth) fetal progenitors produce more CD4+ T cells in the periphery, bringing the CD4:CD8 ratio closer to that produced by adult progenitors. This study further suggests a switch of CD4-CD8- thymocyte precursor potential at or near birth[355].

HSCs from fetal vs. adult can also give rise to cells that differ in their function and phenotype. Peripheral CD4+ T cells derived from fetal thymic precursors produce more IFN-γ

and IL-4 than adult-derived cells in an antigen-specific manner[338]. Human HSC from 18-22 gestational week fetuses has an increased capacity to generate Tregs during thymic maturation than adults[323]. Neonatal CD8+ T cells derived from fetal liver HSCs are inherently more proliferative and preferentially become short-lived effectors, while adult-derived CD8+ T cells form long-lived memory cells after infection[356]. Using a time-stamp method, this group subsequently observed that naïve CD8+ T cell population consists of a heterogeneous population distinguished by their developmental origin, transcriptional and epigenetic profiles[357]. Lastly, Vγ5 γδ T cells are the first wave of γδ T cells is detected in the thymus[48]. Ikuta *et al.* demonstrated that only thymic lobes repopulated with HSCs from the fetal liver can generate Vγ5 γδ T cells but not HSCs from adult bone marrow[358]. Altogether, these observations suggest that there are cell-intrinsic differences in progenitor cells between fetal and adult.

Transcriptome and Epigenetic differences

Epigenetic processes partly contribute preferential neonatal CD4+ T cell differentiation into Th2 effector cells. It was demonstrated that human UCB naïve CD4+ T cells have differential methylation patterns at their IFN-γ promoter regions than adult naïve CD4+ T cells. UCB CD4+ T cells carry a hypermethylation pattern in their IFN-γ promoter regions, and this is reflected with a 5 to 10-fold decrease in IFN-γ production compared to their adult counterparts. In contrast, there was little to no evidence of non-CpG methylation in the IL-4 promoter region in both UCB and adult naïve T cells[359].

Fetal naïve T cells have an increased propensity to differentiate into Tregs even in the absence of high levels of exogenous TGF-β suggestive of additional cell-intrinsic mechanisms such as a poised epigenome for Treg differentiation[324]. Fetal naïve T cells express higher levels of Helios and display higher chromatin accessibility at the Helios locus compared to adult naïve

T cells[327]. Ablation of Helios in fetal naïve T cells subsequently results in poor Treg differentiation and function, suggesting that fetal naïve T cells may have a poised predominant epigenome programmed for immune tolerance.

Recent studies have shown that neonatal CD8+ T cells are poised for rapid effector cell differentiation. CD8+ T cells of early developmental origins displayed increased chromatin accessibility to genes that favor effector cell differentiation such as Tbx21, Id2, Il2ra, Il15ra, and decreased accessibility to genes promoting naïve and memory cell development even without the presence of infection[357]. Furthermore, CD8+ T cells of early developmental origins also express a higher proportion of genes associated with short-term effectors, late effectors, and memory cells. CD8+ T cells of adult origin have a higher expression that characterizes naïve and late memory cells seen in RNA-sequencing[357]. This study corroborated the finding that neonatal T cells generate a comparable lung-localized response against influenza infection as adult T cells but subsequently reduce developing tissue-resident memory cells after the infection[360]. This response is T cell-intrinsic as neonatal T cells highly express transcription factors associated with effector T cell differentiation and function (Blimp1, Id2, and Tbet) as demonstrated using RNA-seq. Neonatal lung effector CD4+ T cells also revealed a T-bet signature, known to drive the generation of terminally differentiated, short-lived effector cells[361].

Ikaros Family of Transcription Factors

The Ikaros (*Ikzf1*) transcription factor was first cloned and characterized in search of a master regulator for T-cell development in 1992 by Georgopoulos *et al.*[362]. Subsequently, the other family members, Helios (*Ikzf2*), Aiolos (*Ikzf3*), Eos (I*kzf4*), and Pegasus (*Ikzf5*), were isolated and found to be functionally diverse. The Ikaros family of proteins are zinc-finger transcription factors. They contain conserved zinc-finger motifs consisting of a zinc atom in

specific coordination with four amino acids that are predominantly cysteine and histidine. This family of transcription factors is characterized by two sets of highly conserved C_2H_2-type (Cysteine-2-Histidine-2) zinc-finger motifs[363]. The first set of zinc-fingers are the amino-terminal (N-terminal) DNA binding zinc-finger domains, which facilitates the binding to specific DNA sequences throughout the genome. The next set of zinc-fingers are the carboxyl-terminal (C-terminal) zinc-finger domains for homo- and heterodimeric protein interaction with other Ikaros family members as well as other transcriptional regulators[364,365]. These proteins can both positively and negatively regulate gene expression through direct interactions with DNA and form transcriptional complexes with other proteins[366]. Ikaros, Aiolos, Helios, and Eos contain four DNA-binding zinc-fingers and are shown to recognize canonical sequence "GGAAA" while Pegasus has been found to recognize distinct DNA sequences "GNNNGNNG" due to its divergent N-terminus zinc finger domains [363,367].

Helios

Helios (*IKZF2*) was cloned in 1998 as a novel dimerization partner of Ikaros. At the chromosomal level, Helios is found to be in complex with Ikaros at centromeric heterochromatin regions of the T cell nuclei[368]. At the nucleosomal level, Helios is also found to associate with proteins of the nucleosome remodeling and histone deacetylase (NuRD) complex in a thymocyte cell line[369].

Helios expression. Helios was detected in all hematopoietic sites of the developing embryo, in adult HSCs, earliest lymphoid progenitors, and subsequently restricted to a subset within the T cell lineage[368]. Using Northern blot, Kelley *et al.* tracked Helios expression in different fetal organs throughout ontogeny. Helios is first detected in the yolk sac blood islands on day 8 of gestation (E8). Subsequently, Helios is detected in the fetal liver by E11. At E13,

Helios is detected in the epithelial lining of the gut and within the liver. Helios is expressed near the center of the thymus, epithelial linings of the esophagus, and the trachea at E16. By E17, Helios expression is found in several epithelial tissues such as the lungs, mouth, salivary glands, and skin but no longer detected in the fetal liver[368]. Although Helios is expressed in the lungs, liver, kidney, and brain at different stages of embryogenesis, it is not detected in adult tissues[368].

During hematopoietic development, Helios, Ikaros, and Aiolos have an overlapping but distinct pattern of expression that might underscore their specific regulatory roles during differentiation[368,370,371]. Helios mRNA is detected in CD4-CD8- DN to CD4+CD8+ DP subsets and declines as these become CD4+ and CD8+ SP thymocytes. Mature T cells in the periphery also have lower *IKZF2* mRNA expression compared to immature thymocytes. However, Helios mRNA is highly detected in skin and gut γδ T cells[368].

Helios was initially thought of as a specific marker for tTreg identification[372]. This conclusion is reached due to the identification of the earliest Foxp3+ T cells in the thymus during the first week of life exclusively express Helios, Foxp3+Helios- cells do not appear in the periphery until after the first week of life. Lastly, iTregs and pTregs do not express Helios. However, Helios is upregulated in both human and mouse CD4+ and CD8+ T cells after activation regardless of Foxp3 status[373]. Another study demonstrated that Helios is expressed by CD4+ T cells differentiating into Th2 and T follicular helper cells (Tfh) without parallel upregulation of Foxp3[374]. Helios is also induced in iTregs generated *in vitro* in the presence of APC and pTregs *in vivo* following antigen-specific immunization[375]. These studies demonstrate that Helios is not specific as a marker to distinguish tTregs from other T cell subsets.

Helios is also expressed by other T cell subsets such as CD8+ T cells and γδ T cells. About 50% of CD8+ Tregs (CD44+CD122+Ly49+) are shown to express Helios in mice[376].

They demonstrate that CD8+ but not CD4+ Tregs displayed reduced numbers in *IKZF2* deficient mice with no apparent signs of autoimmunity until 6-8 months of age. CD8+ Tregs target T helper cells through a Qa-1/peptide-T cell receptor interaction that prevents autoantibody-mediated autoimmune disease[169]. Helios deficient CD8+ Tregs failed to inhibit Tfh cells as demonstrated by increased antigen-specific IgG1 response, increased dsDNA, and Tfh cell number[376]. In addition to that, Helios binds mainly at promoter regions of target genes in both CD4+ and CD8+ Tregs[376]. Roughly one-third of freshly isolated γδ T cells from human peripheral blood express Helios but are Foxp3 negative. However, if Helios is directly involved in the immunoregulatory functions of γδ T cells is still unclear[377]. While the precise role of Helios in γδ T cells remains to be fully characterized, it is observed that the up-regulation of Helios in γδ T cells depends on the cytokine milieu and was more pronounced in response to TCR stimulation as compared to phosphoantigen[377].

The Function of Helios. Initial functional analysis of Helios in tumor cell lines suggested that Helios was a tumor suppressor. Human T cell leukemia and lymphoma cell lines and patients with T-cell acute lymphoblastic leukemia overexpressed short isoforms of Helios, which lacked three of the four N-terminal zinc finger domains[378,379]. Characterization of this short Helios isoform (Hel-5) reflects the loss of repressor function. It also carries dominant-negative effects by associating with full-length isoforms of Ikaros, Aiolos, and Helios, subsequently inhibit their DNA-binding activity[380]. Overexpression of the short Helios isoform lacking the N-terminal DNA binding domain in hematopoietic progenitor cells led to aggressive and transplantable T cell lymphoma in mice.

On the other hand, overexpression of full-length Helios in this model inhibits αβ T cell development at the DN stage within the thymus, resulting in increased frequencies of γδ T cells

and NK cells in the periphery[381]. Overexpression of the full-length Helios isoform in T cells also results in cell death[382]. Studies using the *in vivo* Helios-null mutation system demonstrate that the germline deletion of Helios is embryonically lethal on a B6 background but only almost entirely lethal if the mice were generated on a mixed background (129/Sv: B6)[383]. These Helios deficient mice live at least 22 months of age without overt signs of ill health, defects in T cell function, or Treg development[383]. Overall, these studies suggest that Helios is vital in maintaining normal homeostasis in T cell development.

Helios is important to maintain Treg stability and function[372,384–386]. Helios+ Tregs demonstrated more superior suppressive capability than Helios- Tregs in antigen-specific manner[387]. Treg-specific Helios deficiency led to lower levels of Foxp3 expression and increased effector cytokines such as IFN-γ, TNF, and IL-17 by these cells following immunization[376]. The transfer of Helios deficient Tregs failed to abrogate inflammatory bowel diseases (IBD) induced by effector T cells transfer into *Rag2*$^{-/-}$ mice[376]. Surprisingly, Treg-specific Helios knockout did not exhibit autoimmunity early like Treg defective mice. However, they eventually develop progressive systemic immune activation and hypergammaglobulinemia by 5-6 months of age[385]. Helios regulates IL-2 production by suppressing IL-2 gene transcription in Tregs through the cooperation of Foxp3[388]. Helios does not bind to the *Foxp3* locus in both CD4+ and CD8+ Tregs, while Foxp3 is found to occupy the *Ikzf2* locus in CD4+ Tregs[376,389]. T cell-specific Helios deletion did not affect Foxp3 expression, demonstrating that Helios is not required for Foxp3 expression[372]. Ectopic co-expression of Foxp3 and Helios in CD4+ and CD8+ T cells led to superior suppressive function and delayed xenogeneic graft-versus-host disease compared to Foxp3 alone[390].

Additionally, Helios is implicated in Treg differentiation by being a part of the epigenetic and transcriptional program that lowers the threshold for Treg differentiation and functional commitment. Helios is highly expressed by fetal naïve T cells, and CRISPR-Cas9 mediated deletion of Helios impaired fetal naïve T cell's intrinsic ability to differentiate into iTregs upon TCR activation in the absence of exogenous TGF-β[327]. Helios upregulates Treg-specific genes such as *Il10* and downregulates proinflammatory genes in fetal iTregs. Fetal iTregs deficient in Helios produce lower levels of IL-10 and concurrently increased levels of IFN-γ and IL-2[327]. Microarray data have shown that Foxp3 alone is insufficient to induce a complete Treg gene signature in mouse CD4+ T cells[391]. Expression of full-length Helios can further alter Treg signature and gene expression in cellular pathways[390]. Overall, these data highlight the role of Helios in Treg differentiation, function, and stability. However, mice with Helios deficiency in all T cells do not immediately exhibit autoimmunity (developed at 5-6 months of age) despite the defective suppressive effect of their Treg population[376]. These data suggest that Helios also carries essential functions in non-Treg subsets.

Ikaros

Ikaros is the founding as well as the most studied member of the Ikaros transcription factor family. It is abundantly expressed during early embryonic hematopoiesis, including HSCs, lymphoid, erythroid, and myeloid precursors, as well as various lymphoid and myeloid lineages in mice[362,392–394]. Ikaros can repress and activate gene expression by forming higher-order chromatin binding complexes such as histone deacetylase complexes (HDAC) and SWI/SNF complexes[395,396].

The critical role of Ikaros in hematopoiesis has been investigated in different transgenic mouse models and is implicated to be a lymphocyte-specific lineage factor. In mice that lack all

Ikaros isoforms, demonstrated a devoid of all fetal and most adult lymphoid lineages. Aberrant T cell development after birth and subsequent T cell lymphoma development was observed in these mice[397]. This selective defect in fetal and adult lymphoid compartments suggests that Ikaros are required for fetal HSCs development or differentiation into lymphoid lineages. At the same time, they are partially redundant for adult HSCs differentiation into some lymphoid compartments. In mice that express dominant-negative form of Ikaros (DNA-binding domain deficient) show more severe defects with complete absence of αβ and γδ T cells and NK cells, while their erythroid and myeloid lineages remained intact[370]. These data suggest that the unique pattern of Ikaros expression provides positive signals for lymphocyte differentiation and negative signals for other hematopoietic lineages.

Within mature T cells, Ikaros is known to play a role in CD4+ T cell differentiation. Ikaros inhibits Th1 differentiation by suppressing T-bet and IFN-γ gene expression[398,399]. Ikaros deficiency decreased Th2-specific transcription factors such as GATA-3 and cMAF and a subsequent increase in T-bet and STAT1 expression under Th2 polarizing conditions[398]. T cell-deficient in Ikaros also express lower levels of IL-10, and overexpression of Ikaros restore IL-10 production. Ikaros also directly binds to conserved regulatory regions of the *Il10* locus in Th2 cells, further supporting a role in IL-10 expression[400]. Ikaros is shown to play a role in cytokine responsiveness within CD8+ T cells. Expression of a dominant-negative Ikaros isoform within CD8+ T cells increases CD25 (IL-2Rα) expression and survival in the presence of IL-12[401]. Ikaros also suppresses CD8+ T cells autocrine IL-2 production and subsequent differentiation into IFN-γ producing CTL[402].

Aiolos

Cloned by the Georgopoulos group in 1997, Aiolos has the highest sequence homology to Ikaros and can heterodimerize with Ikaros. However, in contrast to Ikaros, Aiolos expression was not detected in HSCs or precursor cells[371]. The earliest stage where Aiolos was detected was in the pre-B cell and CD4-CD8- DN thymocyte stage. Aiolos expression is subsequently upregulated as these cells terminally differentiate[403]. Loss of Aiolos increases pre-B and immature B cell precursors and subsequent B cell lymphomas while T cell development is relatively unaffected in mice[404].

In mature T cells, Aiolos is implicated in Th17 differentiation by promoting Th17 associated genes, including IL-17a and IL-17f, in part by silencing IL-2 production[405]. Aiolos interacts with a known *Il10* regulator, Ikaros, which has been reported to regulate IL-10 expression in CD4+ T cells[371,400]. Furthermore, Aiolos is required for IL-10 production by CD4+ T cells upon TCR stimulation. However, overexpression of Aiolos did not further upregulate IL-10 production. These data suggest that Aiolos is associated with but not sufficient for IL-10 production by CD4+ T cells[406].

Eos

Eos and Pegasus were cloned around 1999[367,407]. Eos is highly related to Helios and can form heterodimers with the other Ikaros family members and homodimers with itself[367]. Eos expression is not confined to the hematopoietic system as it can also be detected in the liver and the developing nervous system[407]. In the hematopoietic system, Eos is express in myeloid and megakaryocyte cell lines[367]. Within CD4+ T cells, Eos may play a negative role in Th17 polarization as its inhibition by microRNA, miR-17, enhances Th17 differentiation[408]. Eos is a critical mediator of Foxp3-dependent gene silencing in Tregs. Mechanistically, Eos directly

interacts with Foxp3 and induces chromatin modification to suppress Foxp3-dependent gene expression in Tregs. Knocking down Eos in Tregs abrogates their suppressive function *in vivo* and increases effector cytokine gene expression[409].

Pegasus

Pegasus is the most divergent member of the Ikaros family and the least studied among them. Its expression is not restricted to only mature hematopoietic cells but also broadly expressed in the brain, skeletal muscle, heart, liver, and kidney in mice[367]. Pegasus recognizes specific DNA-binding sites from the other Ikaros family of proteins and represses gene expression[367].

Interleukin-10 (IL-10)

IL-10 was initially termed cytokine synthesis inhibitory factor (CSIF) when it was discovered to be produced by Th2 cells and suppress Th1 cytokine production[67]. It was subsequently discovered that IL-10 could be produced by both innate and adaptive immune cells in addition to Th2 CD4+ T cells, such as DC, monocytes, macrophages, B cells, CD8+ T cells, Th1 CD4+ T cells, Tregs, and Th17[410].

Irrespectively of the cellular source, IL-10 is generally accepted as an anti-inflammatory cytokine. IL-10 can inhibit CD4+ effector T cells differentiation and proliferation by downregulating MHC, intracellular adhesion molecule-1 (ICAM-1), and costimulatory molecules CD80/86 on APCs[411–414]. Downregulation of these costimulatory molecules can significantly affect the T cell activating capacity of the APC[413,415]. IL-10 can suppress the production of proinflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-8, M-CSF, IL-12, GM-CSF, TNF, G-CSF by APCs[416–419]. Furthermore, IL-10 can also inhibit IFN-γ production by

Th1 cells by acting on APC[420]. On the other hand, IL-10 can directly act on T cells by inhibiting T cell proliferation and cytokine production[421–423].

The anti-inflammatory role of IL-10 is important in maintaining immune tolerance in the gut. Mutations in IL-10 and its receptors were the first causal genetic defects discovered in very early onset-inflammatory bowel disease (VEO-IBD) pathogenesis[424,425]. VEO-IBD is a subset of IBD patients that developed IBD under six years of age[424]. IL-10 deficient mice developed spontaneous enterocolitis due to a hyperactive immune response against gut microbes[426]. Notably, IL-10 treatment in weanlings prevents IBD development but was not as effective when the same regimen was given in adult mice, suggesting that IL-10 production is vital to maintain tolerance early in life[427].

Alcohol Effects on the Immune System

It is well established that alcohol consumption alters both the innate and the adaptive immune systems in both human and animal models. However, the effects of alcohol are not as clear-cut and are dependent on the duration and the amount of alcohol consumed. Moderate alcohol consumption is associated with immune-stimulatory effects, while heavy or binge drinking results in impaired immune function. Based on the Dietary Guidelines of Americans by the U.S. Department of Health and Human Services and the U.S. Department of Agriculture, moderate alcohol consumption is defined as up to one drink per day for women and up to two drinks per day for men[428]. The Substance Abuse and Mental Health Service Administration (SAMHSA) defines binge drinking as four or more alcoholic drinks for women and five or more alcoholic drinks for men on the same occasion on at least one day in the past month. They further define heavy alcohol use as binge drinking on at least five days or more in the last 30 days[429]. Alcohol can modulate the immune system in a biphasic manner, but these effects remain poorly

understood due to a lack of systematic studies that examine the effects of multiple doses and duration of alcohol intake on the different aspects of the immune system.

Modulation of the Innate Immune System

Monocytes express toll-like receptor 4 (TLR4), a pattern recognition receptor (PRR) capable of recognizing endotoxin LPS on the surface of Gram-negative bacteria[430,431]. Upon TLR activation, monocytes get activated, differentiate, and produce inflammatory cytokines such as IL-1β, IL-12, IL-6, and TNF at the site of infection[432–434]. These events are dependent on the translocation of NFkB into the nucleus, subsequently leading to the production of these proinflammatory cytokines [433,434]. Studies have shown that alcohol can modulate LPS response by monocytes in a dose-dependent manner. Acute treatment of alcohol produces an immunosuppressive response by monocytes. For instance, pre-incubation of human monocytes with 25mM alcohol (~0.1g/dL blood alcohol concentration) for 24 hours inhibits NFkB activation in the presence of LPS, subsequently decreasing the production of TLR4-induced IL-1β, IL-6, and TNF[435]. Acute alcohol exposure led to increased IL-1R-associated kinase-monocyte (IRAK-M) expression, a negative regulator for IRAK-1 in human monocytes, and decreased NFkB activity in human monocytes[436]. Additional studies in monocytes and macrophages demonstrate that acute alcohol exposure induces the expression of heat shock protein 70 (hsp70) in the presence of LPS activation, which subsequently binds NFkB subunit p50 and decreases NFkB activity by inhibiting its nuclear translocation[435]. Overall, acute alcohol exposure of monocyte and macrophages *in vitro* decreases TLR-4 mediated proinflammatory cytokine production by inhibiting the NFkB pathway. Additionally, acute alcohol exposure can also inhibit other PRR signaling pathways such as TLR-8 mediated TNF production while increasing anti-inflammatory cytokine IL-10 by human monocytes[437].

These *in vitro* findings of acute alcohol exposure were recapitulated *in vivo* using mouse models. Serum cytokine levels 2 hours after a single administration of 6g/kg ethanol showed a decrease in IL-6, IL-12, and an increase in IL-10 in response to TLR2/6, TLR4, TLR5, TLR7, and TLR9 agonists administered intraperitoneal or intravenous at the same time as oral gavage of alcohol[438]. Using the same regimen, administering a single dose of alcohol also decreased host resistance to *E.coli*-induced peritonitis[438]. Consumption of 10% (w/v) ethanol *ad libitum* for two days in mice resulted in suppressed DC function by reducing bone marrow DC generation, IL-12 production, costimulatory molecule expression, and impaired capability to promote T cell proliferation[439].

In contrast to immunosuppressive effects by acute alcohol exposure, prolonged alcohol exposure increased inflammatory response. Prolonged incubation (7 days) of human monocytes with alcohol *in vitro* increases LPS-induced TNF production while maintaining IL-10 levels compared to acute exposure[437]. Prolonged alcohol exposure of monocytes and macrophages reduces hsp70 and IRAK-M expression in the presence of LPS activation, which subsequently increases NFkB activity and TNF production[435,436]. In a chronic drinking rodent model, ingesting 6.3% (v/v) ethanol for four weeks increased the serum's proinflammatory cytokines, IL-6 and TNF levels. This response is reflected by increased NFkB activation and subsequent IL-6 and TNF production by hepatic macrophages after LPS challenge *in vitro*[440]. In humans, peripheral monocytes isolated from alcoholic hepatitis patients also demonstrated a significant increase in TNF production in response to LPS challenge *in vitro* compared to healthy controls[441].

Finally, the dose of alcohol also modulates immune function differently. Primary murine alveolar macrophages cultured in increasing amounts (25mM-100mM) of ethanol for 24 hours before the addition of apoptotic cells demonstrated a dose-dependent decrease in efferocytosis,

the process of clearing dead cells[442]. Human monocytes isolated from a 30-day moderate alcohol drinking regimen exhibit increased phagocytic and intracellular bactericidal activity when incubated with fluorescence-labeled *E.coli*[443].

In summary, alcohol can modulate the function of innate immune cells in a dose and time-dependent manner. Acute exposure led to the suppression of innate immune cell function, while prolonged exposure to alcohol led to increased proinflammatory responses by innate immune cells (Figure 7).

Innate Cells	Acute Alcohol Exposure	Chronic Alcohol Exposure
 Monocyte	↓proinflammatory cytokine ↑anti-inflammatory cytokine ↓pathogen clearance	↑proinflammatory cytokine ↑ Phagocytosis
 Macrophages	↓proinflammatory cytokine ↑anti-inflammatory cytokine ↓efferocytosis	
 Dendritic Cells		↓proinflammatory cytokine ↓ DC generation ↓ costimulatory molecules

Figure 7: Modulation of Innate Immune System by Alcohol.

Modulation of the Adaptive Immune System by Alcohol

Alcohol can also modulate cell-mediated and humoral immunity in a dose- and time-dependent manner, although studies with systemic comparison across doses and durations are scarce. Moderate consumption of beer for 30 days in a human study resulted in a significant increase in the number of leukocytes and mature T cells in women[444]. Moderate alcohol consumption is implicated in enhancing the immune response against infection where it is associated with a lower incidence of common cold and better vaccination response [443,445–449]. A cross-sectional study of heavy (90-249 drinks/month), moderate (30-89 drinks/month), or light

(<10 drinks/month) male drinkers demonstrated a dose-dependent effect on both cellular and humoral immunity[450]. In this study, as alcohol consumption increases, there is a decrease in B cells and CD8+ T cell frequency while CD4+ T cell frequency, CD4/CD8 ratio, and IgA and IgM level increase[450]. Another human study across men and women alcohol drinkers (abstainers, light, moderate and heavy drinkers) also found a dose-dependent increase in serum IgA levels[451]. Treatment of a mouse hybridoma cell line with increasing doses of ethanol for 48 hours demonstrated a dose-dependent increase in IgM production[452].

Chronic alcoholics without liver disease demonstrated a decreased CD4/CD8 ratio in their periphery[453]. Multiple chronic drinking rodent models revealed a reduction in total T cell frequency and a progressive loss of both CD4+ and CD8+ T cells[454,455]. In addition to the effects on lymphocyte frequency, chronic alcohol abuse also affects the T cell phenotype. Chronic alcohol users demonstrate a decreased percentage of naïve CD4+ and CD8+ T cells and an increase in memory T cell subsets[456,457]. This phenomenon is recapitulated in a rodent model where chronic ethanol consumption (20%) for up to 6 months causes a decrease in naïve T cell frequency and subsequent increase in memory T cell frequency[458]. Subsequently, the change in T cell subsets by chronic alcohol consumption led to elevated IFN-γ and IL-4 production by these T cells independent of a second costimulatory signal[458]. This shift from naïve to memory phenotype is due to homeostatic-induced proliferation *in vivo* as chronic alcohol consumption can induce lymphopenia[459,460]. The loss of naïve T cells could impair the development of effective response towards vaccines and against infectious pathogens, which may explain the increased susceptibility of chronic alcohol users to different infectious diseases[461–466].

In rodent models of chronic alcohol abuse, there is also an increase in morbidity and mortality against infectious diseases. Mice that consumed 20%(w/v) ethanol for 4-8 weeks

demonstrated impaired immune response against pulmonary influenza as seen in a decrease in pulmonary influenza-specific CD8+ T cell responses, increased mortality and morbidity, as well as virus titers[467]. This phenomenon was also seen in a *Listeria monocytogenes* infection model[468].

In addition to the shift from naïve to memory phenotype, chronic alcohol abuse also led to a significant increase in activated T cell frequency. Adult males who chronically use alcohol increased activated CD8+ T cell frequency as measured by increased HLA-DR expression[457,469]. This observation is recapitulated in rodent models where consumption of 20% (w/v) ethanol for up to 6 months displayed an increased percentage of activated T cells as measured by CD43, Ly6C expression, increased sensitivity to TCR stimulation, and a more robust IFN-γ response upon stimulation[458,459].

In summary, chronic alcohol exposure induces T cell lymphopenia, a shift from naïve to memory T cell phenotype, and increased immunoglobulin production. In contrast, moderate alcohol consumption is implicated in boosting the immune response and causes an increase in lymphocyte frequencies (Figure 8).

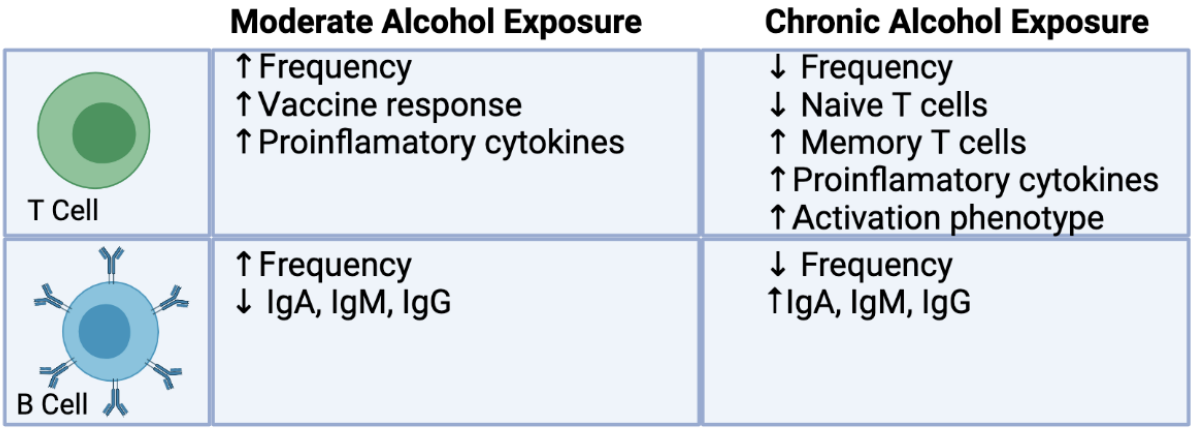

Figure 8: Modulation of Adaptive Immune Cells by Alcohol.

Alcohol Effects on T cell Signaling

Alcohol-related immunosuppression of cell-mediated immunity includes a higher incidence of sepsis, pneumonia, and tuberculosis[461–464]. Alcohol can also suppress T cell proliferation and IL-2 production in response to mitogen, suggesting possible impairment in transmembrane signal-transduction pathways during T cell activation[470–472]. *In vitro* ethanol incubation of human T cells led to decreased proliferative response to anti-CD3 and anti-CD2 accompanied by a decrease in intracellular Ca^{2+} mobilization and IP3 production[473]. This phenomenon is also observed in T cells isolated from alcoholic liver cirrhosis (AC) patients[473]. Furthermore, human primary CD4+ T cells and Jurkat T cells exposed to ethanol led to a dose-dependent decrease in PHA-stimulated IL-2 mRNA and protein levels. The reduction of IL-2 production by CD4+ T cells is due to the inhibition of NFAT upon anti-CD3/CD28 stimulation[474]. Pretreatment of Jurkat and primary CD4+ T cells with ethanol also decreased T cell stimulation mediated phosphorylation of early signaling molecules PLCγ1, Zap70, LAT, and Lck without changing total protein levels. The decrease activation of early TCR signaling molecules may be partly due to a decrease in TCR-activation mediated lipid raft colocalization of PLCγ1, Zap70, LAT, and Lck[474]. These data suggest that ethanol blocks early TCR signaling pathways and subsequently decreases IL-2 production.

Fetal Alcohol Exposure

Despite the well-known teratogenic effects of alcohol on the developing fetus, CDC reported 1 in 9 pregnant women drank alcohol in the past 30 days between 2015-2017. Among these pregnant women, one-third reported their engagement in binge drinking (defined as four or more alcoholic drinks on the same occasion on at least one day in the past month) with an average of 4.5 binge-drinking episodes in that 30-day period[475]. The teratogenic effects of

alcohol on fetuses were first described back in 1967. Lemoine *et al.* described a pattern of congenital disabilities including growth deficiency, low IQ, and psychomotor retardation[476,477]. The term "fetal alcohol syndrome" (FAS) was introduced to the medical community after several publications by Jones *et al.* describing patterns of birth anomalies, including craniofacial abnormalities, growth, and mental deficiencies due to prenatal alcohol exposure[478–480]. However, after years of research, it has become clear that FAS lies near the end of a spectrum of diseases and disorders, resulting in the new term, fetal alcohol spectrum disorders (FASD). The exact number of people with FASD is unknown, but CDC studies have identified that about 0.2 to 1.5 infants in every 1000 births have FAS in the United States[481]. Estimates for FASD based on National Institutes of Health-funded community studies predicted as high as 1-5% of school children in the United States[482–484]. While the most profound and widely known consequences of fetal alcohol exposure (FAE) encompass identifiable neurobehavior and overt dysmorphia outcomes, more recent reporting has uncovered subtle and long-term effects of alcohol on immune function.

FAS children have a higher autoimmune disease, infection, and malignancies rate, implicating significant impairments in cellular immune functions. Children exposed to alcohol *in utero* were associated with an increased risk of developing atopic eczema[485]. In animal models, FAE increases susceptibility to autoimmune diseases such as rheumatoid arthritis and type 2 diabetes[486,487]. Limited alcohol use during gestation increases the risk for neonatal infection by 2.5 fold, while excessive alcohol abuse further increases this predisposition another 3-4 fold[488]. Children exposed to alcohol prenatally have an increased incidence of bacterial infections such as otitis media, meningitis, pneumonia, gastroenteritis, sepsis, urinary tract, and upper respiratory tract infection[489,490]. Case studies also demonstrated an association of FAE with cancer

development, such as ganglioneuroblastoma, neuroblastoma, adrenal carcinoma, and hepatoblastoma[491–494]. FAS children also showed a lower proliferative response of their T cells, lower B and T cell numbers, and hypogammaglobulinemia[490].

Animal models recapitulate the immune deficit associated with FAS in humans as demonstrated by delayed T cell development and function. Marked retardation of thymus development in fetuses exposed to alcohol *in utero* is observed with a reduced thymus size and cellularity[495]. There is also a reduction in thymocyte and T cell proliferative response against mitogen and a decrease in T cell frequencies in both the spleen and the thymus[496–499]. These immune deficits have been shown to persists into adolescence and adulthood. Young adult mice exposed to alcohol *in utero* have decreased contact hypersensitivity and graft-vs-host responses[500]. Several studies also indicated that lymphocyte number and function in response to mitogen stimulation are also reduced in adult rats previously exposed to alcohol *in utero*[501,502]. Similarly, human adolescents exposed to alcohol *in utero* demonstrate impaired immune responses and an increased rate of atopic, hypersensitivity reactions such as skin rashes, asthma, and allergic rhinitis[503]. Adult mice exposed to alcohol only during gestation and nursing period displayed increased subsequent influenza virus infection severity during adulthood, which decreased influenza-specific CD8+ T cells in the lungs[504].

FAE can also modulate cytokine production in the fetus, generating an overall inflammatory cytokine profile. Chronic alcohol usage led to an increase in IL-1β, IL-6, and TNF in both the serum of the fetus and the mother[505]. TNF and IL-6 levels are upregulated in the placenta of pups exposed to alcohol *in utero*[506]. The pattern of cytokine milieu can determine Th differentiation. Therefore, alteration of cytokine profile due to FAE can alter the regulation of Th differentiation and maturation.

In summary, alcohol exposure during development can produce significant alterations in the immune system that can be detrimental and persists throughout life.

Purpose of Dissertation

It has been established that both the innate and adaptive arms of the perinatal and adult immune systems are phenotypically and functionally distinct[507–510]. The overall response of the perinatal immune system is highly tolerogenic, with reduced alloantigen recognition and poor responses against foreign antigens[238,241,257–259]. This tolerant nature is found to be a double-edged sword for infants and newborns. While it allows them to tolerate benign antigens such as food and commensal microbes, newborns are highly susceptible to life-threatening infections as they have low vaccination efficiency[261,262,511]. These life-threatening infections cause 40% of the 3 million annual worldwide neonatal deaths[260]. Therefore, it is essential to understand mechanisms underlying perinatal tolerance to generate better vaccines for infants. As reviewed above, multiple factors can contribute to perinatal tolerance, such as the increased propensity of perinatal T cells to differentiate into Foxp3+ Tregs and intrinsic properties of perinatal T cells that promote an anti-inflammatory response overall. The purpose of my dissertation is to investigate the mechanism by which perinatal T cells can contribute to immune tolerance in infants.

In our lab, we use umbilical cord blood as a model to study the fetal immune system. Our lab demonstrated that UCB T cells differentiate into Foxp3+ T cells upon antigen receptor stimulation. It is unknown if these *ex vivo* generated UCB Foxp3+ T cells carry suppressive functions *in vivo*. Furthermore, these *ex vivo* generated UCB Foxp3+ T cells have stable Foxp3 expression up to 62 days in culture[512]. It is unknown if these *ex vivo* generated UCB Foxp3+ T cells have similar Foxp3 epigenetics status to adult tTregs with stable Foxp3 expression[137,138].

We seek to characterize the Foxp3 epigenetic status of UCB-derived Foxp3+ T cells and determine their *in vivo* function utilizing a xenogeneic-graft-versus-host disease model. Alcohol consumption during pregnancy is known to cause immune dysfunction in infants. Since Foxp3+ Tregs are important for maintaining tolerance and preventing autoimmune diseases, we hypothesized that alcohol impairs the immune functions of neonates by blocking the development of these Foxp3+ T cells and enhancing proinflammatory conditions among infants.

Our lab also demonstrated that UCB naïve CD4+ T cells highly express surface antigen molecules such as CD26 and CD31, while APB naïve CD4+ T cells do not[309]. These data suggest that there are intrinsic differences between UCB and APB naïve CD4+ T cells. To further elucidate other mechanisms contributing to perinatal tolerance, we investigated transcription factors that regulate T cell function and may be differentially expressed between perinatal and adult T cells. We found that Helios, a member of the Ikaros transcription factor family, is significantly upregulated in newborn T cells compared to adult T cells in humans and mice. As reviewed above, Helios is typically associated with Foxp3+ Tregs and is required for Foxp3 stability and suppressive functions. Our findings demonstrated that the majority of perinatal Helios+ T cells do not express Foxp3. Therefore, we seek to characterize the origin and function of these Helios+Foxp3- perinatal T cells.

CHAPTER TWO: MATERIALS AND METHODS

Mononuclear Cell Isolation

Whole Umbilical cord blood (UCB) was kindly donated from Loyola University Medical Center from donors that meet our collection criteria. Donors are excluded if: 1. Evidence of active malignancies; 2. Use of medication that affects the immune system such as glucocorticoids and immunosuppressants; 3. Uncontrolled hyper or hypothyroidism; 4. Presence of autoimmune disease; 5. Presence of active infection. Total UCB was collected into blood collection bags containing a citrate phosphate dextrose solution. Mononuclear cells were enriched by density centrifugation using Lymphopure density gradient medium (Biolegend, San Diego, CA) and red blood cells were lysed with RBC lysis buffer (Biolegend). Pre-term neonate PBMC samples were collected in collaboration with Loyola University Health Center NICU department. The samples were processed similarly as UCB described above.

Antibodies

Antibodies used for flow cytometry were anti-human CD4, CD8, CD25, Foxp3, Zombie Aqua Fixable Viability dye, anti-mouse CD45, CD4, CD8α, CD8β, TCRγδ, TCRβ, Foxp3, IL-10, IFN-γ, anti-human/mouse Helios, anti-human/mouse/rat Foxp3, anti-mouse Ikaros (BioLegend), anti-mouse Foxp3, Eos (ebioscience, ThermoFisher Scientific, Waltham, MA), anti-mouse/human Aiolos (Cell Signaling, Danvers, MA). Functional grade antibodies for cell culture used were all obtained from BioLegend,: anti-human CD3 (clone OKT-3), anti-human CD28 (clone 28.2), anti-mouse CD3 (Clone: 145-2C11), anti-mouse CD28 (clone 37.51), anti-mouse IL-4 (Clone: 11B11), anti-mouse IFNγ (Clone: AN-18), anti-mouse IL-12 (clone C17.8)

Chemicals and Recombinant Proteins

Recombinant human IL-2 and recombinant mouse IL-4 were obtained from PeproTech (Rocky Hill, NJ). Recombinant mouse IL-12 (p70) (BioLegend). Molecular biology grade 200 proof (absolute) Ethanol was obtained from Sigma-Aldrich (St. Louis, MO).

Flow Cytometry

Surface stains were performed using standard staining protocols as described by the manufacturer. Intracellular transcription factor staining was performed using the True Nuclear Transcription Factor Buffer kit (Biolegend) following the manufacturer's protocol. Data were collected on FACS Canto II (BD Biosciences, San Jose, CA) or FACS LSRFortessa (BD Biosciences) and analyzed using FlowJo software (BD Biosciences).

Foxp3+ T cell Induction Culture

Total UCB mononuclear cells were stimulated with recombinant human IL-2 (10ng/ml; >100U/ml) and 0.2µg/ml anti-human CD3 in RPMI 1640 (GE Healthcare Hyclone) supplemented with 10% fetal calf serum, essential- and non-essential amino acids, penicillin/streptomycin and L-glutamine. Cells were split with a media change every 2-3 days, maintaining IL-2 concentrations. In ethanol-treated cultures, indicated concentrations of ethanol (200 proof for molecular biology) was added daily to obtain a final concentration of 50mM or 100mM for the first 5 days of culture. Foxp3+ T cell frequency was analyzed after 13-14 days of culture. For bead-based restimulation, day 14 UCB-derived Foxp3+ T cells (containing both CD4+ and CD8+ subsets) were cultured with polystyrene beads pre-coated with anti-human CD3 with or without anti-human CD28 antibodies (10µg/ml each) at a ratio of 4 beads per 1 T cell. $5x10^4$ cells were cultured for 5 days total in the absence and presence of 10ng/ml IL-2 and other cytokines (10ng/ml IL-4, 50ng/ml IL-6, 10ng/ml IL-1β, 10µM TGF-β receptor kinase inhibitor

SB431542) where indicated. For EtOH total UCB 24-hour bead-based stimulation, total UCB mononuclear cells were cultured in the presence of indicated EtOH with polystyrene beads pre-coated with 10µg/ml anti-human CD3 without IL-2. 24 hours later, cells were harvested and stain for CD25 expression.

Cytokine Profile Analysis

Cell supernatants were collected and analyzed for the expression of major T helper cytokines including IFN-γ, TNF, IL-2, IL-4, IL-5, IL-13, IL-6, IL-9, IL-10, IL-17a, IL-17f, IL-21, IL-22 using LEGENDplex Human Th Cytokine Panel (Biolegend) on BD FACSCanto II (BD Biosciences).

Suppression Assay

Suppressor cells were generated from UCB by stimulating them with anti-CD3 and IL-2 stimulation for 14 days as described above and Foxp3 expression was confirmed by flow cytometry. CD4+ and CD8+ T cells were separated by FACS sorting. tTreg were purified from the same UCB donor using $CD4^{+}CD127^{low}$ $CD25^{+}$ Regulatory T Cell Isolation kit STEMCELL Technologies (Cambridge, MA) and expanded *in vitro* using Immunocult from STEMCELL Technologies in the presence of 10ng/ml IL-2 for 14 days. Both *ex vivo* generated UCB Foxp3+ T cells and tTregs were labeled with 5µM carboxyfluorescein succinimidyl ester (CFSE, Biolegend). For responder cells, unstimulated naïve CD4+ T cells were isolated from allogeneic adult PBMC using naïve CD4+ T cell isolation kit and labeled with 5µM Tag-it violet (Biolegend) proliferation and cell tracking dye. CD14+ enriched cells were isolated using monocyte isolation kit (Biolegend) from the same adult PBMC donor and irradiate at 3000 rad to serve as APCs. Responder cells were stimulated with 0.2µg/ml anti-human CD3 and APCs at a 1:1 ratio, in the presence or absence of indicated ratio of suppressor cells. After 5 days, flow

cytometry was used to gate on all CFSE-negative CTV-positive responder cells to assess percent of proliferation. The percent inhibition of proliferation by suppressor cells for each sample were determined by [(percentage of cells divided without suppressor cells)- (percentage of cells divided with suppressor cells)]/ (percentage of cells divided without suppressor cells).

Xenogeneic Graft versus Host Disease (xGVHD)

xGVHD was established in NSG-S mice by intravenous transfer of total UCB mononuclear cells by tail vein injections. NSG-S mice were subjected to total body irradiation of 2.5gray, 24 hours before mixed $CD4^{+,}$ and $CD8^{+}$ UCB-derived $Foxp3^{+}$ T cells ($1x10^{\wedge 6}$ cells in 200ul PBS) or PBS control injection. 48 hours later, autologous UCB mononuclear cells ($1x10^{\wedge 6}$ cells in 200ul PBS) or PBS alone were injected intravenously by tail vein. Mice were monitored for weight loss and sacrificed when weight dropped to <15% of the original weight or after 35 days post transfer if they did not reach criteria.

Bisulfite Sequencing

Only male donors were used as all female cells carry a methylated allele from one of the X chromosomes. For adult PBMC Treg cells, cells were freshly isolated using the EasySep human $CD4^{+}CD127^{low}$ $CD25^{+}$ Regulatory T Cell Isolation kit (STEMCELL Technologies). UCB-derived Treg cells were induced by culturing total UCB in the presence of anti-CD3 antibody and IL-2 for a total of 14 days and sorted for $CD4^{+}CD25^{+}$ cells on day 14 (only donors that achieved >80% $CD25^{+}Foxp3^{+}$ were used; data not shown). iTreg cells were generated by using $CD4^{+}$ T cells from UCB (CD4 enrichment kit BD iMags) cultured in the presence of IL-2, TGF-β, and ImmunoCult CD3/CD28/CD2 T cell activators (STEMCELL Technologies) for 6 days and sorted for $CD4^{+}CD25^{+}$ (only donors that achieved >80% CD25+Foxp3+ were be used). Effector T cells were cultured in the presence of IL-2 and ImmunoCult CD3/CD28/CD2 T cell

activators (STEMCELL Technologies) for 14 days and sorted for the $CD4^{+}CD25^{-}$ population. DNA isolation was conducted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and bisulfite conversion was conducted following the manufacturer's protocol using Single-step EZ DNA methylation-Direct Kit (Zymo Research, Irvine CA).

Assay for Transposase-Accessible Chromatin (ATAC)-Sequencing

Only male donors were used as all female cells carry a methylated allele from one of the X chromosomes. UCB-derived Foxp3+ cells were induced by culturing total UCB in the presence of anti-CD3 antibody and IL-2 for a total of 14 days and FACs sorted for $CD4^{+}CD25^{+}$ cells on day 14 (only donors that achieved >80% $CD25^{+}Foxp3^{+}$ were used; data not shown). Sorted cells were transported to NuSeq Core Facility at Northwestern University for library preparation and sequencing. Paired-end sequencing was conducted using NextSeq, 37x37bp reads, 200M read pairs. 3' adapter sequences are R1= AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC and R2= AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTA. ATAC-seq reads pre-processing were conducted using Galaxy. The quality of DNA reads in fastq format was evaluated using FastQC, adapters were trimmed using Trim galore. Poor quality reads duplicated reads and reads that aligned to mitochondrial DNA were filtered. The cleaned reads were aligned to *Homo sapiens* genome (hg38) using Bowtie2. Peaks were called and annotated using MACS2. Adult tTreg raw data were mined and process and analyze similarly as our UCB *ex vivo* generated CD4+Foxp3+ samples from published work by Ng *et al*[327].

Mice

C57BL/6 mice were obtained from Jackson Laboratories and bred in-house. Mice were euthanized by CO2 if they are >10 days old or decapitated if <10 days old and dissected by

sterilized scissors to obtain the spleen. Splenocytes were obtained from the different ages indicated where day of birth was called day 0. The spleen was grinded by Frosted Microscope Slides (Fisher Scientific, Waltham, MA) to obtain splenocytes. The cells were then washed once with 2% FCS RPMI and filtered through a cell strainer (DOT Scientific, Burton, MI) to obtain a single-cell suspension. Time pregnancies were set up to collect E17.5/18.5 fetal samples. The day after time pregnancy set up was set as E0.5.

Naïve CD4+ T Cell Isolation

Naïve CD4+ T cells were isolated from UCB or APB mononuclear cells via negative selection using EasySep™ Human Naïve CD4+ T cell Enrichment Kit (STEMCELL Technologies). Naïve CD4+ T cells were isolated from total splenocytes via negative selection using MojoSort™ Mouse CD4+ Naïve T Cell Isolation Kit (BioLegend)

CRISPR-Cas9 Editing

CRISPR-Cas9 editing was conducted using Neon transfection system (ThermoFischer Scientific) according to manufacturer's protocol. Briefly, UCB naïve CD4+ T cells were isolated and activated with 5µg/ml anti-human CD3 and 5µg/ml anti-human CD28 in the presence of 10ng/ml IL-2 for 3 days. After 3 days of activation, cells were harvested and resuspended in buffer R at $4x10^5$ cells/10µl. 610ng of gRNA was incubated with 1µg Invitrogen TrueCut Cas9 Protein v2 (ThermoFischer Scientific) for 15-20 minutes, room temperature, before adding 5µl of cell mixture to generate a final concentration of $2x10^5$ cells per 10µl transfection reaction. The cell and Cas9/gRNA complex mixture were pipette into Neon 10µl tip and transfected using program #24 (1600V/10ms/3 pulses). After transfection, cells were immediately transferred into a 48-well containing 1ml of pre-warmed antibiotic free RPMI 1640 supplemented with 10% fetal calf serum, essential- and non-essential amino acids, and L-glutamine for 2 days. After 2 days,

cells were checked for knockout efficiency and ready for downstream experiments. Human *ikzf2* and non-target gRNA was designed using GUIDES. gRNA was then generated using Invitrogen GeneArt Precision gRNA Synthesis Kit (ThermoFischer Scientific) according to manufacturer's protocol.

T Helper Cell Differentiation

Isolated human naïve CD4+ T cells from UCB or APB were maintained in IL-7 for 7 days in IL-7, then were stimulated with 5µg/ml anti-human CD3 and 5µg/ml anti-human CD28 and differentiated using human Th1 Differentiation Kit for 5 days (CDK001, R&D Systems, Minneapolis, MN) according to manufacturer's protocol. 5 days after Th1 polarization, the cells were harvested, washed, and plated at $1x10^6$/ml in the absence or presence of 50ng/ml phorbol 12-myristate 13-acetate (PMA, Fischer Scientific) and 1µM Ionomycin (Sigma-Alrich) for 4 hours. After stimulation, the supernatants were harvested and analyzed for cytokine expression using Legendplex Human Th Cytokine Panel (BioLegend).

For CRISPR-cas9 gene editing experiments, isolated human naïve CD4+ T cells from UCB were subjected to Th0 or Th1 culture conditions 48 hours after transfection. $2x10^5$/ml cells were cultured in IL-2 (10ng/ml; >100U/ml) for Th0 and Human Th1 Differentiation kit (CDK001, R&D Systems) according to manufacturer's protocol for 5 days. After 5 days in culture, cells were harvested, washed, and restimulated with PMA and Ionomycin for 4 hours at $1x10^6$ cells/ml.

Isolated mouse naïve CD4 T cells or total splenocytes (5-day old neonates) were cultured at $1x10^6$ per ml in 96 well flat bottom, non-treated plate (CELLTREAT, Fisher Scientific) coated with 5µg/ml anti-mouse CD3 and 2µg/ml soluble anti-mouse CD28, under neutral, Th1 or Th2 conditions for the first three days. For the first three days of culture, neutral conditions: no

additional cytokines or antibodies, Th1: 10ng/ml IL-12, 10µg/ml anti-IL-4, Th2: anti-IFNγ 10µg/ml, anti-IL-12 2µg/ml, IL-4 10ng/ml. After three days, the cells were equally divided into 96 round bottom tissue culture treated plate (DOT Scientific) uncoated with anti-CD3 and continue culture for additional two more days. The cells were supplemented with these cytokines for the rest of the culture, neutral conditions: 10ng/ml IL-2; Th1 condition: 10ng/ml IL-12 and 10ng/ml IL-2; Th2 conditions: 10ng/ml IL-4. At day 5, the cells were harvested, washed, and subjected to PMA and Ionomycin stimulation for 5 hours. The cells were plated at $1x10^6$ cells/ml and 1X Monensin (Biolegend) was added at final two hours of the restimulation culture. After restimulation, the cells were stained for intracellular IL-10 and Helios.

Intraepithelial Lymphocytes (IEL) and Lamina Propria Lymphocytes (LPL) Isolation

IEL were isolated from the mouse small intestine and colon as described by Yamamoto *et al* with modifications[513]. Briefly, the small intestine and colon were removed, and fecal matter, fat, mesentery lymph nodes and payer's patches were removed. The intestinal tissues were open longitudinally and cut into 1cm pieces. The tissues were incubated in calcium- and magnesium-free 10% HBSS supplemented with fetal calf serum, 10mM HEPES and 1mM Dithiothreitol (DTT, Sigma Alrich) rocking for 30 minutes in 37˚C. The tissues were then vortex vigorously and intestinal IEL were obtained by filtration of the supernatant through 100µm filter. To isolate LPL, the remaining tissues were further cut into smaller pieces, and incubated in 10% HBSS supplemented with 0.1mg/ml DNAseI (Roche, Basel, Switzerland), 2mg/ml Collagenase D (Sigma Aldrich) and 0.17U/ml Dispase (Gibco, Thermo Fisher Scientific), rocking for 30 minutes in 37˚C. The digest was stopped with 10% HBSS supplemented with 5mM EDTA and cells were obtained by filtration of the supernatant through 100µm filter. Purified IEL were

obtained by harvesting the cell interphase of 40/70% Percoll (Cytiva, Marlborough, MA) centrifugation and LPL were obtained from 40/80% Percoll centrifugation.

Generation of Humanized Mice

NSG-S mice were subjected to total body irradiation of 2.5gray, 24 hours before CD34+Lineage- HSCs (using anti-human lineage antibody cocktail: CD3, CD14, CD16, CD19, CD20, CD56, Biolegend) FACS sorted from UCB were transferred via tail vein injection intravenously. A total of around 10,000-100,000 HSCs were injected in 200µl PBS depending on the donor and final sorted HSCs number. The mice were monitored and human CD45 reconstitution were checked 4 weeks after transfer. After human CD45 cells reconstitution is confirmed, humanized mice were sacrifice between 10-21 weeks post UCB HSCs transfer.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (San Diego, CA). The following designation was used throughout the dissertation: *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

CHAPTER THREE: RESULTS

Section I: Epigenetic and Functional Analysis of UCB-derived Foxp3+ T Cells.

The developing human fetus has a unique ability to tolerate benign and necessary antigens, including self, environmental, and food antigens that are transferred across the placenta[511]. Multiple mechanisms contribute to fetal tolerance, one of which is the strong tendency of fetal CD4+ T cells to differentiate into Foxp3+ regulatory T cells (Treg)[258]. The tolerant nature of fetal immunity is beneficial to prevent excessive inflammation against the vast array of foreign antigens that they encounter after birth. However, this also makes them vulnerable to succumb to infections caused by invading pathogens[514]. For this reason, neonatal immunity needs to adapt to the complex demands of tolerance against harmless environmental antigens, while maintaining protective immune responses against pathogens[515]. A deeper understanding of the nature of the developing human immune system will provide pivotal knowledge to develop safe and effective strategies to protect infants from infection.

Generation of Foxp3+ T cells from Human UCB

To understand the cellular mechanisms that establish fetal tolerance, our lab utilizes umbilical cord blood (UCB) as a source of full-term fetal immune cells. In agreement with previous reports, UCB and adult peripheral blood (APB) displayed similar frequencies of pre-existing CD4+ T cells that express Foxp3, an essential transcription factor for the development and maintenance of Tregs, at day 0 (Figure 9A)[512,516]. Since Foxp3 plays a pivotal role in the establishment of immunological tolerance and the perinatal immunity is tolerant biased, and the

majority of UCB T cells carry a naïve phenotype[517–519], we hypothesized that UCB T cells preferentially differentiate into Foxp3+ T cells upon stimulation.

We stimulated total UCB mononuclear cells with anti-CD3 antibodies in the presence of IL-2. Activated human CD4+ T cells can transiently express Foxp3 up to one week after stimulation and takes two weeks to lose Foxp3 expression[102,520]. Therefore, to assess stable Foxp3 expression by UCB T cells, we continued this culture with IL-2 medium for 2 weeks. We found that both UCB CD4+ and CD8+ T cells acquire prototypic Treg cell markers, CD25 and Foxp3, while only a few APB CD4+ and CD8+ T cells under the same culture conditions acquire them (Figure 9B, C). These Foxp3+ T cells are not expanded from pre-existing Tregs, as removal of CD4+CD25+ T cells (majority of CD4+CD25+ cells also express Foxp3[521,522]) from UCB did not reduce the frequency of Foxp3+ T cell induction after anti-CD3 stimulation (Figure 9D, E).

Furthermore, these UCB-derived Foxp3+ T cells also express Helios, a transcription factor strongly associated with Foxp3+ Tregs and is important to maintain Treg stability and function[372,384–386] (Figure 9F). These data demonstrated that UCB T cells differentiate into Foxp3+ T cells *de novo* upon antigen receptor stimulation. Further mechanistic studies found that CD14+CD36hi monocytes in UCB provide TGF-β and retinoic acid to UCB T cells to promote Foxp3+ T cell differentiation (conducted by Dr. Jessica Lee MD, Ph.D)[512].

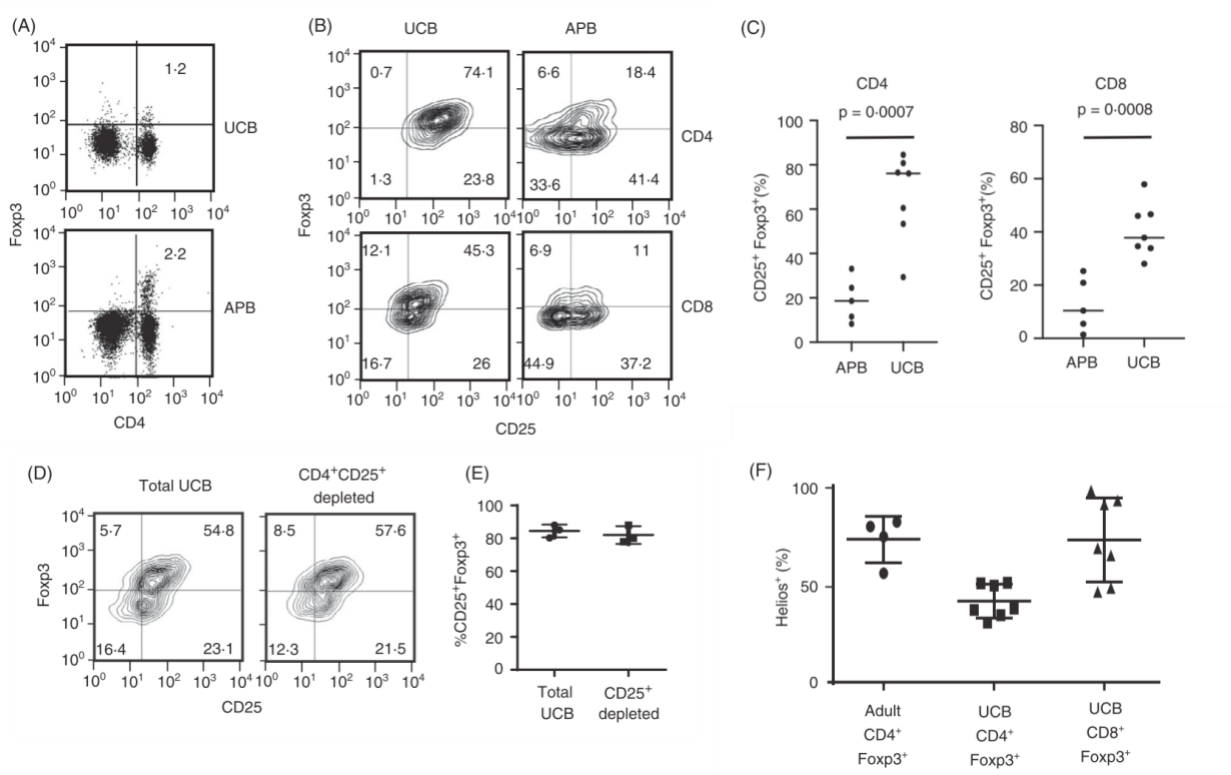

Figure 9: Induction of Foxp3+ T Cells from UCB. A) Foxp3 expression among CD4 T cells from freshly isolated UCB and APB mononuclear cells. B, C) Frequency of cells expressing CD25 and Foxp3 by CD4 and CD8 T cells after anti-CD3 and IL-2 stimulation of UCB or APB total mononuclear cells for 12-15 days. N=5-7, 2-tailed Student's t-test. D, E) Frequency of cells expressing CD25 and Foxp3 from stimulation of total UCB mononuclear cells or CD4+CD25+ depleted UCB with anti-CD3 and IL-2 for 12-15 days. F) Helios frequency within freshly isolated APB CD4+Foxp3+ T cells and UCB-derived Foxp3+ CD4 and CD8 T cells. N=4-7. UCB or APB mononuclear cells were subjected to anti-CD3 stimulation in the presence of IL-2 for 13-15 days. The culture was split throughout and replenish with IL-2 media to maintain IL-2 concentration. Data and Figures by Dr. Jessica Lee MD, Ph.D. from Lee *et al.* [512].

Regulatory Functions of Foxp3+ T Cells from Human UCB *In Vitro*

To determine if these *ex vivo* generated Foxp3+ T cells from UCB carry regulatory functions, we conducted a standard *in vitro* suppression assay to assess if they can suppress effector T cell proliferation by measuring CellTrace Violet dilution. Both *ex vivo* generated UCB CD4+, and CD8+ Foxp3+ T cells can suppress effector T cell proliferation in a dose-dependent manner, comparable to preexisting UCB isolated tTregs (Figure 10)[512]. These data demonstrated that UCB-derived Foxp3+ T cells are functional regulatory T cells *in vitro*.

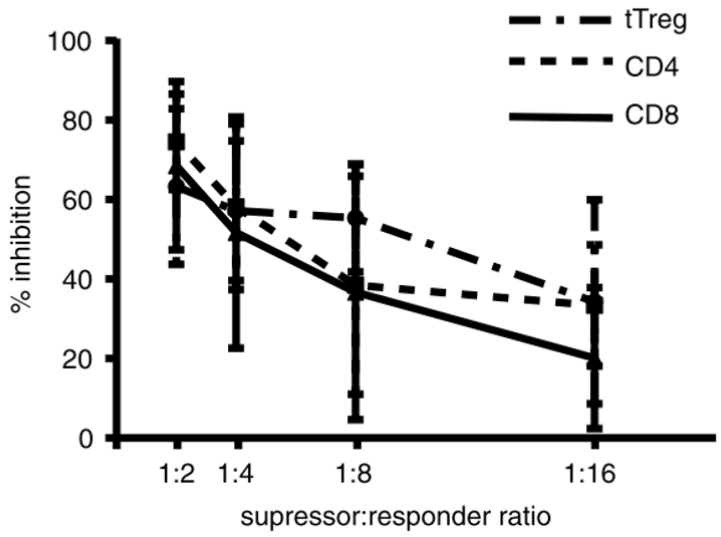

Figure 10: Regulatory Function of UCB-Derived Foxp3+ T Cells *In Vitro*. UCB-derived Foxp3+ T cells were induced as described in Figure 9 and used as suppressor cells. Preexisting tTregs were isolated using a Treg isolation kit from the autologous donor of UCB-derived Foxp3+ T cells and expanded in culture were also used as suppressor cells. CellTrace Violet labeled allogeneic adult naïve CD4 T cells were used as responder cells and cultured with CFSE labeled suppressor cells at indicated ratios in the presence of irradiated CD14+ enriched cells from the same adult PBMC donor to serve as APCs. Figure from Lee *et al.* [512].

Regulatory Functions of Foxp3+ T cells from Human UCB *In Vivo*

To determine if these *ex vivo* generated UCB-derived Foxp3+ T cells also carry regulatory function in an *in vivo* setting, we utilize a xenogeneic graft-versus-host disease (xGVHD) in NSG-S mice. This is a well-established xGVHD model by adoptively transferring human peripheral blood mononuclear cells (PBMC) into NSG-S immunodeficient mice[523,524]. Since NSG-S mice lack T, B, and NK cells, xenogeneic transplants of human PBMC causes acute GVHD-like syndrome that results in death by 20-50 days[524]. Others have shown that adult tTregs can reduce lethal GVHD in this system[525,526].

We utilized NSG-S mice, which share the background as NSG mice but provide human growth factor support of myeloid lineages, allowing for a higher rate of engraftment[527,528]. We

hypothesized that if UCB-derived Foxp3+ T cells are suppressive *in vivo*, we should see a delay in xGVHD development using this system.

To test if these *ex vivo* generated UCB-derived Foxp3+ T cells are suppressive *in vivo,* we intravenously injected sub-lethally irradiated (2.5Gy, day 0) NSG-S mice with a final volume of 200µl with UCB-derived Foxp3+ T cells (1x10^6 cells) or PBS control (i.v. lateral tail vein). 48 hours later, an equal number of autologous UCB mononuclear cells in 200µl PBS or PBS alone were injected. The development of xGVHD was monitored 5 days a week by observing overall mouse health conditions such as body weight and general appearances (fur and skin conditions). Mice were given xGVHD "scores" adapted from the scoring system developed by Cooke et al[529]. The experiment model is depicted in Figure 11.

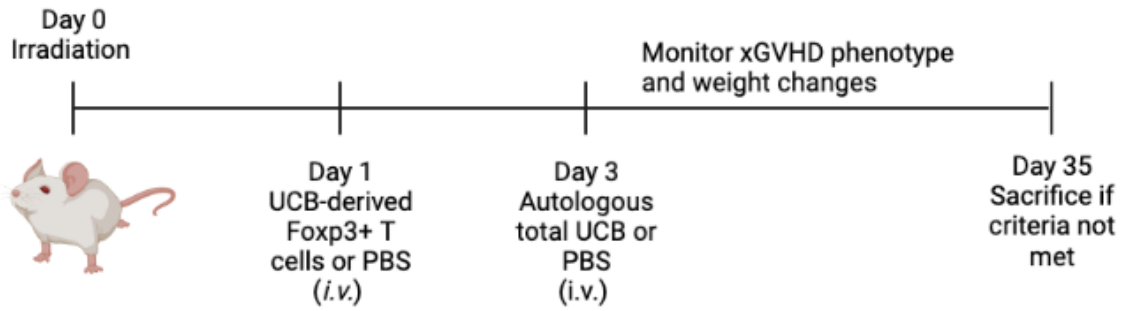

Figure 11: Xenogeneic Graft-Versus-Host Disease (xGVHD) Experimental Model. NSG-S mice were sub-lethally irradiated at day 0 and allowed to rest for 24 hours. PBS alone or $1x10^6$ UCB-derived Foxp3+ T cells were generated as described in Figure 9 and injected 24 hours post-irradiation. 48 hours after the first injection, PBS alone or $1x10^6$ autologous total UCB mononuclear cells in PBS were injected. Mice were monitored for weight change and xGVHD phenotype up to day 35 and sacrificed if they lost>15% of original body weight measured at day 0.

Using this xGVHD model, we found that the transfer of total UCB mononuclear cells led to rapid xGVHD development. Mice lost weight approximately 10 days after total UCB transfer and reached the criteria for euthanasia (>15% weight lost) no later than day 18 (day 16 ± 1.4). Control mice that received PBS alone remained healthy throughout the experiment days

(observation stopped at day 35). The transfer of equal amounts of *ex vivo* generated UCB-derived Foxp3+ T cells (CBT) alone did not cause xGVHD. Pretreatment of UCB-derived Foxp3+ T cells (CBT) 48 hours before the transfer of autologous total UCB mononuclear cells significantly delayed the onset of xGVHD in these mice (day 21.6 ± 3.1, Figure 12).

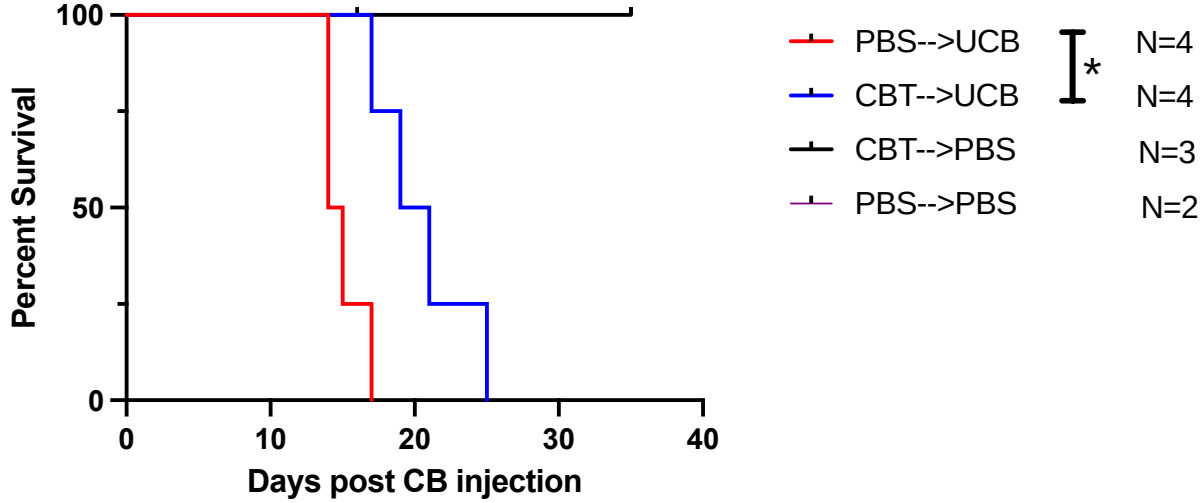

Figure 12: The Effect of UCB-derived Foxp3+ T Cells Pre-Transfer on xGVHD Induced by Autologous UCB. NSG-S mice were injected with $1x10^6$ UCB-derived Foxp3+ T cells (Cord blood-derived T cells, CBT) or PBS 48 hours before injecting $1x10^6$ autologous UCB mononuclear cells or PBS intravenously. Mice were monitored for xGVHD phenotype and weight loss of more than 15% original weight as criteria. Data combined from two independent experiments demonstrating percent of mice survive across indicated days post autologous UCB injection, *$p<0.05$, Log-rank (Mantel-Cox) test.

In addition, mice that received UCB-derived Foxp3+ T cells alone or PBS control gradually increased their weight throughout the experiment. Mice that received total UCB mononuclear cells demonstrated weight loss around 10 days post transfer and then rapidly lost weight until they hit criteria (>15% weight loss). Mice pretreated with UCB-derived Foxp3+ T cells 48 hours before total autologous UCB injection had less weight loss on average (Figure 13). Together these data demonstrated that *ex vivo* generated Foxp3+ T cells from UCB carry suppressive functions *in vivo.*

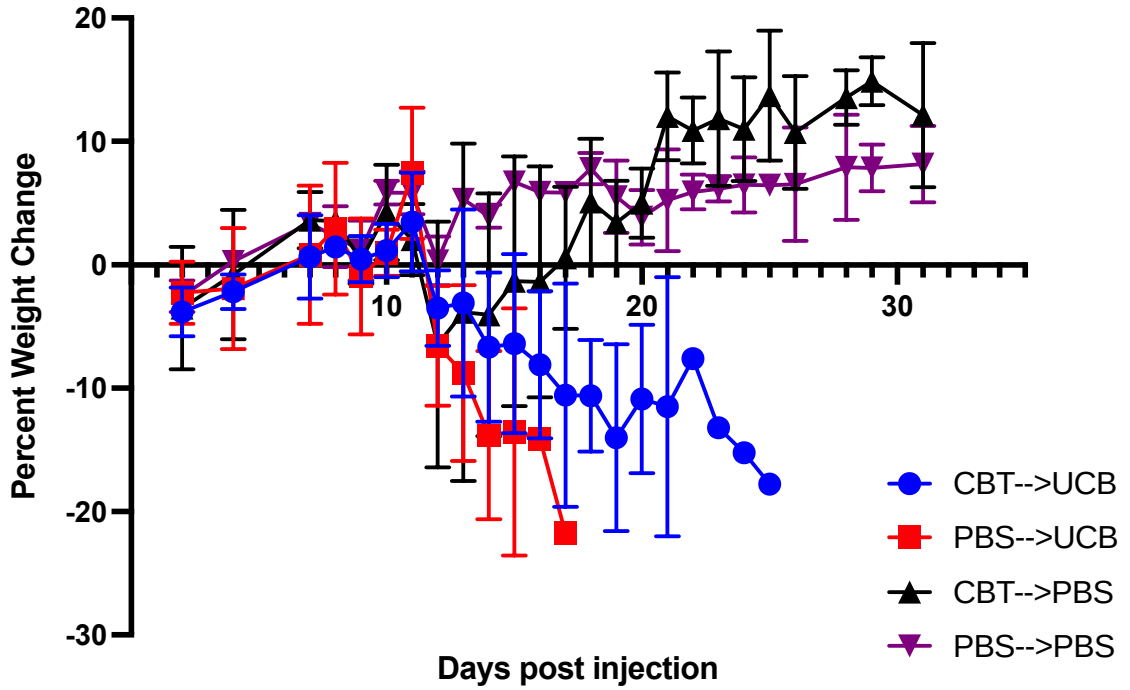

Figure 13: The Effect of UCB-Derived Foxp3+ T Cells (CBT) Pre-Transfer on xGVHD Induced by Autologous UCB. NSG-S mice were injected with $1x10^6$ UCB-derived Foxp3+ T cells or PBS 48 hours before injecting $1x10^6$ autologous UCB mononuclear cells or PBS intravenously. Mice were monitored for xGVHD phenotype and weight loss of more than 15% original weight as criteria. Data combined from two independent experiments.

The transfer of human PBMC in the xGVHD model leads to subsequent human mononuclear cell infiltration in peripheral organs and elevation of Th1 pre-dominant cytokine levels in the serum[530,531]. To determine if the delay of xGVHD development by UCB-derived Foxp3+ T cell (CBT) transfer is accompanied by a decrease in circulating proinflammatory cytokines produced by engrafted human PBMC, we measure cytokine levels in the serum of mice collected when they hit criteria (immediately before sacrifice). We found that mice that received PBS alone or CBT then PBS had little to no detection of Th1 and Th2 cytokine in their serum. Mice that received PBS then UCB had elevated levels of Th1 cytokines as demonstrated by others. Lastly, mice pretreated with CBT before UCB transfer also had similar levels of Th1 and Th2 cytokines in the serum. These data demonstrated that although CBT pretransfer

enhanced survival and decreased weight loss, Th1 and Th2 cytokine production was similar to UCB transfer alone (Figure 14).

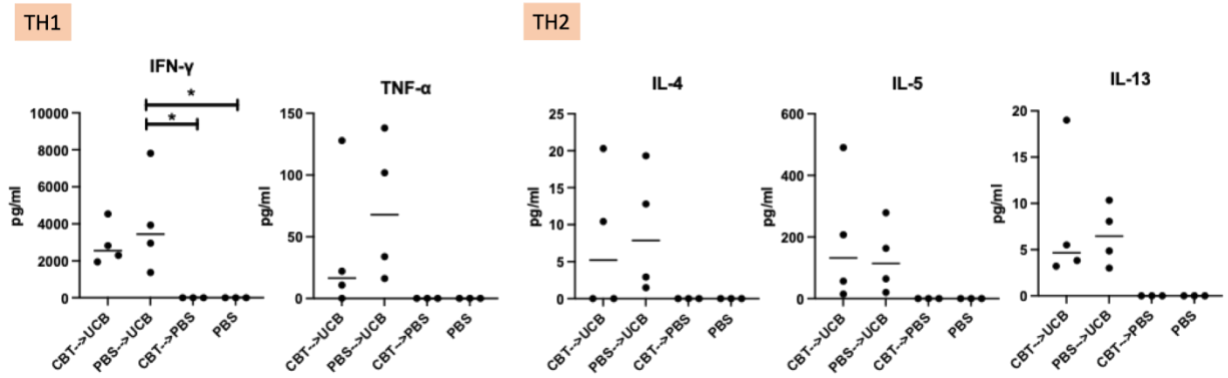

Figure 14: Serum Cytokine Analysis for xGVHD. NSG-S mice were injected with $1x10^6$ UCB-derived Foxp3+ T cells (CBT) or PBS 48 hours before injecting $1x10^6$ autologous UCB mononuclear cells or PBS intravenously. Mice were monitored for xGVHD phenotype and weight loss of more than 15% original weight as criteria. Serum was collected by spinning down peripheral blood collected via cheek bleed at the day of sacrifice and cytokine profile was analyzed using LEGENDplex Human T Helper Cytokine panel. Data combined from two independent experiments. One-way ANOVA, Tukey's multiple comparisons test, *$p<0.05$.

Patients with GVHD have been reported to have elevated serum levels of the commonly known anti-inflammatory cytokine, IL-10. High IL-10 levels were associated with fatal outcomes in these patients[532]. We next assess if pretreatment of CBT will reduce serum levels of IL-10 in our xGVHD model. We found that serum levels of IL-10 in mice pretreated with CBT were significantly reduced compared to mice that received UCB alone (131.7±40.7pg/ml vs 53.1±21.2 pg/ml, Figure 15). These data suggest that CBT pretreatment may ameliorate xGVHD by reducing IL-10 levels.

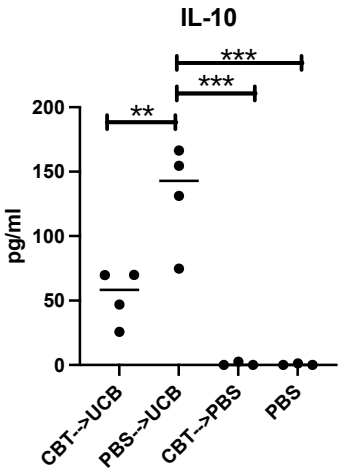

Figure 15: Serum IL-10 Levels for xGVHD. NSG-S mice were injected with $1x10^6$ UCB-derived Foxp3+ T cells (CBT) or PBS 48 hours before injecting $1x10^6$ autologous UCB mononuclear cells or PBS intravenously. Mice were monitored for xGVHD phenotype and weight loss of more than 15% original weight as criteria. Serum was collected by spinning down peripheral blood collected via cheek bleed at the day of sacrifice, and cytokine profile was analyzed using LEGENDplex Human T Helper Cytokine panel. Data combined from two independent experiments. One-way ANOVA, Tukey's multiple comparisons test, **p<0.01, ***p<0.001.

Epigenetic Regulation of *Foxp3* in UCB-derived Foxp3+ T Cells

We demonstrated that UCB T cells differentiate into Foxp3+ T cells upon antigen receptor stimulation without exogenous TGF-β addition and that they carry regulatory functions comparable to pre-existing tTregs. Furthermore, these *ex vivo* generated UCB-derived Foxp3+ T cells also significantly delayed xGVHD mediated by UCB transfer *in vivo.* Previous lab members have shown that Foxp3 expression by UCB-derived Foxp3+ T cells is relatively stable, where it was sustained up to 62 days in culture[512]. These UCB-derived Foxp3+ T cells also do not express CD127, the IL-7 receptor α chain that is known to be absent on Treg cells[533]. The majority of the preexisting Tregs are produced in the thymus (tTregs), but mature naïve T cells can also differentiate into Foxp3+ Tregs in the periphery in the presence of TGF-β, retinoic acid, and IL-2[103,104]. These *ex vivo* generated UCB-derived Foxp3+ T cells also express Helios; a transcription factor initially thought to be a specific marker for tTreg identification (Figure 9)[372].

This prompted us to investigate if UCB-derived Foxp3+ T cells resemble pre-existing tTregs or peripherally induced pTregs.

Foxp3 was discovered as the master transcription factor for Tregs. A single *Foxp3* gene mutation in the X-chromosome led to spontaneous autoimmune disease development in Scurfy mice and IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) disease in human[95–97]. Since the discovery of Foxp3 as the master transcription factor of Tregs and plays such a critical role in Treg biology, extensive studies have been conducted to understand the molecular mechanisms that govern and regulate the induction of this transcription factor. Three CNS intronic enhancer element, CNS1, CNS2, and CNS3 at the *Foxp3* locus has been identified in addition to the promoter[129]. These enhancer regions have been shown to differentially contribute to tTreg and pTreg differentiation. CNS1 region is shown to be important for iTreg/pTreg development. CNS2 is crucial for the maintenance of Foxp3 expression where it is highly enriched with CpG sites that can be epigenetically regulated through methylation[136]. It has been shown that the stable expression of *Foxp3* by tTregs is contributed by the fully demethylated CpG sites on CNS2[137,138]. Lastly, CNS3 controls *de novo* Foxp3 expression and tTreg differentiation[129].

Since we observed relatively stable Foxp3 expression by UCB-derived Foxp3+ T cells, we asked if the CNS2 region of *Foxp3* in UCB-derived Foxp3+ T cells is highly demethylated like those of adult tTregs. To test this, we conducted genomic bisulfite sequencing to identify 5-methylcytosine residues on a gene sequence[534]. We compared the *Foxp3* CNS2 region of CD4+ UCB-derived Foxp3+ T cells (generated as described in Figure 9), adult CD4+ tTregs, UCB CD4+ effector T cells as well as UCB *in vitro* induced iTregs (Treg generated *in vitro* in the presence of TGF-β). Only male donors were used as female donors carry a methylated allele

from one of the X chromosomes. As reported previously in the literature, we found that *Foxp3* CNS2 CpG sites in adult tTregs (APB tTregs) are highly demethylated (high frequency of demethylated CpG sites) and contained a range of demethylated frequencies based on donor variability. Similarly, on our hands, we observe that the CNS2 enhancer region is highly methylated (low frequency of demethylated CpG sites) in both Foxp3- effector T cells as well *in vitro* generated Tregs (iTregs)[135,138,139]. Surprisingly, we found that although Foxp3 expression in CD4+ UCB-derived Foxp3+ T cells (CD4 CBT) is relatively stable, their CNS2 enhancer region is highly methylated (low frequency of demethylated CpG sites) as well, just like their UCB effector T cells and iTreg counterparts (Figure 16). These data suggest that Foxp3 expression in UCB-derived Foxp3+ T cells is regulated differently than adult tTregs.

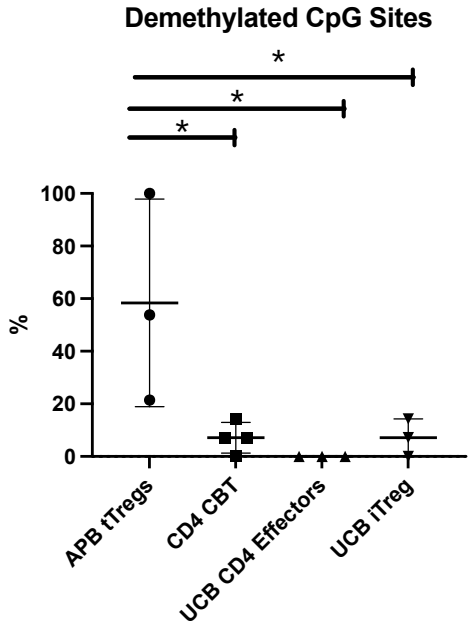

Figure 16: Frequency of Demethylated CpG Sites of Foxp3 CNS2 Region as Determined by Bisulfite Sequencing. Bisulfite conversion was conducted on genomic DNA isolated by the indicated T cell subsets. Each dot represents one donor (N=3-4), analyzed by One-way ANOVA, Tukey's multiple comparison test, *$p<0.05$.

To investigate how *Foxp3* in UCB-derived Foxp3+ T cells may be regulated, we conducted Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) to assess

genomic chromatin accessibility of the *Foxp3* region. It has been previously demonstrated that there is greater chromatin accessibility at the *Foxp3* locus in Tregs compared to effector T cells[149]. Since we observed UCB-derived Foxp3+ T cells have differential methylated status in CNS2 compared to adult tTregs, we sought to determine if CNS regions within the *Foxp3* locus have differential chromatin accessibility between the two populations.

UCB-derived Foxp3+ cells were generated as described in Figure 9. Only male donors that achieved >80% CD25+Foxp3+ were sorted for CD4+CD25+ on day 14 post-induction and transported to Northwestern University NuSeq Core facility for ATAC-Sequencing library preparation and sequencing. Adult CD4+ tTreg ATAC-sequencing raw data were obtained from a previously published source and analyzed with our sequencing samples using Galaxy and mapped against *homo sapien* genome hg38[327]. After pre-processing, chromatin accessibility was visualized using the Integrative Genomics Viewer (IGV) for ATAC signals at the gene of interest[535]. CNS and promoter sequences of *Foxp3* locus were obtained from Zheng *et al* and blasted onto IGV for visualization[129]. ATAC-seq data show that UCB-derived CD4+ Foxp3+ T cells (CBT) have less chromatin accessibility than adult CD4+ tTreg samples as demonstrated by lower signal peaks at the promoter, CNS1, CNS2, and CNS3 (Figure 17).

Together, our bisulfite sequencing and ATAC-sequencing data further suggest that Foxp3 expression by *ex vivo* generated UCB-derived Foxp3+ T cells are epigenetically regulated differently than preexisting adult tTregs.

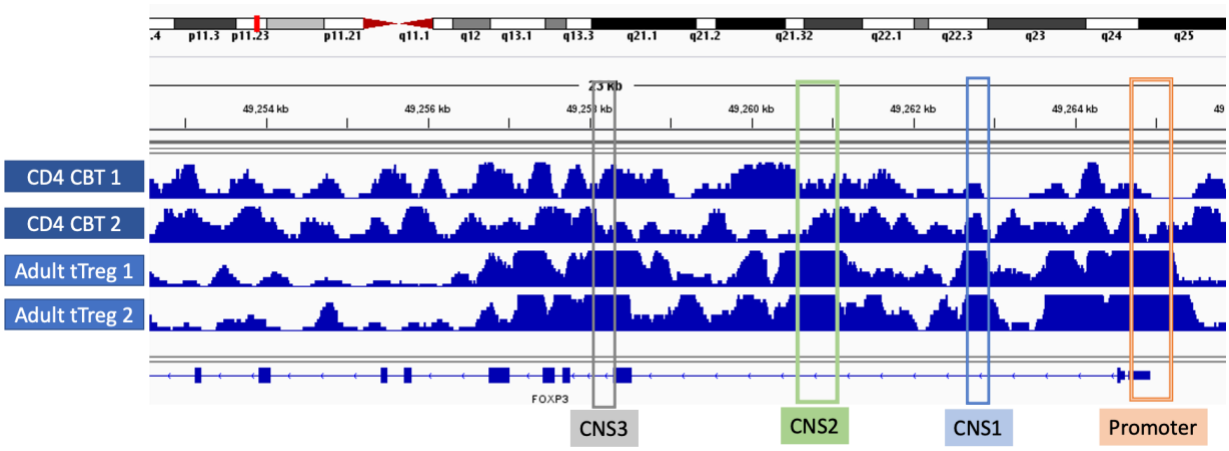

Figure 17: Chromatin Accessibility Within *Foxp3* Locus by UCB-Derived CD4+ Foxp3+ T Cells and Adult Ttreg Using ATAC-Seq. CD4+CD25+ cells were sorted from male UCB-derived Foxp3+ T cells (CBT) after induction culture as described in Figure 9 was sent for ATAC-sequencing transposase reaction, library preparation, and sequencing at NuSeq Core, Northwestern University. Preprocessed sequencing data were visualized using the Integrative Genomics Viewer for chromatin accessibility. Tracks show ATAC signals at the human *Foxp3* locus and the promoter and CNS regions are indicated in colored boxes.

When we look at regions that are unique to UCB-derived Foxp3+ T cells (CBT) within the *Foxp3* locus, we found 3 regions that are more accessible in UCB-derived Foxp3+ T cells than adult CD4+ tTregs as demonstrated by higher ATAC signals (Red boxes, Figure 18). These regions are annotated in Figure 18 as region 1 (Chrx:49265521bp-49265983bp), region 2 (Chrx: 49255141-49256310bp) and region 3 (Chrx: 49252706-49254283).

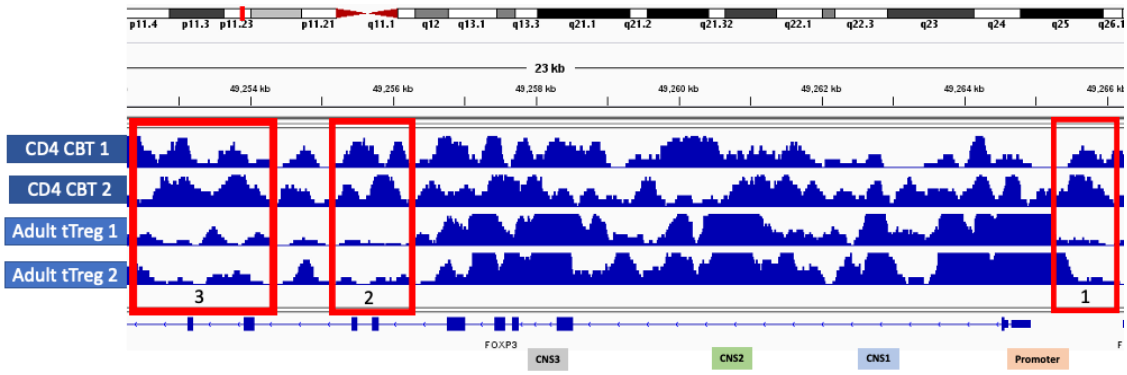

Figure 18: Unique Region of Chromatin Accessibility Within *Foxp3* Locus by UCB-Derived CD4+ Foxp3+ T Cells. CD4+CD25+ cells were sorted from male UCB-derived Foxp3+ T cells (CBT) after induction culture as described in Figure 9 was sent for ATAC-sequencing transposase reaction, library preparation, and sequencing at NuSeq Core, Northwestern University. Preprocessed sequencing data were visualized using the Integrative Genomics Viewer for chromatin accessibility. Tracks show ATAC signals at the human *foxp3* locus and the promoter and CNS regions are indicated in colored boxes. Red boxes indicate regions that are accessible in CBT but not in adult tTregs and are annotated as 1, 2 and 3.

Using NCBI Blast, region 1 was not conserved in the mouse *Foxp3* locus nor within chromosome X, while two ranges within regions 2 (79% and 80% homology, Figure 19) and 3 (69% and 74% homology, Figure 20) are conserved in the mouse *Foxp3* locus. Furthermore, like human *Foxp3*, region 2 ranges from exon 6 and 7 in mouse *Foxp3* and region 3 spans exon 8 and 9 (Figure 18 and 21).

A) Range 1

Alignment statistics

Score	Expect	Identities	Gaps	Strand
157 bits(173)	5e-35	154/195(79%)	3/195(1%)	Plus/Minus

```
Region 2     239    TGCCCCCCAGCAGTCTGAGTCTGCCACCACCAGTCCTGGGGTCGCTCACCACAGATGAAG  298
                    ||||||  |||||||    |||||||  ||  |   | | ||||||  |||||||||||| ||
Mu_Foxp3     22054  TGCCCCTCAGCAGTTGAAGTCTGCTGCCTCTGGACTTGGGGTA-CTCACCACAGATGGAG  21996

Region 2     299    CCTTGGTCAGTGCCATTTTCCCAGCCAGGTGGGCCTGCATGGCACTCAGCTTCTCCTTCT  358
                    ||||||  |||  |||||  ||||||||||||||||||||||  ||  |  |||||||||||  |
Mu_Foxp3     21995  CCTTGGCCAGCGCCATCTTCCCAGCCAGGTGGGCCTGCATAGCTCCCAGCTTCTCCTTTT  21936

Region 2     359    CCAGCACCAGCTGT-GAAATG-GCACAAACATGAGGCCTCAGCCTGGCCCTTCTCTGCCA  416
                    |||||  ||||||||  |   |||   |||  ||   ||  |||  ||   |||||  |  |||||  |  ||
Mu_Foxp3     21935  CCAGCTCCAGCTGTGGGGATGTACACCAAAGTGGGGCTTCCACCTGGTCGTTCTCAGGCA  21876

Region 2     417    CATCTTTGCCCAGGC  431
                    ||  ||||||||  ||
Mu_Foxp3     21875  CACTTTTGCCCATGC  21861
```

B) Range 2

Alignment statistics

Score	Expect	Identities	Gaps	Strand
171 bits(189)	2e-39	158/197(80%)	5/197(2%)	Plus/Minus

```
Region 2     566    GGCATTACCTGCTGCTCCAGAGACTGTACCATCTCTCTCTGGAGGAGACATTGTGCCCTG  625
                    |||||||||||||||||||||||||||  ||||  ||||||||||||||  ||  ||  |||  ||
Mu_Foxp3     21793  GGCATTACCTGCTGCTCCAGAGACTGCACCACTTCTCTCTGGAGGAGGCACTGGGCCTTG  21734

Region 2     626    CCCTTCTCATCCAGAAGATGGTCCGCCTGGCAGTGCCTAAGTAGGGAGAAGATTCCATGC  685
                    ||  ||||||||||| |||||  ||  ||  ||||||||||| ||||  ||||||||||||||||
Mu_Foxp3     21733  CCTTTCTCATCCAGGAGATGATCTGCTTGGCAGTGCCTGAGTAAGGAGAAGATTCCATGT  21674

Region 2     686    AGGTGACCACGACAGGCCTGGTC---TGGCTCAATG-CTCTGAATGGGGAGGGCCCAGAC  741
                    ||  ||||||  |   ||  |  ||  |   |||||  |  ||   ||  |   |  ||||  ||||  ||||
Mu_Foxp3     21673  AGCTGACCATGGTGGGTCAGGCCTCTTGGCTTAGTGTATCAG-TTAGGGACGGCCTAGAC  21615

Region 2     742    CCTCTGGGAGTTCTCTC  758
                    |||  ||||||  |  ||||
Mu_Foxp3     21614  CCTTTGGGAGATTTCTC  21598
```

Figure 19: Sequence Alignment of CBT Region 2 Against Mouse *Foxp3*. Sequence alignment and statistics between UCB-derived Foxp3+ T cell (CBT) region 2 from Figure 18 with mus musculus scurfin (*Foxp3*) gene using NCBI Blast (NC_000086.8:7445915-7461482 Mus musculus strain C57BL/6J chromosome X, GRCm39, discontiguous megablast program). Two separate alignments were found between CBT region 2 and mouse *Foxp3*. A) Range 1 consists of 239-431 nucleotide of region 2, B) Range 2 consists of 566-758 nucleotide of region 2.

A) Range 1

Alignment statistics

Score	Expect	Identities	Gaps	Strand
188 bits(208)	1e-44	405/587(69%)	39/587(6%)	Plus/Minus

```
Region 3   407    TGCCCTGCTTACCCAGCGGATGAGCGTGGCGTAGGTGAAAGGGGGTCGCATGTTGTGGAA  466
                  ||||||||||||||| ||||| || ||||| ||||||||||||||||||||| ||||||
Mu_Foxp3   25079  TGCCCTGCTTACCCATCGGATAAGGGTGGCATAGGTGAAAGGGGGTCGCATATTGTGGTA  25020

Region 3   467    CTTGAAGTAGTCCATGTTGTGGAGGAACTCTGTCAGAGGGTGGGGATGAATCAAGCCCCA  526
                  ||||||||||||||||||||||| |||||||| |||| ||| || ||  ||| ||  ||
Mu_Foxp3   25019  CTTGAAGTAGTCCATGTTGTGGAAGAACTCTG-CAGAAGGTTGGCATAGATC-AGAGCCT  24962

Region 3   527    TGCAGGACCTCCTAGCTAGCTCCCTGTCCCCTCCCCCTACAAGGTGAGTCTACAGGCCTG  586
                  |    | ||||||  |   || ||   | |||    || ||    || || | ||| ||
Mu_Foxp3   24961  T---TGGCCTCCTCACCCCCTACC--CCACCTTTGTCTGCATCAGGAATCCA-AGGTCTC  24908

Region 3   587    AGATCTCACCGTCAACACC------------CGTGTCCACGGGCACA---GGTACTGTTT  631
                  |  ||  |||   | ||||               ||| | |  ||||    |||||||||
Mu_Foxp3   24907  AATTCCTACCACTAGCACCAAGGGGGGAGGGACAGTCTATGCTCACACATAGTACTGTTT  24848

Region 3   632    GCTGAGCACCTGACATAAGTTGTATCATTTATTCTTTGCACCACTTCTGCCAAAATAGTT  691
                   ||||| ||| | | |||| ||| ||     |||||||||  ||| ||||| |||||| |
Mu_Foxp3   24847  ACTGAGTACCCGCCGTAAGCTGTGTC-----TTCTTTGCAGTACTCCTGCC-AAATAGCT  24794

Region 3   692    CTCCCCGAGGTTGAAAAGAAGCGGAGTAACTTGCACACCAAAGG-ATGCAAG-AGGTTAA  749
                  |||| | |  || ||||| ||   ||||| ||| ||| |||||| ||| ||| || | ||
Mu_Foxp3   24793  CTCCTC-ACTTTAAAAAGGAGTCAAGTAATTTGTACATCAAAGGCATGTAAGCAGCTCAA  24735

Region 3   750    ATGGCAGAGCCAGGATGACAGTCAAGGTCTCTGATCCCTGCTAAGCCCACAGGCCAGGCC  809
                   ||||| ||||||  |||||   ||  ||| || |||||| ||| | ||||  |||||||
Mu_Foxp3   24734  GTGGCATAGCCAGCCTGACACCAAATATCTATGGTCCCTGGTAAACTCACAACCCAGGCC  24675

Region 3   810    TGGTGGAGAGCAGTGGATAGGTGAGCTCGGGCGAATCCACCCCGATTTTCCTTGGTCAGG  869
                  ||   |   | || || | ||| |||| ||  || | || || ||  ||||| |    ||
Mu_Foxp3   24674  TGACAGGTGGGAGAGGGTTGGTTAGCTTGGTTGAGTGCA-CCTGA-CTTCCTGGAGGTGG  24617

Region 3   870    GGA-GGAAAGGAGGTGCTC-CTGGAATTACTTAGCAGGGTCCCTCCCTTCTGATGGCCGA  927
                  ||| ||||  |||| |||  |||| | |||| ||  ||  |||||||  || || | ||
Mu_Foxp3   24616  GGACGGAAGAGAGG-GCTCTTTGGACTCACTTGGCCAGGCTCCTCCCTCATGGTGACTGA  24558

Region 3   928    ATATAGTAGCTGGAGTCCAGAGTGGGTGAGGCATGGCCCCAATCCCC  974
                  ||  ||||||  ||  ||| | | ||| |||| |||| |||||||||
Mu_Foxp3   24557  ATGAAGTAGC-AGATGCCA-AATAGGTCAGGCCTGGCTCCAATCCCC  24513
```

B) Range 2

Alignment statistics

Score	Expect	Identities	Gaps	Strand
125 bits(138)	1e-25	150/202(74%)	4/202(1%)	Plus/Minus

```
Region 3   1205   CTTACCTGGGAATGTGCTGTTTCCATGGCTACCCCACAGGTGCCTCCGGACAGCAAACAG  1264
                  || ||||||||| |  || || |||||||| ||||| |||||||||| || ||||||||
Mu_Foxp3   24363  CTGACCTGGGAAGGAACTATTGCCATGGCTTCCCCAGAGGTGCCTCCGCACTGCAAACAG  24304

Region 3   1265   ---GCTGTCAGGGGCCTCCCGGGGGCCAGACCAGGCTGGGACGACAGGGCCTTGGCTGCC  1321
                     || ||| || |||||||| ||  |||||||||| ||||  |||  ||| ||  | |
Mu_Foxp3   24303  GCCGCCGTCTGGAGCCTCCCGAGGAGCAGACCAGGCCGGGAGCACACTGCCCTGAGTACT  24244

Region 3   1322   AGCAGCTACGATGCAGCAGGAGCCCTTGTCGGATGATGCCTGGGTGAGGGGGAGAGG-CT  1380
                   |  |||||||||||||| |||| ||||||   ||| ||||| | |   ||||| ||  |
Mu_Foxp3   24243  GGTGGCTACGATGCAGCAAGAGCTCTTGTCCATTGAGGCCTGAGGGGTTGGGAGGGGTGT  24184

Region 3   1381   GGTGACCCAGAGGCTTAAACTT  1402
                  ||| | | |||  ||||| |||
Mu_Foxp3   24183  GGTAATCGAGACACTTAAGCTT  24162
```

Figure 20: Sequence Alignment of CBT Region 3 Against Mouse *Foxp3*. Sequence alignment and statistics between UCB-derived Foxp3+ T cell (CBT) region 3 from Figure 18 with mus musculus scurfin (*Foxp3*) gene using NCBI Blast (NC_000086.8:7445915-7461482 Mus musculus strain C57BL/6J chromosome X, GRCm39, discontiguous megablast program). Two separate alignments were found between CBT region 3 and mouse *Foxp3*. A) Range 1 consists of 407-974 nucleotide of region 3, B) Range 2 consists of 1205-1402 nucleotide of region 3.

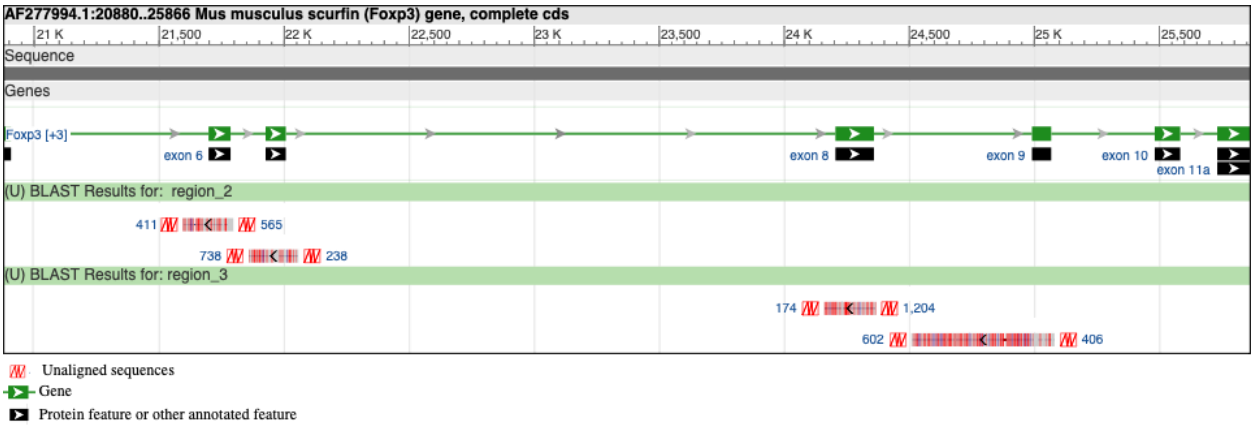

Figure 21: Graphics of blast results for CBT region 2 and region 3 against mouse *Foxp3*. CBT region 2 spans from exon 6 and 7 in mouse *Foxp3* and region 3 spans exon 8 and 9.

These findings could allow the generation of transgenic mice to study if these regions can regulate Foxp3 expression, particularly in perinatal T cells. However, the identification of UCB-derived Foxp3+ T cells murine counterpart has yet to be identified.

Foxp3 Stability by UCB-derived Foxp3+ T Cells under Inflammatory Conditions

Several studies in the inflammatory settings suggested that Foxp3 expression by Tregs can be lost during inflammatory responses. For instance, IL-6 led to a decrease in Foxp3 expression from sorted CD4+CD25+ Tregs and subsequently increases IL-17 production in these cells *in vitro*[536]. Tumor necrosis factor (TNF) has been shown to decrease *Foxp3* mRNA and protein expression by the Tregs[537]. TNF and IL-6 led to Foxp3 degradation and subsequent loss of function in Tregs[538]. We asked if *ex vivo* generated UCB-derived Foxp3+ T cells lose Foxp3 expression when subjected to anti-human CD3 and anti-human CD28 coated beads (10ug/ml) and 10ng/ml IL-2 restimulation in the absence or presence of other inflammatory cytokines. We added 10ng/ml IL-4, 50ng/ml IL-6, 10ng/ml IL-1β, or 10μM TGF-β receptor kinase inhibitor SB431542 in the presence of IL-2 and harvested the cells after 5 days of restimulation.

We found that Foxp3 frequency in both CD4+ and CD8+ T cell subsets did not significantly change in the presence of the indicated cytokines compared to controls (NT). The

addition of SB431542, TGF-β receptor 1 kinase inhibitor, showed a decreasing trend in Foxp3 frequency within CD4 T cells (Figure 22). This is plausible because TGF-β1 has been shown to maintain Foxp3 expression in Tregs and a defect in TGF-β1 signaling in Tregs is associated with a decrease in Foxp3 expression and suppressive capability[133]. These data demonstrated that Foxp3 expression by *ex vivo* generated UCB-derived Foxp3+ T cells are stable in the presence of inflammatory cytokines.

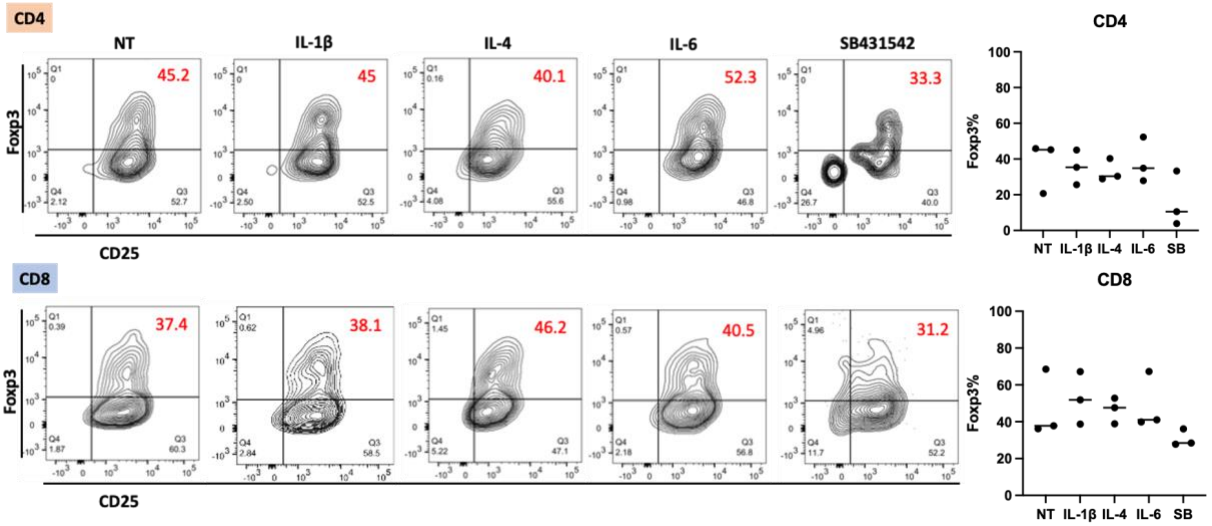

Figure 22: Foxp3 Frequency Within CD4+ And CD8+ UCB-Derived Foxp3+ T Cells After Restimulation in the Absence or Presence of Other Cytokines. Representative FACS plot of CD25 and Foxp3 expression and summary of Foxp3 frequency by UCB CD4+ and CD8+ Foxp3+ T cells after restimulation in the absence or presence of other cytokines. After Foxp3+ induction culture as described in Figure 9, UCB-derived Foxp3+ T cells were harvested and cultured in the presence of beads coated with 10ug/ml anti-human CD3 and anti-human CD28 for 5 days in the presence of 10ng/ml IL-2 and in addition to other indicated cytokines (NT= IL-2 alone, SB=SB431542). After 5 days, cells were harvested and intracellularly stained for Foxp3. Each dot indicates one donor (N=3), analyzed by one-way ANOVA, Dunnett's multiple comparisons test.

Effects of Alcohol on UCB-derived Foxp3+ T Cell Generation

Next, we sought to determine if Foxp3 induction by UCB T cells will be inhibited under inflammatory conditions early in the differentiation process. So far, we found that UCB T cells differentiate into Foxp3+ T cells upon antigen receptor stimulation and carry suppressive

functions in vitro and in vivo. Foxp3 expression by these UCB T cells is epigenetically regulated differently from those of adult tTregs. Foxp3 expression by these *ex vivo* generated UCB Foxp3+ T cells are also stable even after restimulation in the presence of inflammatory cytokines.

One of the factors that can promote an overall inflammatory state in the fetus is prenatal alcohol exposure[505]. Chronic alcohol usage by mothers causes an increase in serum proinflammatory cytokines such as IL-1β, IL-6, and TNF in the fetus. Alcohol is a known environmental factor that causes immune dysfunctions in infants. Alcohol consumption during pregnancy can lead to fetal alcohol spectrum disorders (FASDs), which include abnormalities in development and cognitive function, as well as immune dysfunction[498,539,540]. Limited alcohol use during gestation increases the risk for neonatal infection by 2.5 fold, while excessive alcohol abuse further increases this predisposition another 3-4 fold[488]. This immune deficit may induce significant and long-term defects in immunity and increase the susceptibility of individuals with fetal alcohol exposure (FAE) to autoimmune diseases and bacterial infections[486–488,490]. Notably, in animal models, FAE increases susceptibility to autoimmune diseases such as rheumatoid arthritis and type 2 diabetes[486,487]. Since Foxp3+ T cells are important for maintaining tolerance and preventing autoimmune disease, we hypothesized that alcohol impairs the immune functions of neonates by blocking the development of Foxp3+ T cells from UCB and enhancing proinflammatory conditions among infants.

To determine the effects of alcohol exposure in fetal tolerance, we tested if the presence of ethanol (EtOH) affects Foxp3+ T cell induction from total UCB mononuclear cells. Using the same culture system established as described in Figure 9, we added increasing concentrations of EtOH during the first 5 days of the UCB Foxp3+ induction culture. The addition of EtOH did not cause apparent toxicity or cell death as the total number of cells in the wells with or without

EtOH are comparable at the end of the 2-week culture (Figure 23). To test if EtOH may cause cell death earlier in the culture, we also counted cell numbers on days 3 and 5. Total cell numbers were not significantly different in the absence or presence of 100mM EtOH at both days 3 and 5 (Figure 24).

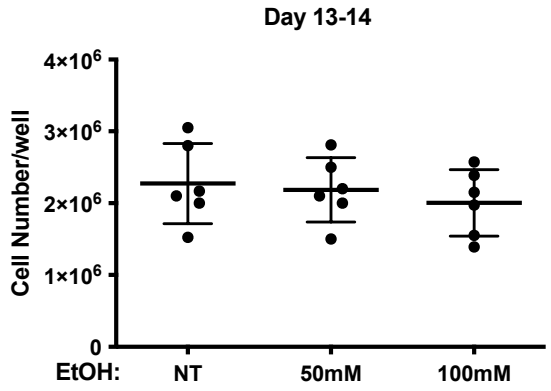

Figure 23: Total Cell Number After UCB Foxp3+ T Cell Induction Culture in the Absence or Presence of Ethanol. Total umbilical cord blood mononuclear cells were subjected to Foxp3+ induction culture as described in Figure 9, with or without ethanol at the indicated concentrations for the first five days of the culture. On days 13-14, the culture was harvested, and cell counts were obtained. N=6 UCB donors.

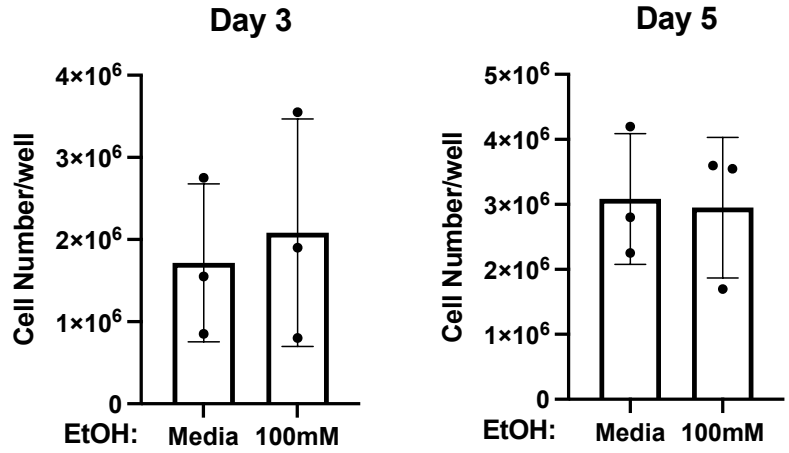

Figure 24: Total Cell Number from Day 3 And Day 5 Of UCB Foxp3+ T Cell Induction Culture in the Presence or Absence of Ethanol. Total umbilical cord blood mononuclear cells were subjected to Foxp3+ induction culture as described in Figure 9, with or without ethanol at the indicated concentrations for the first three or five days of the culture. Ethanol was added daily up to the day of harvest (3- or 5-days total spike treatment) to achieve the indicated EtOH concentration. At days 3 or 5, cell numbers were calculated. N=3 UCB donors.

When we assessed the frequency of CD25+Foxp3+ within CD4+ and CD8+ T cell population after Foxp3+ T cell induction culture as described in Figure 9, there was a significant decrease in CD25+Foxp3+ frequency when 100mM EtOH was added in the induction culture in comparison to media alone in both CD4+ and CD8+ T cell subsets (Figure 25).

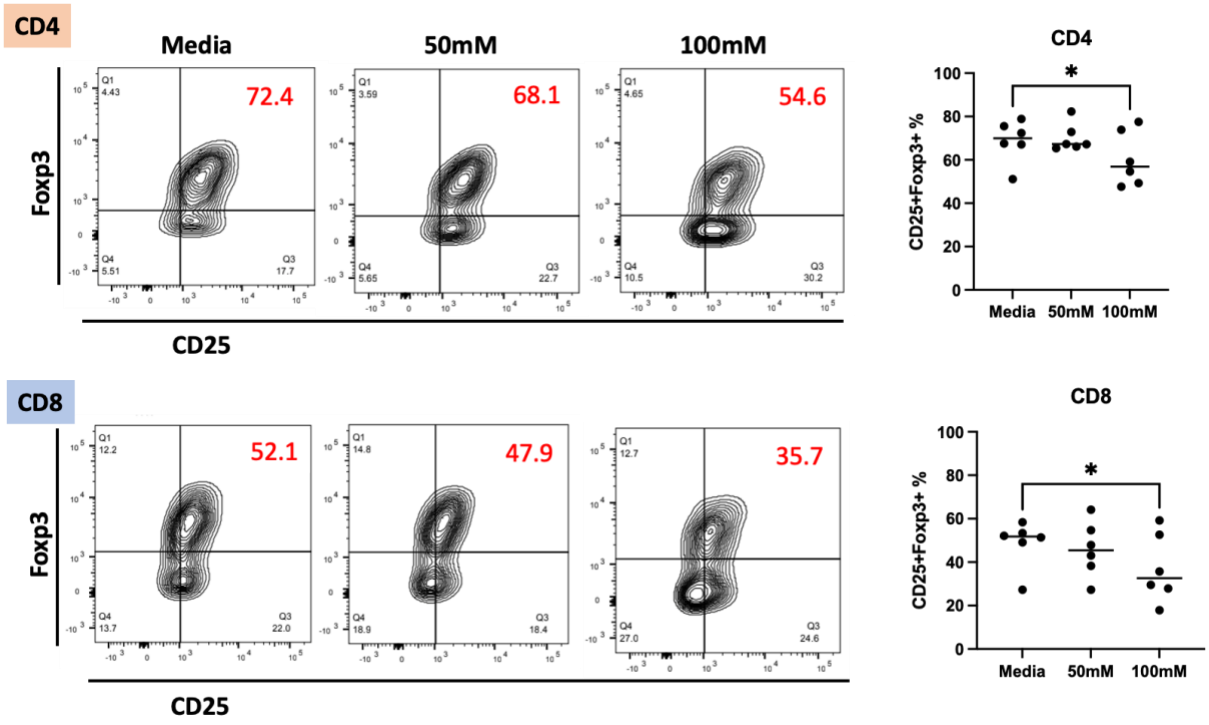

Figure 25: Frequency of CD25+Foxp3+ Within CD4+ And CD8+ T Cells at The End of UCB Foxp3+ Induction Culture in the Absence or Presence of Ethanol. Left: Representative FACS plot of CD25 and Foxp3 within CD4+ and CD8+ T cells after UCB Foxp3+ induction culture in absence or presence of indicated EtOH concentration for first five days of culture. Right: Frequency plots of CD25+Foxp3+ within CD4+ and CD8+ T cells after UCB Foxp3+ induction culture in absence or presence of indicated EtOH concentration. Total umbilical cord blood mononuclear cells were cultured in the presence of anti-human CD3 and IL-2 for 13-14 days with or without EtOH of the indicated concentration. N=6 independent UCB donors. *$p<0.05$ paired t test.

Next, we ask if the presence of EtOH affects the total cell number of CD25+Foxp3+ T cells after the induction culture. We observed a significant decrease in total CD4+CD25+Foxp3+ T cell number but did not see a significant decrease in CD8+CD25+Foxp3+ T cells at 100mM

EtOH concentration (Figure 26). Due to donor variability in cell number, we normalized each treatment cell number to its media control counterpart.

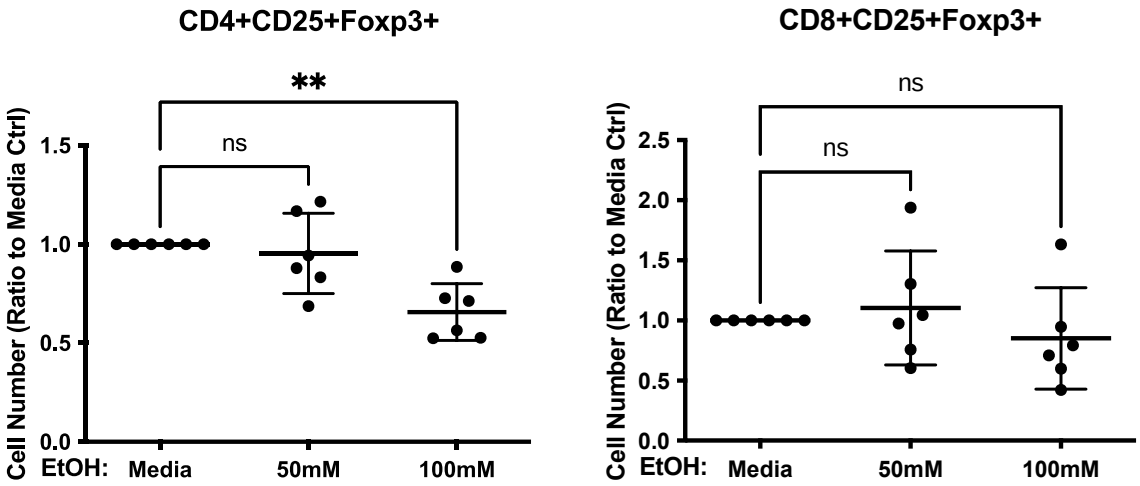

Figure 26: Total Cell Number of CD4+CD25+Foxp3+ And CD8+CD25+Foxp3+ Tregs at the end of UCB Foxp3+ Induction Culture With or Without EtOH. Total umbilical cord blood mononuclear cells were cultured in the presence of anti-human CD3 and IL-2 for 13-14 days with or without ethanol of the indicated concentrations for the first five days of the culture. On days 13-14, the culture was harvested and stained CD4, CD8, CD25, and Foxp3. Cell number is reflected as a ratio to respective media control of the same donor. N=6 UCB donors. **p<0.01, One-way ANOVA, Dunnett's multiple comparisons test.

Furthermore, 100mM EtOH significantly decreased Foxp3 expression in both CD4+ and CD8+ T cells as seen by a decrease in mean fluorescence intensity (MFI) at the end of the Foxp3+ T cell induction culture (Figure 27). Therefore, the presence of 100mM ethanol decreased Foxp3 protein expression within the cells. Altogether, these data demonstrate that EtOH can inhibit UCB Foxp3+ T cell induction.

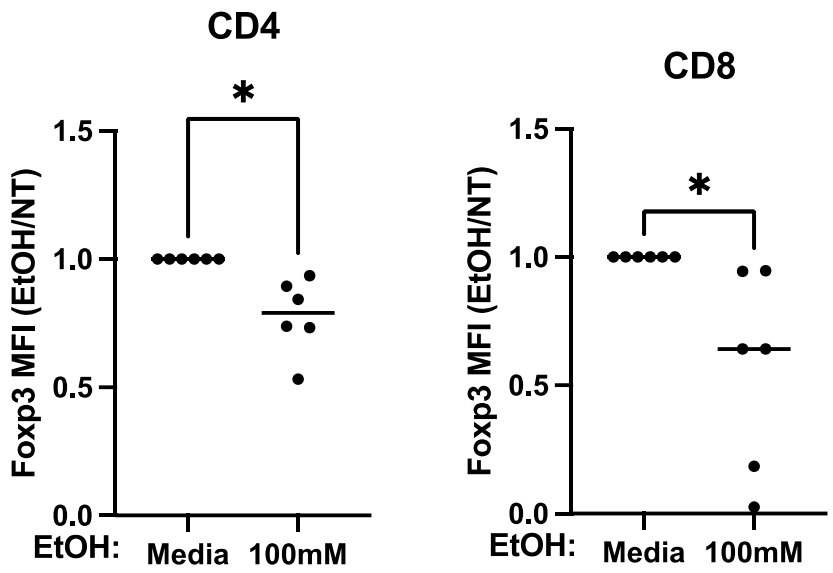

Figure 27: Mean Fluorescence Intensity (MFI) Of Foxp3 at the End of Foxp3+ Treg Induction Culture With or Without EtOH. Total umbilical cord blood mononuclear cells were cultured in the presence of anti-human CD3 and IL-2 for 13-14 days with or without ethanol of the indicated concentrations for the first five days of the culture. On days 13-14, the culture was harvested and stained for CD4, CD8, CD25, and Foxp3. MFI is reflected as a ratio of ethanol treatment to media control (NT). N=6 umbilical cord blood donors, *$p<0.05$, paired t-test.

A Potential Mechanism Underlying UCB-derived Foxp3+ T Cells Induction Inhibition by EtOH

It has been shown previously that IL-2 is required for TGF-β to induce naïve CD4+CD25- T cells to become CD25+ and express Foxp3[105]. IL-2 signaling is initiated by binding with high affinity to the trimeric IL-2R that consists of CD25, also known as IL-2 receptor alpha (IL-2Rα), IL-2Rβ, and common γ chain[541–543]. IL-2Rβ and the common γ chain can also form a dimer and bind IL-2 with low affinity. $CD25^{-/-}$ mice develop severe autoreactivity and develop autoimmune disorders[544]. This lymphoproliferative syndrome was partly attributed to a deficiency in Treg cells[545]. We hypothesized that EtOH inhibits UCB-derived Foxp3+ induction by blocking CD25 expression early in the culture.

To determine if the presence of EtOH changes CD25 expression on UCB T cells, total UCB mononuclear cells were cultured with beads coated with anti-human CD3 for 1 day in the presence and absence of EtOH without exogenous IL-2 (as IL-2 can promote CD25 expression).

After 1 day, the cells were harvested and stained for CD25 expression on both CD4 and CD8 T cells.

In the presence of anti-CD3 stimulation, CD25 expression is induced in both CD4+ and CD8+ T cells after 1 day (NT, Figure 28). EtOH produces a significant dose-dependent decrease in both CD25 frequency and expression (gMFI) within CD4+ and CD8+ T cell population especially in the presence of 100mM EtOH. The expression of CD25 appears to be more sensitive to EtOH treatment in the CD8+ T cell subset, where 50mM EtOH led to a significant decrease in both CD25 frequency and expression (Figure 28). These data suggest that EtOH inhibits UCB-derived Foxp3+ T cell induction by blocking CD25 expression early on during Foxp3+ T cell induction culture.

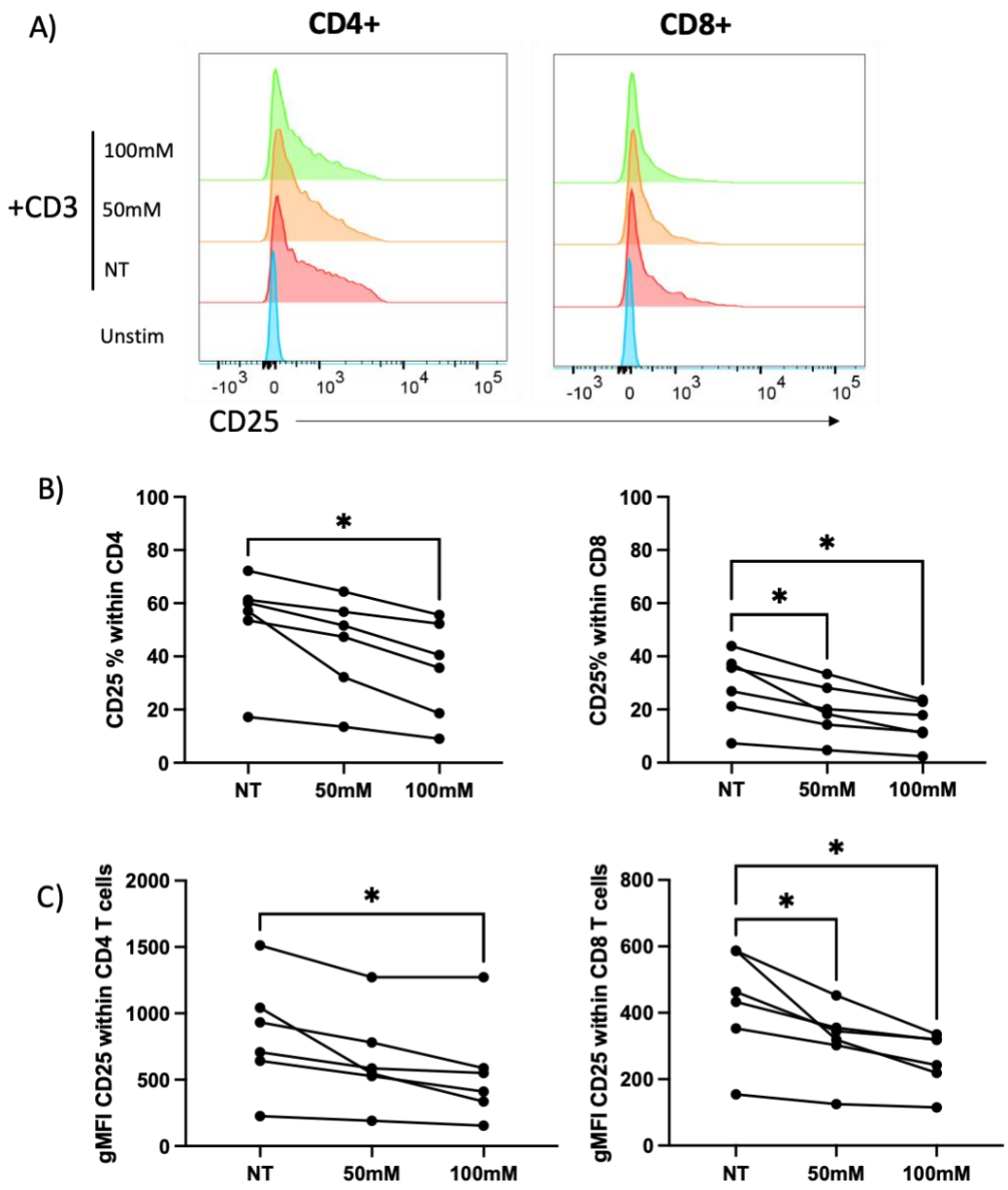

Figure 28: CD25 Frequency and Expression in UCB T Cells in the Absence and Presence of EtOH After CD3 Stimulation. A) Representative histogram of CD25 expression by CD4+ and CD8+ T cells 1 day after CD3 stimulation. B) CD25 frequency within CD4+ and CD8+ T cells. C) CD25 expression as reflected by geometric mean fluorescent intensity within CD4+ and CD8+ T cells. Total UCB were cultured in the presence of anti-CD3 coated beads for 1 day. Ethanol was added to achieve indicated final concentration at the time of culture. After 1 day, the cells were harvested and stain for CD25 within CD4+ and CD8+ T cells. NT= media control, 50mM, and 100mM indicate the final concentration of EtOH in the media. N=6 independent UCB donors. Analyzed with one-way ANOVA, Dunnett's multiple comparisons test, *$p<0.05$.

Section II: Helios Expression in Perinatal T Cells

The perinatal immune system is phenotypically and functionally distinct from the adult immune system, which is highly tolerantly biased. In murine models, one of the main differences is murine neonatal T cells appeared to be heavily biased toward a Th2 profile against both primary and secondary responses *in vitro* and *in vivo* compared to adult T cells[307,546]. A Th2 biased response is critical for tolerance establishment, especially during the first few days of birth, while a Th1 response has been associated with allograft rejection[547,548]. This Th2 biased response by neonates rapidly declines to adult T cell levels by day 5 after birth[308]. In human perinatal T cells, we have previously shown that UCB naïve CD4+ T cells differ from adult naïve CD4+ T cells in terms of surface antigen expression as well as cytokine production[309]. Freshly isolated UCB naïve CD4+ T cells do not exhibit a Th2 biased response but produce less IFN-γ compared to adult naïve CD4+ T cells[309]. Furthermore, UCB T cells produce higher levels of IL-10, an anti-inflammatory cytokine, upon antigen receptor stimulation compared to adult T cells[310]. These data demonstrate that perinatal T cells are biased towards a tolerant and anti-inflammatory response.

Helios Expression in Human Perinatal and Adult T Cells

To investigate if there are transcription factors that could contribute to the tolerant nature of the perinatal immune system, we screened for transcription factors known to regulate T cell function and cytokine productions. As Helios is generally considered a transcription factor expressed by a subset of CD4+ Foxp3+ Tregs, and plays a critical role in maintaining Treg stability and suppressive function[372,384–386], we investigated if UCB has a higher frequency of Helios+ T cells.

We previously found that our *ex vivo* generated UCB-derived Foxp3+ T cells co-express Helios after our 14-day induction culture, like freshly isolated adult CD4+ Foxp3+ tTregs (Figure 9)[512]. However, when we analyzed Helios and Foxp3 expression in freshly isolated UCB and APB total mononuclear cells, a significantly higher frequency of UCB CD4+ T cells (46.9±27.2%) express Helios without Foxp3 co-expression, in comparison to APB CD4+ T cells (8.1±4.1%). About 3±3.8% of UCB CD4+ T cells express both Foxp3 and Helios while about 15±7.6% adult CD4+ T cells are Foxp3+Helios+ (Figure 29). This demonstrates that a majority of Helios+ UCB CD4+ T cells do not express Foxp3, while a majority of adult CD4+Helios+ T cells are Foxp3+. This is in agreement with previous findings, where fetal naïve CD4+ T cells express higher levels of Helios compared to adult naïve CD4+ T cells[327]. Additionally, a significantly higher frequency of UCB CD8+ T cells express Helios compared to APB CD8+ T cells (79.3±8.43% and 25.5±12.35% respectively, Figure 29).

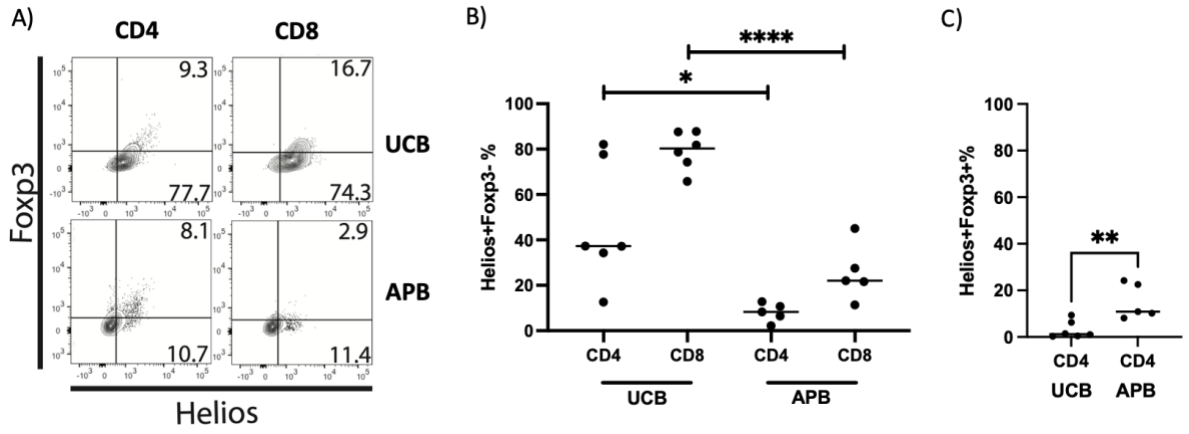

Figure 29: Helios and Foxp3 Expression in UCB And APB CD4+ and CD8+ T Cell Population. A) Representative FACS plot of Helios and Foxp3 expression by UCB and APB CD4+ and CD8+ T cell population. B) Helios+Foxp3- frequency summary by UCB and APB CD4+ and CD8+ T cells. C) Helios+Foxp3+ frequency summary by UCB and APB CD4+ T cells. Freshly isolated UCB and APB mononuclear cells were stained for Helios and Foxp3 frequency within CD3+CD4+ and CD3+CD8+ T cells. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 unpaired t test. Each dot represents individual donors (APB N=5, UCB N=6)

The striking difference in Helios frequency between UCB and APB T cells led us to hypothesize that Helios+ T cell frequency decreases with age. In collaboration with the Loyola Neonatal Intensive Care Unit, we obtained blood samples from 8 pre-term (<30 weeks' gestation) donors and tracked their Helios expression during the first three weeks of life during their routine blood work. Helios+Foxp3- by CD4+ T cells started at a relatively high frequency (26.0±20.3%) and showed a decreasing trend of significantly lower at 3 weeks after birth (5.8±3.2%). The Helios+Foxp3- by CD8+ T cells also started at a relatively higher frequency (48.7±27.0%) and decreased significantly by 3 weeks after birth (15.1±19.1%, Figure 30).

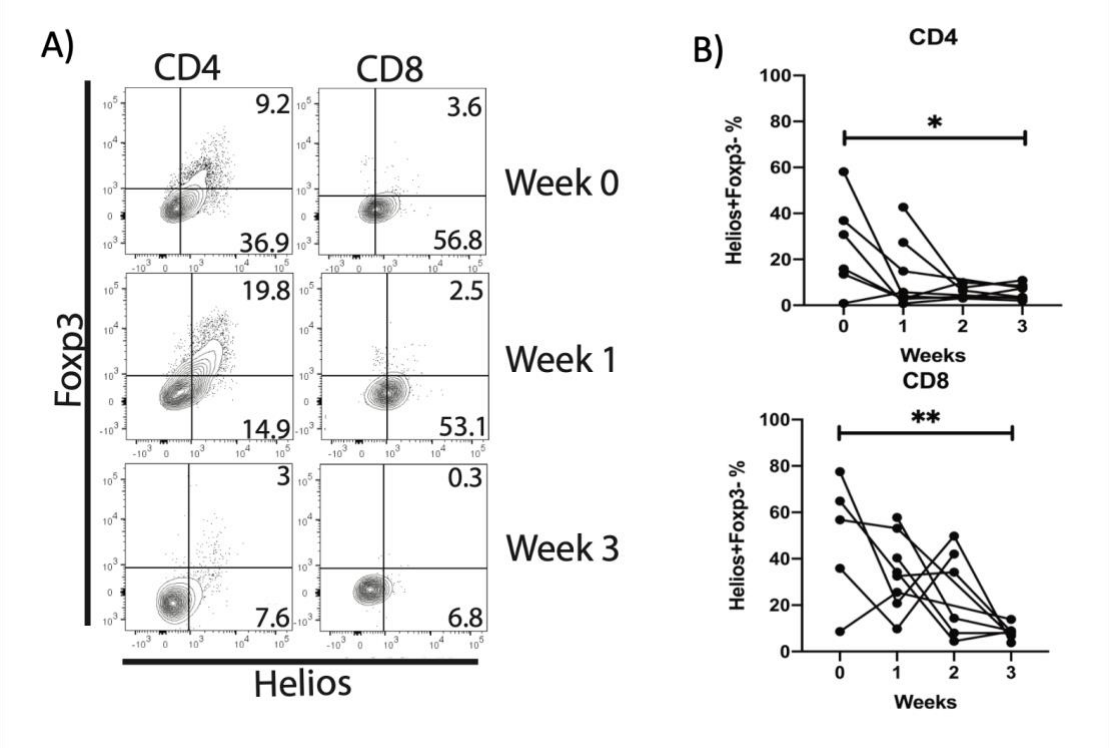

Figure 30: Frequency of Helios Within CD4 and CD8 T Cells from Preterm Neonate's Peripheral Blood Collected Up To 3 Weeks After Birth. A) Representative FACS plot of Helios and Foxp3 expression by preterm neonates CD4 and CD8 T cells. B) Summary plot of Helios+Foxp3- frequency by CD4 and CD8 T cells in preterm neonate's peripheral blood. Freshly isolated mononuclear cells were harvested from pre-term donors and stained for Helios and Foxp3 frequency within CD4 and CD8 T cells. *p<0.05, **p<0.01 unpaired t-test comparing to week 0. Each dot represents an individual donor, and the line tracks the same donor. N=5-8 each week, depending on the donor.

Helios Expression in Mouse Splenic T Cells

To determine if we observe a similar phenotype in mice, we analyzed Helios and Foxp3 expression of splenic CD4+, CD8+ αβ T cells of different ages ranging from 0 to 5-day old neonates, 7 weeks (50 days) after birth as well as >6 months old. In agreement with previous reports, the detection of Foxp3+ Tregs started to appear in the periphery around 3 days after birth[93,94]. These CD4+ Foxp3+ T cells also express Helios and increase gradually from 2.8±0.4 to 11.1±0.5% at 7 weeks old. Billingham and Medawar demonstrated that tolerance against foreign antigens could be established *in utero*[241]. These data suggest that other Foxp3- T cells could contribute to the tolerant nature of the perinatal immune system prior to the appearance of Tregs in the periphery. As observed in humans, most CD4+ and CD8+ T cells from 0-day old neonate splenocytes express Helios do not co-express Foxp3 (34.7±11.4% and 76.6±15.5% respectively). Helios+ T cell frequency rapidly decreases after birth by 2-4 days, reaching to adult levels for both CD4+ and CD8+ T cells (7.2±0.6% and 7.5±1.3% respectively, Figure 31).

These data demonstrate that neonates have a higher frequency of Helios+ T cells that do not co-express Foxp3 in both CD4+ and CD8+ T cell subset and this frequency decreases with age in both humans and mice. Furthermore, these Helios+ T cells are detected in the periphery before the appearance of Helios+Foxp3+ Tregs, suggesting that these Helios+Foxp3- T cells may be a non-Treg mechanism that promotes perinatal tolerance.

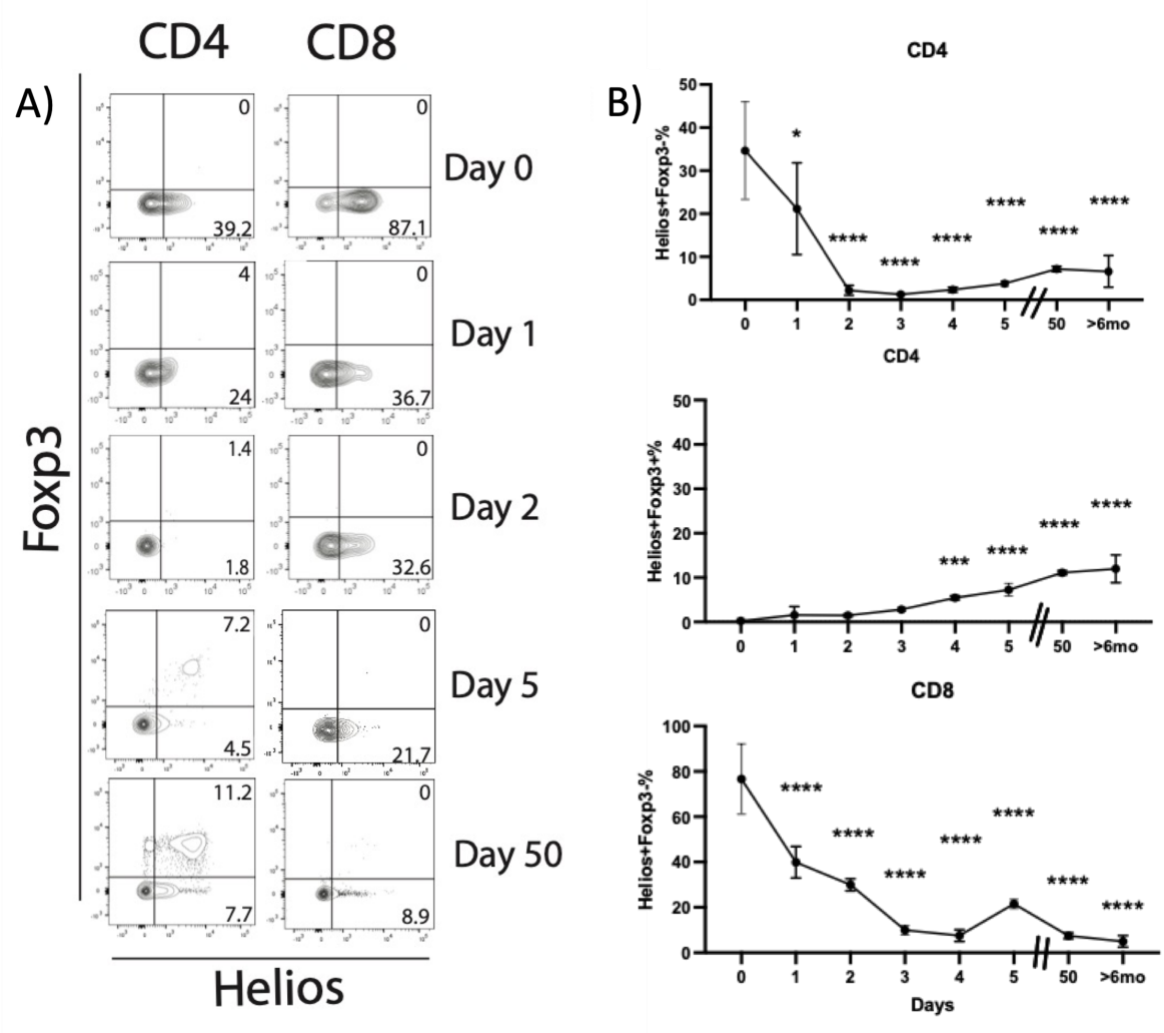

Figure 31: Helios and Foxp3 Expression Within Splenic CD3+γδTCR- CD4+ and CD8+ T Cell Population in Mice. Splenocytes were harvested from mice of indicated ages and stain for Helios and Foxp3 within CD4+ and CD8+ αβ T cells. αβ T cells were gated as CD3+γδTCR- population. A) Representative FACS plots of indicated age group. B) Plot represents mean and standard deviation of N=3-7 individual mice or pooled neonatal mice. At earlier neonatal days, splenocytes were pooled from littermates to obtain enough cells for staining and is counted as one donor. All value on the x-axis indicates days except for >6mo where it represents >6-month-old adult. Analyzed by ordinary one-way ANOVA, Dunnett's multiple comparisons test comparing each age against 0-day old *p<0.05, ****p<0.0001.

In mice, γδ T cells are more highly represented in neonatal stage compared to αβ T cells[353]. γδ T cells are disproportionately important for immune protection in younger mice compared to adult mice in an age-dependent manner. As a majority of peripheral T cells in mice before 5 days of age are γδ T cells, we next determined if murine γδ T cells express Helios and

also display an age-dependent decrease in Helios frequency as observed in αβ T cells. In fact, we observed a high frequency of γδ T cells in the spleen express Helios but not Foxp3 by 0-day old neonates (91±13.4%). The frequency of Helios+Foxp3- γδ T cells rapidly decreases to adult levels between 2-4 days after birth (37.4±3.9% at 50 days old, 15.7±3.4% at 6 months old, Figure 32C). The majority of splenic γδ T cells are CD8+ or CD4-CD8- DN (Figure 32A). When we further investigated Helios+ Foxp3- frequency within the different splenic γδ T cell subset, we found that Helios frequency decreases in both CD8+ and DN γδ T cells after birth. Strikingly, we found that while adult CD8+ γδ T cells express a very low frequency of Helios, they express significantly higher frequency of Foxp3+ without co-expression of Helios compared to perinatal CD8+ γδ T cells in the spleen. This phenomenon is unique to CD8+ γδ T cells, as Foxp3 is not detected by CD4-CD8- DN γδ T cells at any time points (Figure 32D).

In summary, we found that a higher frequency of αβ and γδ T cells express Helios in neonatal mice, and Helios frequency rapidly decreases a few days after birth to adult levels.

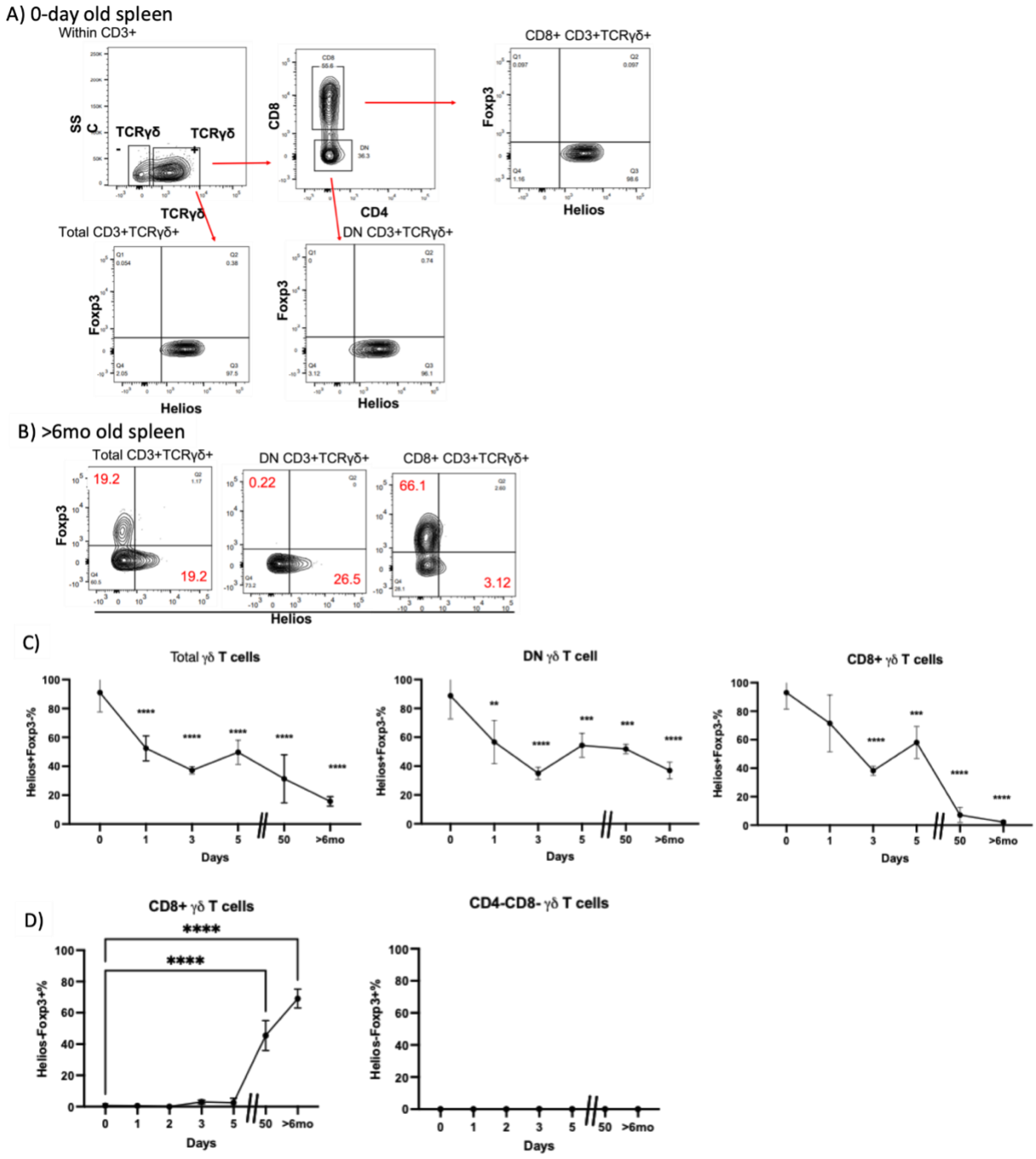

Figure 32:Helios and Foxp3 Expression Within Splenic CD3+γδTCR+ T Cell Population in Mice. A) Gating strategy for total CD3+γδTCR+ T cells, CD8+ CD3+ γδTCR+ and CD4-CD8- (DN) CD3+γδTCR+. The same gating strategy is applied for all indicated age groups. B) Representative FACS plots for Helios and Foxp3 by adult total CD3+γδTCR+ T cells, CD8+ CD3+ γδTCR+ and CD4-CD8- (DN) CD3+γδTCR+ in the spleen. C) Kinetics of Helios+Foxp3- by total CD3+γδTCR+ T cells, DN CD3+γδTCR+ T cells and CD8+ CD3+γδTCR+ T cells in the spleen from neonates through adulthood. D) Kinetics of Helios-Foxp3+ by CD8+ and DN CD3+γδTCR+ T cells in the spleen. Splenocytes were harvested from mice of indicated ages and

stained for Helios and Foxp3. The plot represents the mean and standard deviation of N=3-7 individual mice or pooled neonatal mice. At earlier neonatal days, splenocytes were pooled from littermates to obtain enough cells for staining and counting as one donor. All value on the x-axis indicates days except for >6mo where it represents >6-month-old adult. Analyzed by ordinary one-way ANOVA, Dunnett's multiple comparisons test comparing each day against 0-day old **p<0.01, ***p<0.001, ****p<0.0001.

Helios Expression in Mouse Thymocytes

These Helios differences between perinatal and adult T cells may be pre-determined at the hematopoietic stem cell (HSC) level, as hypothesized by the concept that the immune system develops in a "layered" fashion. This hypothesis suggests that unique hematopoietic stem cells (HSCs) give rise to distinct lymphocyte lineages at different stages of development. These distinct populations of cells may co-exist for a period of time[323,356]. This prompted us first to examine if the rapid reduction of Helios+ T cells we observed in the spleen in neonatal mice has a developmental origin in the thymus. To test this, we obtained thymocytes from fetuses in embryonic days (E) 17.5/18.5, 0, 1, 3, and 5-day old neonates and compared their Helios and Foxp3 expression to adult mice (>6 months of age). Figure 33 demonstrates the gating strategy to examine Helios and Foxp3 expression in CD4+ and CD8+ single-positive (SP) population in the thymus.

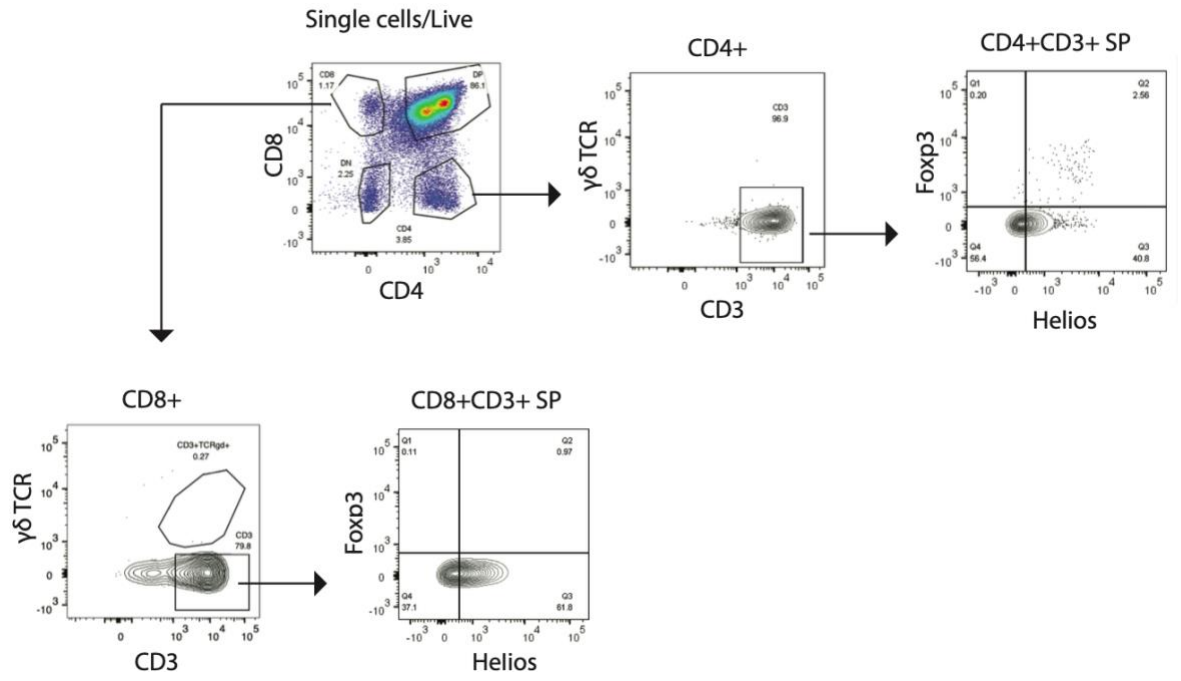

Figure 33: Gating Strategy of Helios and Foxp3 Expression in Different Thymocyte Population. This strategy is applied for all thymocyte analyses for the different age groups.

The frequency of Helios+Foxp3- expressing CD4+ and CD8+ SP thymocytes is highest at E17/18.5 (75.32±3.71% and 96.1±1.08% respectively). We observed a significant decrease in Helios+Foxp3- CD4+ SP thymocytes starting at birth (day 0, 43.73±2%) and rapidly decline to adult levels (35.28±12.88%) by day 1 after birth (32.56±2.27%). Like previous reports, we detected the emergence of Foxp3+ CD4+ Tregs in the thymus around 3 days after birth. These Foxp3+ CD4+ Tregs also express Helios and can be detected through adulthood (2.17±0.66%at day 3, 3.44±0.77% at >6 months old, Figure 34A).

In contrast, the frequency of Helios+ CD8+ SP thymocytes significantly decreased only by 3 days after birth (78.28±2.17%) and is the lowest in adults (60.13±10.93%, Figure 34B). These data demonstrate that Helios expression in both CD4+ and CD8+ thymocytes is the highest before birth and rapidly declines after birth, like what we observed in the periphery (Figure 31 and 34).

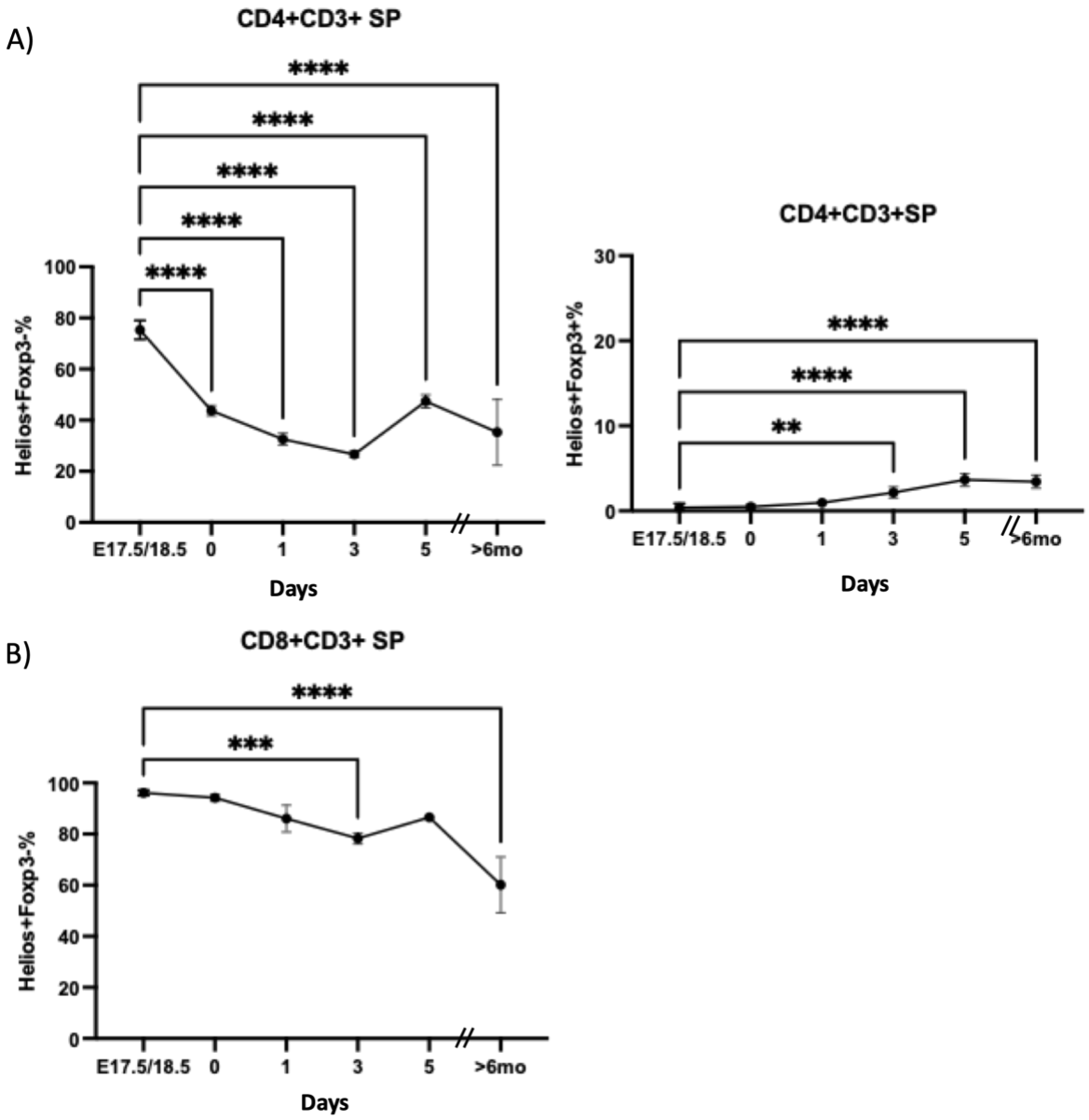

Figure 34: Helios and Foxp3 Frequency and Kinetics in Murine Perinatal and Adult CD4+CD3+ SP And CD8+CD3+SP Thymocytes At the Indicated Ages. A) Helios+Foxp3- and Helios+Foxp3+ frequency by CD4+ SP thymocytes. B) Helios+Foxp3- frequency by CD8+ SP thymocytes. CD4 and CD8 SP are gated as CD4+CD3+ and CD8+CD3+ after gating for live and single cells. Analyzed by ordinary one-way ANOVA, Dunnett's multiple comparisons test comparing each timepoints to E17.5/E18.5, **p<0.01, ***p<0.001, ****p<0.0001. Each data point represents mean and standard deviation of N=3-4 pooled samples or individual mice. All value on the x-axis indicates days except for >6mo where it represents >6-month-old adult.

To determine if perinatal γδ T cells in the thymus also express Helios, we analyzed Helios and Foxp3 expression by γδ T cells in the thymus at the indicated ages. γδ T cells in the thymus are predominantly DN but a minor population of CD8+ γδ T cells have been reported[549]. Therefore, we analyzed Helios frequency by total γδ T cells (CD3+γδTCR+), DN γδ T cells (CD4-CD8-CD3+γδTCR+) as well as CD8+ γδ T cells. In our hands, we detected CD8+γδ T cells in the thymus, especially in fetal and neonatal thymocytes, but almost undetectable in adults. Within total γδ T cells, the highest Helios frequency was detected before birth, E17.5/18.5 (96.2±2.72%). The frequency of Helios expressing total γδ T cells significantly decreases at day 0, 3 and 5 after birth. Helios frequency within adult (>6mo) total γδ T cells is significantly lower (60.1±12.9%) compared to fetal thymocytes. When we assessed Helios expression within CD8+γδ T cells, we saw a significant decrease in Helios frequency by 3 days after birth. Lastly, looking within CD4-CD8- DN γδ T cells population, Helios frequency is only significantly lower in adults compared to perinatal thymocytes (Figure 35C).

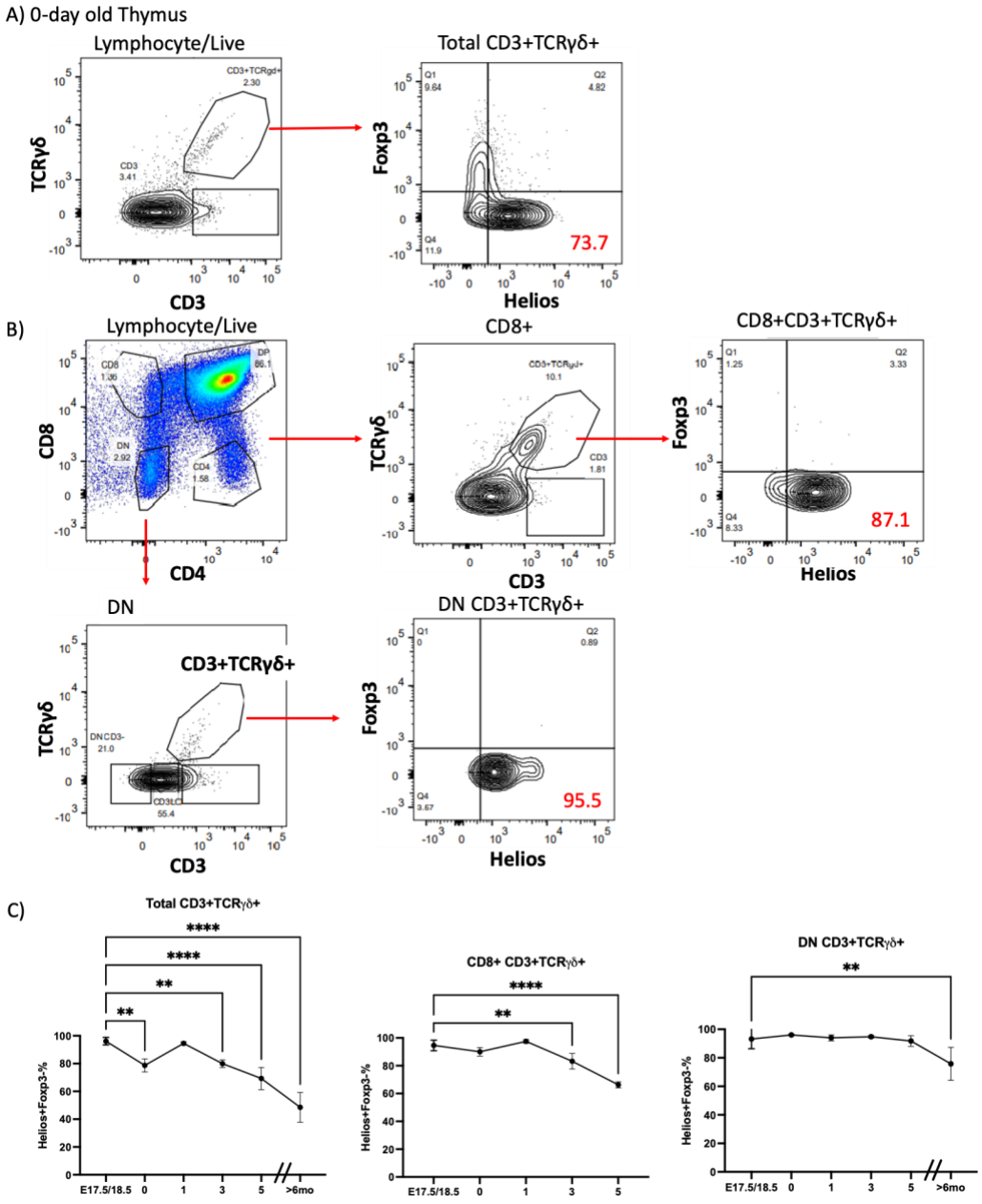

Figure 35: Helios Kinetics in Murine Perinatal and Adult CD3+γδTCR+ Thymocytes at the Indicated Ages. A and B) Gating strategy for Helios and Foxp3 expression by total CD3+γδTCR+, CD8+ CD3+γδTCR+ and DN CD3+γδTCR+ in the thymus. FACS plots are from 0-day old thymus sample. The same strategy is applied for all analysis of different age groups. C) Helios+Foxp3- frequency by total γδ T cells (CD3+γδTCR+), DN γδ T cells (CD4-CD8-CD3+γδTCR+) and CD8+ γδ T cells (CD8+CD3+γδTCR+) in the thymus of indicated ages. Analyzed by ordinary one-way ANOVA, Dunnett's multiple comparisons test comparing each time point to E17.5/E18.5, **p<0.01, ****p<0.0001. Each data point represents the mean and standard deviation of N=3-4 individual mice. All value on the x-axis indicates days except for >6mo where it represents >6-month-old adult.

Overall, the frequency of Helios in CD4+ SP, CD8+ SP and γδ TCR+ thymocytes are higher than the spleen across all the time points, but the kinetics of Helios+ T cells are similar between both the spleen and the thymus. Both newborn splenocytes and thymocytes started with high Helios frequency and their Helios frequency rapidly decreased to adult levels within the first week of birth (Figure 36). Altogether, these data demonstrate that perinatal αβ and γδ T cells express a higher frequency of Helios and the frequency of Helios+ T cells decreases with age both in the thymus and the spleen.

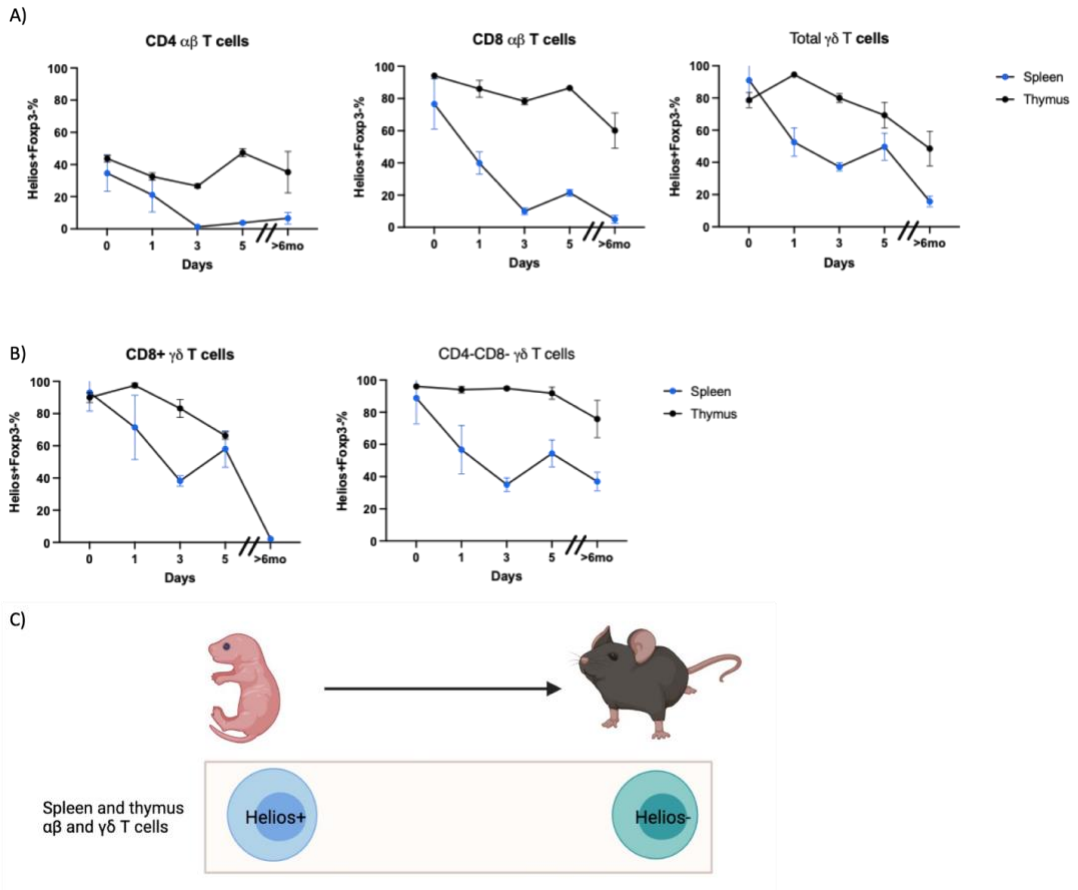

Figure 36: Helios+Foxp3- Frequency Comparison by Splenic and Thymic αβ T Cells and γδ T Cells of Perinatal and Adult Mice. A) Helios+Foxp3- frequency by CD4+ and CD8 αβ T cells and total γδ T cells in the spleen and thymus. B) Helios+Foxp3- frequency by CD8+ and CD4-CD8- DN γδ T cells in the spleen and thymus. Each data point represents mean and standard deviation of N=3-7 individual mice or pooled neonatal samples. All value on the x-axis indicates days except for >6mo where it represents >6-month-old adult. C) Summary schematics of Helios expression by αβ and γδ T cells in the spleen and thymus from perinatal stage to adulthood.

Helios Expression in Gut Associated T Cells

The intestinal immune surveillance network is an integrated part of the organ, enabling it to balance host protection against pathogens and maintain tissue homeostasis. T cells reside in three main compartments in the intestine, namely gut-associated lymphoid tissue (GALT), lamina propria (LP), and the epithelium (IEL). These frontline T cells are primarily heterogenous and can be distinguished into two major subsets (type a and type b) based on T cell receptor and co-receptor expression[550,551]. "Type a" cells consist of conventional thymus-selected antigen-experienced T cells that can also be found in the peripheral blood, spleen, and secondary lymphoid organs. These T cells express TCRαβ+ and are MHC class I or class II restricted CD4+ and CD8αβ+ T cells. "Type b" T cells consist of unconventional T cells that express CD8αα homodimer and can express either TCRαβ+ or TCRγδ+ (Figure 37). A large proportion of the gut IEL compartment consists of these CD8αα TCRαβ+ or TCRγδ+ "type b" T cells.

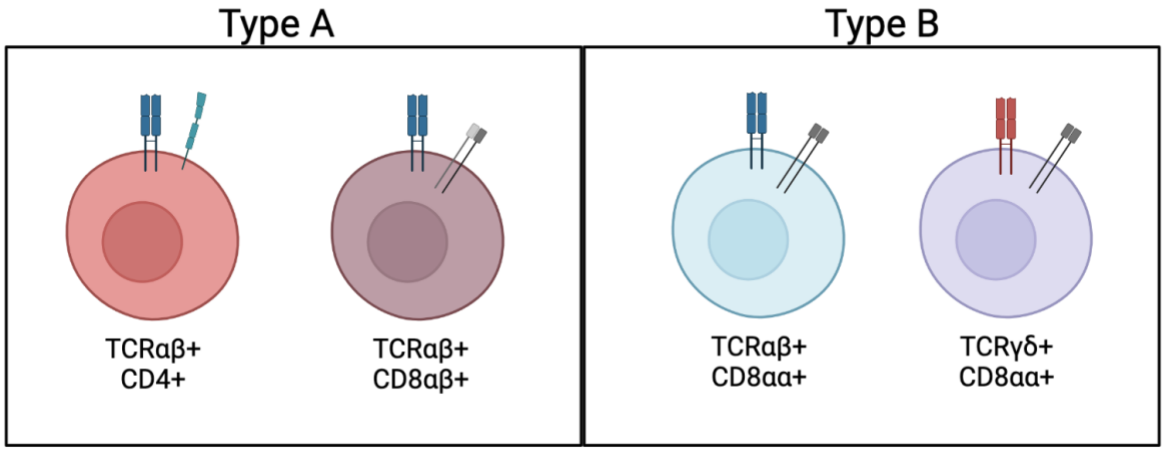

Figure 37: Type A and B Gut Associated T Cells.

"Type b" T cells have been implicated to be of perinatal origin. These "type b" T cells can be detected at fetal stages, but major colonization of the gut epithelium by these cells occurred perinatally. The detection of Vγ7+ γδ T cells in the gut IEL fraction suggests that γδ TCR+ IELs originate from the fetal origin as Vγ7 is detected in the thymus at E13[51]. It is also

implicated that αβ TCR+ IELs are of fetal origin as αβ TCR+ can be detected in both IEL and LPL of human fetal intestine[552,553]. Furthermore, grafting of adult thymi into athymic nude mice led to virtually no γδ TCR+ IELs and relatively less CD8αα αβ TCR+ IELs compared to fetal thymi[554]. These data suggest that progenies of fetal thymocytes are the major source of CD8αα γδ and αβ T cell subsets in the intestinal IELs. Since we observed a higher frequency of Helios+ γδ and αβ T cells in both the spleen and thymus of neonatal mice, we seek to determine if gut-associated "type b" γδ+ and αβ+ T cells express Helios due to their implicated fetal origin.

The gut immune system undergoes significant changes throughout life in terms of numbers and subset compositions since it is exposed to a wide variety of food and microbial antigens[555]. First, we wanted to determine if we could detect "type a" and "type b" gut T cells in both neonates and adult mice. We isolated LPL and IEL fractions from small intestines (SI) of 5-day old neonates and adult mice (6-12 weeks old) and assessed their cell proportions. Due to the isolated mononuclear cell number, panel design, and fluorophore restrain, we stained for γδ TCR and CD3 and gated CD3+ γδTCR- as CD3+αβ T cell population. We verified that majority of CD3+ γδTCR- contains cells that stained for αβ TCR population in both the gut IEL and LPL fraction (Figure 38). Therefore, from here onwards, αβ T cell population in the gut will be represented under CD3+ γδTCR- fraction.

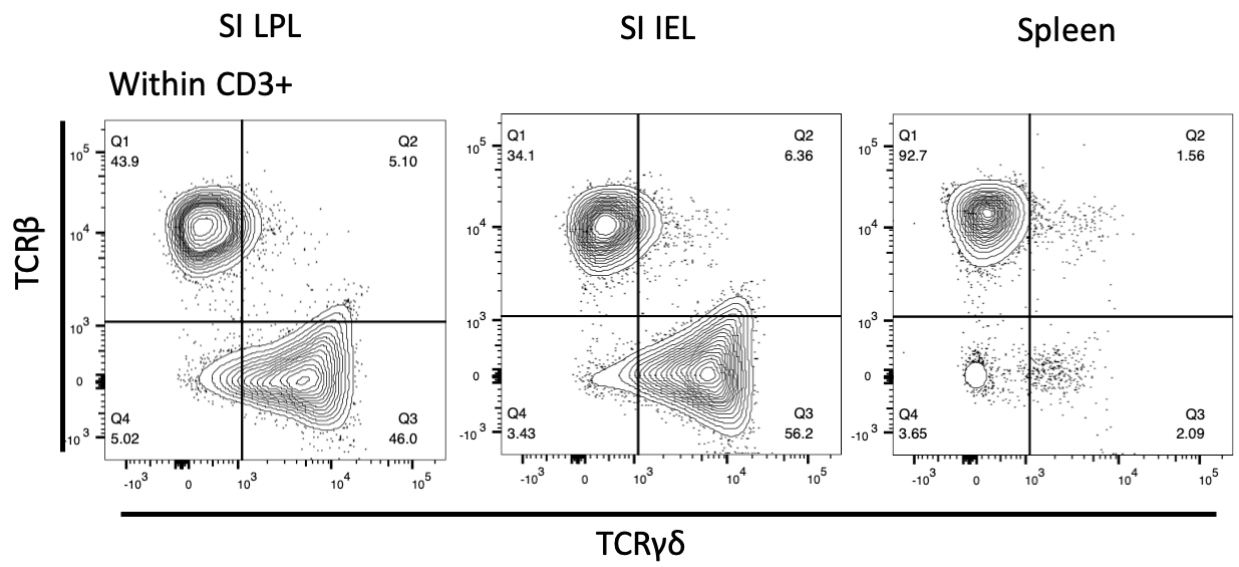

Figure 38: TCRαβ and γδ Expression by CD3+ T Cells within SI LPL, SI IEL and Spleen of Adult Mice.

When we compared the frequency of αβ T cells and γδ T cells in the SI IEL and SI LPL between 5-day old neonates and adults, we observed that the frequency of αβ T cells (CD3+ γδTCR-) is similar in the SI IEL but significantly higher in 5-day old neonatal SI LPL compared to adult SI LPL (Figure 39A). In contrast, the frequency of γδ T cells (CD3+ γδTCR+) is significantly higher in adult SI IEL than 5-day old neonates, while it is similar in the SI LPL (Figure 39B).

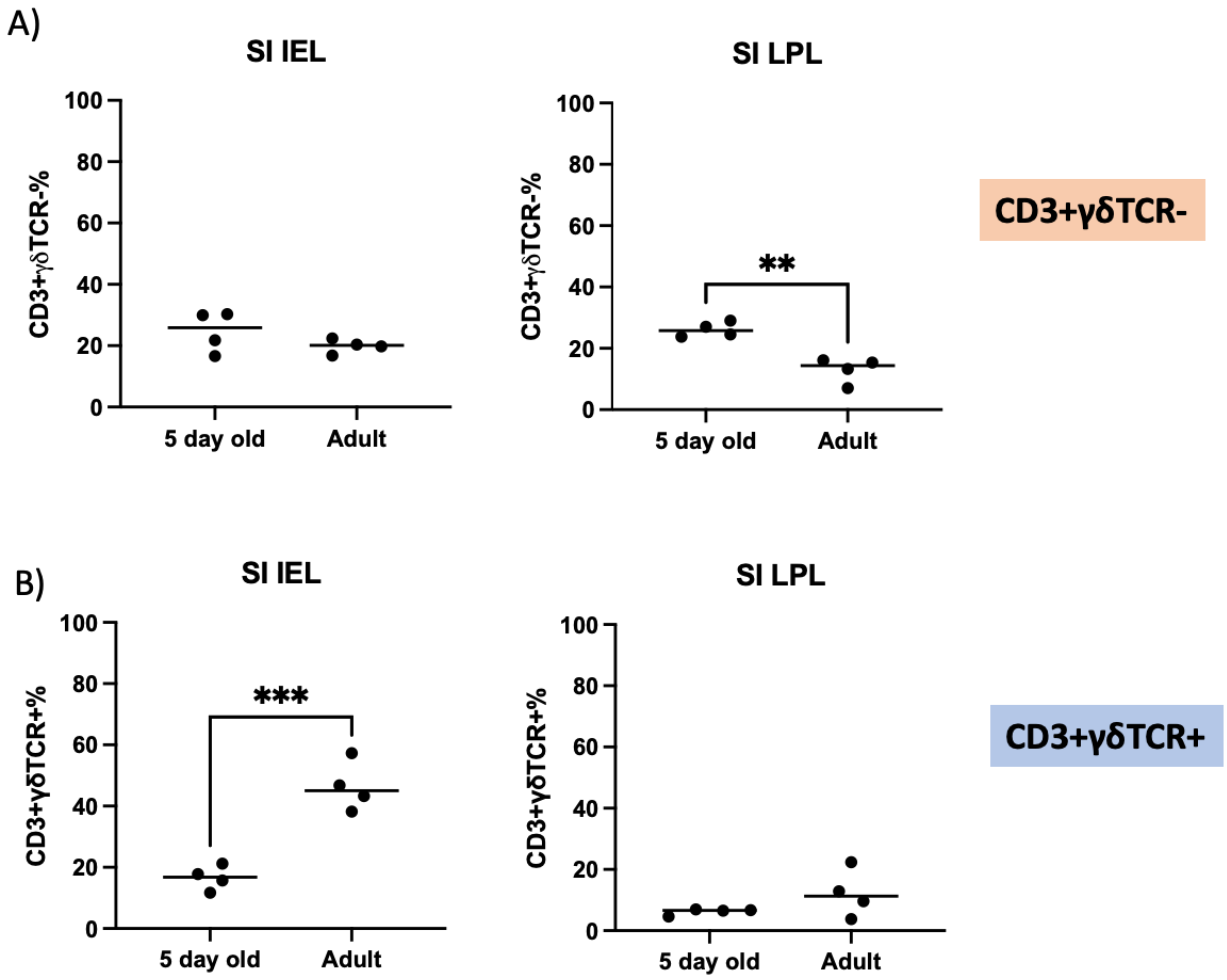

Figure 39: Frequency of αβ and γδ T cells Among CD45+ Cells in Neonatal and Adult SI LPL and IEL. A) Frequency of αβ T cells reflected by the frequency of CD3+ γδTCR- gated within CD45 population in the SI IEL and LPL of 5-day old neonates and adult. B) Frequency of γδ T cells as reflected by the frequency of CD3+ γδTCR+ gated within CD45 population in the SI IEL and LPL of 5-day old neonates and adult. The small intestine intraepithelial layer and lamina propria are processed to isolate mononuclear cell subsets and stained for different surface antigen markers to determine their frequency. Each dot represents an individual experiment with either individual or pooled SI of the indicated age group. Analyzed by unpaired t-test, **p<0.01, ***p<0.001. N=4 individual experiments.

It was previously reported that gut-associated αβ TCR+ and γδ TCR+ IELs predominantly express CD8αα homodimer[170,556]. In the human fetal intestine, nearly 50% of fetal IELs express an antigen-experienced and CD8αα phenotype[552]. We further stained adult and neonatal LPL and IEL for other surface markers, including CD4, CD8α, and CD8β. Gating strategies are depicted in Figure 40.

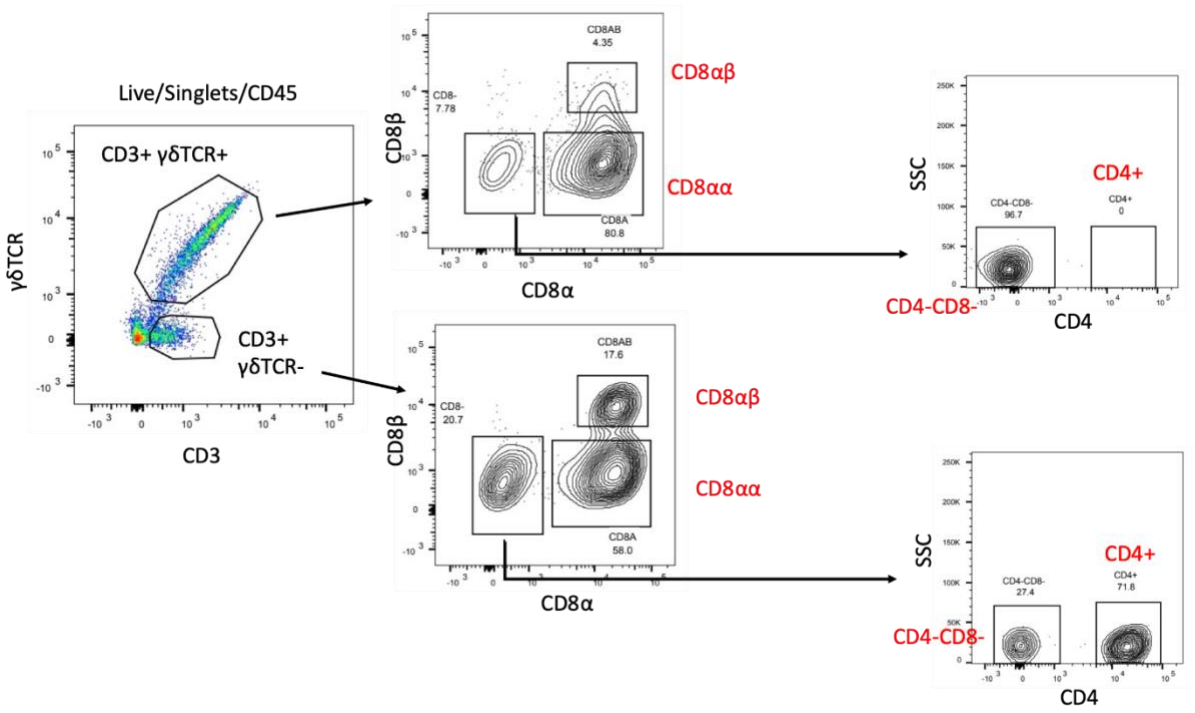

Figure 40: Gating Strategy of CD4, CD8αα, CD8αβ, and CD4-CD8- Subsets Within CD3+γδTCR+ and CD3+γδTCR- Population. The same gating strategy was applied to both SI IEL and SI LPL.

Within the αβ T cell subsets, both SI IEL and LPL in 5-day old neonates are predominantly enriched with CD4+ subsets (46.7±15.48% and 64.27±6.59% respectively). Neonatal SI IEL CD4+ frequency within αβ T cell subsets is significantly higher than adult SI IEL (14.15±3.81%). Still, it is not significantly different between neonatal SI LPL and adult SI LPL (50.24±11.59%). The population of CD8αα αβ T cells is significantly lower in 5-day old SI IEL (7.15±4.07%) and LPL (2.64±1.64%) in comparison to adult SI IEL (60.64±11.65%) and LPL (24.43±8.5%). On the other hand, CD8αβ subset frequency within αβ T cells is not significantly different between neonates (22.48±9.43%) and adult SI IEL (14.41±8.11%). Lastly, 5-day old neonate SI IEL has significantly lower CD4-CD8- αβ T cell frequency compared to adult SI IEL (3.4±2.32% and 6.55±1.76% respectively). At the same time, they are not significantly different in the SI LPL compartment (Figure 41). In addition to that, there is also a

clear difference between SI IEL and LPL fraction particularly in the adult gut CD8αα αβ T cells where there was an enrichment in the SI IEL fraction but not as prominent in the LPL fraction.

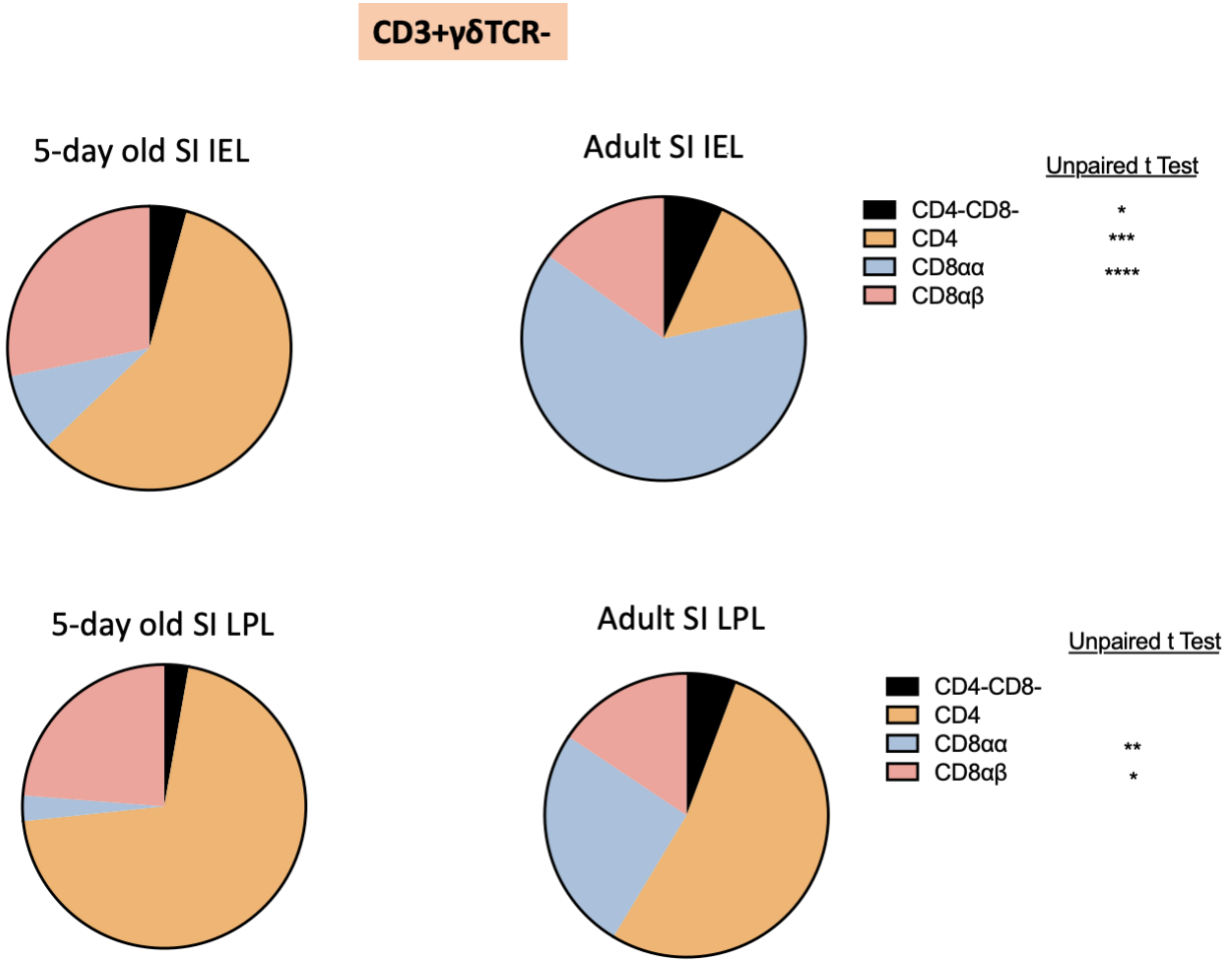

Figure 41: Proportion of CD4, CD8αα, CD8αβ and CD4-CD8- Within αβ T Cells in 5-day Old and Adult SI IEL and LPL. The small intestine intraepithelial layer and lamina propria is processed to isolate mononuclear cell subsets and stained for different surface antigen markers to determine their frequency. αβ T cells are gated as CD3+ γδTCR- within CD45 population in the SI IEL and LPL of 5-day old neonates and adults. Within the αβ T cells subset, the average frequency of each subset is obtained from N=4-7 individual experiments and plotted as a pie chart. Analyzed by unpaired t-test comparing each subsets between 5-day old neonates and adult, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Within the γδ T cells subset in the gut, the SI IEL are predominantly enriched with CD8αα expressing cells in both neonates and adults. However, the frequency of CD8αα subset is significantly higher in adult SI IEL (86.33±3%) in comparison to 5-day old neonates (71.13±3.32%). Gut CD8αα and CD4-CD8- γδ T cells have been the most studied population,

but a novel CD8αβ γδ T cell subset has just been recently identified and characterized in the human gut. This CD8αβ γδ T cell subset has cytotoxic potential and is negatively correlated with disease severity in inflammatory bowel disease (IBD), suggesting a potential regulatory role in IBD[557]. We detected CD8αβ γδ T cells in both neonates and adult gut, with a significantly higher frequency in 5-day old neonates SI IEL and LPL (11.05±2.92% and 7.6±2.07% respectively) compared to adult SI IEL and LPL (2.94±1.12% and 2.94±1.95% respectively). Both neonatal SI IEL and LPL has a higher frequency of CD4-CD8- γδ T cell subsets (12.52±2.87% and 41.54±1.64% respectively) compared to adults SI IEL and LPL (6.41±1.4% and 10.22±2.43% respectively) where strikingly in the neonatal SI LPL, CD4-CD8- subsets reconstitute almost half of all γδ T cells. We detected a minimal frequency of CD4 expressing γδ T cells in both neonates and adults SI IEL and LPL (Figure 42).

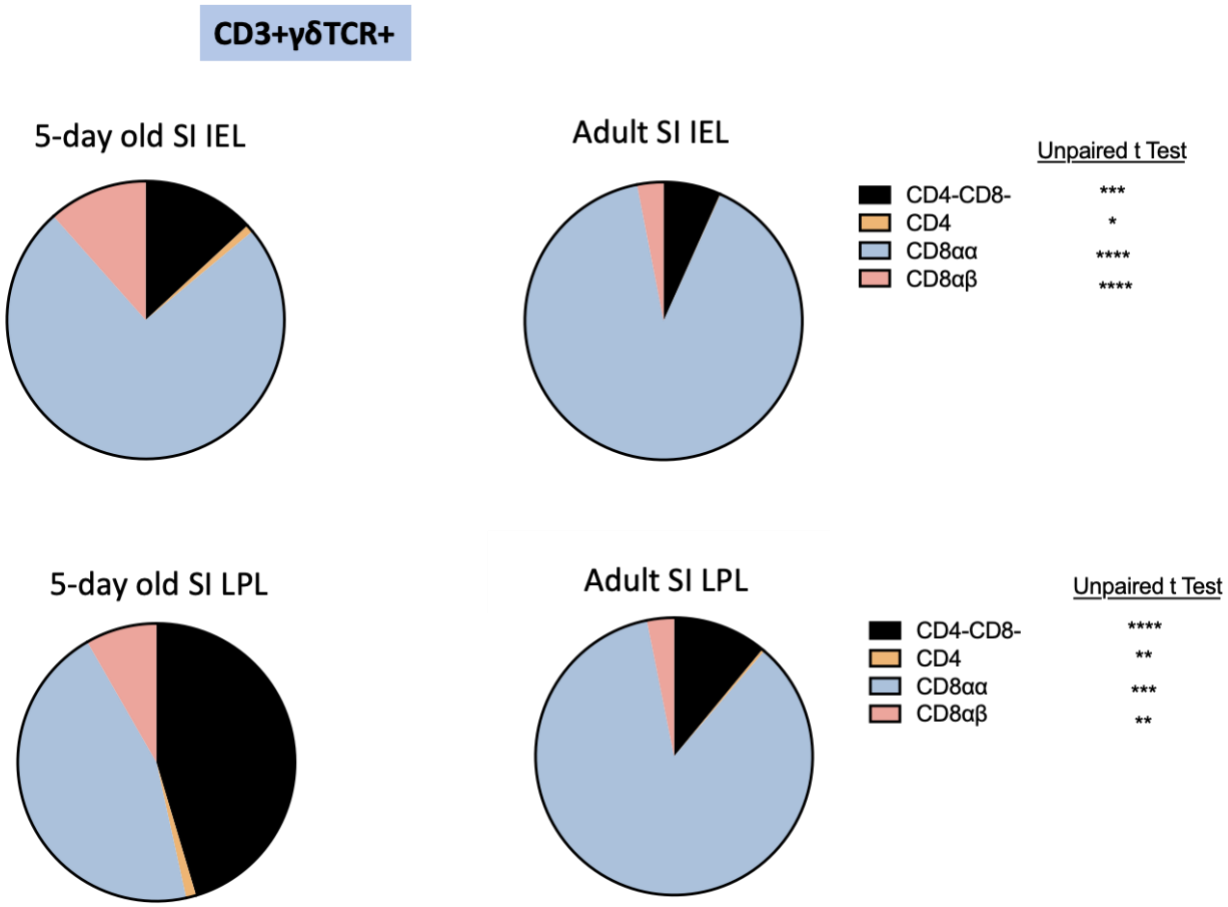

Figure 42: Proportion of CD4, CD8αα, CD8αβ, and CD4-CD8- Within γδ T Cells in 5-day Old and Adult SI IEL and LPL. The small intestine intraepithelial layer and lamina propria were processed to isolate mononuclear cell subsets and stained for different surface antigen markers to determine their frequency. γδ T cells are gated as CD3+ γδTCR+ within CD45 population in the SI IEL and LPL of 5-day old neonates and adults. Within the γδ T cells subset, the average frequency of each subset is obtained from N=4-7 individual experiments and plotted as a pie chart. Analyzed by unpaired t-test comparing each subsets between 5-day old neonates and adults, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Helios expression in gut-associated "type a" and type b" T cells. After we demonstrated that we could successfully detect the different types of gut mucosal T cells in both 5-day old neonates and adults SI IEL and LPL fractions, we next checked Helios expression within the different gut T cell subsets. Since gut-associated "type b" γδ+ and αβ+ T cells has implications of perinatal origin, we hypothesized that "type b" γδ+ and αβ+ T cells express the high frequency of Helios throughout adulthood while "type a" CD4 and CD8 αβ+ T cells do not.

In the SI IEL “type a” T cell fraction, we found that the frequency of Helios+Foxp3- within CD4+ T cells of 5-day old neonates and adults are not significantly different (20.1±20.83% vs 9.43±4.1%). In contrast, Helios+Foxp3- frequency within the CD8αβ αβ T cells are significantly higher in 5-day old IEL in comparison to adult SI IEL (54.87±10.5% vs 17.06±9.77%). On the other hand, within the SI IEL “type b” T cell fraction, which compromises of CD8αα γδ and αβ T cells, the frequency of Helios+Foxp3- is not significantly different between neonates and adults CD8αα γδ T cells (89.03±14.76% in neonates vs. 69.3±20.61% in adults). There is no significant difference between neonatal and adult CD8αα αβ T cells in their Helios+Foxp3- frequency (95.7±5.73% in neonates vs. 71.26±20.32% in adults) in the SI IEL fraction (Figure 43). However, it is important to note that the frequency of CD8αα αβ T cell subset in the gut of 5 day old neonates is very low as observed in Figure 41 and in agreement with previously published studies[558].

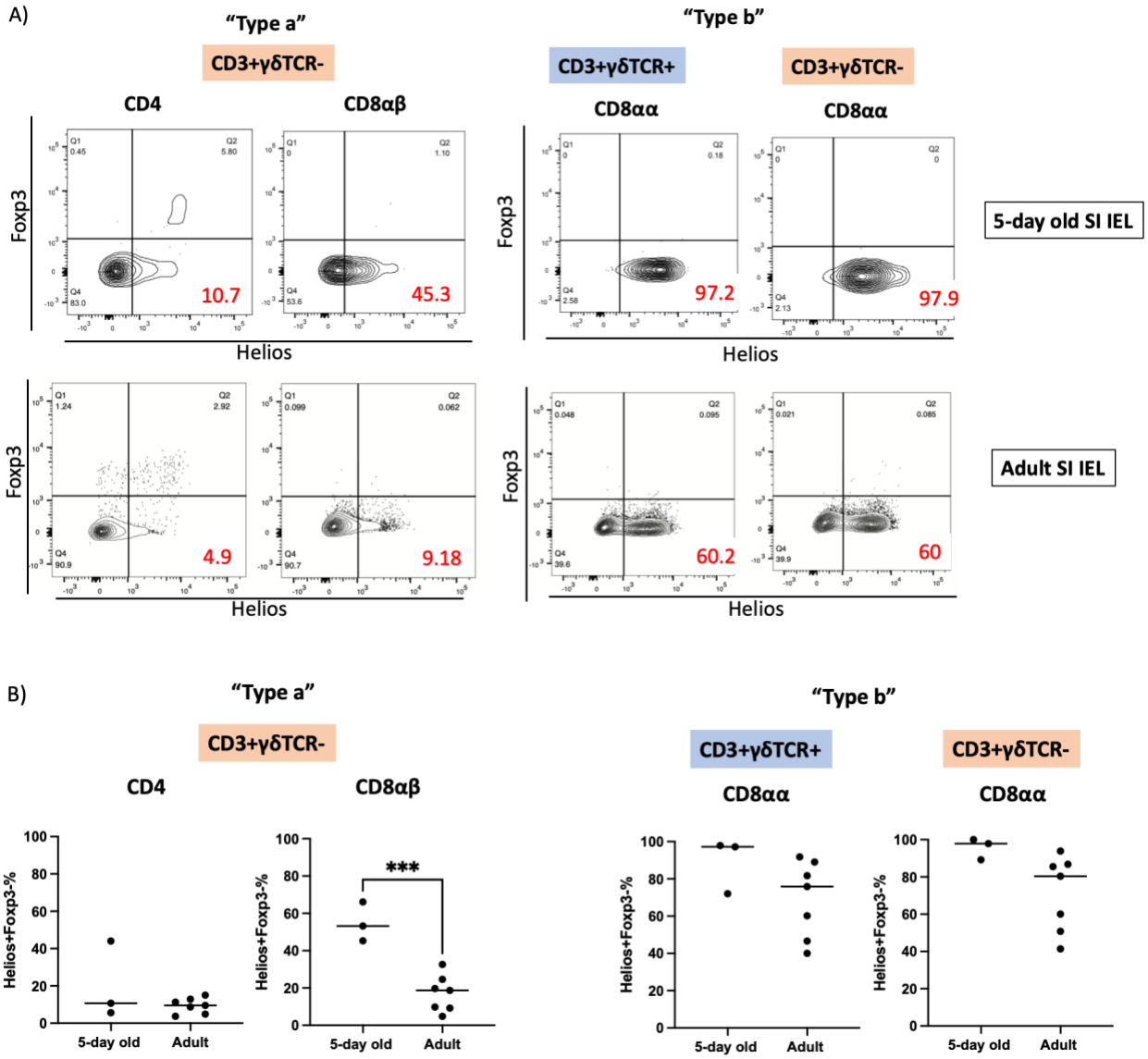

Figure 43: Helios and Foxp3 Expression Within "Type A" and "Type B" SI IEL Associated T Cells. A) Representative flow plots of Helios and Foxp3 expression within CD4, CD8αβ αβ T cells and CD8αα αβ and γδ T cells from small intestine IEL fraction isolated from 5-day old neonates or adult. B) Summary plots comparing the frequency of Helios+Foxp3- expression within CD4, CD8αβ αβ T cells (type a) and CD8αα αβ and γδ T cells (type b) from small intestine IEL fraction isolated from 5-day old neonates or adult. αβ T cells are gated as CD3+ γδTCR- gated within CD45 population and γδ T cells are gated as CD3+ γδTCR+ within CD45 population in the SI IEL. N=3 individual experiments of pooled neonatal gut (6-9 pups per experiment) and N=7 individual experiments for adult gut samples. They were analyzed by unpaired t test ***p<0.001.

In the SI LPL "type a" T cell fraction, the frequency of Helios+Foxp3- within CD4+ T cells of 5-day old neonates and adult are not significantly different (9.33±5.39% vs

10.39±5.32%). Helios+Foxp3- frequency within the CD8αβ αβT cell subset is significantly higher in 5-day old SI LPL in comparison to adult SI LPL (39.4±8.46% vs 10.02±4.47%). Within the SI LPL "type b" T cell fraction, the frequency of Helios+Foxp3- is not significantly different between neonates and adults CD8αα γδ T cells (93.6±5.43% in neonates vs 86.65±8.12% in adults). There is also no significant difference in Helios+Foxp3- frequency between neonates and adult SI LPL CD8αα αβ T cells. (86.73±7.34% and 87.93±9.68% respectively, Figure 44). Like the SI IEL fraction, neonatal SI LPL CD8αα αβ T cells frequency is very low (Figure 41)[558].

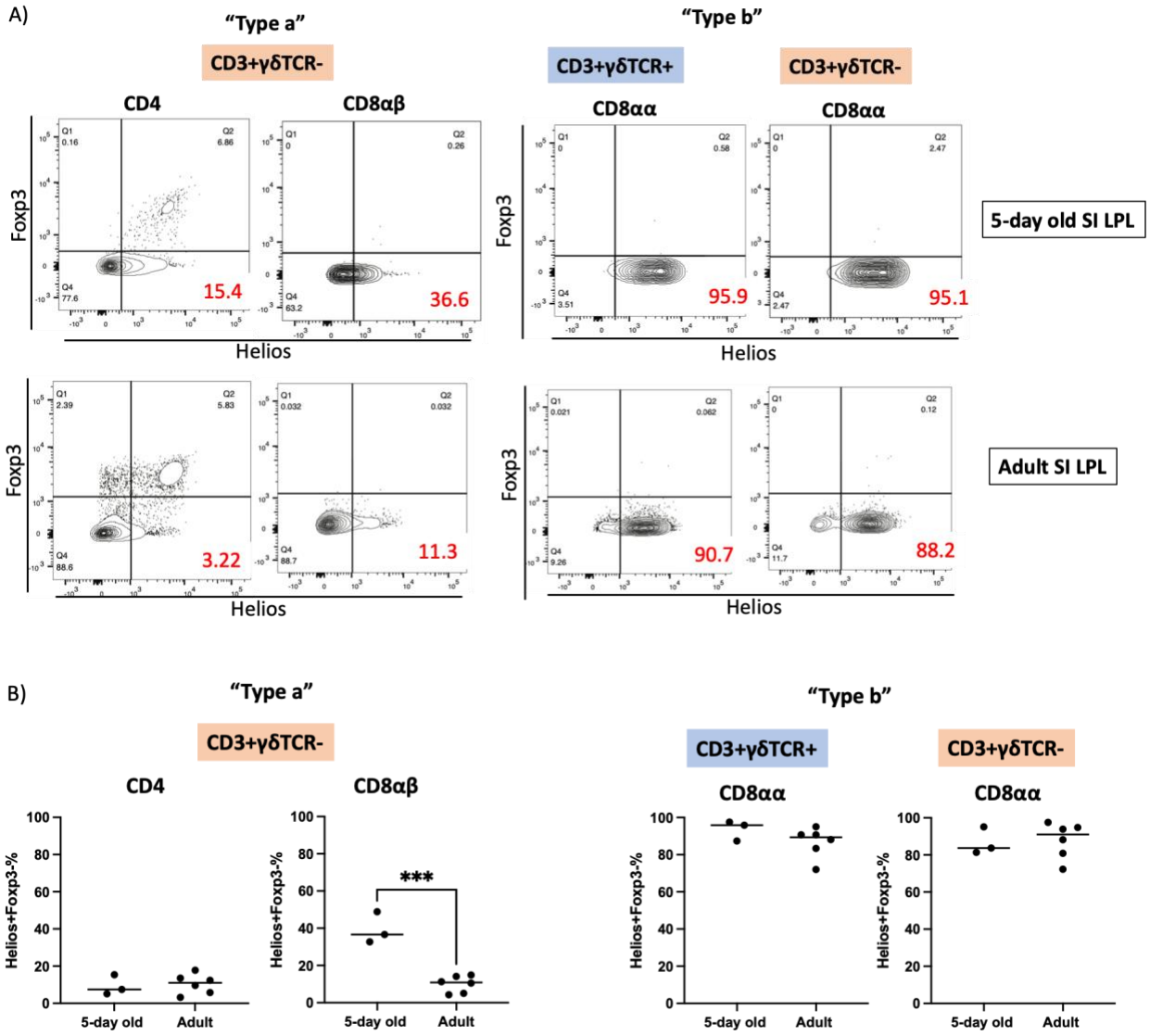

Figure 44: Helios and Foxp3 Expression Within "Type A" and "Type B" SI LPL Associated T Cells. A) Representative flow plots of Helios and Foxp3 expression within CD4, CD8αβ αβ T cells and CD8αα αβ and γδ T cells from small intestine LPL fraction isolated from 5-day old neonates or adult. B) Summary plots comparing the frequency of Helios+Foxp3- expression within CD4, CD8αβ αβ T cells (type a) and CD8αα αβ and γδ T cells (type b) from small intestine LPL fraction isolated from 5-day old neonates or adult. αβ T cells are gated as CD3+ γδTCR- gated within CD45 population and γδ T cells are gated as CD3+ γδTCR+ within CD45 population in the SI LPL. N=3 individual experiments of pooled neonatal gut (6-9 pups per experiment) and N=6 individual experiments for adult gut samples. Analyzed by unpaired t test ***p<0.001.

When we compared Helios+Foxp3- frequency between "type a" and "type b" T cell subsets in the SI IEL and LPL, "type b" T cells (both CD8αα αβ and γδ T cells) express

significantly higher frequency of Helios+Foxp3- than "type a" T cells (CD8αβ and CD4 αβ T cells) in both neonates and adults (Figure 45). These data suggest that Helios expression is limited to T cells of perinatal origin and is not induced by the gut microenvironment as not all gut-associated T cells express Helios.

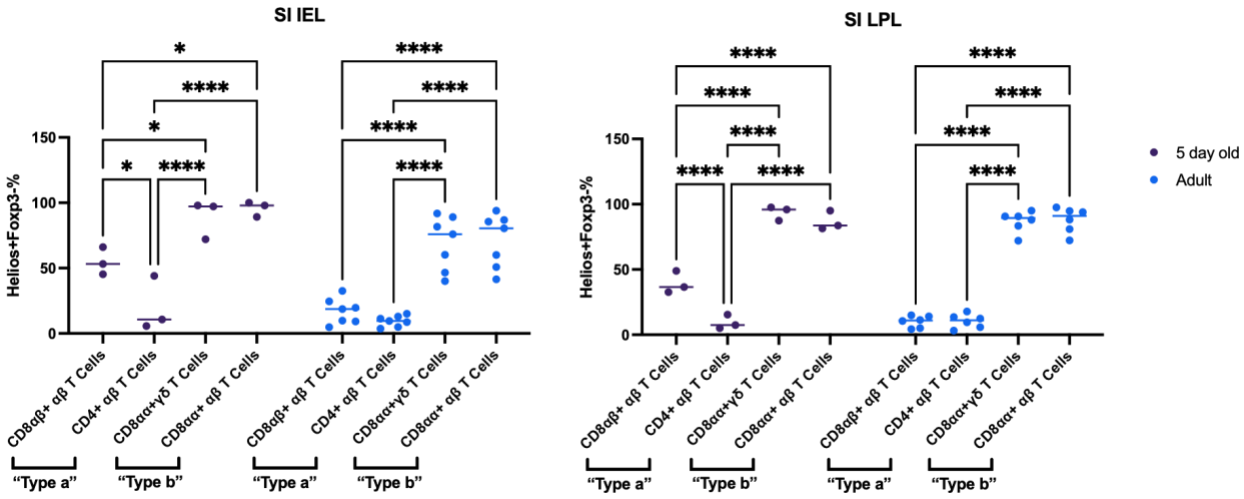

Figure 45: Comparison of Helios+Foxp3- Frequency Between "Type A" and "Type B" T Cells in SI IEL and LPL by Neonates and Adults. Summary plots comparing the frequency of Helios+Foxp3- between "type a": CD8αβ αβ T cells, CD4 αβ T cells and "type b": CD8αα γδ T cells and CD8αα αβ T cells by 5 day old and adult SI IEL and LPL. αβ T cells are gated as CD3+ γδTCR- gated within CD45 population, and γδ T cells are gated as CD3+ γδTCR+ within CD45 population in the SI IEL and LPL. N=3 individual experiments of pooled neonatal gut (6-9 pups per experiment) and N=6-7 individual experiments for adult gut samples. Analyzed by 2way ANOVA, Tukey's multiple comparisons test, *p<0.05, ****p<0.0001.

Helios expression in "type c" gut T cells. We detected the presence of a less studied γδ T cell subset both in the SI IEL and LPL of neonates and adults. These γδ T cells express CD8αβ and will be categorized as "type c" gut-associated T cells hereinafter (Figure 42). This group of CD8αβ γδ T cells carries a striking phenotype where neonates express a higher frequency of Helios+Foxp3- in both SI IEL and LPL and significantly decreases in both adult SI IEL and LPL. However, there is a significant increase in the frequency of Helios and Foxp3 co-expression by these CD8αβ γδ T cells, particularly in adult SI IEL and a trend of increase by CD8αβ γδ T cells in adult SI LPL in comparison to neonates (Figure 46).

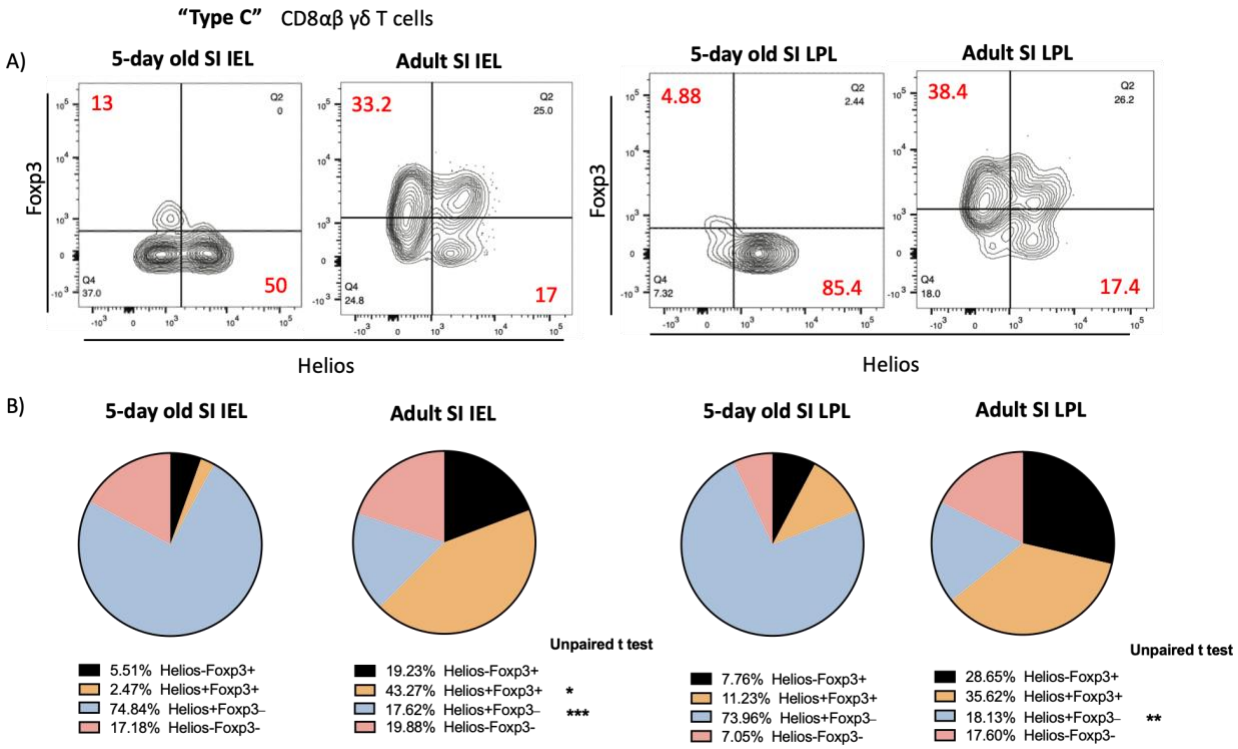

Figure 46: Helios and Foxp3 Expression by CD8αβ γδ "Type C" Gut Associated T Cells. The small intestine intraepithelial layer and lamina propria are processed to isolate mononuclear cell subsets and stained for Helios and Foxp3 expression. CD8αβ+ subset was gated within CD3+ γδTCR+ population in the SI IEL and LPL of 5-day old neonates and adults. Within CD8αβ+γδ T cells subset, the average frequency of Helios-Foxp3+, Helios+Foxp3+, Helios+Foxp3- and Helios-Foxp3- is obtained from N=6-7 adult mice and N=3 individual experiments for 5-day old, pooled neonate samples and plot as a pie chart. They were analyzed by unpaired t-test comparing each subsets between 5-day old neonates and adult SI IEL or SI LPL, *p<0.05, **p<0.01, and ***p<0.001.

Helios expression in fetal gut T cells. It was previously demonstrated that gut intraepithelial γδ T cells can be detected in rat intestines as early as 1 day after birth[559]. Next, we asked if we could also detect Helios+Foxp3- T cells in fetal gut IEL and LPL fraction (whole intestine). To test this, we set up timed pregnancies and obtained gut tissue from fetuses between E17.5 and 18.5. During this period, the majority of fetal gut αβ T cells are CD4-CD8- while the majority of γδ T cells express CD8αα (Figure 47).

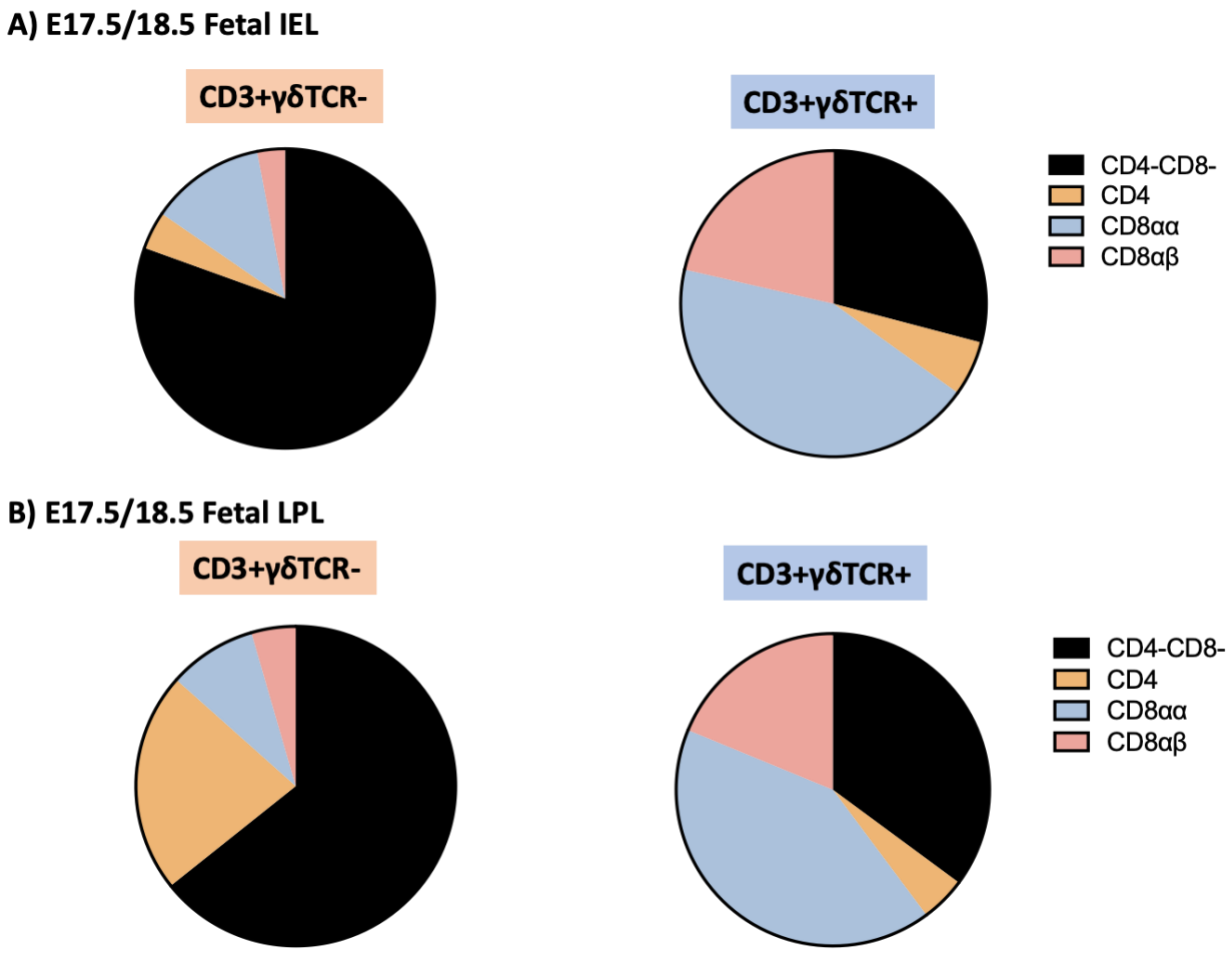

Figure 47: Proportion of CD4, CD8αα, CD8αβ and CD4-CD8- Within αβ and γδ T Cells in E17.5/18.5 Fetal IEL and LPL. The whole intestine (small and large) intraepithelial layer and lamina propria is processed to isolate mononuclear cell subsets and stained for different surface antigen markers to determine their frequency. αβ T cells are reflected as CD3+ γδTCR- while γδ T cells are gated as CD3+ γδTCR+ within CD45 population in the fetal IEL and LPL fraction. The average frequency of each subset is obtained from N=4 individual experiments and plotted as a pie chart. Fetal gut was pooled from the same litter (N=7-11 pups).

Next, we analyzed Helios expression by "Type b" CD8αα γδ T cells in both the fetal IEL and LPL. We found that both fetal IEL and LPL CD8αα γδ T cells express a high frequency of Helios+Foxp3- (80.84±18.83% and 78.72±12.23% respectively, Figure 48). We are unable to analyze CD8αα αβ T cells in the fetal gut due to low cell number.

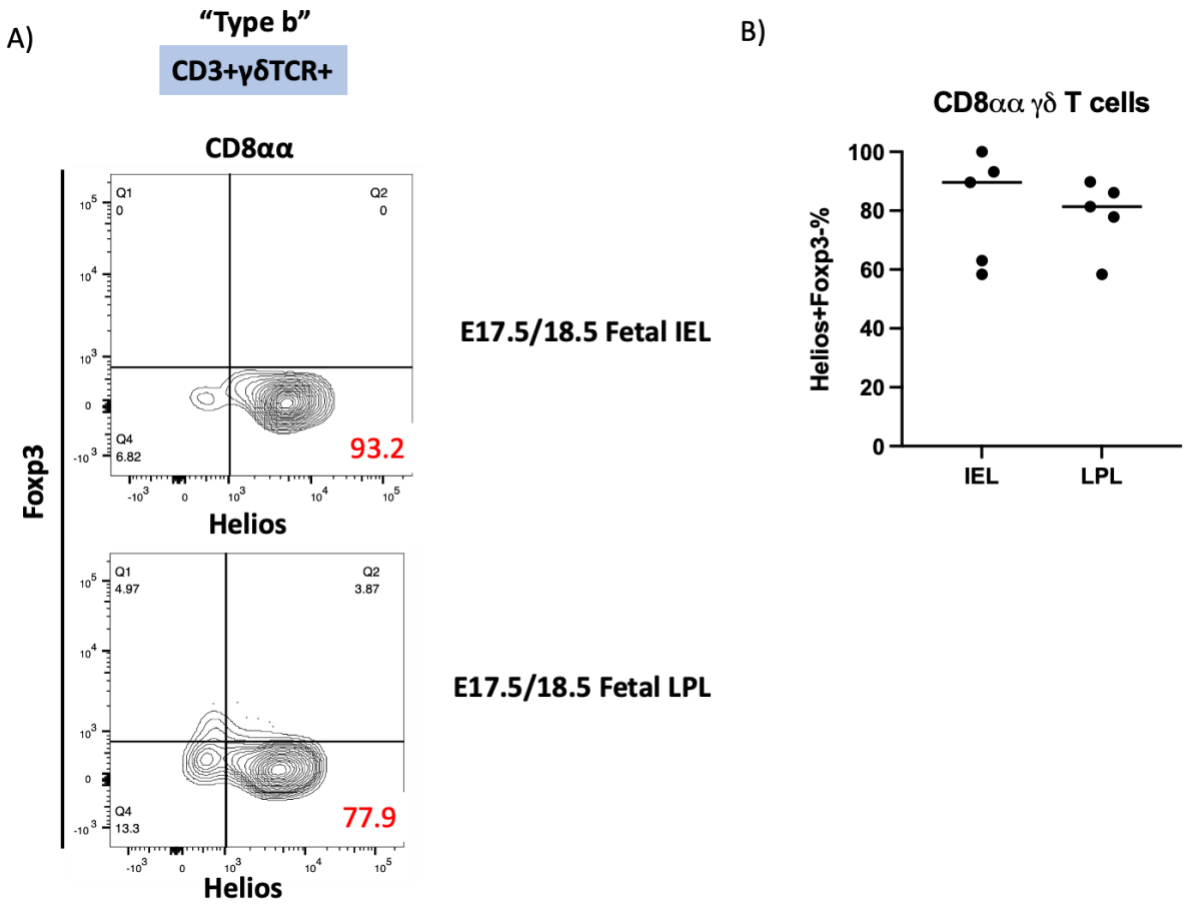

Figure 48: Helios and Foxp3 Expression Within CD8αα γδ T Cells in E17.5/18.5 Fetal IEL and LPL. A) Representative FACS plot of Helios and Foxp3 expression by CD8αα γδ T cells in the IEL and LPL. B) Summary plot reflecting Helios+Foxp3- frequency within CD8αα γδ T cells in the IEL and LPL. Whole intestine (small and large) intraepithelial layer and lamina propria is processed to isolate mononuclear cell subsets and stained for different surface antigen markers to determine their frequency. γδ T cells are gated as CD3+ γδTCR+ within CD45 population in the fetal IEL and LPL fraction. N=5 individual experiment, fetal gut was pooled from the same litter (N=7-11 pups).

In summary, Helios+Foxp3- can be detected in gut CD8αα γδ T cells as early as E17.5/18.5 fetal stage. Helios+Foxp3- frequency is higher in "type b" T cells than "type a" T cells in both SI IEL and LPL in both neonates and adults. Altogether, these data suggest that "type b" perinatal origin gut T cells in both SI IEL and LPL express high Helios+Foxp3- frequency and maintains throughout adulthood. On the other hand, within the "type a" T cells, neonatal gut associated CD8αβ αβ T cells express higher frequency of Helios+Foxp3- but

significantly decreases in adult, while CD4+ T cells express similar Helios+Foxp3- frequency between 5-day neonates and adult. Lastly, “type c” CD8αβ γδ T cells highly express Helios+Foxp3- during the neonatal stage, at levels similar to those of “type b” gut associated T cells. However, their Helios+Foxp3- frequency significantly decreases in adults with the subsequent emergence of Helios+Foxp3+ and Foxp3+ populations instead (Figure 49).

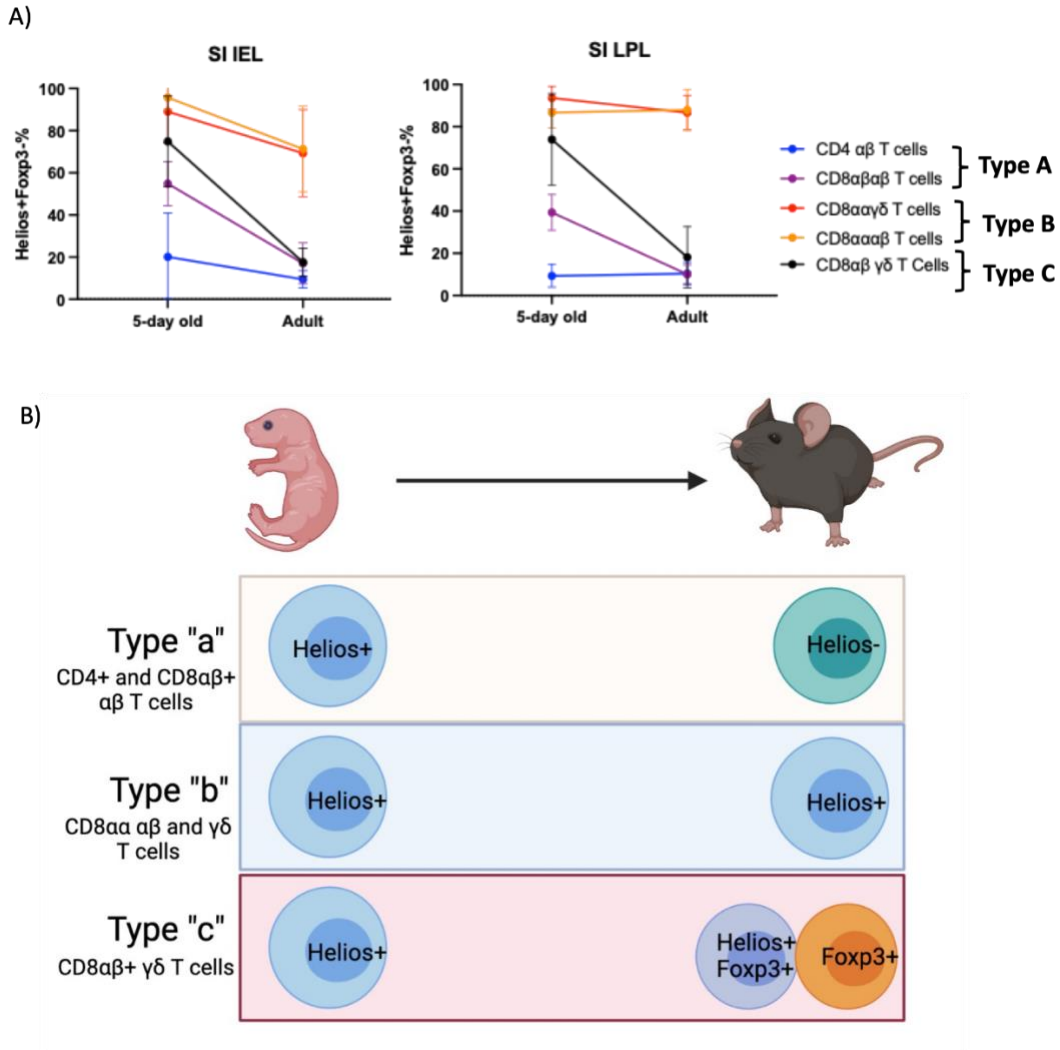

Figure 49: Helios+Foxp3- Frequency and Kinetics by “Type A”, “Type B” and “Type C” T Cells in SI IEL and SI LPL in 5-day old Neonates and Adult. A) Helios+Foxp3- frequency by “type a”, “type b” and “type c” T cells in SI IEL and LPL by 5-day old neonates and adults. B) Summary diagram of Helios and Foxp3 kinetics by indicated gut-associated T cell subsets from perinatal stage to adulthood.

Ikaros Transcription Factor Family Expression in Gut Associated T Cells

The Ikaros zinc finger (IKZF) transcription factors are known regulators of immune cell development. Furthermore, these transcription factors are differentially expressed by different T cell subsets and can play a prominent role in T cell differentiation. For instance, Ikaros expression has been shown to negatively regulate Th1 differentiation by silencing T-bet expression and IFNγ production[398,399]. Helios expression has been shown to maintain the function and stability of Foxp3+ Tregs. One of the mechanisms is by repressing IL-2 expression in Tregs, which is one of Treg's suppressive mechanisms to compete for IL-2 secreted by responder T cells[388,560]. Aiolos has also been shown to directly repress IL-2 expression in CD4 T cells under Th17 differentiation conditions[405].

We observed that perinatal T cells express a higher frequency of Helios while adult T cells do not. Furthermore, gut-associated "type b" T cells implicated in perinatal origin also highly express Helios and maintained Helios expression through adulthood. However, if other IKZF transcription factors such as Ikaros and Aiolos follows similar kinetics as Helios is unknown. We next seek to compare Ikaros, Aiolos, and Helios expression in "type a", "type b" and "type c" gut-associated T cells by 5-day old neonates and adult mice.

Within "type a" gut-associated T cells, the predominant IKZF transcription factor combination is Aiolos+ Helios- and Ikaros+ (A+H-I+). We do not observe a significant difference in any of the IKZF transcription factor combination by CD8αβ αβ T cells between 5-day old neonates and adult SI IEL. Due to fluorophore constraints, we analyzed CD8- αβ T cell fraction to include both CD4 and CD4-CD8- population. Based on our previous findings, the majority of CD8- cells consist of CD4+ T cell subset (Figure 40). In the CD8- αβ T cell fraction, the Aiolos+ Helios- Ikaros+ (A+H-I+) IKZF transcription factor combination is significantly

higher in neonates than their adult counterpart (59±20.4% and 33.25±13.23% respectively, Figure 50).

Within “type b” gut associated T cells, the predominant IKZF transcription factor combination is Aiolos+ Helios+ and Ikaros+ (A+H+I+) for both CD8αα αβ and γδ T cells. The frequency of Aiolos+ Helios+ Ikaros+ (A+H+I+) within CD8αα γδ T cells is significantly higher in neonates than adults SI IEL (70.3±15.5% and 53.5±11.27% respectively). Additionally, there is a significantly higher frequency of Aiolos+ Helios+ but Ikaros- (A+H+I-) in adult SI IEL. CD8αα γδ T cell population in adults (23±6%) compared to neonates (3.67±1.53%). Like their “type b” CD8αα γδ T cell counterpart, about 22±4.32% of adult SI IEL CD8αα αβ T cells express Aiolos+ Helios+ (A+H+I-) alone (Figure 50). Analysis for 5-day old neonates was unavailable due to the low frequency of CD8αα αβ T cells.

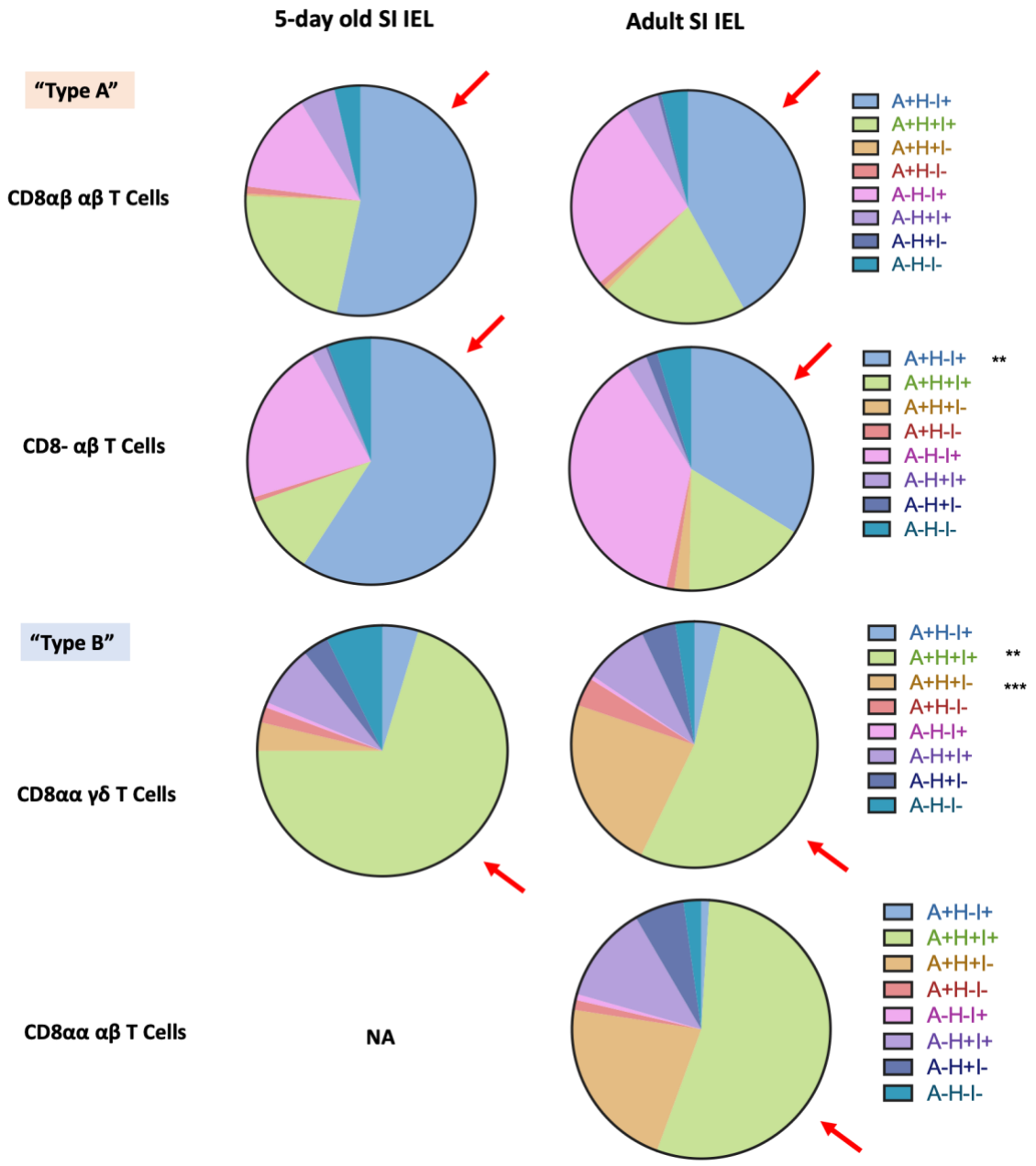

Figure 50: Ikaros Transcription Factor Proportions Within "Type A" and "Type B" Gut Associated T Cells in 5-day Old Neonates and Adult SI IEL. Pie charts reflecting the average frequency of transcription factor combination (A: Aiolos, H: Helios and I: Ikaros). Small intestines were processed to isolate mononuclear cell subsets from the IEL fraction and stained for different surface and intracellular markers to determine their frequency. αβ T cells are gated as CD3+ γδTCR- and γδ T cells are gated as CD3+ γδTCR+ within CD45 population. Within αβ and γδ T cells subset, the average frequency of each subset is obtained from N=3-6 individual experiments and plot as a pie chart. Neonates were pooled from the same litter of 6-9 pups and adults are individual mouse. Analyzed by 2way ANOVA, Sidak's multiple comparisons test, comparing each individual combination between 5-day old neonates and adult, **p<0.01, ***p<0.001.

In summary, we found that “type b” gut associated T cells predominantly express the combination of Aiolos, Helios, and Ikaros (A+H+I+) IKZF transcription factors in both neonates and adults. On the other hand, “type a” gut associated T cells predominantly express Aiolos and Ikaros (A+H-I+) in both neonates and adults. Consistent with our previous findings, the major difference between “type a” and “type b” gut T cells is that Helios is mainly expressed by “type b” T cells in both neonates and adult.

The second predominant IKZF transcription factor combination in adult “type b” gut associated T cells is Aiolos+ Helios+ Ikaros- (A+H+I-). This population is significantly lower in 5-day old neonate “type b” T cells. On the other hand, a big proportion of “type a” T cells also express Ikaros alone without Helios and Aiolos. This population demonstrates a trend of higher frequency in adult in comparison to 5-day old neonates. A summary depiction of the changes in the major IKZF transcription factor combination by type a and type b T cells are shown in Figure 51.

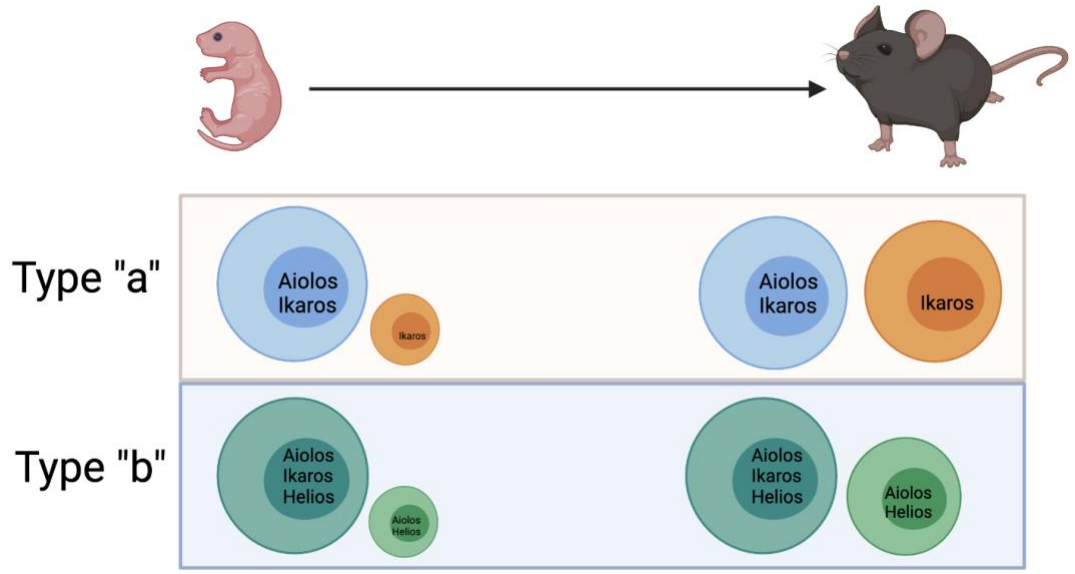

Figure 51: Summary Diagram of Aiolos, Ikaros and Helios Expression by "Type A" and "Type B" Gut Associated T Cells in 5-Day Old Neonates and Adult SI IEL. Within "type a" T cells, one of the predominant IKZF transcription factor combination is Aiolos+ Ikaros+ in both neonates and adult gut. A smaller subset of Ikaros+ "type a" T cells can be detected in neonates, with increasing frequency in adults. On the other hand, "type b" T cells were more enriched with Aiolos+ Ikaros+ Helios+ T cells that maintained through adulthood. A smaller subset of Aiolos+ Helios+ "type b" T cells can be detected in neonates and can be found at a higher frequency in the adult gut.

Ikaros Transcription Factor Family Expression in Splenic T Cells

We observed that Helios expression in splenic T cells started high in newborns and rapidly decrease a few days after birth. Since we observed differences in between "type a" and "type b" gut T cells in Ikaros transcription factor family expression, we next investigate IKZF transcription factor profile in splenic T cells. We hypothesized that splenic T cells expresses similar IKZF transcription factor profile as gut associated "type a" T cells.

In the spleens of 5-day old neonates and adults, majority of CD8+ αβ T cells and γδ T cells express CD8αβ heterodimer co-receptor and not CD8αα as we observed in gut associated "type b" T cells (Figure 52A). Due to fluorophore constraints, CD8- population will consists of CD4 and CD4-CD8- populations for αβ T cells and mainly CD4-CD8- population for γδ T cells (Figure 52B).

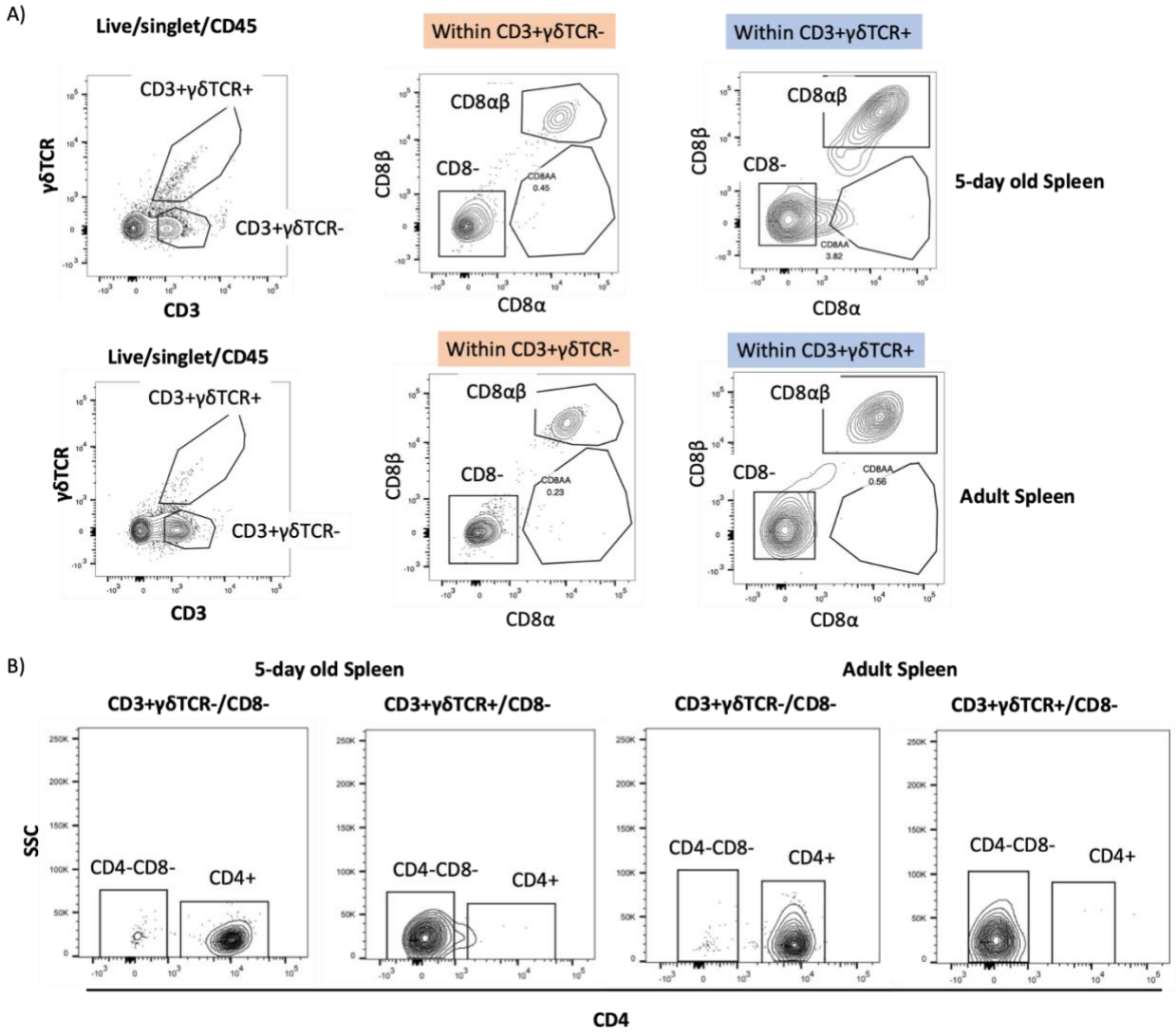

Figure 52: Gating Strategy for CD8αβ and CD8- for αβ T Cells and γδ T Cells in the Spleen. A) Gating strategy for IKZF transcription factor profile in the spleen αβ (CD3+γδTCR-) and γδ T cells (CD3+γδTCR+) for 5-day old neonates and adult. B) CD4+ and CD4-CD8- (DN) profile gated within CD8- population of αβ and γδ T cells in neonatal and adult spleen.

Within splenic αβ T cells, neonatal CD8αβ and CD8- αβ T cells express the IKZF combination Aiolos+ Helios- and Ikaros+ (A+H-I+), with a significant higher frequency than adults. Neonatal CD8αβ T cells also express significantly higher frequency of Aiolos+ Helios+ and Ikaros+ (A+H+I+) combination in comparison to adults. Another major proportion of splenic αβ T cells express Ikaros alone, with no significant difference between neonates and adults.

Both neonatal CD8αβ and CD8- γδ T cells express a significantly higher frequency of the IKZF transcription factor combination Aiolos+ Helios+ and Ikaros+ (A+H+I+) in comparison to adults. In both adult splenic αβ and γδ T cells, there is a significant higher frequency in cells that do not express any of the Ikaros transcription factor tested (A-H-I-) in comparison to neonatal splenic αβ and γδ T cells (Figure 53).

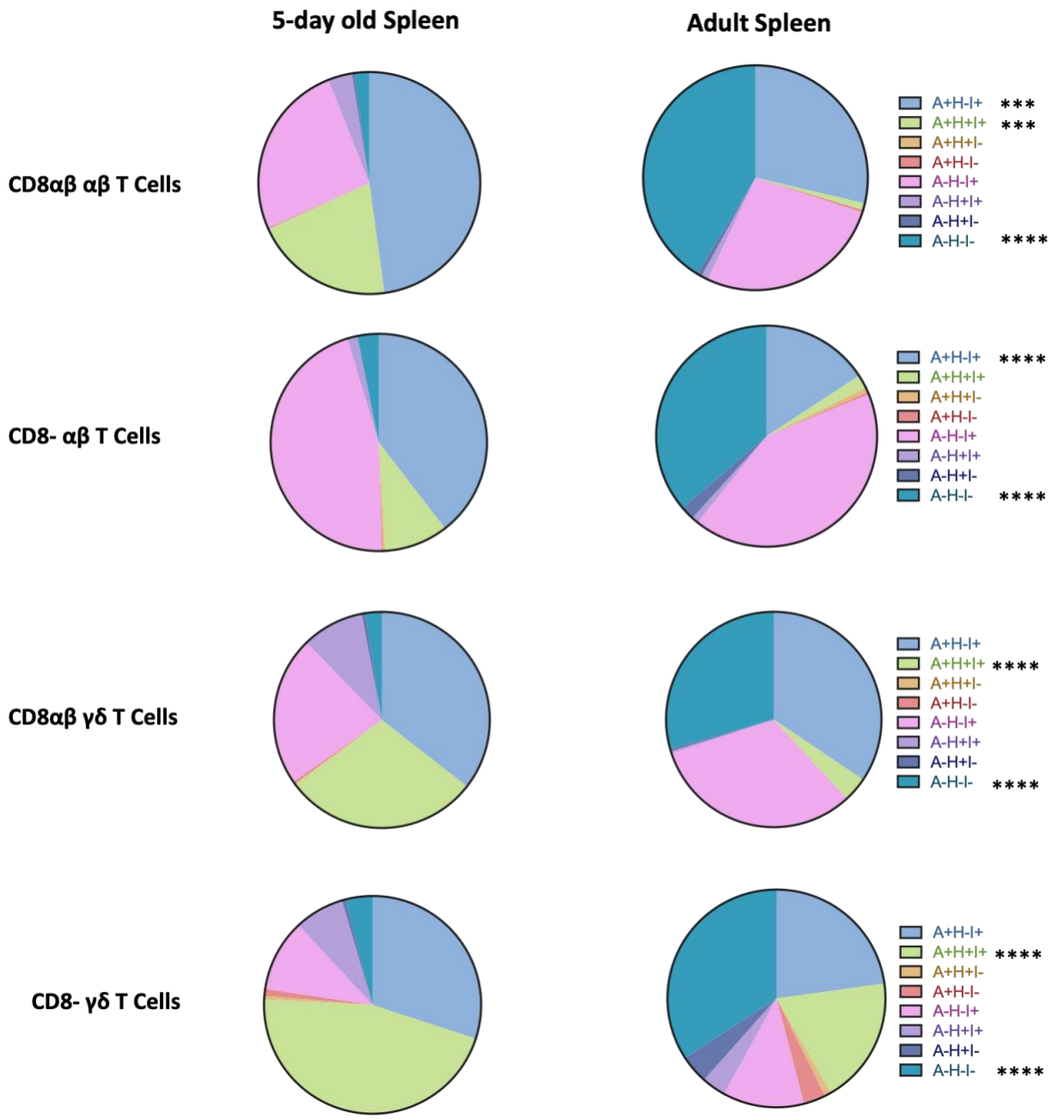

Figure 53: Ikaros Transcription Factor Proportions by Splenic αβ and γδ T Cells. Pie charts reflecting the average frequency of transcription factor combination (A: Aiolos, H: Helios and I: Ikaros). Splenocytes were harvested and stained for different surface and intracellular markers to determine their frequency. αβ T cells are gated as CD3+ γδTCR- and γδ T cells are gated as CD3+ γδTCR+ within CD45 population. Within αβ and γδ T cells subset, the average frequency of each subset is obtained from N=3-6 individual experiments and plot as a pie chart. Neonates were pooled from the same litter of 2-3 pups and adults are individual mouse. Analyzed by 2way ANOVA, Sidak's multiple comparisons test, comparing each individual combination between 5-day old neonates and adult, ***p<0.001, ****p<0.0001.

This prompted us to investigate if Aiolos- Helios- and Ikaros- phenotype is enriched within the memory T cell population in adult spleen. When we stained for CD44 and CD62L along with Aiolos, Helios and Ikaros, we did not observe a correlation between Aiolos- Helios- and Ikaros- with memory T cell population as assessed by CD44 and CD62L expression (Figure 54).

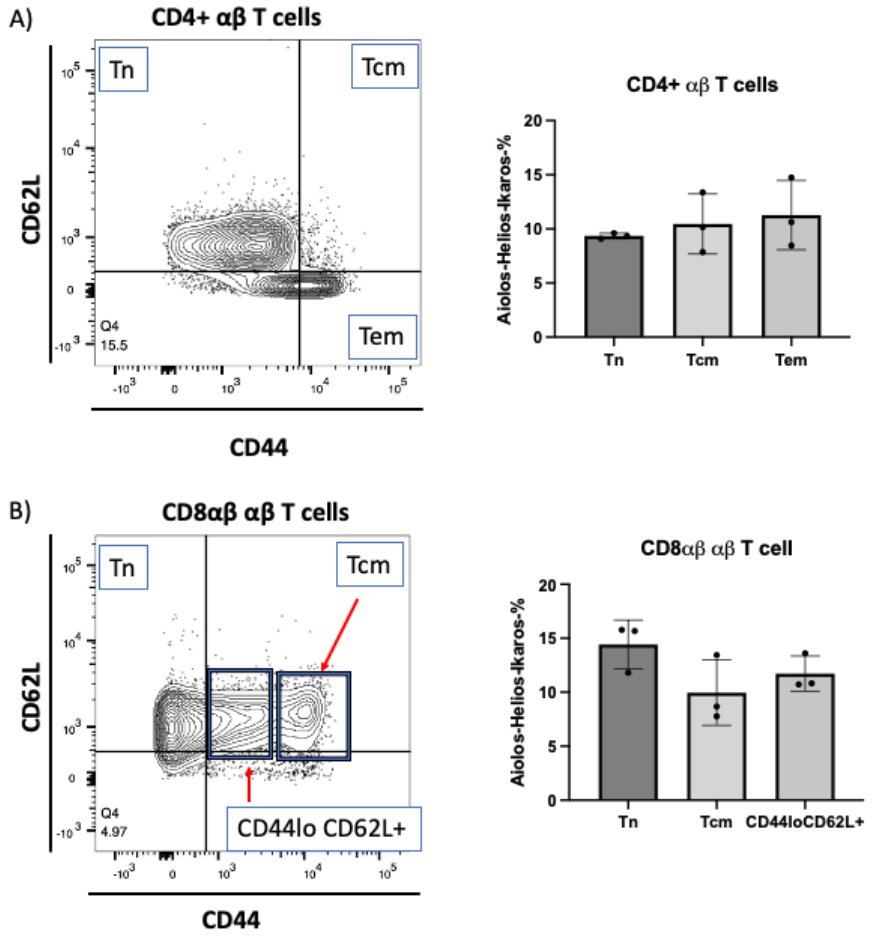

Figure 54: Frequency of Aiolos- Helios- and Ikaros- Within Splenic Naïve and Memory αβ T Cell Subsets. Representative FACS plot of CD44 and CD62L and frequency of Aiolos- Helios- and Ikaros- by A) CD4+ and B) CD8αβ+ αβ T cells in adult spleen. Splenocytes were harvested and stained for different surface and intracellular markers to determine their frequency. αβ T cells are gated as CD3+ γδTCR- within live and single cells. Tn= naïve T cells as reflected by CD44-CD62L+, Tcm= T central memory as reflected by CD44+CD62L+ and Tem= T effector memory as reflected by CD44+CD62L-. N=3 individual mouse.

In summary, like what we observed with "type a" T cells in the neonatal gut, the predominant IKZF transcription factor combination is Aiolos+ Helios- and Ikaros+ (A+H-I+) in neonatal splenic αβ T cells. Unlike the gut, there is a significant increase in adult αβ T cells that do not express any of the three IKZF transcription factors tested. On the other hand, neonatal γδ T cells in the periphery were more enriched with Aiolos+ Ikaros+ Helios+ (A+H+I+) cells and these cells significantly decreases in adults. Unlike the gut, there is an increase in a subset of peripheral splenic γδ T cells that do not express any of the three IKZF transcription factor tested in adults. Summary diagram depicted in Figure 55.

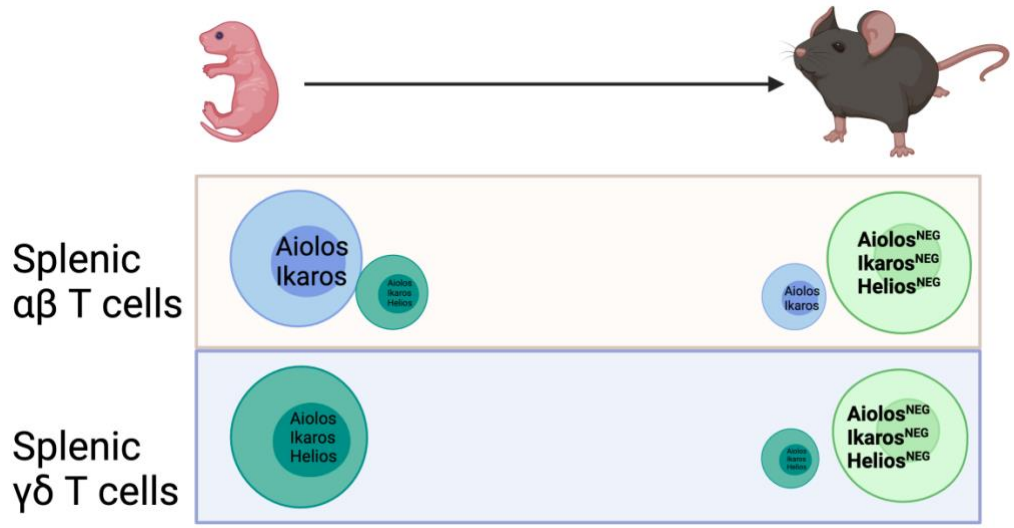

Figure 55: Summary Diagram of Aiolos, Ikaros and Helios Expression by αβ and γδ T cells in 5-day old Neonates and Adult Splenocytes.

Helios Expression in UCB HSC-derived T Cells using Humanized Mouse Model

Since we observed that T cells of perinatal origin express higher Helios+Foxp3- frequency in both human and mice, we next asked if Helios+ T cells may arise from perinatal origins. It is known that fetal and adult HSC can give rise to different types of T cells that differ in their phenotype and function[323,356,357]. To test this, we generated humanized mice by transferring CD34+Lineage- hematopoietic stem cells (HSC) isolated from UCB into NSG-S

mice after sublethal total body irradiation. Human hematopoietic cell reconstitution was checked by staining cheek bleed samples collected from the mice at 4-, 9- and 19/20-weeks post HSC transfer with anti-human CD45. We demonstrated that UCB HSC can successfully reconstitute NSG-S mice and human CD45+ cells can be detected in the periphery as early as 4 weeks post transfer and maintained up to 19 weeks post transfer prior to sacrifice. Similar to previous reports, the transfer of UCB CD34+ HSC can successfully reconstitute the different hematopoietic lineages (Figure 56)[561].

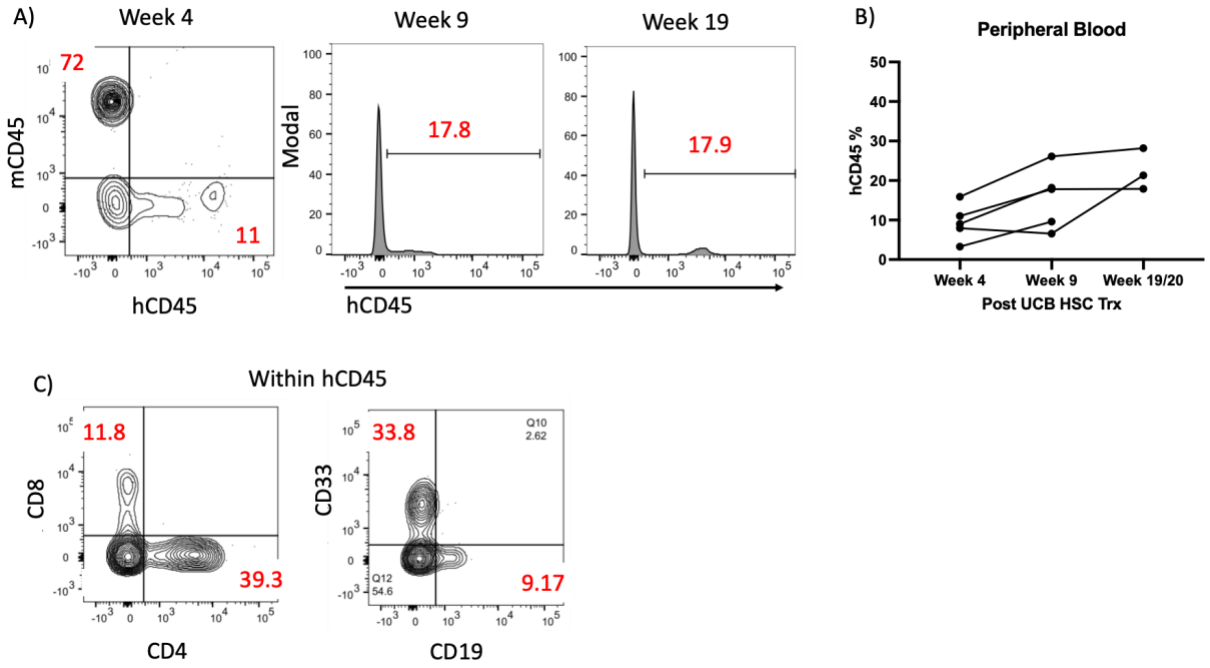

Figure 56: Detection of Human CD45, CD4, CD8, CD19 and CD33 in Peripheral Blood of NSG-S mice that Received UCB HSC. NSG-S mice received sublethal total body irradiation and CD34+Lin- sorted HSC from UCB 24 hours later. A, B) Representative FACS plot and frequency of human CD45 cells were measured by staining peripheral blood for anti-hCD45 at indicated weeks post HSC transfer. C) Representative FACS plot of the different human hematopoietic lineages within human CD45+ cells in the peripheral blood at 4 weeks post UCB HSC transfer.

After establishing this humanized mouse model, we sought to determine if CD4+ and CD8+ T cells generated *in vivo* in UCB-HSC humanize mice express Helios. We found that both UCB HSC-derived CD4+ and CD8+ T cells express Helios in splenocytes harvested from

humanized mice between 10-21 weeks post UCB HSC transfer (Figure 57). UCB HSC-derived CD4+ T cells express around 49.92±30% Helios+Foxp3-, like what we observed in our UCB samples (46.9±27.2%, Figure 29B). About 83.18±11.84% of UCB HSC-derived CD8+ T cells express Helios without co-expressing Foxp3 like what we observed previously with freshly isolated UCB mononuclear samples (79.3±8.43%, Figure 29B). These data demonstrates that UCB HSC-derived T cells express and maintain Helios *in vivo* using NSG-humanized mouse model. This implicated that Helios+ T cells arise from perinatal progenitor origin and potentially the low frequency of Helios+ T cell observed in adults is due to a switch in HSC pool after birth.

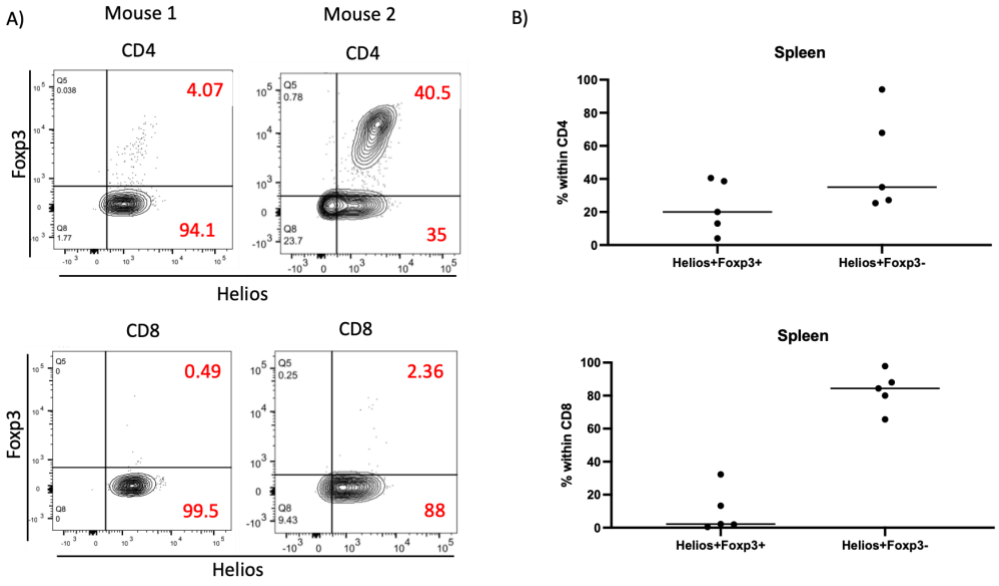

Figure 57: Helios and Foxp3 Expression by CD4+ and CD8+ T Cells in UCB-HSC Humanized Mice. NSG-S mice received sublethal total body irradiation and CD34+Lin- sorted HSC from UCB 24 hours later. Mice were checked for human CD45 reconstitution and harvested between 10-21 weeks post HSC transfer. A) Representative FACS plot of Helios and Foxp3 by CD4+ and CD8+ T cells gated within anti-human CD45 and CD3 population in the spleen. B) Summary plot for Helios and Foxp3 frequency within CD4 and CD8 T cells in the spleen of UCB-HSC humanized mice.

Section III: Potential Function of Helios in Perinatal T Cells

Helios and IL-10 Expression by CD4+ T Cells Under Th1 Polarizing Conditions

The neonatal immune system is phenotypically and functionally distinct from the adult immune system. The overall response of the neonatal immune system is high tolerogenic, with reduced alloantigen recognition and poor responses against foreign antigens[238,241,257–259]. This propensity for tolerance is important in preventing excessive inflammation when neonates are first exposed to benign antigens such as food and maternal antigens[511].

One mechanism that accounts for this increased tolerance is the Th2-biased response generated by the neonatal immune system. Murine neonatal T cells differentiate into IL-4 producing Th2 cells in the presence of allogeneic cells, while IL-2 and IFN-γ production by Th1 cells are defective[562]. While human UCB T cells do not show a Th2 biased response, they demonstrated a defective Th1 response. For instance, under neutral conditions, UCB T cells produce lower levels of the proinflammatory cytokine IFN-γ while producing higher levels of the anti-inflammatory cytokine interleukin-10 (IL-10) compared to APB T cells[309,310].

In our previous studies, we demonstrated that UCB naïve T cells differ in their surface antigen expression compared to APB naïve T cells[309]. Our lab also demonstrated that UCB naïve CD4+ T cells are capable of producing Th1 and Th2 type cytokine to levels comparable to adult naïve CD4+ T cells and they are not preprogrammed to produce Th2 cytokines under non-polarizing conditions[309]. However, strikingly we found that UCB naïve CD4+ T cells produce significantly higher levels of IL-10 in the presence of Th1 polarizing conditions compared to APB naïve CD4+ T cells, particularly without PMA and ionomycin restimulation. This is not because UCB naïve CD4+ T cells are incapable of producing IFN-γ when subjected to Th1 conditions, as UCB naïve CD4+ T cells produce higher IFN-γ than APB naïve CD4+ T cells

upon PMA and ionomycin restimulation (Figure 58). These data demonstrated that under Th1 inflammatory conditions, UCB naïve CD4+ T cells produce high levels of IL-10. This was also demonstrated in studies that looked at newborns with mothers infected with Malaria, a Th1 predominant response, during pregnancy[563]. These data suggest intrinsic differences between UCB T cells and APB T cells in their effector differentiation processes.

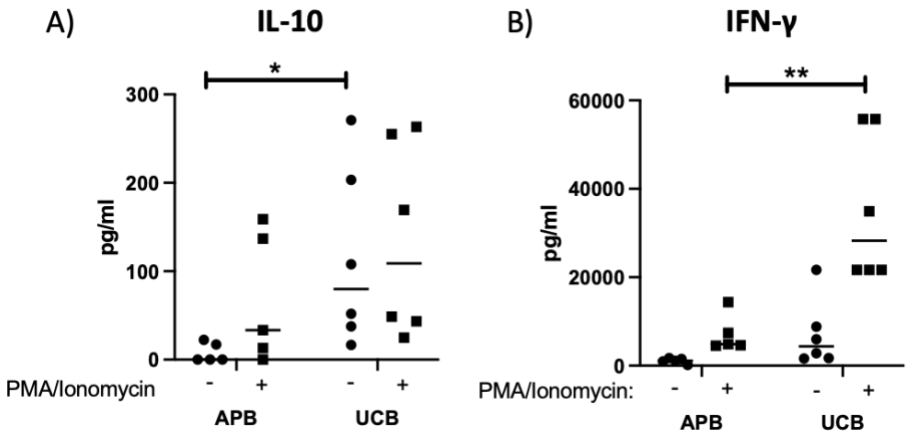

Figure 58: IL-10 and IFN-γ Production by APB and UCB Naïve CD4+ T Cells After Th1 Polarization. Naïve CD4+ T cells were cultured in the presence of Th1 polarizing conditions for 5 days. After 5 days, cells were harvested and restimulated with or without PMA and Ionomycin for 4 hours. After 4 hours, supernatants were collected, and cytokine levels such as A) IL-10 or B) IFN-γ were assayed using the Human Th cytokine Legendplex Panel and analyzed by unpaired t test, *$p<0.05$, **$p<0.001$. These data were generated by Ramiah Jacks Ph.D.

To determine if we also observe IL-10 production by mouse neonatal T cells under Th1 inflammatory conditions, total splenocytes from 5-day old neonates were subjected to Th0, Th1 and Th2 conditions for 5 days. After Th polarization, the cells were harvested and restimulated with PMA and ionomycin in the presence of monensin and stained for IL-10 expression by CD4+ T cells. We found that neonatal CD4+ T cells produce IL-10 after PMA and Ionomycin restimulation in Th0, Th1 and Th2 conditions. However, a significantly higher frequency of neonatal CD4+ T cells express IL-10 under Th1 and Th2 conditions even without PMA and ionomycin restimulation than neutral Th0 conditions. These data demonstrate that murine

neonatal CD4+ T cells produce IL-10 under both Th1 and Th2 inflammatory conditions (Figure 59).

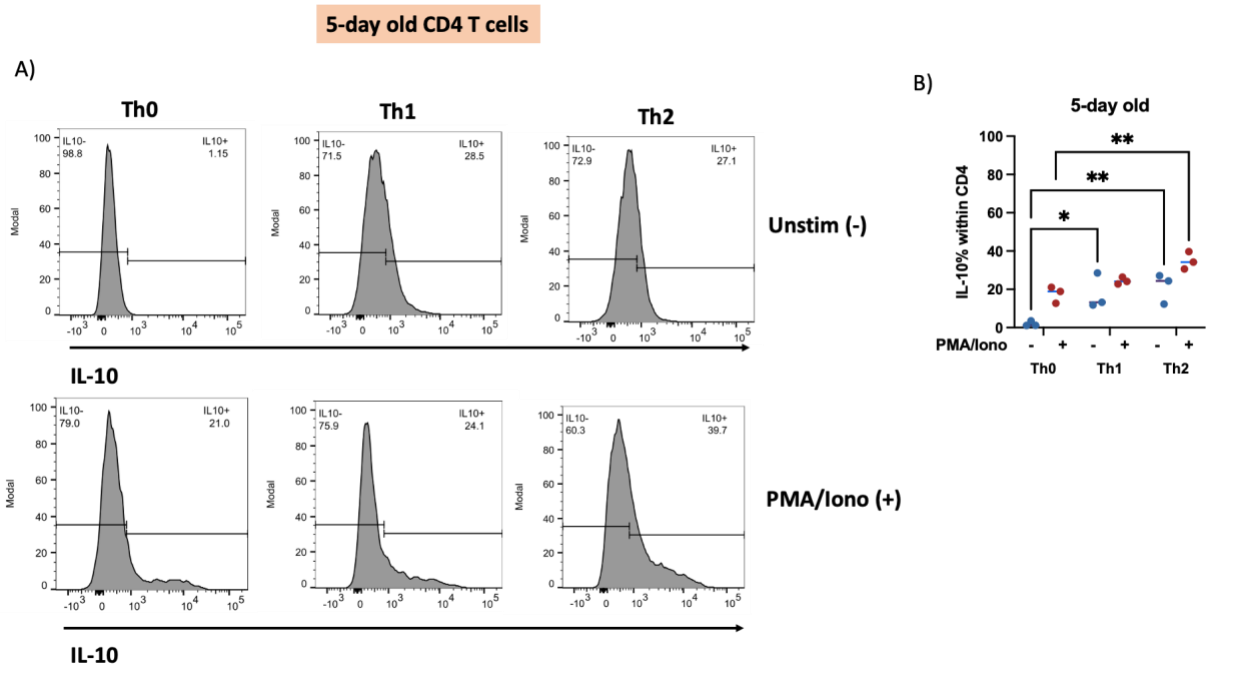

Figure 59: IL-10 Expression by Neonatal CD4+ T cells After Th0, Th1 and Th2 Conditions. A) Representative histogram of IL-10 expression and B) summary of IL-10 frequency within 5-day old neonatal CD4+ T cells in the absence or presence of PMA and Ionomycin stimulation with monensin for 4 hours after 5 days of Th0, Th1 or Th2 culture condition. Each dot represents individual mouse (N=3), analyzed by 2way ANOVA, Sidak's multiple comparisons test. *p<0.05, **p<0.01.

We observed that a higher frequency of perinatal T cells expressed Helios without co-expression of Foxp3 in both mice and humans and the frequency of these Helios+ T cells rapidly declines early on in life to adult levels in the periphery. In humans, UCB naïve CD4+ T cells differ in IL-10 production under Th1 conditions compared to adults. We also found a significantly higher frequency of IL-10 producing CD4+ T cells under inflammatory conditions in neonatal mice. Furthermore, other IKZF transcription factors such as Ikaros and Aiolos have been reported to promote IL-10 production by CD4+ T cells[400,406]. While Helios has been reported to form heterodimers with Ikaros, it is unknown if Helios promotes IL-10 production in

T cells[368]. With these data, we hypothesized that high Helios expression in perinatal T cells contributes to tolerance by promoting IL-10 production under inflammatory conditions.

To determine if there is a correlation of Helios expression and IL-10 production under inflammatory conditions, we checked for Helios and IL-10 expression after subjecting 5-day old total splenocytes to Th0, Th1 and Th2 culture conditions. We observed a significantly higher frequency of Helios expressing CD4+ T cells after Th1 polarizing conditions than Th0 and Th2 (Figure 60).

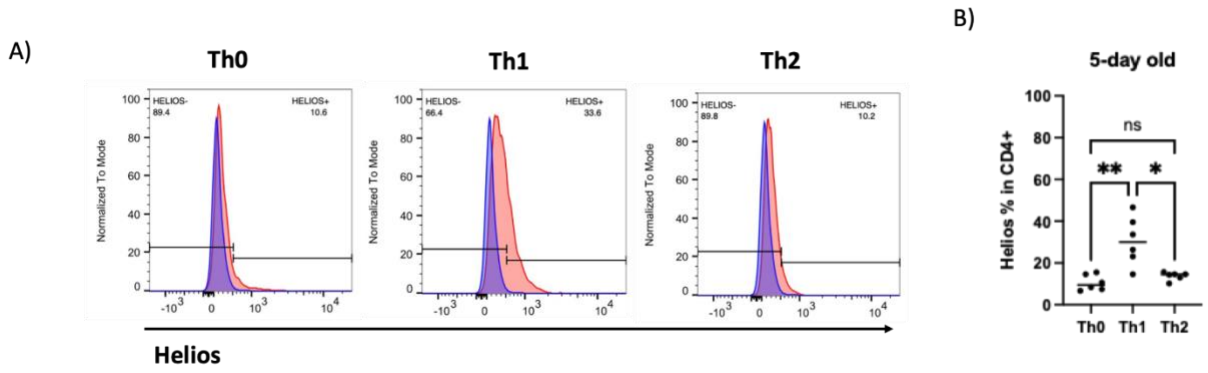

Figure 60: Helios Frequency Within CD4+ T Cells from 5-day old Neonatal Splenocytes. A) Representative histograms and B) summary plot of Helios frequency within CD4+ T cells from 5-day old neonate splenocytes after indicated Th culture conditions for 5 days. Blue line indicates isotype control and red line indicate fully stained samples. Each dot represents individual mouse (N=6), analyzed by one-way ANOVA, Tukey's multiple comparisons test, *p<0.05, **p<0.01.

Next, we compared the frequency of IL-10 producing CD4+ T cells within CD4+Helios+ and CD4+Helios- populations. After indicated Th polarization culture, the cells were harvested and subjected to PMA and Ionomycin restimulation. Upon PMA and ionomycin restimulation, there is a significantly higher frequency of IL-10 producers within CD4+ T cells that express Helios than those that do not express Helios after Th1 polarizing conditions (Figure 61). These data demonstrated that under Th1 conditions, expression of Helios in CD4+ T cells that correlates with IL-10 production.

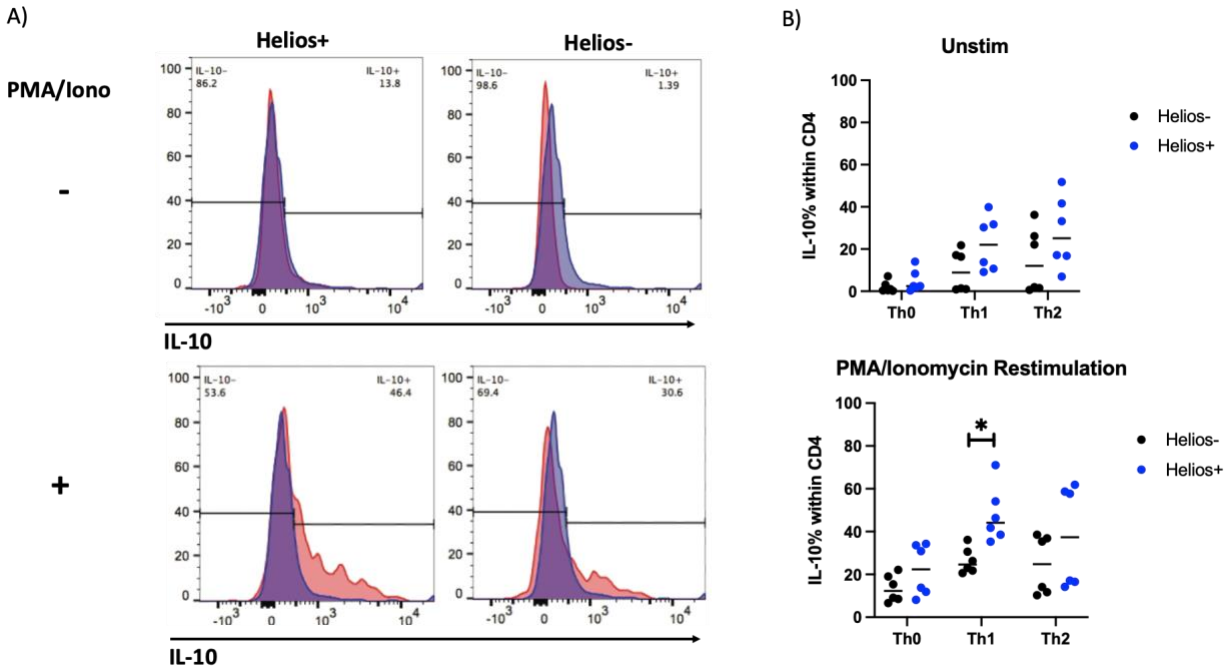

Figure 61: IL-10 Frequency Within Helios+ and Helios- CD4+ T Cells from 5-day Old Neonatal Splenocytes. A) Representative IL-10 histogram gated within Helios+ or Helios- CD4+ T cells in the absence or presence of PMA and Ionomycin restimulation after Th1 polarization. Blue lines indicate isotype control, and red lines indicate fully stained samples. B) Summary plot of IL-10 producing CD4+ T cells by Helios- and Helios+ subsets under indicated Th conditions in the presence or absence of PMA and Ionomycin restimulation. Total splenocytes were isolated from 5-day old neonates and subjected to Th0, Th1 or Th2 culture conditions for 5 days. After 5 days, cells were harvested and subjected to PMA and Ionomycin restimulation in the presence of monensin. Each dot indicates individual mouse (N=6), analyzed by 2way ANOVA, Sidak's multiple comparisons test, *$p<0.05$.

It was previously reported that Helios is upregulated in both CD4+ and CD8+ T cells after activation in both humans and mice[373]. To investigate whether the higher IL-10 frequency by CD4+ Helios+ T cells is due to Helios representing more activated cells and producing cytokines, we compared IFN-γ frequency by Helios+ and Helios- CD4+ T cells in the same culture. Without PMA and Ionomycin restimulation, there is a significantly higher frequency of IFN-γ producing CD4+ T cells by the Helios+ subset compared to Helios- subset in both Th1 and Th2 polarizing culture conditions. However, after Th1 polarizing conditions, both Helios+ and Helios- CD4+ T cells contain a similar frequency of IFN-γ producing cells after PMA and Ionomycin restimulation. There is a significantly higher IFN-γ producing CD4+ T cell frequency

within the Helios+ subset under Th2 polarizing conditions after PMA and Ionomycin restimulation. These data indicate Helios expression correlates with IL-10 producing CD4+ T cells under Th1 inflammatory conditions after PMA and Ionomycin restimulation and is not because Helios negative cells are incapable of producing cytokines under Th1 conditions (Figure 62).

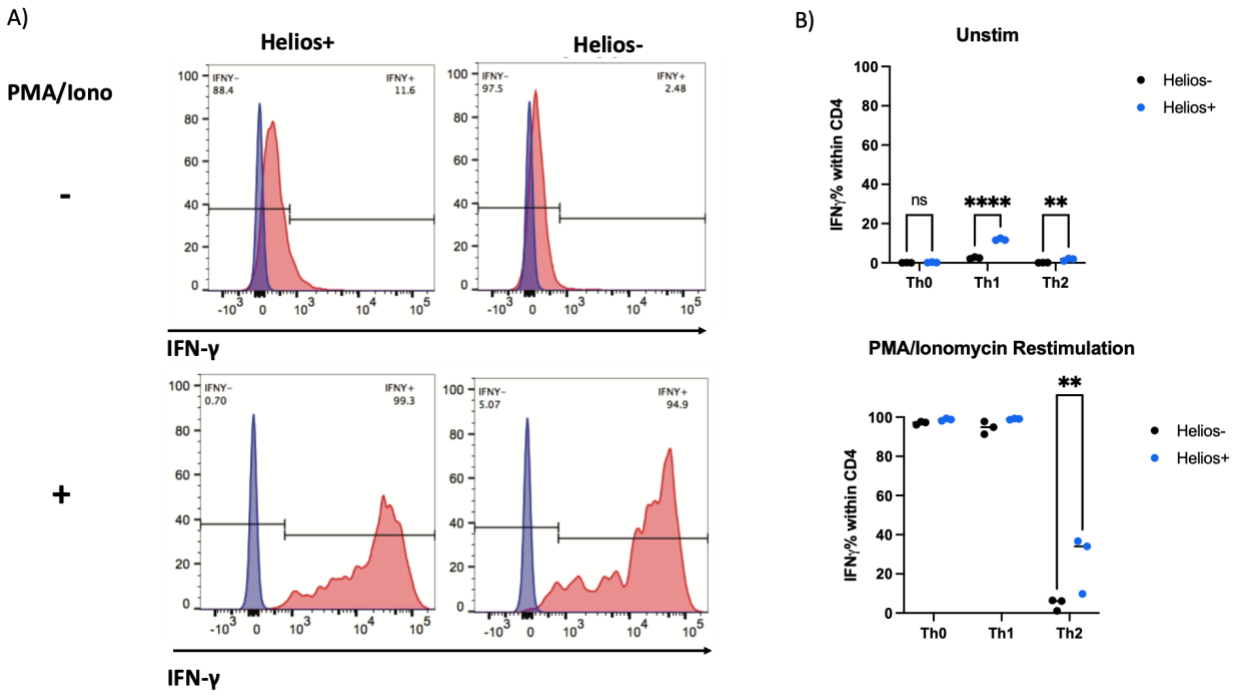

Figure 62: IFN-γ Frequency Within Helios+ and Helios- CD4+ T Cells From 5-day old Neonatal Splenocytes. A) Representative IFN-γ histogram gated within Helios+ or Helios- CD4+ T cells in the absence or presence of PMA and Ionomycin restimulation after Th1 polarization. Blue lines indicate isotype control, and red lines indicate fully stained samples. B) Summary plot of IFN-γ producing CD4+ T cells by Helios- and Helios+ subsets under indicated Th conditions in the presence or absence of PMA and Ionomycin restimulation. Total splenocytes were isolated from 5-day old neonates and subjected to Th0, Th1 or Th2 culture conditions for 5 days. After 5 days, cells were harvested and subjected to PMA and Ionomycin restimulation in the presence of monensin. Each dot indicates individual mouse (N=3), analyzed by 2way ANOVA, Sidak's multiple comparisons test, *p<0.05.

Requirement of Helios in IL-10 Production Under Th1 Polarizing Conditions

Since we observe that there is a correlation in Helios expression and IL-10 production by CD4+ T cells in mice, we next tested if Helios promotes IL-10 production under Th1 polarizing

conditions by knocking out Helios in human UCB T cells. We designed guide RNAs (gRNA) against Helios or non-target as a control. We found that guide 3 (g3) against human *ikzf2* successfully knockout Helios at efficiency between 15-30% by both CD4+ and CD8+ UCB T cells. After checking that Helios is successfully knockout in these cells, the transfected cells were harvested and subjected to Th1 polarizing conditions for 5 days to test if Helios promotes IL-10 production under Th1 conditions. While Helios was knockout in a proportion of both CD4+ and CD8+ T cells, we did not consistently observe a decrease in IL-10 production by these cells (Figure 63).

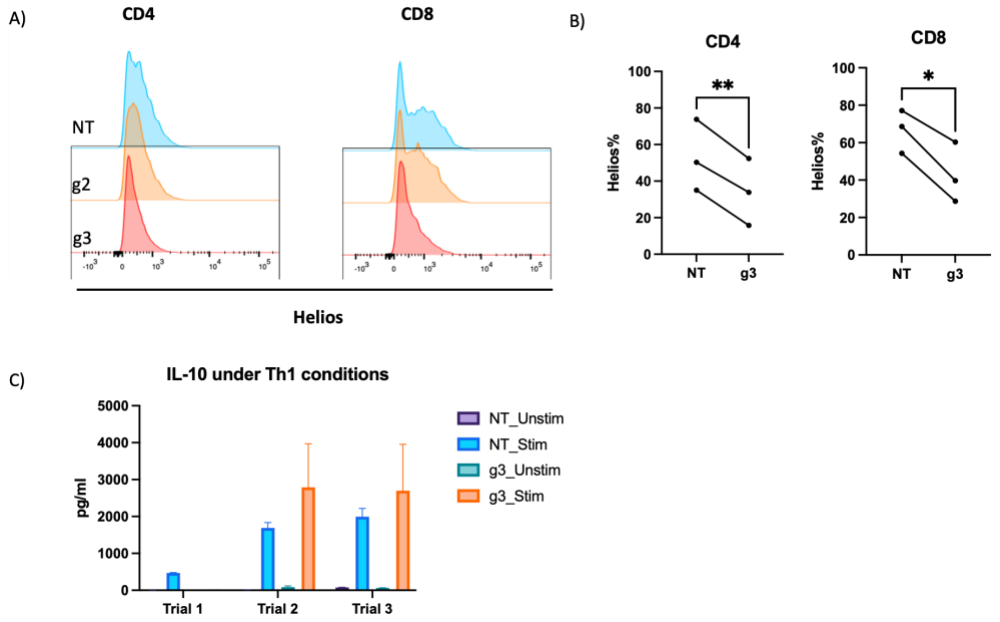

Figure 63: Helios Knockout in UCB T Cells and its Effect in IL-10 Production Under Th1 Conditions. A) Representative histogram and B) summary frequency plots for Helios expression by CD4+ and CD8+ T cells 2 days after electroporation with NT, guide 2 (g2) or guide 3 (g3) against human *ikzf2*. C) IL-10 levels in supernatants from unstimulated or PMA and Ionomycin restimulated conditions from cells transfected with NT gRNA or *Ikzf2* g3 after being subjected to 5 days of Th1 culture conditions. Total T cells were isolated from UCB and stimulated for 3 days with anti-CD3 and CD28 in the presence of IL-2. After three days, the cells were harvested and transfected with NT or *ikzf2* gRNA with Cas9 protein using Neon electroporation system. The cells were allowed to rest for two days before checking knockout efficiency for Helios and then subsequently cultured under Th1 polarizing conditions for 5 days. After 5 days, the cells were harvested and restimulated with PMA and Ionomycin for 4 hours and supernatants were collected and analyzed for cytokine production using Legendplex kit and analyzed by paired t-test *$p<0.05$, **$p<0.01$.

Since we did not observe a decrease in IL-10 production in the UCB total T cell culture even when we observed a reduction in Helios expression, we next tested our hypothesis using purified naïve CD4+ T cells isolated from UCB. Similarly, while we observed a decrease in Helios expression after CRISPR cas9, IL-10 production was not affected in knockout conditions (Figure 64). These data demonstrate that while Helios correlates with IL-10 expression under Th1 inflammatory conditions, it is not required for IL-10 production.

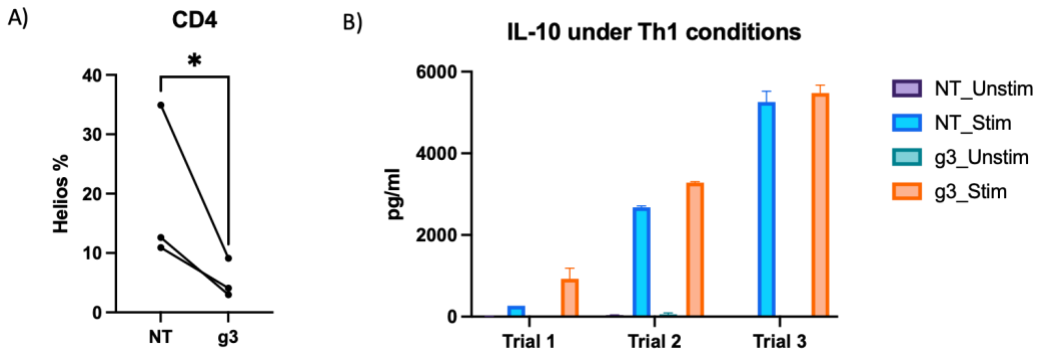

Figure 64: Helios Knockout in UCB Naïve CD4+ T Cells and its Effect in IL-10 Production Under Th1 Conditions. A) Summary frequency plot for Helios expression by CD4+ T cells 2 days after electroporation with NT or guide 3 (g3) against human *ikzf2*. B) IL-10 levels in supernatants from unstimulated or PMA and Ionomycin restimulated conditions from cells cells transfected with NT gRNA or *Ikzf2* g3 and subjected to 5 days of Th1 culture conditions. Naïve CD4+ T cells were isolated from UCB and stimulated for 3 days with anti-CD3 and CD28 in the presence of IL-2. After three days, the cells were harvested and transfected with NT or *ikzf2* gRNA with Cas9 protein using Neon electroporation system. The cells were allowed to rest for two days before checking knockout efficiency for Helios and then subsequently cultured under Th1 polarizing conditions for 5 days. After 5 days, the cells were harvested and restimulated with PMA and Ionomycin for 4 hours and supernatants were collected and analyzed for cytokine production using Legendplex kit. Analyzed by ratio paired t test *$p<0.05$.

Helios Suppresses Effector Cytokine Production by Perinatal T Cells

Most of the studies related to the function of Helios have been focused on within the Treg subset. Treg-specific Helios knockout subsequently increased effector cytokine production such as IFN-γ, TNF, and IL-17[376]. Fetal iTregs differentiated from cells that are Helios deficient also demonstrate concurrent increased levels of IFN-γ and IL-2[327]. We previously demonstrated that freshly isolated UCB naïve CD4+ T cells produce significantly lower levels of IFN-γ compared

to adult naïve CD4+ T cells[309]. With these data, we hypothesized that perinatal T cells express high levels of Helios to suppress effector cytokine production to promote an overall tolerant state. To test this, we knockout Helios from UCB naïve CD4+ T cells using CRISPR-cas9 gene editing system and restimulated them with PMA and Ionomycin to assess the effect of Helios in cytokine productions by UCB CD4+ T cells. We found that compared to cells that received non-target (NT) gRNA control, Helios knockout led to a significant increase in IFN-γ (638.4pg/ml ± 773.7pg/ml vs 910.5pg/ml ±785.6pg/ml) and IL-13 (339.3pg/ml ± 134pg/ml vs 555.9pg/ml ± 172.1pg/ml) production (Figure 65). In agreement with our data from Th1 polarization, we did not see a difference between NT gRNA and *ikzf2* gRNA (g3) in IL-10 production (Figure 63-65). These data demonstrate that Helios expression in UCB T cells suppresses effector cytokine production such as IFN-γ and IL-13 but is not required for IL-10 production.

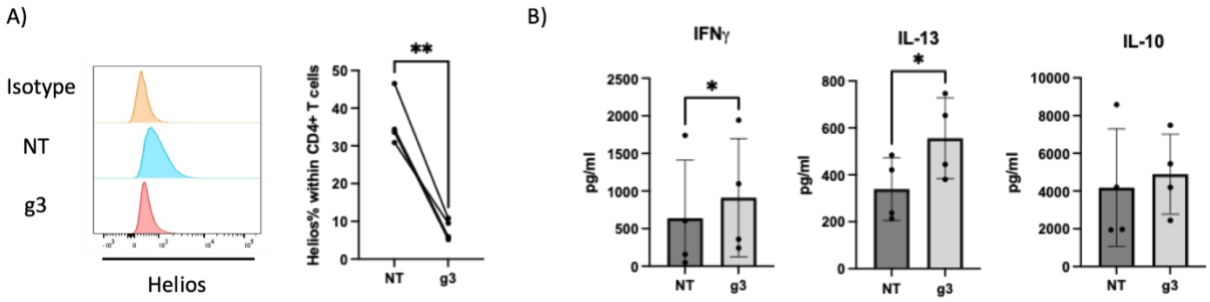

Figure 65: Cytokine Production by Helios Knockout UCB CD4+ T Cells After PMA and Ionomycin Restimulation. A) Representative histogram and summary of Helios frequency within CD4+ T cells 2 days after electroporation. B) Cytokine levels by cells that received NT or IKZF2 g3 after PMA and Ionomycin restimulation 2 days post electroporation. Naïve CD4+ T cells were isolated from UCB and stimulated for 3 days with anti-CD3 and CD28 in the presence of IL-2. After three days, the cells were harvested and transfected with NT or *ikzf2* gRNA (g3) with Cas9 protein using Neon electroporation system. The cells were allowed to rest for two days before checking knockout efficiency for Helios and then subsequently restimulated with PMA and Ionomycin for 4 hours. Supernatants were collected and analyzed for cytokine production using Legendplex kit. N=4 individual donors, analyzed by paired t-test *p<0.05, **p<0.01.

CHAPTER FOUR: DISCUSSION

Summary of Results

It is well established that the perinatal immune system is highly tolerogenic. This tolerant nature allows fetuses and newborns to tolerate benign antigens such as food, maternal antigens, and commensal bacteria. However, it also increases a newborn's susceptibility to life-threatening infections. My dissertation aims to investigate how perinatal T cells can contribute to immune tolerance in infants. A better understanding of the perinatal immune system will provide pivotal knowledge to develop strategies to protect infants from infectious diseases while establishing immune homeostasis with commensal microbes.

Using UCB as a source for full-term immune cells to study perinatal tolerance, we demonstrated that UCB CD4+ and CD8+ T cells differentiate into Foxp3+ T cells upon antigen receptor stimulation without the addition of exogenous TGF-β. In contrast, adult CD4+ and CD8+ T cells have a significantly lower frequency of Foxp3+ T cell differentiation even though they were subjected the same culture system set up (Figure 9). One of the main functions of Foxp3+ Tregs is to suppress conventional T cell proliferation to prevent an excessive inflammatory response. This prompted us to investigate if these *ex vivo* generated Foxp3+ T cells from UCB also carry regulatory functions. We demonstrated that *ex vivo* generated UCB CD4+ and CD8+ Foxp3+ T cells carry suppressive functions comparable to their preexisting UCB tTreg counterpart using an *in vitro* suppression assay system (Figure 10). Furthermore, these UCB-derived Foxp3+ T cells also significantly delayed xGVHD mediated by the transfer of autologous total UCB mononuclear cells, demonstrating regulatory functions *in vivo* (Figure 12).

Interestingly, we found that the pre-treatment of *ex vivo* generated UCB Foxp3+ T cells did not decrease any other proinflammatory cytokines in the serum except for a significant decrease in anti-inflammatory cytokine, IL-10, in this xGVHD model (Figure 14, 15).

We also found that these *ex vivo* generated UCB-derived Foxp3+ T cells maintain relatively stable Foxp3 expression up to 62 days in culture, and Foxp3 expression is stable upon restimulation in the presence of inflammatory cytokines (Figure 22). We demonstrated that Foxp3 expression in UCB-derived Foxp3+ T cells is relatively stable after differentiation when the cells were subjected to restimulation in the presence of inflammatory cytokines such as IL-1β, IL-4, and IL-6. However, we observe a decreasing trend in Foxp3 expression when we block TGF-β signaling using TGF-β receptor 1 kinase inhibitor SB431542 (Figure 22). These data suggest that TGF-β signaling is involved in Foxp3 maintenance in UCB-derived Foxp3+ T cells, similar to previous reports regarding the role of TGF-β1 signaling in Treg Foxp3 expression and suppressive functions[133].

We next investigated if these UCB-derived Foxp3+ T cells resemble preexisting tTregs or peripherally induced pTregs by assessing their epigenetic status of Foxp3. It was previously shown that the CNS2 region of *Foxp3* is highly demethylated to allow stable expression of Foxp3 by tTregs[137,138]. In contrast, the CNS2 region of *Foxp3* is highly methylated in Foxp3+ *in vitro* induced Tregs (iTregs) and Foxp3- effector T cells as well. Consequently, Foxp3 expression in iTregs is very unstable and is lost when TGF-β is removed[135]. Using genomic bisulfite sequencing, we found that the CNS2 region of *Foxp3* by UCB-derived CD4+ Foxp3+ T cells is highly methylated like CD4+ effector T cells and their iTreg counterpart (Figure 16). These data suggest that although UCB-derived Foxp3+ T cells maintain stable Foxp3 expression like preexisting tTregs, their maintenance of Foxp3 expression may not come from *Foxp3* CNS2

demethylation. This prompted us to investigate other epigenetic mechanisms that could contribute to the Foxp3 stability by our *ex vivo* generated UCB-derived Foxp3+ T cells. We next conducted ATAC-seq on UCB-derived Foxp3+ T cells to assess genomic chromatin accessibility at the *Foxp3* locus compared to tTregs. We found that UCB-derived CD4+ Foxp3+ T cells have less chromatin accessibility than CD4+ tTreg samples at the promoter, CNS1, CNS2 and CNS3 regions of *Foxp3* (Figure 17). We further found UCB-derived Foxp3+ T cell unique region in *Foxp3* locus that is more accessible than tTregs (Figure 18). These unique regions potentially could be regions that regulate Foxp3 expression and maintenance in these cells. Altogether, our bisulfite sequencing and ATAC-seq data demonstrate that UCB-derived Foxp3 T cells are epigenetically regulated differently from preexisting tTregs.

Fetal alcohol exposure is one of the factors that can promote an overall inflammatory state in the fetuses and can led to persistent immune deficit into adulthood[486–488,490,505]. While we demonstrated that Foxp3 expression by UCB-derived Foxp3+ T cells is stable after differentiation, previous lab members demonstrated that UCB-derived Foxp3+ T cell differentiation could be inhibited in the presence of proinflammatory cytokines IL-4 and IL-12 (Jessica Lee MD, Ph.D.). Therefore, we hypothesized that the presence of EtOH would block UCB-derived Foxp3+ T cell differentiation. Indeed, we observed that the presence of 100mM EtOH for the first five days of the induction culture significantly inhibits both CD4+ and CD8+ UCB-derived Foxp3+ T cell differentiation without causing apparent toxicity or cell death (Figure 23-25). Mechanistically, EtOH inhibits IL-2Rα (CD25) expression in both UCB CD4+ and CD8+ T cells early during the culture (Figure 28). IL-2 signaling is required for TGF-β mediated Foxp3 induction by naïve CD4+ T cells[105]. Our data demonstrated that EtOH can

inhibit CD25 expression and block IL-2 signaling, subsequently decreasing Foxp3+ T cell differentiation in our induction culture.

While Foxp3+ T cells are one of the contributing factors of the tolerant biased perinatal immune system, we next investigated if other transcription factors expressed by perinatal Foxp3 negative conventional T cells also contribute to perinatal tolerance. We found that a higher frequency of UCB CD4+ and CD8+ T cells expresses the Ikaros transcription factor family protein, Helios, without co-expression of Foxp3 compared to adult CD4+ and CD8+ T cells (Figure 29). Helios is generally considered a transcription factor expressed by Foxp3+ Tregs and is essential for Treg stability and function[372,384–386]. Subsequently, we found that the high frequency of Helios+Foxp3- expressing CD4+ and CD8+ T cells decreased by 3 weeks after birth (Figure 30).

Next, we investigated if this phenotype also occurs in mice. We found that the frequency of Helios+Foxp3- CD4+ and CD8+ αβ T cells is significantly higher in the spleen of newborn mice compared to the adult. The frequency of Helios rapidly decreases by 2-3 days after birth to adult levels. Furthermore, the appearance of Helios+Foxp3+ Tregs in mice did not appear in the periphery until three days after birth (Figure 31). These data demonstrate that the presence of Helios+ αβ T cells in the periphery predates the appearance of Tregs in mice and may contribute to perinatal tolerance establishment prior to Treg development in the periphery.

Additionally, it is known that γδ T cells are highly represented in the neonatal stage compared to αβ T cells in mice[353]. We next investigated if peripheral γδ T cells also express Helios. Strikingly, we found a significantly higher frequency of (T cells in newborn mice express Helios without Foxp3 co-expression than adults (Figure 32). This high frequency of Helios in the periphery also rapidly decreases to adult levels between 2-3 days after birth, as we observed with

αβ T cells. Majority of splenic γδ T cells are CD8+ or DN. While Helios is highly express by both subsets during the perinatal stage and decreases by 2-3 days after birth, we found a striking increase in Foxp3 expression by adult CD8+ γδ T cells compared to neonates (Figure 32).

Next, we investigated if the differences in Helios between perinatal and adult T cells has a developmental origin in the thymus. We found that the frequency of Helios+Foxp3- of CD4+ SP, CD8+ SP as well as total γδ TCR+ thymocytes is significantly higher before birth (E17.5/18.5) and decreases by 3 days after birth (Figure 34, 35). We also observed that the frequency of Helios+Foxp3- by CD4+SP, CD8+SP, as well as γδ TCR+ thymocytes, are higher than splenic T cells across all timepoints even though the kinetics of Helios+ T cell frequency is similar (Figure 36).

Our data so far demonstrated that the frequency of Helios+Foxp3- expression started high in the perinatal stage and rapidly decreased after birth in both αβ and γδ T cells in the periphery. These data suggest that high Helios expression is limited to perinatal T cells in both humans and mice. Next, we sought to investigate if Helios expression is maintained in T cells that have been shown to have a fetal developmental origin. These T cells consist of gut-associated "type b" CD8αα γδ T cells as well as CD8αα αβ T cells[51,554]. We found that Helios was highly expressed by these "type b" gut-associated T cells compared to "type a" gut-associated T cells that mainly consists of conventional CD4+ and CD8αβ+ αβ T cells in both neonate and adult SI IEL and LPL fraction (Figure 45).

Furthermore, Helios expression can be detected by "type b" T cells as early as fetal stage E17.5 and maintained through adulthood (Figure 48). Additionally, we found a different γδ T cell subset expressing CD8αβ and categorized them as "type c" gut-associated T cells based on their Helios and Foxp3 kinetics. These "type c" γδ T cells in the gut are different from "type a"

and “type b” gut T cells because neonatal CD8αβ+ γδ T cells express a high frequency of Helios without co-expression of Foxp3. However, the adult CD8αβ+ γδ T cells have a significantly higher frequency of Helios+Foxp3+ as well as a trend of higher frequency of Foxp3+ alone population compared to neonates (Figure 46). We also observed a similar phenotype by adult splenic CD8αβ+ γδ T cells where they express significantly higher Foxp3 compared to neonates (Figure 32).

It is known that Helios can form homodimer or heterodimers with other Ikaros transcription factor proteins. We next investigated if other Ikaros transcription factors such as Aiolos and Ikaros follow Helios kinetics or if this is specifically unique to Helios. We found that gut-associated “type b” T cells predominantly express all three IKZF transcription factors Helios, Aiolos, and Ikaros, while “type a” T cells predominantly express Aiolos and Ikaros without Helios. These data further suggest that Helios expression is unique to perinatal origin “type b” T cells (Figure 50). When we assessed the IKZF transcription factor profile in the periphery, we found that 5-day old neonatal splenic αβ T cells predominantly express Aiolos and Ikaros without Helios, like those of “type a” gut-associated T cells. This follows our previous finding where splenic αβ T cells lose Helios expression significantly by 3 days after birth.

Furthermore, splenic γδ T cells in 5-day old neonates have a significantly higher frequency of Aiolos+ Helios+ and Ikaros+ expression compared to adults. The observation of Helios+ γδ T cells at 5 days after birth is consistent with our previous findings where splenic γδ T cells have a higher frequency of Helios compared to both CD4+ and CD8+ splenic αβ T cells 5 days after birth (Figure 36). Strikingly, we observed a significant increase in both splenic αβ and γδ T cells that do not express any of the IKZF transcription factors tested in adults (Figure 53).

Our data demonstrate that Helios expressing T cells is developmentally controlled and limited to those developing during the perinatal stage. To test this hypothesis, we generated humanized mice by transferring UCB isolated HSCs into immunodeficient NSG-S mice. We found that CD4+ and CD8+ T cells generated from UCB HSCs retain high levels of Helios expression (Figure 57). These data suggest that Helios expressing T cells are derived from fetal HSCs.

We next sought to investigate the function of Helios in perinatal T cells. Our previous studies demonstrate that UCB naïve T cells carry self-intrinsic differences phenotypically and functionally compared to adult naïve T cells[309]. UCB naïve CD4+ T cells produce significantly higher levels of IL-10 in the presence of Th1 polarizing conditions compared to adult naïve CD4+ T cells, and this is not due to the lack of Th1 differentiation by UCB naïve CD4+ T cells as they produce significantly higher levels of IFN-γ after Th1 polarization in the same culture compared to adult naïve CD4+ T cells (Figure 58). We hypothesized that Helios promotes IL-10 production under Th1 polarizing conditions in neonatal T cells. We found that Helios frequency is the highest among CD4+ T cell subsets after Th1 polarization (Figure 60).

Furthermore, Helios expression correlates with CD4+ T cells that produce IL-10 under Th1 polarizing conditions after PMA and Ionomycin restimulation while IFN-γ frequency is similar between Helios+ and Helios- CD4+ T cells (Figure 61, 62). To test if Helios is required for Th1 mediated IL-10 production by perinatal T cells, we designed guide RNA against human *IKZF2* to knockout Helios in UCB T cells. We found that while Helios can successfully be knocked out in UCB T cells, IL-10 production was not affected in Helios knockout conditions after Th1 polarization (Figure 63, 64). These data suggest that Helios correlates with IL-10 production under Th1 conditions but is not required for IL-10 production. Previous literature

suggested that Helios expression by Foxp3+ Tregs may suppress effector cytokine production such as IFN-γ, TNF and IL-17[376]. We hypothesized that perinatal T cells express Helios to suppress their effector cytokine production as well. Indeed, we found that when we knockout Helios from UCB naïve CD4+ T cells, certain effector cytokine production increases in comparison to non-target guide RNA controls (Figure 65).

Regulatory Functions of UCB-derived Foxp3+ T Cells *In Vitro* and *In Vivo*

We demonstrated that both CD4+ and CD8+ T cells from UCB differentiate into Foxp3+ T cells upon antigen receptor stimulation. When we assess if these UCB-derived Foxp3+ T cells carry suppressive function *in vitro*, we found that both UCB CD4+ and CD8+ *ex vivo* generated Foxp3+ T cells suppress effector T cell proliferation in a dose-dependent manner comparable to autologous UCB preexisting CD4+CD25+ tTreg population.

Traditionally, Tregs have been considered a stable T helper lineage, carrying suppressive functions and a terminally differentiated phenotype. However, many studies have indicated that Tregs retain some degree of plasticity and acquire different transcriptional programs similar to the effector T cell population to regulate Th1, Th2, or Th17 responses[564]. For example, Th1-type Treg express chemokine receptors CXCR3 and transcription factor T-bet, while Th17-type Treg express CCR6 and RORγ[565,566]. The acquisition of T-bet by Tregs is necessary to control Th1 inflammation *in vivo*[126]. T-bet+ CXCR3+ Tregs are required to control type 1 diabetes, a Th1 predominant autoimmune disease[567]. These responses are associated with the expression of specific Th-associated transcription factors and chemokine receptor expression[568]. Characterization conducted by previous lab members demonstrated that these UCB *ex vivo* generated Foxp3+ T cells differ in their surface chemokine receptor phenotype from those of pre-existing adult tTregs. A majority of both UCB-derived CD4+ and CD8+ Foxp3+ T cells express

Th1-type phenotype, CXCR3+ and T-bet+ while majority of adult tTregs are Th17-like Tregs that express CCR6+[569]. Therefore, although UCB-derived Foxp3+ T cells carry comparable suppressive functions like their autologous tTregs counterparts *in vitro*, potentially *in vivo*, these UCB Foxp3+ T cells may be generated in the periphery upon antigen receptor stimulation to localize to Th1 inflammation sites and regulate Th1 responses specifically. Furthermore, human neonates are deficient in their Th1 responses (as reviewed above)[309]. A Th2 biased response in mice during the first week of life is critical for tolerance establishment, while Th1 response contributes to allograft rejection[547,548]. We demonstrated that adult CD4+ and CD8+ T cells have a significantly lower Foxp3+ T cell differentiation frequency than UCB, even though they were subjected to the same Foxp3+ T cell induction culture set up (Figure 9). Therefore, these UCB-derived Foxp3+ T cells may be explicitly generated during the perinatal stage as an additional mechanism to suppress Th1 response and promote tolerance establishment.

Using xGVHD model, we demonstrated that pre-treatment of UCB-derived Foxp3+ T cells significantly delayed xGVHD development mediated by total autologous UCB mononuclear cell transfer. The transfer of human PBMC in the xGVHD model is a Th1 predominant response with the majority of CD4+ T cells expressing IFN-γ[530,531]. These data demonstrated that UCB-derived Foxp3+ T cells carries suppressive functions *in vivo* in a Th1-type predominant response xGVHD model. Like previous reports, we observed high levels of IFN-γ in the serum with our xGVHD model. However, while the transfer of UCB-derived Foxp3+ T cells significantly delays xGVHD development, we did not observe any significant suppression of Th1 type cytokine levels in the serum (Figure 14). This could be because the serum was collected when mice hit criteria (lost <15% of original weight after cell transfer). Potentially we might see a difference between total UCB mononuclear cell transfer vs. UCB-

derived Foxp3+ T cell pretransfer group if we collect serum earlier on before the mice hit xGVHD criteria.

Strikingly, we observed that the UCB-derived Foxp3+ T cell pretreatment group has a significantly lower level of IL-10 in the serum than the group that received autologous UCB mononuclear cells alone. This result was counterintuitive because IL-10 is known to be an anti-inflammatory cytokine. However, patients with GVHD have been reported to have elevated serum levels of IL-10 and were significantly associated with fatal outcomes[532]. Furthermore, IL-10 administration accelerates GVHD in mice while IL-10 neutralization diminishes GVHD mediated by allogeneic donor grafts[570]. Using an xGVHD model, Abraham *et al* demonstrated that IL-10 overexpression leads to expansion of human T cells and subsequent exacerbation of xGVHD development in these mice[571]. It is still unclear how UCB-derived Foxp3+ T cells suppress IL-10 levels in our xGVHD model.

Rapid Kinetics of UCB Mononuclear Cells-mediated xGVHD

Numerous studies have utilized the xGVHD model to investigate new clinical regimens to decrease GVHD. It is one of the significant causes of morbidity and mortality following allogeneic bone marrow transplantation in patients. Typically, human adult PBMCs were transferred into NSG mice, and a range of cell numbers was transferred ($2x10^6$ to $3x10^7$). The median survival in NSG mice that receive $2x10^6$ adult PBMC is 56 days[530]. In our xGVHD model, we used total UCB mononuclear cells instead. We consistently observed that mice that received $1x10^6$ total UCB mononuclear cells hit criteria no later than 18 days post transfer (Figure 12). This is not due to the type of NSG mice we used (NSG-S instead of NSG for better human cell engraftment) because the transfer of the same amount of adult PBMC into NSG-S

mice did not manifest xGVHD as rapidly as total UCB mononuclear cell transfer in our hands (data not shown).

GVHD is known to be initiated by donor T cells; the rapid induction of xGVHD by UCB transfer may be due to these factors by UCB T cells[572]: First, UCB contained a higher frequency of CD45RA naïve T cells compared to APB[517–519]. It was previously demonstrated that the transfer of allogeneic naïve T cells led to a more severe GVHD development in mice[573]. Second, we demonstrated that UCB naïve CD4+ T cells have enhanced effector cytokine production than adult naïve CD4+ T cells under Th polarizing conditions. UCB naïve CD4+ T cells produce significantly higher levels of IFN-γ than adult naïve CD4+ T cells under Th1 polarizing conditions[309]. It is known that proinflammatory Th1 cytokines such as IL-2 and IFN-γ can exacerbate GVHD by promoting T cell expansion as well as enhancing cellular damage[574]. Lastly, we also found that UCB naïve CD4+ T cells produce significantly higher levels of IL-10 under Th1 polarizing conditions than adult naïve CD4+ T cells (Figure 58). Overexpression of recombinant IL-10 in NSG mice that receive $1x10^7$ adult PBMC succumbed to xGVHD between 15 to 21 days compared to control mice that received adult PBMC alone are free from GVHD for around 30 days[571]. It is interesting to note that overexpression of IL-10 exacerbates adult PBMC mediated xGVHD to the kinetics like what we observed when we transfer total UCB mononuclear cells in our xGVHD model (median survival 18 days). Therefore, the transfer of total UCB mononuclear cells potentially initiates xGVHD more rapidly due to their higher IL-10 production by CD4+ T cells under Th1 inflammatory conditions. However, it is still unclear what is the cellular source of IL-10 production in our xGVHD model.

Our data demonstrated that antigen receptor stimulation of total UCB leads to the generation of suppressive Foxp3+ T cells *in vitro* in the presence of IL-2 after 14 days of culture.

However, the transfer of total UCB into NSG-S mice causes xGVHD manifestation *in vivo*. One potential explanation could be that the generation of UCB Foxp3+ T cells is slower than the activation of UCB T cells that produces IL-10. In our hands, UCB naïve CD4+ T cells can produce high levels of IL-10 (Figure 65) upon antigen receptor stimulation in the presence of IL-2 as early as day 5 in culture. These data suggest that under similar culture conditions, UCB naïve CD4+ T cells can produce IL-10 earlier than the generation of stable Foxp3+ T cells that carries suppressive functions. It has been demonstrated that APB mononuclear cells activated with anti-CD3 in the presence of IL-10 led to an initial inhibition of CD8+ T cell proliferation followed by a proliferative phase between days 4 to days 9 in culture. These CD8+ T cells are biased towards a type 2 response and produce IL-4[575]. Previous lab member (Jessica Lee M.D., Ph.D.) also demonstrated that IL-4 could further inhibit Foxp3+ T cell induction by UCB. Therefore, potentially in our xGVHD model mediated by total UCB transfer, UCB CD4+ T cells produce high levels of IL-10 upon alloreactive activation *in vivo*. The high levels of IL-10 subsequently lead to the expansion of UCB CD8+ T cells that produce IL-4 and inhibit UCB Foxp3+ T cell generation *in vivo*.

Epigenetic Regulation of *FOXP3* by UCB-derived Foxp3+ T Cells

We demonstrated that UCB-derived Foxp3+ T cells could be generated upon antigen receptor stimulation without adding exogenous TGF-β in our induction culture system. We further demonstrated that a subset of CD14+CD36^{hi} monocytes is important in generating these Foxp3+ T cells and acts as the cellular source of TGF-β and retinoic acid[569]. This is similar to the requirement for mature naïve T cells in the periphery to differentiate into pTregs[103,104]. Furthermore, we also demonstrated that UCB-derived Foxp3+ T cells from our induction culture are not coming from the expansion of preexisting tTregs, as depletion of CD4+CD25+ prior to

culture set up still generate the same frequency for Foxp3+ T cells[569]. This prompts us to investigate if Foxp3 regulation in UCB-derived Foxp3+ T cells is like that of tTregs or pTregs.

Using bisulfite sequencing and ATAC-seq, we found that the epigenetic landscape of Foxp3 by UCB-derived CD4+ Foxp3+ T cells are different from tTregs (Figure 16,17). Bisulfite sequencing demonstrated that *Foxp3* CNS2 region of UCB-derived Foxp3+ T cells is highly methylated like those of CD4+ effector T cells or *in vitro* differentiated Tregs (iTregs). Highly methylated CNS2 contributes to the instability of Foxp3 expression in iTregs[137,138]. However, we observed that UCB-derived Foxp3+ T cell maintain their Foxp3 expression for a long time in our culture system (up to 62 days in culture)[512]. Together with our ATAC-seq data, Foxp3 expression by UCB-derived Foxp3+ T cells is regulated differentially than tTregs. Our ATAC-seq data further demonstrate unique chromatin open regions found in the *Foxp3* locus of UCB-derived Foxp3+ CD4+ T cells compared to adult CD4+ Foxp3+ tTregs. These gene regions were conserved in mouse *Foxp3* locus as well. These findings could allow the generation of transgenic mice to study if these regions can regulate Foxp3 expression, particularly in perinatal T cells. However, UCB-derived Foxp3+ T cells murine counterpart has yet to be identified.

One potential mechanism that maintains Foxp3 stability in our *ex vivo* generated Foxp3+ T cells from UCB is the transcription factor Helios. Helios has been previously reported to be important for Treg Foxp3 stability and function[372,384–386]. Treg-specific Helios knockout subsequently leads to a decrease in Foxp3 expression[376]. Interestingly, Helios does not bind to the *Foxp3* locus in both CD4+ and CD8+ Tregs, while Foxp3 is found to occupy the *Ikzf2* locus in CD4+ Tregs[376,389]. T cell-specific Helios deletion did not affect Foxp3 induction, demonstrating that Helios is not required for Foxp3 expression[372]. These data suggest that while Helios is not required for Foxp3 induction, it may be maintaining Foxp3 expression through

some other unknown mechanisms. After the 14-day induction culture, we found that both UCB CD4+ and CD8+ Foxp3+ T cells also highly express Helios (Figure 9F).

Further testing will be required if Helios indeed supports Foxp3 stability in these cells. Potentially, Helios could be knockout using the CRISPR-Cas9 system after the induction culture to test if Helios is required to maintain Foxp3 expression in UCB-derived Foxp3+ T cells. Additionally, the role of Helios in Foxp3 expression by CD8+ T cells is unknown.

Helios has been reported previously to enhance differentiation of human fetal naïve CD4+ T cells into Foxp3+ Tregs without the addition of exogenous TGF-β. They demonstrated that Helios knockout impairs the capability of fetal naïve CD4+ T cells to differentiate into Foxp3+ Tregs, and this impairment is rescued with the addition of TGF-β[327]. One difference with this study is they differentiate Foxp3+ Tregs using purified naïve CD4+ T cells stimulated in the presence of anti-CD3/CD28/CD2 tetramers and check Foxp3 induction by 6 days of culture. In our hands, bead stimulation with UCB purified T cells yields a lower frequency of Foxp3+ T cells at the end of the 14-day induction culture. Future studies to dissect if different types of Foxp3+ T cells are generated from perinatal T cells based on culture conditions (i.e., the presence of other cell subsets such as APCs) and their subsequent requirement for Helios to express and maintain Foxp3 are needed.

Alcohol Effects on UCB-derived Foxp3+ T Cell Differentiation

Implications of the Inhibition of UCB-derived Foxp3+ T Cell Differentiation by Alcohol

Our data demonstrated that EtOH presence during the first five days of culture significantly inhibits Foxp3+ T cells induction from both UCB CD4+ and CD8+ T cells as reflected by the significant decrease in CD25+ Foxp3+ frequency within CD4+ and CD8+ T cells. This aligns with previous reports where FAE promotes an overall inflammatory state in the

fetus. In animal models, FAE increases the development of autoimmune diseases such as rheumatoid arthritis and type 2 diabetes[486,487]. FAE also subsequently leads to an increase in proinflammatory cytokine levels in the fetus[505]. Foxp3+ Tregs play a significant role in regulating allergic reactions by inducing and maintaining immune tolerance against allergens. The percentage of Foxp3+ Tregs is significantly decreasing in allergic children (atopic dermatitis and/or food allergy) compared to healthy controls[576]. In humans, alcohol consumption during pregnancy was associated with a dose-dependent increased risk of atopic dermatitis during early infancy[577]. Since Foxp3+ T cells play a vital component in peripheral immune tolerance, our data demonstrated that it is plausible that prenatal alcohol exposure inhibits the generation of Foxp3+ T cells *in vivo* during the perinatal stage and subsequently leads to an increase in allergies and autoimmune disease development.

Potential Mechanisms Underlying the Inhibition of UCB-derived Foxp3+ T Cell Induction by Alcohol

When we assessed if EtOH induces toxicity in the culture and leads to cell death, we did not see any significant differences in total UCB mononuclear cell numbers early on or at the end of the culture (Figure 23 and 24). This suggests that EtOH may be altering important pathways that are required for Foxp3+ T cell differentiation. We observed that EtOH also significantly decreases Foxp3 expression as reflected by the mean fluorescent intensity in both CD4+ and CD8+ T cells (Figure 27). Levels of Foxp3 expression have been previously shown to reflect their regulatory functions in transplantation settings. Foxp3 expression levels are directly associated with their potential in preventing allograft rejection through the production of regulatory cytokines and suppressing effector T cell activation[578].

While the mechanism for Foxp3 induction in CD4+ T cells has been heavily studied, there is still a lot to explored regarding CD8+ Foxp3+ T cell differentiation. In the absence of

DCs, it was shown that murine splenic CD8+ T cells upregulate Foxp3 most optimally in the presence of antigen, IL-2, TGF-β, and retinoic acid[579]. In our hands, induction of Foxp3 is significantly inhibited in both CD4+ and CD8+ UCB T cells in the presence of a TGF-β receptor I kinase inhibitor, demonstrating that TGF-β signaling is also required for Foxp3 induction by CD8+ T cells in humans[512]. Furthermore, it has been shown previously that IL-2 signaling is required for TGF-β to induce naïve CD4+CD25- T cells to become CD25+ and express Foxp3[105]. Indeed, we found that EtOH significantly inhibits CD25 (IL-2Rα) expression, a component of the IL-2 receptor important for IL-2 signaling, in both CD4+ and CD8+ UCB T cells early on in the culture (Figure 28).

One potential mechanism by which EtOH is inhibiting CD25 expression is through its modulation of Phospholipase D (PLD) signaling. PLD is a phosphodiesterase that catalyzes the hydrolysis of phosphatidylcholine, a major phospholipid in the plasma membrane, into phosphatidic acid (PA) and choline. There are two closely related members in the PLD family, PLD1 and PLD2, with different subcellular localizations[580]. Primary alcohol such as 1-butanol and EtOH are excellent substrates for PLD and are preferred over water by at least 400-fold[581]. In the presence of primary alcohol, PLD favors transphosphatidylation over hydrolysis, significantly reducing the production of PA while yielding phosphatidyl alcohol (i.e., phosphatidyl ethanol, PEth). In early TCR signaling studies using Jurkat T cell lines, PLD is induced in the presence of TCR cross-linking and PMA activation, subsequently inducing transcription factor AP-1, a transcription factor involved in the regulation of growth and proliferation[582–585]. Jurkat treated with EtOH produce PEth upon activation, and anti-CD3 induced AP-1 activity is blocked[585]. PA has been shown to induce CD25 expression on T cells with equal potency as saturating doses of IL-2[586]. Additionally, PLD1 and PLD2 double KO

CD4+ T cells have less CD25 upregulation after stimulation than WT CD4+ T cells[587]. Therefore, in the presence of EtOH, it is possible that the reduction of PA production by UCB T cells decreases their CD25 expression. This decrease in CD25 expression early in our UCB culture subsequently inhibits Foxp3 induction by CD4+ and CD8+ UCB T cells.

CD25 Expression Differences between CD4+ and CD8+ T Cells

Upon antigen receptor stimulation, CD25 is induced in both CD4+ and CD8+ T cells. However, we found that consistently across all our donors, the frequency and gMFI of CD25 is higher in CD4+ than those of CD8+ UCB T cells 24 hours after stimulation. This could be due to intrinsic intracellular signaling differences between CD4+ and CD8+ T cells. Both CD4 and CD8 molecules acts as coreceptors to enhance antigen responsiveness mediated by TCR. It is found that TCR/CD4 cross-linking led to greater tyrosine phosphorylation of intracellular substrates compared to TCR/CD8 cross-linking. Cross-linking CD4 also initiated greater kinase activity compared with CD8 cross-linking in an *in vitro* kinase assay[588]. Furthermore, TCR/CD8 signaling is implicated to depend more on a PKC-mediated signaling pathway, as the addition of PKC specific inhibitor significantly attenuated IL-2 production by CD8+ T cells, whereas CD4+ T cells were far less affected[588]. Hence, it is plausible that the overall lower CD25 induction by CD8+ T cells after activation is due to intrinsic signaling differences between CD4+ and CD8+ T cells.

Implications of High Helios Expression in Perinatal T Cells

The Role of Helios in Tolerance

It is well established that the perinatal immune system is tolerance biased. We found that perinatal T cells in both humans and mice highly express Helios without the expression of Foxp3. The frequency of these Helios+ T cells rapidly decreases after birth (Figure 29-32).

Furthermore, Helios+ αβ T cells and γδ T cells can be detected in the periphery prior to the appearance of Foxp3+ Tregs in neonatal mice. Helios$^{-/-}$ mice also present significant fatality within the first week of postnatal life[383]. This suggests that Helios+ αβ T cells and γδ T cells may contribute to perinatal tolerance prior to existence of Tregs in the periphery since autoimmunity does not develop prior to Treg appearance at 3 days after birth in mice[87,93,94]. The absence of autoimmunity in the first few days of life was not due to the impaired function of perinatal T cells, as perinatal T cells can generate appropriate immune responses under certain conditions[276–279].

γδ T cells predate the development of αβ T cells, leading to a predominance of γδ T cells in the periphery the first few days after birth in mice[192]. In Billingham and Medawar's seminal perinatal tolerance experiment, allogeneic cells were injected into the fetus at E 15-16[241]. They observed subsequent tolerance against skin graft specific to that mouse strain but rejected skin graft from an unrelated strain (not previously exposed). At E15-16, the frequency of γδ thymocytes reaches its peak[192]. Foxp3+ Tregs were not detected in mice until around three days after birth[93,94]. These data suggest that tolerance establishment against allogeneic cells introduced during E15-16 are predominantly mediated by γδ T cells prior to the appearance of Foxp3+ Tregs. While γδ T cells are considered unconventional T cells where they can recognize antigens in a non-MHC restricted manner, they possess a large diversity of complementary determinant region 3 (CDR3) of their TCR due to germline VDJ rearrangement[589]. αβ T cells undergo endogenous ligand driven positive and negative selection in the thymus before entering the periphery. However, the role of ligand-mediated selection in γδ T cells is still unclear. Furthermore, Allogeneic cells injected within 24 hours after birth homes to the thymus, suggesting a role in the thymocyte selection process[590]. Therefore, the introduction of allogeneic

cells during the fetal stage in Billingham and Medawar's model may lead to the selection and expansion of γδ T cells expressing TCR specific to the injected allogeneic cells and establish tolerance.

While most of the Helios related studies are focused in Foxp3+ Treg populations, several studies have suggested the association of Helios with tolerogenic T cell responses in both the thymus and periphery. Helios+ T cells have also been shown to be induced in the periphery against self-antigen and food antigens. Using an autoimmune gastritis mouse model, Ross *et al.* demonstrated Helios was expressed in H^+/K^+ ATPase-specific CD4+Foxp3- T cells in the periphery following H^+/K^+ ATPase presentation in the paragastric lymph nodes. These CD4+Helios+ cells have less survival and proliferation in response to peptide *in vitro* and produce very little IL-2 compared to CD4+Helios- cells[591]. Another study observed that Helios+Foxp3- CD4 T cells are generated predominantly in Peyer patches (PP) upon physiological uptake of dietary proteins and exhibited a proapoptotic phenotype with co-expression of programmed cell death protein 1 (PD-1). They also showed that patients with Crohn's disease have lower Helios+Foxp3- CD4 T cell frequency in their PP compared to healthy controls, implicating the importance of these cells to maintain intestinal homeostasis in response to food antigens[592]. These studies demonstrate that Helios expression in non-Tregs can contribute to maintaining immune tolerance against food or autoantigen. Our observation of Helios+ αβ and γδ T cells in the perinatal stage may be cells that are specific to self-antigen and generated before Treg appearance to maintain tolerance against self. Future studies investigating the TCR repertoire and specificity of Helios+ αβ and γδ T cells is needed to determine if perinatal Helios+ T cells are limited to self-antigen specific T cells.

Additionally, the perinatal immune system is thought to be Th2 biased, particularly in mice. For instance, neonatal T cells produce higher levels of IL-4 compared to adult T cells, and this Th2 biased response rapidly declines by 5 days after birth to adult levels[307,308]. A Th2 biased response is also critical for establishing tolerance, especially during the first few days of birth. In contrast, Th1 cytokines have been associated with allograft rejection[547,548]. Using a neonatal model of tolerance where allogeneic splenocytes were injected into mice within 24 hours after birth, allospecific Th2 biased response with high IL-4 and low IFN-γ levels was observed, and antigen-specific tolerance was established[593]. On the other hand, alloantigen exposure during adulthood triggers a Th1 biased response[593]. Furthermore, redirecting the priming response into Th1 biased response by injecting IFN-γ during neonatal alloantigen exposure subsequently led to antigen rejection in this model[51]. These data demonstrated that Th2 biased response is important for establishing perinatal tolerance. The generation of tolerance against foreign antigen was not due to the generation of Foxp3+ Tregs. Helios expression was induced in CD4+ T cells differentiating into Th2 *in vivo* without parallel upregulation of Foxp3[374]. It is plausible that the high frequency of Helios in perinatal T cells is associated with the Th2 biased response in perinatal mice prior to the appearance of Foxp3+ Tregs in the periphery to promote tolerance.

Helios+ T cells are Limited to Perinatal Origin

The "layered immune system" hypothesis states that perinatal T cells and adult T cells differ in their ontogeny and arise in succession from different waves of HSCs. This concept is applied to the observation that γδ T cells and B cells lineages in mice have different ontogeny and arise from different waves in succession[352,353]. It was also demonstrated that HSCs from fetal and adult give rise to T cells with different functions and phenotype[323,356]. Fetal-derived CD4+ T cells have been shown to preferentially become Tregs and made Th2 cytokines upon

stimulation[323,594]. Our observation that Helios is highly expressed in both human and mouse perinatal T cells compared to adults suggests that Helios+ T cells may be generated from HSC of perinatal origin. In fact, we demonstrated that Helios+ CD4+ and CD8+ αβ T cells could be generated *in vivo* using humanized mouse model by transferring UCB HSCs into NSG-S mice (Figure 57). These Helios+ T cells were maintained in the periphery for up to 21 weeks post HSC transfer. One caveat of a humanized mouse model is the development of human T cells in a mouse thymic environment. It is still unknown if there are thymic factors that regulates the generation of Helios+ T cells and contribute to the Helios differences we observed between perinatal and adult T cells. Additionally, further testing is required to compare if AB-HSC humanized mice can also generate Helios+ T cells.

The next evidence that suggests Helios expression is limited to perinatal origin is the detection of Helios specifically within "type b" CD8αα αβ and γδ T cells in the gut. Helios expression in "type b" T cells can be detected in the perinatal stage and is maintained through adulthood (Figure 45). "Type b" gut-associated T cells have been demonstrated to arise from perinatal progenitor cells Helios expression is maintained through adulthood [51,554]. These data suggest that Helios+ T cells are limited to those coming from perinatal origin.

When we investigated if the kinetics of Helios+ T cells we observed in the spleen is also reflected in the thymus, we saw that both αβ and γδ thymocytes also express high frequency of Helios and is significantly lower in adults. However, one striking observation is that Helios expression in the thymus across all stages is consistently higher than the spleen. This suggests that there may be extrathymic factors that further regulates Helios+ T cells that exit into the periphery. Alternatively, Helios+ T cells may also migrate to non-lymphoid organs after they exit the thymus. Further testing by transferring fetal liver HSC or adult BM HSC into

immunodeficient neonate and adult Rag2$^{-/-}$ mice will allow us to test the hypothesis whether Helios discrepancy between perinatal and adult T cells is due to progenitor cell differences or extrathymic factor.

The Role of Helios in Self-renewal

In acute myeloid leukemia, Helios drives leukemia stem cell self-renewal and inhibits myeloid differentiation[595]. γδ T cells of perinatal origin such as murine dendritic epidermal T cells (DETCs) and γδ IEL have the capability for life-long self-renewal in tissues independently of circulating precursors[596]. It was demonstrated that "type b" gut-associated T cells, consisting of CD8αα αβ and γδ T cells, arise from progenitor cells of perinatal origin[51,554]. We only observe high Helios expression specifically in "type b" gut-associated T cells and their expression maintained through adulthood. Helios expression by these "type b" T cells may contribute to their self-renewal properties. Furthermore, we do not observe high expression of Helios by "type a" conventional T cells in the gut. These data demonstrated that Helios is not required for T cell localization to the gut, or the gut microenvironment induces its expression.

TCR Repertoire by Helios+ T Cells

It was previously reported that the perinatal TCR repertoire in human and mice are skewed towards those with higher self-reactivity based on higher CD5 detection, a surrogate marker for TCR avidity for self-pMHC[346,597]. This was due to thymocytes bearing TCR with low affinity against self-peptide are not efficiently selected into the neonatal repertoire, while stronger TCR signals accompany both conventional and Treg selection. TCR with high self-reactivity has been linked to more promiscuous binding to different foreign peptide-MHC complexes and higher affinity binding against foreign antigens [347–349]. Thus, the limited TCR repertoire in neonates endows them with a greater ability to respond to multiple foreign antigens.

In the thymus, Helios is shown to differentiate CD4+ thymocytes' response to weak or strong self-pMHC stimulation. Helios is expressed at low levels in the DP stage but was further downregulated during positive selection of CD4+ thymocytes that bind self-pMHC weakly. On the other hand, Helios was upregulated in strongly self-reactive CD4+ single positive thymocytes particularly when elimination of self-reactive thymocytes was inhibited in Bim deficient mice[598]. TCR repertoire analysis comparing Helios+ and Helios- Treg demonstrate very little overlap[599]. We observe that Helios expression is restricted to T cells of perinatal origin (both in the periphery and the gut). The IEL population appears to be enriched with T cells that bear autoreactive TCR. Although no specific self-peptide ligand has yet been identified, several lines of evidence support the idea that this population is autoreactive. For instance, IEL CD8αα αβ T cells express Vβ TCR that recognizes endogenous antigen (Mls antigen in mice bearing Mls allele)[600]. Recognition of self-antigen in the context of class I MHC is required for the selection and differentiation of CD8αα αβ T cells in the IEL using TCR transgenic mice[601]. Autoreactive γδ T cells have been shown to escape negative selection in the neonatal thymus, but not in adults, and undergo extrathymic differentiation in the intestinal epithelium[602]. Hence, the high frequency of Helios+ perinatal T cells in the spleen and the gut may reflect T cells with higher self-reactivity, and this was due to the differences in thymic selection in the perinatal versus adult stage.

Additionally, not 100% of "type b" gut-associated T cells express Helios especially in the adult gut (around 70%, Figure 45). This observation could be attributed to "induced" IELs instead of naturally arising IEL, where CD8αα can be upregulated by conventional CD8αβ+ and CD4+ αβ T cells in the intestine[603,604]. Furthermore, the detection of Vγ2 and Vγ4 chain in addition to Vγ7 on the IEL γδ T cells suggests that γδ IELs consist of γδ T cells from different

developmental waves based on their Vγ chain usage[49,20]. Potentially Helios+ γδ IELs may be confined to certain Vγ chain usage. Future studies to investigate the TCR repertoire differences between Helios+ and Helios- perinatal T cells (both in the periphery and the gut) will be needed.

Helios Renders Perinatal T Cells to be Less Reactive

As reviewed above, the perinatal immune system is functional where they can generate inflammatory responses like their adult counterpart. This suggests that strong and redundant mechanisms must exist to preserve a tolerogenic biased immune response. Some of the known cell intrinsic mechanisms to promote tolerance response are such as the increased capacity of fetal T cells to differentiate into Tregs, due to higher sensitivity to respond to TGF-β[324]. Helios expression by fetal naive T cells has been suggested to generate a poised epigenome for enhanced Treg differentiation[327].

Another implication of high Helios expression in perinatal T cells is to suppress effector cytokine production by perinatal T cells. Freshly isolated UCB naïve CD4+ T cells have been shown to produce significantly lower levels of IFN-γ compared to adult naïve CD4+ T cells[309]. Neonatal CD8+ T cells also produce significantly less granzyme B, IFN-γ, and IL-2 compared to adult CD8 T cells[316]. In Tregs, Helios deficiency led to increased effector cytokine production such as IFN-γ, TNF and IL-17[376]. Helios expression is also found to suppress IL-2 gene transcription in Tregs[388]. Hence it is possible that perinatal T cells and gut-associated “type b” T cells express high levels of Helios to prevent excessive inflammatory cytokine production against foreign antigen when establishing tolerance is critical. Our data suggest a novel function of Helios in perinatal T cells where it suppresses effector cytokine production upon T cell activation (Figure 65).

Furthermore, we also demonstrated that mouse neonatal CD4+ T cells subjected to Th1 polarizing conditions express significantly higher Helios frequency than Th0 or Th2 conditions (Figure 60). Helios expression also correlated with IL-10 producing CD4+ T cells under Th1 polarizing conditions in neonates (Figure 61). These data suggest that neonatal T cells may also upregulate Helios under Th1 inflammatory condition as a feedback mechanism to limit inflammation.

We initially hypothesized that Helios is required for IL-10 production under Th1 inflammatory conditions as we observed a correlation of Helios with IL-10 producing CD4+ T cells under Th1 polarization in mice (Figure 61). However, when we knockout Helios in UCB naïve CD4+ T cells and subjected them to Th0 or Th1 conditions, we did not observe a decrease in IL-10 production (Figure 63, 64). Additionally, we also did not observe any effect on Helios knockout when UCB T cells were restimulated immediately without further culturing in Th0 or Th1conditions (Figure 65). This suggests that Helios is not required for IL-10 production by human CD4+ T cells. Alternatively, the Ikaros transcription factor family is known to form heterodimers with other proteins in the family[368,371,400].

Ikaros and Aiolos have been reported to promote IL-10 production by CD4+ T cells[400,406]. Helios-null mice have minimal impact on T cell development and function, in contrast to hyperproliferative T cell response and T lymphomas development observed with dominant-negative Helios overexpression[381,383]. This suggests that other Ikaros family members can compensate for Helios in T cells. Therefore, the lack of apparent IL-10 effects by Helios knockout may be compensated by other family members. Future experiments will be needed to test the effect of double and triple knockouts using gRNA against Helios, Aiolos, and Ikaros.

In gut mucosal tissue, we found that perinatal origin "type b" T cells expressed Helios since fetal stages and maintained it throughout adulthood. "Type b" gut-associated γδ and αβ IEL demonstrates an "activated yet resting" phenotype where expression of conventional cytokines such as IFN-γ, IL-I2, IL-4 and IL-10 are very low[605]. An increase in effector cytokine production by Helios deficient UCB CD4+ T cells further suggests that "type b" gut-associated T cells may also express Helios as a mechanism to suppress effector cytokine production to prevent excessive inflammation. This is important because the epithelial tract of the gut is constantly confronted with a plethora of foreign and potentially harmful antigens. The gastrointestinal tract immune system must constantly distinguish harmless dietary proteins and commensal microbes and respond accordingly to harmful pathogens. Hence it is plausible that these perinatal origin "type b" T cells reconstitute the gut early on to promote the establishment of immune homeostasis against commensal microbes and harmless food antigens that the newborns are exposed after birth.

It has been reported that Ikaros transcription factor family of proteins can both repress and activate gene expression by the formation of higher-order chromatin binding complexes. Helios has been reported to be associated with proteins of the Mi-2β/NuRD complex[369]. Depending on the context in the chromatin, Mi-2/NuRD complexes can either activate or repress gene transcription[606,607]. We observed that when we knockout Helios in UCB CD4+ T cells, there is a significant increase in certain effector cytokines such as IFN-γ and IL-13 production upon PMA and ionomycin restimulation. It was previously reported that intracellular pathogens like Toxoplasma gondii could repress STAT1 transcription and block IFN-γ dependent transcription by recruiting Mi-2/NuRD to STAT1-dependent promoters[608]. Furthermore, inactivation of NuRD by knocking out a NuRD-specific component, metastasis-associated protein 2 (Mta2), led to

hyper induction of IL-2, IL-4, and IFN-γ by T cells[609]. Altogether, these data suggest that Helios may suppress IFN-γ production in UCB T cells via NuRD complexes.

Why Does Helios+ T Cells Decrease Rapidly After Birth in the Periphery?

We observed that the frequency of Helios+ T cells rapidly decrease in the periphery by 2-3 days after birth in mice, and by 3 weeks after birth in humans to adult levels. While we do not have human fetal T cell Helios data, Ng *et al* demonstrated that fetal naïve T cells from 18-23 gestational week expressed Helios. This enhances the preferential differentiation of fetal naïve T cells towards a Treg cell fate[327]. Together with our data, this demonstrates that Helios+ T cells are present as early as 18-23 gestational weeks in fetuses and progressively decline by 3 weeks after birth in human. After birth, the decline of Helios+ T cells may be essential for the gradual switch from the tolerant biased perinatal immune response into an immune system capable of generating effector functions.

One potential explanation for this progressive decrease of Helios+ T cells is that Helios+ perinatal T cells arise from distinct HSC lineage from adult T cells. Their frequency decreases in the periphery when they are superseded by "adult-type" T cells. In fact, phenotypically similar immune cells of the developing fetal immune system and those from adults have been shown to be functionally and transcriptionally different[327,356,357,360,361]. Immune cell phenotype has also been shown to drastically change between birth (UCB) and postnatal peripheral blood[610]. Using single-cell mapping, Bunis *et al* demonstrated that lymphoid cells, myeloid cells as well as HSCs from fetal, perinatal and adult transition occur progressively along a continuum of maturity instead of the layering of fetal lineage alongside a distinct adult lineage[611]. Their study also provides evidence that T cell populations of different developmental stages are characterized by a progressive downregulation of fetal genes and upregulation of adult genes from fetal through

adulthood. It is plausible that Helios+ T cells are confined in the perinatal stage of development and subsequently replaced by Helios- T cells in the periphery as part of the perinatal to the adult immune system transition process.

Another potential explanation is the presence of steroid hormones estrogen and progesterone that has potent immunomodulatory effects throughout gestation and shortly after birth in newborns. Estrogen and progesterone are implicated to be critical to maternal tolerance against the fetus while little is known about their effects on fetal T cells[612]. Estrogen has been shown to promote Treg induction and expansion *in vitro* and *in vivo*[613,614]. Progesterone also promotes differentiation of UCB naïve T cells into Tregs while it has little effect on adult T cells, suggesting that sensitivity to progesterone is lost in adults. Estrogen and progesterone have been detected in UCB plasma[615,616]. The highest concentration of progesterone was noted during the first 24 hours of life and progressively decreases by 72 hours after birth to 0-16ng/ml[615]. Estrogen can regulate HSC homeostasis and affect their number and mobilization. During pregnancy, estrogen can increase HSC self-renewal in the bone marrow to support extramedullary hematopoiesis to support a rapid expansion of maternal blood volume[617]. Overall, the presence of estrogen and progesterone during pregnancy and the rapid decrease of estrogen and progesterone right after birth in newborns, it is possible that these hormones may influence the generation of Helios+ T cells, specifically in the perinatal stage. While most studies regarding progesterone and estrogen have been focused on maternal side of the maternal-fetal interface of pregnancy, future investigations are required to understand how exposure of these hormones can affect the perinatal immune system development and their role in perinatal tolerance.

Implications of "Type C" γδ T Cells

In the gut, we found a subset of less characterized γδ T cells that express CD8αβ receptor, which we categorize as "type c" gut-associated T cells. Neonatal SI IEL and LPL CD8αβ γδ T cells express a high frequency of Helios+Foxp3-, and this frequency significantly decreases in adults. These "type c" T cells subsequently express Helios+Foxp3+ or Foxp3+ alone in adults (Figure 46). In the spleen, γδ T cells are either DN or express CD8αβ (Figure 52). While the Helios+Foxp3- frequency in both splenic DN and CD8αβ γδ T cells decreases from neonates to adulthood, splenic CD8αβ γδ T cells in adults also demonstrate a subsequent increase in Foxp3+ expression alone (Figure 32). The increase in Foxp3 frequency by gut and splenic CD8αβ γδ T cells suggests that Foxp3 expression is induced in the periphery and accumulates with age.

Not much is known about Foxp3 expressing γδ T cells and how these cells can be generated. However, many studies have reported *in vitro* generated Foxp3+ γδ T cells can carry immunoregulatory functions. One study reported that freshly isolated mouse splenic total γδ T cells do not express Foxp3. However, the addition of TGF-β in the presence of anti-γδTCR stimulation subsequently lead to the induction of Foxp3 by these γδ T cells[618]. These *in vitro* generated Foxp3+ γδ T cells carriy suppressive functions, where it inhibits proliferation of responder T cells and decreases their IL-2 production[618]. In humans, the induction of suppressive Foxp3+ γδ T cells in the presence of TGF-β and IL-15 upon antigen receptor stimulation has been reported[619]. Another report demonstrated that Vitamin C further increased TGF-β-induced Foxp3 expression, stability, and suppressive capability of human γδ T cells. These Foxp3+ γδ T cells has hypomethylation at their *Foxp3* CNS2 region[620]. The detection of CD8αβ γδ T cells have also been reported in the human gut mucosa and was implicated to play an important role in gut homeostasis as their frequency is negatively correlated with inflammatory bowel disease

severity[557]. Potentially, the "type c" CD8αβ γδ T cells we observe may induce Foxp3 expression *in vivo* upon antigen receptor stimulation and necessary cytokine signals and reflect a population of antigen experienced γδ T cells in adults. Further studies are required to determine if these Foxp3+ CD8αβ γδ T cells carry suppressive functions.

Implications of IKZF Transcription Factor Profile

The majority of gut "type b" associated T cells express Helios, Aiolos, and Ikaros from neonates and maintain through adulthood (Figure 50). While all three transcription factors have been described individually in T cell differentiation, their expression in gut-associated T cells remained unexplored. To the best of our knowledge, a comprehensive analysis of these three transcription factors together in neonates and adult T cells has not been conducted. In addition to the role of Helios in tolerance as discussed above, some of the known functions by Ikaros and Aiolos in T cells implicates some contribution to perinatal tolerance. For instance, Ikaros inhibits Th1 differentiation by suppressing T-bet expression and IFN-γ production[398,399]. Furthermore, Ikaros deficiency also decreases GATA-3 expression, a Th2 associated transcription factor, as well as Th2 cytokines such as IL-4, IL-5 and IL-13, under Th2 polarizing conditions[398]. Furthermore, T cells with Ikaros deficiency also produce lower levels of anti-inflammatory cytokine, IL-10 and an increase in Th1 cytokines such as IL-2 and IFN-γ upon antigen receptor stimulation[621]. These data suggest that Ikaros is important an important regulator in Th2 cell differentiation and responses. Aiolos may contribute to neonatal tolerance where it is associated with IL-10 production by CD4+ T cells[406]. Aiolos is shown to be able to form heterodimers with Ikaros, which is known to bind the *Il10* locus[371,400,406].

The main difference between gut associated "type a" vs "type b" T cells is the expression of Helios, where "type a" predominantly express Aiolos and Ikaros while "type b" T cells

express Helios, Aiolos and Ikaros (Figure 50). This further suggests that Helios expression is restricted to T cells of perinatal origin. Within the "type b" T cells, the adult gut has a significant increase in a population that expresses Aiolos and Helios without Ikaros. Based on known function of Ikaros in CD4+ T cells, it is possible that this proportion of Helios+ Aiolos+ Ikaros- "type b" T cells are the "induced" IEL and carry different function than those of Helios+ Aiolos+ Ikaros+ "type b" T cell population. Future experiments using single cell RNA-seq will allow us to elucidate how the different Ikaros transcription family factor combinations may regulate the function of these T cells in the gut. Additionally, it will be interesting to test if Helios+ Aiolos+ Ikaros+ "type b" T cells arise from perinatal origin by transferring fetal liver HSCs vs adult BM HSCs into $Rag2^{-/-}$ mice to assess if solely perinatal HSCs will give rise to Helios+ Aiolos+ and Ikaros+ "type b" T cells.

When we investigate the Ikaros transcription factor family profile in the spleen between neonates and adults, we observe that in neonates αβ and γδ T cells, we can still detect a proportion of cells that express Helios+ Aiolos+ and Ikaros+ in 5-day old neonates. Like "type a" T cells in the gut, a big proportion of 5-day old neonatal splenic αβ T cells express Aiolos and Ikaros without Helios (Figure 53). This corresponds to our observation where the frequency of Helios+ CD4+ and CD8+ αβ T cells rapidly decreases by 2-3 days after birth. It would be interesting to investigate if other Ikaros transcription factor profiles differ prior to 5 days after birth.

A significant larger proportion of neonatal γδ T cells express Helios+ Aiolos+ and Ikaros+ and this population significantly decreases in adult γδ T cells in the spleen (Figure 53). This suggests that there may be a switch in the γδ T cell pool in the periphery from perinatal origin vs adult origin. In humans, γδ V gene usage is different between UCB and adult[304].

Additionally, UCB and adult γδ T cells display differences in cytokine production regardless of V gene usage where UCB γδ T cells produce higher levels of IL-10 in comparison to adults[306]. In mice, while both Vγ1 and Vγ4 γδ T cells can be detected in the adult spleen and LN[44,52,53], they are implicated in carrying different functions. Vγ1+ γδ T cells are shown to promote CD4+ T cells into a Th2 type response while Vγ4+ γδ T cells promote a Th1 type response following Coxsackievirus B3 infection[622]. In a different study, Vγ1+ γδ T cells enhance allergic airway inflammation by promoting Th2 cytokines in the airway while Vγ4+ γδ T cells suppress allergic airway inflammation[623]. Future testing will be required to determine if there is an association with Ikaros family protein expression profile with Vγ chain usage by neonates and adult γδ T cells in the spleen.

Another striking observation is the appearance of both αβ and γδ T cells that do not express Aiolos, Helios or Ikaros in the adult spleen. This is not correlated to the naïve and memory proportions in the adult spleen (Figure 54). We do not observe a significant emergence of this population in adult gut-associated “type a” or “type b” T cells, suggesting that it is unique to αβ and γδ T cells in the periphery.

It is known that the Ikaros transcription factor family proteins can form homodimer with themselves or heterodimer with each other to carry out their regulatory functions. While we detected Helios, Ikaros and Aiolos mostly in perinatal origin gut “type b” T cells in both neonates and adults, we do not know the level of expression, as well as if these transcription factors are functioning in these T cells as homodimers or heterodimers. Further experiments will be needed to determine their mRNA and protein levels via RT-PCR or western blots from purified “type b” T cells in the gut. To determine if Helios, Ikaros and Aiolos are forming homodimers or heterodimers in these T cells, co-immunoprecipitation can be performed. One

limitation for these experiments is the low cell number after sorting for “type b” T cells in the gut.

Concluding Remarks

The tolerogenic biased nature of perinatal immune system is beneficial for infants to establish immune homeostasis against an onslaught of harmless antigens they encounter after birth. The tolerance against harmless foreign antigens such as food and commensal bacteria is established during a critical time window of development. Failure to establish tolerance can cause allergic disease or inflammation in adulthood. However, increased tolerance renders newborns highly susceptible to life-threatening infections, which cause 40% of the 3 million annual worldwide neonatal deaths[260]. Therefore, understanding the mechanisms that contribute to perinatal immune tolerance is important to develop strategies for better vaccines development and establishing immune homeostasis against harmless antigens to prevent atopic diseases.

In this study, we demonstrated that there are multiple mechanisms by which T cells can contribute to immune tolerance in newborns. Firstly, most perinatal T cells express the transcription factor Helios, and Helios decreases effector cytokine production upon T cell activation. Second, perinatal T cells differentiate into a unique subset of Foxp3+Helios+ T cells that carries immunoregulatory functions but are epigenetically and phenotypically distinct from canonical adult Foxp3+ tTregs. These Foxp3+ T cells differentiation requires the presence of CD36hi monocytes. Our data also suggest that Helios+ T cells are limited to perinatal origin as: 1) UCB HSC-derived T cells generated in a humanized mouse model express and maintained Helios, and 2) perinatal origin “type b” gut-associated T cells, important for the maintenance of gut homeostasis, express Helios in the fetal stage and maintained their expression through adulthood (Figure 66).

Altogether, both cell-intrinsic (Helios) and extrinsic (CD36hi monocytes) mechanisms promote the tolerogenic nature of the perinatal immune system.

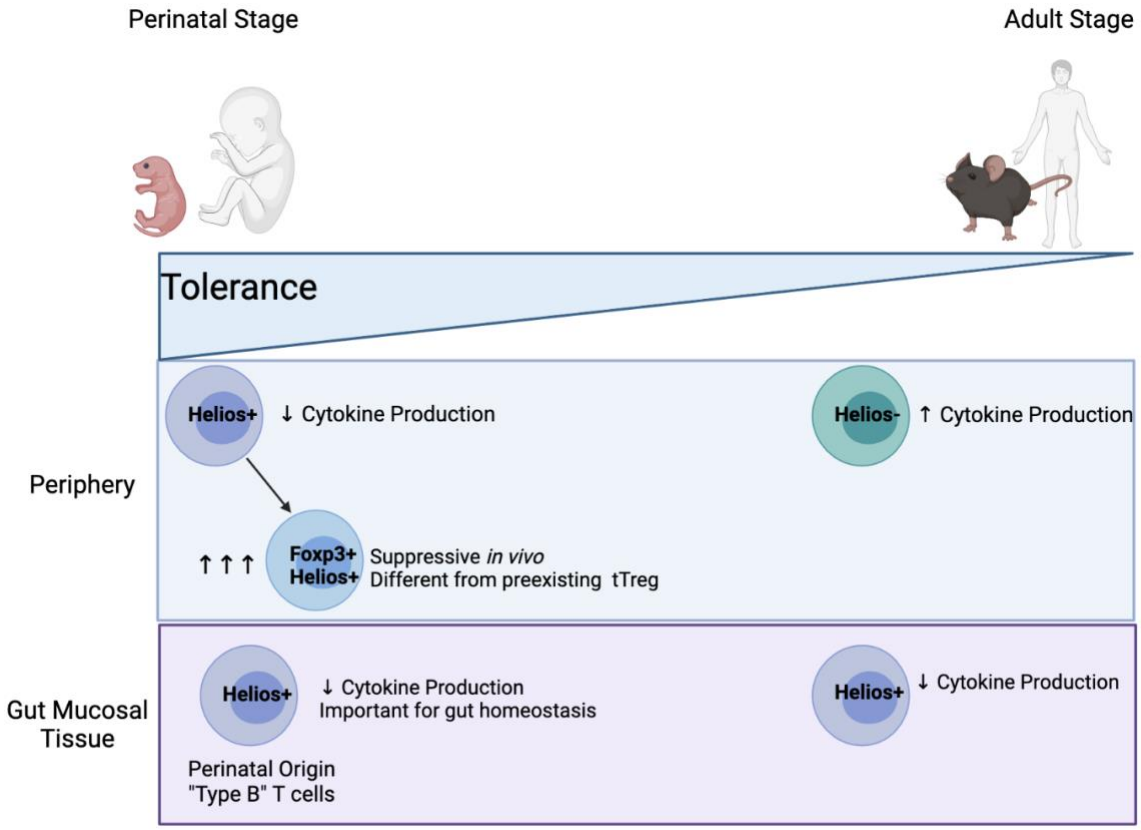

Figure 66: Working Model of Helios+ T Cells in Perinatal Immune Tolerance.

REFERENCE LIST

1. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 2000;404(6774):193-197. doi:10.1038/35004599

2. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. 1997;91(5):661-672. doi:10.1016/S0092-8674(00)80453-5

3. Miller JFAP. The discovery of thymus function and of thymus-derived lymphocytes. *Immunol Rev*. 2002;185:7-14. doi:10.1034/j.1600-065X.2002.18502.x

4. Miller JFAP. Effect of neonatal thymectomy on the immunological responsiveness of the mouse. *Proc R Soc London Ser B Biol Sci*. 1962;156(964):415-428. doi:10.1098/rspb.1962.0048

5. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annu Rev Immunol*. 2003;21(1):139-176. doi:10.1146/annurev.immunol.21.120601.141107

6. Donskoy E, Goldschneider I. Thymocytopoiesis is maintained by blood-borne precursors throughout postnatal life. A study in parabiotic mice. *J Immunol*. 1992;148(6):1604-1612. http://www.ncbi.nlm.nih.gov/pubmed/1347301. Accessed April 13, 2020.

7. Frey JR, Ernst B, Surh CD, Sprent J. Thymus-grafted SCID mice show transient thymopoiesis and limited depletion of V beta 11+ T cells. *J Exp Med*. 1992;175(4):1067-1071. doi:10.1084/jem.175.4.1067

8. Benz C, Bleul CC. A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision. *J Exp Med*. 2005;202(1):21-31. doi:10.1084/jem.20050146

9. Heinzel K, Benz C, Martins VC, Haidl ID, Bleul CC. Bone Marrow-Derived Hemopoietic Precursors Commit to the T Cell Lineage Only after Arrival in the Thymic Microenvironment. *J Immunol*. 2007;178(2):858-868. doi:10.4049/jimmunol.178.2.858

10. Reinherz EL, Meuer SC, Schlossman SF. The delineation of antigen receptors on human T lymphocytes. *Immunol Today*. 1983;4(1):5-8. doi:10.1016/0167-5699(83)90094-4

11. Lind EF, Prockop SE, Porritt HE, Petrie HT. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J Exp Med*. 2001;194(2):127-134. doi:10.1084/jem.194.2.127

12. Kurd N, Robey EA. T-cell selection in the thymus: A spatial and temporal perspective. *Immunol Rev*. 2016;271(1):114-126. doi:10.1111/imr.12398

13. Petrie HT, Scollay R, Shortman K. Commitment to the T cell receptor-alpha beta or -gamma delta lineages can occur just prior to the onset of CD4 and CD8 expression among immature thymocytes. *Eur J Immunol*. 1992;22(8):2185-2188. doi:10.1002/eji.1830220836

14. Capone M, Hockett RD, Zlotnik A. Kinetics of T cell receptor β, γ, and δ rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44+CD25+ Pro-T thymocytes. *Proc Natl Acad Sci U S A*. 1998;95(21):12522-12527. doi:10.1073/pnas.95.21.12522

15. Godfrey DI, Kennedy J, Mombaerts P, Tonegawa S, Zlotnik A. Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3-CD4-CD8- thymocyte differentiation. *J Immunol*. 1994;152(10):4783-4792. http://www.ncbi.nlm.nih.gov/pubmed/7513723.

16. Mombaerts P, Clarke AR, Rudnicki MA, et al. Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature*. 1992;360(6401):225-231. doi:10.1038/360225a0

17. Groettrup M, Ungewiss K, Azogui O, et al. A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor β chain and a 33 kd glycoprotein. *Cell*. 1993;75(2):283-294. doi:10.1016/0092-8674(93)80070-U

18. Von Boehmer H, Bonneville M, Ishida I, et al. Early expression of a T-cell receptor β-chain transgene suppresses rearrangement of the V(γ4) gene segment. *Proc Natl Acad Sci U S A*. 1988;85(24):9729-9732. doi:10.1073/pnas.85.24.9729

19. Shinkai Y, Koyasu S, Nakayama KI, et al. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science (80-)*. 1993;259(5096):822-825. doi:10.1126/science.8430336

20. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell*. 1994;76(1):17-27. doi:10.1016/0092-8674(94)90169-4

21. De Magistris MT, Alexander J, Coggeshall M, et al. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell*. 1992;68(4):625-634. doi:10.1016/0092-8674(92)90139-4

22. Mandal M, Crusio KM, Meng F, et al. Regulation of lymphocyte progenitor survival by the proapoptotic activities of Bim and Bid. *Proc Natl Acad Sci U S A*. 2008;105(52):20840-20845. doi:10.1073/pnas.0807557106

23. Teh HS, Kisielow P, Scott B, et al. Thymic major histocompatibility complex antigens and the αβ T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature*. 1988;335(6187):229-233. doi:10.1038/335229a0

24. Kappler JW, Roehm N, Marrack P. T cell tolerance by clonal elimination in the thymus. *Cell*. 1987;49(2):273-280. doi:10.1016/0092-8674(87)90568-X

25. Ueno T, Saito F, Gray DHD, et al. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J Exp Med*. 2004;200(4):493-505. doi:10.1084/jem.20040643

26. Kwan J, Killeen N. CCR7 Directs the Migration of Thymocytes into the Thymic Medulla. *J Immunol*. 2004;172(7):3999-4007. doi:10.4049/jimmunol.172.7.3999

27. Liston A, Lesage S, Wilson J, Peltonen L, Goodnow CC. Aire regulates negative selection of organ-specific T cells. *Nat Immunol*. 2003;4(4):350-354. doi:10.1038/ni906

28. Derbinski J, Gäbler J, Brors B, et al. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med*. 2005;202(1):33-45. doi:10.1084/jem.20050471

29. Smith KM, Olson DC, Hirose R, Hanahan D. Pancreatic gene expression in rare cells of thymic medulla: Evidence for functional contribution to T cell tolerance. *Int Immunol*. 1997;9(9):1355-1365. doi:10.1093/intimm/9.9.1355

30. Yano M, Kuroda N, Han H, et al. Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. *J Exp Med*. 2008;205(12):2827-2838. doi:10.1084/jem.20080046

31. Gallegos AM, Bevan MJ. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J Exp Med*. 2004;200(8):1039-1049. doi:10.1084/jem.20041457

32. Koble C, Kyewski B. The thymic medulla: A unique microenvironment for intercellular self-antigen transfer. *J Exp Med*. 2009;206(7):1505-1513. doi:10.1084/jem.20082449

33. Cowan JE, Parnell SM, Nakamura K, et al. The thymic medulla is required for Foxp3+ regulatory but not conventional CD4+ thymocyte development. *J Exp Med*. 2013;210(4):675-681. doi:10.1084/jem.20122070

34. Aschenbrenner K, D'Cruz LM, Vollmann EH, et al. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat Immunol*. 2007;8(4):351-358. doi:10.1038/ni1444

35. Bautista JL, Lio C-WJ, Lathrop SK, et al. Intraclonal competition limits the fate determination of regulatory T cells in the thymus. *Nat Immunol*. 2009;10(6):610-617. doi:10.1038/ni.1739

36. Annunziato F, Cosmi L, Liotta F, et al. Phenotype, localization, and mechanism of suppression of CD4+CD25+ human thymocytes. *J Exp Med*. 2002;196(3):379-387. doi:10.1084/jem.20020110

37. Martín-Gayo E, Sierra-Filardi E, Corbí AL, Toribio ML. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood*. 2010;115(26):5366-5375. doi:10.1182/blood-2009-10-248260

38. McCaughtry TM, Wilken MS, Hogquist KA. Thymic emigration revisited. *J Exp Med*. 2007;204(11):2513-2520. doi:10.1084/jem.20070601

39. Boursalian TE, Golob J, Soper DM, Cooper CJ, Fink PJ. Continued maturation of thymic emigrants in the periphery. *Nat Immunol*. 2004;5(4):418-425. doi:10.1038/ni1049

40. Xu X, Zhang S, Li P, Lu J, Xuan Q, Ge Q. Maturation and emigration of single-positive thymocytes. *Clin Dev Immunol*. 2013;2013:282870. doi:10.1155/2013/282870

41. Melichar HJ, Narayan K, Der SO, et al. Regulation of γδ versus αβ T lymphocyte differentiation by the transcription factor SOX13. *Science (80-)*. 2007;315(5809):230-233. doi:10.1126/science.1135344

42. Lauritsen JPH, W. WG, Lee S-Y, et al. Differential induction of Id3 signals lineage divergence, Notchindependent differentiation, and functional maturation of γδ T cells. *Immunity*. 2009;31(4):565-575. doi:10.1016/j.immuni.2009.07.010.Differential

43. Haks MC, Lefebvre JM, Lauritsen JPH, et al. Attenuation of γδTCR signaling efficiently diverts thymocytes to the αβ lineage. *Immunity*. 2005;22(5):595-606. doi:10.1016/j.immuni.2005.04.003

44. Ito K, Bonneville M, Takagaki Y, et al. Different γδ T-cell receptors are expressed on thymocytes at different stages of development. *Proc Natl Acad Sci U S A*. 1989;86(2):631-635. doi:10.1073/pnas.86.2.631

45. Garman RD, Doherty PJ, Raulet DH. Diversity, rearrangement, and expression of murine T cell gamma genes. *Cell*. 1986;45(5):733-742. doi:10.1016/0092-8674(86)90787-7

46. Asarnow DM, Kuziel WA, Bonyhad M, Tigelaar RE, Tucker PW, Allison JP. Limited diversity of γδ antigen receptor genes of thy-1+ dendritic epidermal cells. *Cell*. 1988;55(5):837-847. doi:10.1016/0092-8674(88)90139-0

47. Bonneville M, Janeway CA, Ito K, et al. Intestinal intraepithelial lymphocytes are a distinct set of γδ T cells. *Nature*. 1988;336(6198):479-481. doi:10.1038/336479a0

48. Havran WL, Allison JP. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature*. 1988;335(6189):443-445. doi:10.1038/335443a0

49. Heyborne KD, Cranfill RL, Carding SR, Born WK, O'Brien RL. Characterization of gamma delta T lymphocytes at the maternal-fetal interface. *J Immunol*. 1992;149(9):2872-2878. http://www.ncbi.nlm.nih.gov/pubmed/1401918.

50. Itohara S, Farr AG, Lafaille JJ, et al. Homing of a γδ thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. *Nature*. 1990;343(6260):754-757. doi:10.1038/343754a0

51. Carding SR, Kyes S, Jenkinson EJ, et al. Developmentally regulated fetal thymic and extrathymic T-cell receptor γδ gene expression. *Genes Dev*. 1990;4(8):1304-1315. doi:10.1101/gad.4.8.1304

52. Pereira P, Gerber D, Huang SY, Tonegawa S. Ontogenic development and tissue distribution of vγl-expressing γ/δ T lymphocytes in normal mice. *J Exp Med*. 1995;182(6):1921-1930. doi:10.1084/jem.182.6.1921

53. Takagaki Y, DeCloux A, Bonneville M, Tonegawa S. Diversity of γδ T-cell receptors on murine intestinal intraepithelial lymphocytes. *Nature*. 1989;339(6227):712-714. doi:10.1038/339712a0

54. Ribot JC, Lopes N, Silva-Santos B. γδ T cells in tissue physiology and surveillance. *Nat Rev Immunol*. October 2020:1-12. doi:10.1038/s41577-020-00452-4

55. Meuer SC, Schlossman SF, Reinherz EL. Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector T cells recognize products of different major histocompatibility complex regions. *Proc Natl Acad Sci U S A*. 1982;79(14):4395-4399. doi:10.1073/pnas.79.14.4395

56. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986;136(7):2348-2357. http://www.ncbi.nlm.nih.gov/pubmed/2419430. Accessed November 29, 2018.

57. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science (80-)*. 1993;260(5107):547-549. doi:10.1126/science.8097338

58. Smeltz RB, Chen J, Ehrhardt R, Shevach EM. Role of IFN-gamma in Th1 differentiation: IFN-gamma regulates IL-18R alpha expression by preventing the negative effects of IL-4 and by inducing/maintaining IL-12 receptor beta 2 expression. *J Immunol*. 2002;168(12):6165-6172. doi:10.4049/jimmunol.168.12.6165

59. Afkarian M, Sedy JR, Yang J, et al. T-bet is a STATI-induced regulator for IL-12R expression in naïve CD4+ T cells. *Nat Immunol*. 2002;3(6):549-557. doi:10.1038/ni794

60. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 2000;100(6):655-669. doi:10.1016/S0092-8674(00)80702-3

61. Suzuki Y, Orellana MA, Schreiber RD, Remington JS. Interferon-γ: The major mediator of resistance against Toxoplasma gondii. *Science (80-)*. 1988;240(4851):516-518. doi:10.1126/science.3128869

62. Murray HW, Rubin BY, Carriero SM, Harris AM, Jaffee EA. Human mononuclear phagocyte antiprotozoal mechanisms: oxygen-dependent vs oxygen-independent activity against intracellular Toxoplasma gondii. *J Immunol*. 1985;134(3):1982-1988. http://www.ncbi.nlm.nih.gov/pubmed/2981929. Accessed April 15, 2020.

63. Melzer T, Duffy A, Weiss LM, Halonen SK. The gamma interferon (IFN-gamma)-inducible GTP-binding protein IGTP is necessary for toxoplasma vacuolar disruption and induces parasite egression in IFN-gamma-stimulated astrocytes. *Infect Immun*. 2008;76(11):4883-4894. doi:10.1128/IAI.01288-07

64. Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD, Paul WE. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J Immunol*. 2008;181(5):2943-2951. http://www.ncbi.nlm.nih.gov/pubmed/18713964. Accessed April 15, 2020.

65. Gajewski TF, Fitch FW. Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J Immunol*. 1988;140(12):4245-4252. http://www.ncbi.nlm.nih.gov/pubmed/2967332. Accessed April 21, 2020.

66. Zheng WP, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell*. 1997;89(4):587-596. doi:10.1016/S0092-8674(00)80240-8

67. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*. 1989;170(6):2081-2095. doi:10.1084/jem.170.6.2081

68. Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem*. 1997;272(34):21597-21603. doi:10.1074/jbc.272.34.21597

69. Zhu J, Min B, Hu-Li J, et al. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat Immunol*. 2004;5(11):1157-1165. doi:10.1038/ni1128

70. Gurish MF, Bryce PJ, Tao H, et al. IgE Enhances Parasite Clearance and Regulates Mast Cell Responses in Mice Infected with Trichinella spiralis . *J Immunol*. 2004;172(2):1139-1145. doi:10.4049/jimmunol.172.2.1139

71. Coffman RL, Seymour BWP, Hudak S, Jackson J, Rennick D. Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. *Science (80-)*. 1989;245(4915):308-310. doi:10.1126/science.2787531

72. Urban JF, Noben-Trauth N, Donaldson DD, et al. IL-13, IL-4Rα, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis. *Immunity*. 1998;8(2):255-264. doi:10.1016/S1074-7613(00)80477-X

73. Woodruff PG, Modrek B, Choy DF, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med*. 2009;180(5):388-395. doi:10.1164/rccm.200903-0392OC

74. Dougherty RH, Sidhu SS, Raman K, et al. Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma. *J Allergy Clin Immunol*. 2010;125(5):1046-1053.e8. doi:10.1016/j.jaci.2010.03.003

75. Aggarwal S, Ghilardi N, Xie M-H, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem*. 2003;278(3):1910-1914. doi:10.1074/jbc.M207577200

76. Liang SC, Tan XY, Luxenberg DP, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med*. 2006;203(10):2271-2279. doi:10.1084/jem.20061308

77. Miyamoto M, Prause O, Sjöstrand M, Laan M, Lötvall J, Lindén A. Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways. *J Immunol*. 2003;170(9):4665-4672. doi:10.4049/jimmunol.170.9.4665

78. Rovedatti L, Kudo T, Biancheri P, et al. Differential regulation of interleukin 17 and interferon γ production in inflammatory bowel disease. *Gut*. 2009;58(12):1629-1636. doi:10.1136/gut.2009.182170

79. Sato K, Suematsu A, Okamoto K, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med*. 2006;203(12):2673-2682. doi:10.1084/jem.20061775

80. Nograles KE, Zaba LC, Guttman-Yassky E, et al. Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways. *Br J Dermatol*. 2008;159(5):1092-1102. doi:10.1111/j.1365-2133.2008.08769.x

81. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005;6(11):1123-1132. doi:10.1038/ni1254

82. Ivanov II, McKenzie BS, Zhou L, et al. The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. *Cell*. 2006;126(6):1121-1133. doi:10.1016/j.cell.2006.07.035

83. Stritesky GL, Yeh N, Kaplan MH. IL-23 Promotes Maintenance but Not Commitment to the Th17 Lineage. *J Immunol*. 2008;181(9):5948-5955. doi:10.4049/jimmunol.181.9.5948

84. Wei L, Laurence A, Elias KM, O'Shea JJ. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem*. 2007;282(48):34605-34610. doi:10.1074/jbc.M705100200

85. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology*. 1970;18(5):723-737. http://www.ncbi.nlm.nih.gov/pubmed/4911896. Accessed April 16, 2020.

86. Sakaguchi S, Wing K, Miyara M. Regulatory T cells - A brief history and perspective. *Eur J Immunol*. 2007;37(SUPPL. 1). doi:10.1002/eji.200737593

87. Nishizuka Y, Sakakura T. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science*. 1969;166(3906):753-755. http://www.ncbi.nlm.nih.gov/pubmed/5823314. Accessed November 28, 2018.

88. Penhale WJ, Farmer A, McKenna RP, Irvine WJ. Spontaneous thyroiditis in thymectomized and irradiated Wistar rats. *Clin Exp Immunol*. 1973;15(2):225-236. http://www.ncbi.nlm.nih.gov/pubmed/4543427. Accessed April 16, 2020.

89. Penhale WJ, Stumbles PA, Huxtable CR, Sutherland RJ, Pethick DW. Induction of diabetes in PVG/c strain rats by manipulation of the immune system. *Autoimmunity*. 1990;7(2-3):169-179. doi:10.3109/08916939008993389

90. Fowell D, Mason D. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. *J Exp Med*. 1993;177(3):627-636. doi:10.1084/jem.177.3.627

91. Penhale WJ, Irvine WJ, Inglis JR, Farmer A. Thyroiditis in T cell-depleted rats: suppression of the autoallergic response by reconstitution with normal lymphoid cells. *Clin Exp Immunol*. 1976;25(1):6-16. http://www.ncbi.nlm.nih.gov/pubmed/791546. Accessed April 16, 2020.

92. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995;155(3):1151-1164. http://www.ncbi.nlm.nih.gov/pubmed/7636184. Accessed April 16, 2020.

93. Asano M, Toda M, Sakaguchi N, Sakaguchi S. *Autoimmune Disease as a Consequence of Developmental Abnormality of a T Cell Subpopulation*. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2192701/pdf/je1842387.pdf. Accessed January 12, 2019.

94. Suri-Payer E, Amar AZ, McHugh R, Natarajan K, Margulies DH, Shevach EM. Post-thymectomy autoimmune gastritis: fine specificity and pathogenicity of anti-H/K ATPase-reactive T cells. *Eur J Immunol*. 1999;29(2):669-677. doi:10.1002/(SICI)1521-4141(199902)29:02<669::AID-IMMU669>3.0.CO;2-J

95. Chatila TA, Blaeser F, Ho N, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest*. 2000;106(12):R75-81. doi:10.1172/JCI11679

96. Wildin RS, Ramsdell F, Peake J, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*. 2001;27(1):18-20. doi:10.1038/83707

97. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 2001;27(1):20-21. doi:10.1038/83713

98. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299(5609):1057-1061. doi:10.1126/science.1079490

99. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+T regulatory cells. *J Immunol*. 2017;198(3):993-998. doi:10.1038/ni909

100. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003;4(4):330-336. doi:10.1038/ni904

101. Gavin MA, Torgerson TR, Houston E, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A*. 2006;103(17):6659-6664. doi:10.1073/pnas.0509484103

102. Wang J, Ioan-Facsinay A, van der Voort EIH, Huizinga TWJ, Toes REM. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol*. 2007;37(1):129-138. doi:10.1002/eji.200636435

103. Li MO, Sanjabi S, Flavell RA. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity*. 2006;25(3):455-471. doi:10.1016/j.immuni.2006.07.011

104. Sun C-M, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med*. 2007;204(8):1775-1785. doi:10.1084/jem.20070602

105. Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA. IL-2 Is Essential for TGF-β to Convert Naive CD4 + CD25 − Cells to CD25 + Foxp3 + Regulatory T Cells and for Expansion of These Cells . *J Immunol*. 2007;178(4):2018-2027. doi:10.4049/jimmunol.178.4.2018

106. Selvaraj RK, Geiger TL. A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-β. *J Immunol*. 2007;179(2):1390.3-1390. doi:10.4049/jimmunol.179.2.1390-b

107. Martinez GJ, Zhang Z, Chung Y, et al. Smad3 differentially regulates the induction of regulatory and inflammatory T cell differentiation. *J Biol Chem*. 2009;284(51):35283-35286. doi:10.1074/jbc.C109.078238

108. Laurence A, Tato CM, Davidson TS, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity*. 2007;26(3):371-381. doi:10.1016/j.immuni.2007.02.009

109. Atarashi K, Tanoue T, Shima T, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science*. 2011;331(6015):337-341. doi:10.1126/science.1198469

110. Lathrop SK, Bloom SM, Rao SM, et al. Peripheral education of the immune system by colonic commensal microbiota. *Nature*. 2011;478(7368):250-254. doi:10.1038/nature10434

111. Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell*. 2012;150(1):29-38. doi:10.1016/j.cell.2012.05.031

112. Chen WJ, Jin W, Hardegen N, et al. Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3. *J Exp Med*. 2003;198(12):1875-1886. doi:10.1084/jem.20030152

113. Apostolou I, Von Boehmer H. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med*. 2004;199(10):1401-1408. doi:10.1084/jem.20040249

114. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med*. 1998;188(2):287-296. doi:10.1084/jem.188.2.287

115. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol*. 2000;164(1):183-190. doi:10.4049/jimmunol.164.1.183

116. Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by CD25+CD4+ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med*. 2000;192(2):303-309. doi:10.1084/jem.192.2.303

117. Read S, Malmström V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25+CD4+ regulatory cells that control intestinal inflammation. *J Exp Med*. 2000;192(2):295-302. doi:10.1084/jem.192.2.295

118. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4+CD25+ T cells with regulatory properties from human blood. *J Exp Med*. 2001;193(11):1303-1310. doi:10.1084/jem.193.11.1303

119. Oderup C, Cederbom L, Makowska A, Cilio CM, Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology*. 2006;118(2):240-249. doi:10.1111/j.1365-2567.2006.02362.x

120. Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-β in CD4+CD25+ regulatory T cell function. *Eur J Immunol*. 2004;34(11):2996-3005. doi:10.1002/eji.200425143

121. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4+CD25+ regulatory T cells is mediated by cell surface-bound transforming growth factor β. *J Exp Med*. 2001;194(5):629-644. doi:10.1084/jem.194.5.629

122. Nakamura K, Kitani A, Fuss I, et al. TGF-β1 Plays an Important Role in the Mechanism of CD4 + CD25 + Regulatory T Cell Activity in Both Humans and Mice . *J Immunol*. 2004;172(2):834-842. doi:10.4049/jimmunol.172.2.834

123. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med*. 1999;190(7):995-1004. doi:10.1084/jem.190.7.995

124. Zhang X, Koldzic DN, Izikson L, et al. IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. *Int Immunol*. 2004;16(2):249-256. doi:10.1093/intimm/dxh029

125. Belkaid Y. Regulatory T cells and infection: a dangerous necessity. *Nat Rev Immunol*. 2007;7(11):875-888. doi:10.1038/nri2189

126. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol*. 2009;10(6):595-602. doi:10.1038/ni.1731

127. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *J Immunol*. 2017;198(3):981-985. doi:10.1126/science.1079490

128. Iizuka-Koga M, Nakatsukasa H, Ito M, Akanuma T, Yoshimura A. Induction and maintenance of regulatory T cells by transcription factors and epigenetic modifications. *J Autoimmun*. 2017;83:113-121. doi:10.1016/J.JAUT.2017.07.002

129. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 2010;463(7282):808-812. doi:10.1038/nature08750

130. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, Tone M. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol*. 2008;9(2):194-202. doi:10.1038/ni1549

131. Xu L, Kitani A, Stuelten C, McGrady G, Fuss I, Strober W. Positive and Negative Transcriptional Regulation of the Foxp3 Gene is Mediated by Access and Binding of the Smad3 Protein to Enhancer I. *Immunity*. 2010;33(3):313-325. doi:10.1016/j.immuni.2010.09.001

132. Takimoto T, Wakabayashi Y, Sekiya T, et al. Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. *J Immunol*. 2010;185(2):842-855. doi:10.4049/jimmunol.0904100

133. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-β1 maintains suppressor function and Foxp3 expression in CD4 +CD25+ regulatory T cells. *J Exp Med*. 2005;201(7):1061-1067. doi:10.1084/jem.20042276

134. Josefowicz SZ, Niec RE, Kim HY, et al. Extrathymically generated regulatory T cells control mucosal T H 2 inflammation. *Nature*. 2012;482(7385):395-399. doi:10.1038/nature10772

135. Floess S, Freyer J, Siewert C, et al. Epigenetic Control of the foxp3 Locus in Regulatory T Cells. Marrack P, ed. *PLoS Biol*. 2007;5(2):e38. doi:10.1371/journal.pbio.0050038

136. Mohn F, Weber M, Rebhan M, et al. Lineage-Specific Polycomb Targets and De Novo DNA Methylation Define Restriction and Potential of Neuronal Progenitors. *Mol Cell*. 2008;30(6):755-766. doi:10.1016/j.molcel.2008.05.007

137. Li X, Liang Y, LeBlanc M, Benner C, Zheng Y. Function of a Foxp3 cis-Element in Protecting Regulatory T Cell Identity. *Cell*. 2014;158(4):734-748. doi:10.1016/J.CELL.2014.07.030

138. Polansky JKK, Kretschmer K, Freyer J, et al. DNA methylation controls Foxp3 gene expression. *Eur J Immunol*. 2008;38(6):1654-1663. doi:10.1002/eji.200838105

139. Baron U, Floess S, Wieczorek G, et al. DNA demethylation in the humanFOXP3 locus discriminates regulatory T cells from activated FOXP3+ conventional T cells. *Eur J Immunol*. 2007;37(9):2378-2389. doi:10.1002/eji.200737594

140. Zorn E, Nelson EA, Mohseni M, et al. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood*. 2006;108(5):1571-1579. doi:10.1182/blood-2006-02-004747

141. Ohkura N, Hamaguchi M, Morikawa H, et al. T Cell Receptor Stimulation-Induced Epigenetic Changes and Foxp3 Expression Are Independent and Complementary Events Required for Treg Cell Development. *Immunity*. 2012;37(5):785-799. doi:10.1016/j.immuni.2012.09.010

142. Josefowicz SZ, Wilson CB, Rudensky AY. Cutting Edge: TCR Stimulation Is Sufficient for Induction of Foxp3 Expression in the Absence of DNA Methyltransferase 1. *J Immunol*. 2009;182(11):6648-6652. doi:10.4049/jimmunol.0803320

143. Toker A, Engelbert D, Garg G, et al. Active Demethylation of the Foxp3 Locus Leads to the Generation of Stable Regulatory T Cells within the Thymus . *J Immunol*. 2013;190(7):3180-3188. doi:10.4049/jimmunol.1203473

144. Yang R, Qu C, Zhou Y, et al. Hydrogen Sulfide Promotes Tet1- and Tet2-Mediated Foxp3 Demethylation to Drive Regulatory T Cell Differentiation and Maintain Immune Homeostasis. *Immunity*. 2015;43(2):251-263. doi:10.1016/j.immuni.2015.07.017

145. Nair VS, Oh KI. Down-regulation of Tet2 prevents TSDR demethylation in IL2 deficient regulatory T cells. *Biochem Biophys Res Commun*. 2014;450(1):918-924. doi:10.1016/j.bbrc.2014.06.110

146. Wang L, Liu Y, Han R, et al. Mbd2 Promotes Foxp3 Demethylation and T-Regulatory-Cell Function. *Mol Cell Biol*. 2013;33(20):4106-4115. doi:10.1128/mcb.00144-13

147. Long M, Park SG, Strickland I, Hayden MS, Ghosh S. Nuclear Factor-κB Modulates Regulatory T Cell Development by Directly Regulating Expression of Foxp3 Transcription Factor. *Immunity*. 2009;31(6):921-931. doi:10.1016/j.immuni.2009.09.022

148. Ruan Q, Kameswaran V, Tone Y, et al. Development of Foxp3+ Regulatory T Cells Is Driven by the c-Rel Enhanceosome. *Immunity*. 2009;31(6):932-940. doi:10.1016/j.immuni.2009.10.006

149. Kitagawa Y, Ohkura N, Kidani Y, et al. Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. *Nat Immunol*. 2016;18(2):173-183. doi:10.1038/ni.3646

150. Cai S, Han HJ, Kohwi-Shigematsu T. Tissue-specific nuclear architecture and gene expession regulated by SATB1. *Nat Genet*. 2003;34(1):42-51. doi:10.1038/ng1146

151. Kitagawa Y, Ohkura N, Kidani Y, et al. Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. *Nat Immunol*. 2017;18(2):173-183. doi:10.1038/ni.3646

152. Kondo M, Tanaka Y, Kuwabara T, Naito T, Kohwi-Shigematsu T, Watanabe A. SATB1 Plays a Critical Role in Establishment of Immune Tolerance. *J Immunol*. 2016;196(2):563-572. doi:10.4049/jimmunol.1501429

153. Lee W, Lee GR. Transcriptional regulation and development of regulatory T cells. *Exp Mol Med*. 2018;50(3):e456. doi:10.1038/emm.2017.313

154. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J Exp Med*. 2005;202(5):637-650. doi:10.1084/jem.20050821

155. Gately MK, Wolitzky AG, Quinn PM, Chizzonite R. Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cell Immunol*. 1992;143(1):127-142. doi:10.1016/0008-8749(92)90011-D

156. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Cutting Edge: Type I IFNs Provide a Third Signal to CD8 T Cells to Stimulate Clonal Expansion and Differentiation. *J Immunol*. 2005;174(8):4465-4469. doi:10.4049/jimmunol.174.8.4465

157. Flynn S, Toellner KM, Raykundalia C, Goodall M, Lane P. CD4 T cell cytokine differentiation: The B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. *J Exp Med*. 1998;188(2):297-304. doi:10.1084/jem.188.2.297

158. Brehm MA, Daniels KA, Welsh RM. Rapid Production of TNF-α following TCR Engagement of Naive CD8 T Cells. *J Immunol*. 2005;175(8):5043-5049. doi:10.4049/jimmunol.175.8.5043

159. Lowin B, Hahne M, Mattmann C, Tschopp J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature*. 1994;370(6491):650-652. doi:10.1038/370650a0

160. Henkart PA. Lymphocyte-mediated cytotoxicity: Two pathways and multiple effector molecules. *Immunity*. 1994;1(5):343-346. doi:10.1016/1074-7613(94)90063-9

161. Kägi D, Ledermann B, Bürki K, et al. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature*. 1994;369(6475):31-37. doi:10.1038/369031a0

162. Walsh CM, Matloubian M, Liu CC, et al. Immune function in mice lacking the perforin gene. *Proc Natl Acad Sci U S A*. 1994;91(23):10854-10858. doi:10.1073/pnas.91.23.10854

163. Van Den Broek MF, Kägi D, Ossendorp F, et al. Decreased tumor surveillance in perforin-deficient mice. *J Exp Med*. 1996;184(5):1781-1790. doi:10.1084/jem.184.5.1781

164. Cosmi L, Liotta F, Lazzeri E, et al. Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. *Blood*. 2003;102(12):4107-4114. doi:10.1182/blood-2003-04-1320

165. Brimnes J, Allez M, Dotan I, Shao L, Nakazawa A, Mayer L. Defects in CD8 + Regulatory T Cells in the Lamina Propria of Patients with Inflammatory Bowel Disease . *J Immunol*. 2005;174(9):5814-5822. doi:10.4049/jimmunol.174.9.5814

166. Tennakoon DK, Mehta RS, Ortega SB, Bhoj V, Racke MK, Karandikar NJ. Therapeutic Induction of Regulatory, Cytotoxic CD8 + T Cells in Multiple Sclerosis . *J Immunol*. 2006;176(11):7119-7129. doi:10.4049/jimmunol.176.11.7119

167. Bisikirska B, Colgan J, Luban J, Bluestone JA, Herold KC. TCR stimulation with modified anti-CD3 mAb expands CD8+ T cell population and induces CD8+CD25+ Tregs. *J Clin Invest*. 2005;115(10):2904-2913. doi:10.1172/JCI23961

168. Hu D, Ikizawa K, Lu L, Sanchirico ME, Shinohara ML, Cantor H. Analysis of regulatory CD8 T cells in Qa-1-deficient mice. *Nat Immunol*. 2004;5(5):516-523. doi:10.1038/ni1063

169. Kim HJ, Verbinnen B, Tang X, Lu L, Cantor H. Inhibition of follicular T-helper cells by CD8 + regulatory T cells is essential for self tolerance. *Nature*. 2010;467(7313):328-332. doi:10.1038/nature09370

170. Guy-Grand D, Cerf-Bensussan N, Malissen B, Malassis-Seris M, Briottet C, Vassalli P. Two gut intraepithelial CD8+ lymphocyte populations with different T cell receptors: A role for the gut epithelium in T cell differentiation. *J Exp Med*. 1991;173(2):471-481. doi:10.1084/jem.173.2.471

171. Lai Y-G, Hou M-S, Hsu Y-W, et al. IL-15 Does Not Affect IEL Development in the Thymus but Regulates Homeostasis of Putative Precursors and Mature CD8αα+ IELs in the Intestine. *J Immunol*. 2008;180(6):3757-3765. doi:10.4049/JIMMUNOL.180.6.3757

172. Jabri B, Ebert E. Human CD8+ intraepithelial lymphocytes: a unique model to study the regulation of effector cytotoxic T lymphocytes in tissue. *Immunol Rev*. 2007;215(1):202-214. doi:10.1111/J.1600-065X.2006.00481.X

173. Boll G, Reimann J. Lamina propria T cell subsets in the small and large intestine of euthymic and athymic mice. *Scand J Immunol*. 1995;42(2):191-201. doi:10.1111/J.1365-3083.1995.TB03645.X

174. Van Kerckhove C, Russell GJ, Deusch, Kai. Reich K, Bhan AK, DerSimonian H, Brenner MB. Oligoclonality of human intestinal intraepithelial T cells. *J Exp Med*. 1992;175(1):57-63. doi:10.1084/JEM.175.1.57

175. Regnault A, Cumano A, Vassalli P, Guy-Grand D, Kourilsky P. Oligoclonal repertoire of the CD8 alpha alpha and the CD8 alpha beta TCR-alpha/beta murine intestinal intraepithelial T lymphocytes: evidence for the random emergence of T cells. *J Exp Med*. 1994;180(4):1345-1358. doi:10.1084/JEM.180.4.1345

176. Leishman AJ, Gapin L, Capone M, et al. Precursors of functional MHC class I- or class II-restricted CD8alphaalpha(+) T cells are positively selected in the thymus by agonist self-peptides. *Immunity*. 2002;16(3):355-364. doi:10.1016/S1074-7613(02)00284-4

177. Cruz D, Sydora BC, Hetzel K, Yakoub G, Kronenberg M, Cheroutre H. An opposite pattern of selection of a single T cell antigen receptor in the thymus and among intraepithelial lymphocytes. *J Exp Med*. 1998;188(2):255-265. doi:10.1084/JEM.188.2.255

178. Saurer L, Seibold I, Rihs S, Vallan C, Dumrese T, Meuller C. Virus-induced activation of self-specific TCR alpha beta CD8 alpha alpha intraepithelial lymphocytes does not abolish their self-tolerance in the intestine. *J Immunol*. 2004;172(7):4176-4183. doi:10.4049/JIMMUNOL.172.7.4176

179. Poussier P, Ning T, Banerjee D, Julius M. A Unique Subset of Self-specific Intraintestinal T Cells Maintains Gut Integrity. *J Exp Med*. 2002;195(11):1491. doi:10.1084/JEM.20011793

180. Chien YH, Becker DM, Lindsten T, Okamura M, Cohen DI, Davis MM. A third type of murine T-cell receptor gene. *Nature*. 1984;312(5989):31-35. doi:10.1038/312031a0

181. Brenner MB, McLean J, Dialynas DP, et al. Identification of a putative second T-cell receptor. *Nature*. 1986;322(6075):145-149. doi:10.1038/322145a0

182. Kranz DM, Saito H, Heller M, et al. Limited diversity of the rearranged T-cell γ gene. *Nature*. 1985;313(6005):752-755. doi:10.1038/313752a0

183. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science*. 1998;279(5357):1737-1740. doi:10.1126/SCIENCE.279.5357.1737

184. Flament C, Benmerah A, Bonneville M, Triebel F, Mami-Chouaib F. Human TCR-gamma/delta alloreactive response to HLA-DR molecules. Comparison with response of TCR-alpha/beta. *J Immunol*. 1994;153(7).

185. Kierkels G, Scheper W, Meringa A, et al. Identification of a tumor-specific allo-HLA-restricted γδTCR. *Blood Adv*. 2019;3(19):2870-2882. doi:10.1182/BLOODADVANCES.2019032409

186. Uldrich A, Le nours J, Pellicci D, et al. CD1d-lipid antigen recognition by the γδ TCR. *Nat Immunol*. 2013;14(11):1137-1145. doi:10.1038/NI.2713

187. Tanaka Y, Sano S, Nieves E, et al. Nonpeptide ligands for human gamma delta T cells. *Proc Natl Acad Sci U S A*. 1994;91(17):8175-8179. doi:10.1073/PNAS.91.17.8175

188. Constant P, Davodeau F, Peyrat M, et al. Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. *Science (80-)*. 1994;264(5156):267-270. doi:10.1126/SCIENCE.8146660

189. Koning F, Stingl G, Yokoyama WM, et al. Identification of a T3-associated γδ T cell receptor on thy-1+ dendritic epidermal cell lines. *Science (80-)*. 1987;236(4803):834-837. doi:10.1126/science.2883729

190. Goodman T, Lefrançois L. Expression of the γ-δ T-cell receptor on intestinal CD8+ intraepithelial lymphocytes. *Nature*. 1988;333(6176):855-858. doi:10.1038/333855a0

191. Trejdosiewicz LK, Smart CJ, Oakes DJ, et al. Expression of T-cell receptors TcR1 (γ/δ) and TcR2 (α/β) in the human intestinal mucosa. *Immunology*. 1989;68(1):7-12.

192. Itohara S, Nakanishi N, Kanagawa O, Kubo R, Tonegawa S. Monoclonal antibodies specific to native murine T-cell receptor γδ: Analysis of γδ T cells during thymic ontogeny and in peripheral lymphoid organs. *Proc Natl Acad Sci U S A*. 1989;86(13):5094-5098. doi:10.1073/pnas.86.13.5094

193. McVay LD, Jaswal SS, Kennedy C, Hayday A, Carding SR. The generation of human gammadelta T cell repertoires during fetal development. *J Immunol*. 1998;160(12):5851-5860. http://www.ncbi.nlm.nih.gov/pubmed/9637496.

194. Ismail AS, Severson KM, Vaishnava S, et al. γδ intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. *Proc Natl Acad Sci U S A*. 2011;108(21):8743-8748. doi:10.1073/pnas.1019574108

195. Bandeira A, Mota-Santos T, Itohara S, et al. Localization of γ/δ T cells to the intestinal epithelium is independent of normal microbial colonization. *J Exp Med*. 1990;172(1):239-244. doi:10.1084/jem.172.1.239

196. Komano H, Fujiura Y, Kawaguchi M, et al. Homeostatic regulation of intestinal epithelia by intraepithelial γδ T cells. *Proc Natl Acad Sci U S A*. 1995;92(13):6147-6151. doi:10.1073/pnas.92.13.6147

197. Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R. Protection of the intestinal mucosa by intraepithelial γδ T cells. *Proc Natl Acad Sci U S A*. 2002;99(22):14338-14343. doi:10.1073/pnas.212290499

198. Dalton JE, Cruickshank SM, Egan CE, et al. Intraepithelial γδ+ Lymphocytes Maintain the Integrity of Intestinal Epithelial Tight Junctions in Response to Infection. *Gastroenterology*. 2006;131(3):818-829. doi:10.1053/j.gastro.2006.06.003

199. Inagaki-Ohara K, Chinen T, Matsuzaki G, et al. Mucosal T Cells Bearing TCRγδ Play a Protective Role in Intestinal Inflammation. *J Immunol*. 2004;173(2):1390-1398. doi:10.4049/jimmunol.173.2.1390

200. Kühl AA, Pawlowski NN, Grollich K, Loddenkemper C, Zeitz M, Hoffmann JC. Aggravation of intestinal inflammation by depletion/deficiency of γδ T cells in different types of IBD animal models. *J Leukoc Biol*. 2007;81(1):168-175. doi:10.1189/jlb.1105696

201. Bhagat G, Naiyer AJ, Shah JG, et al. Small intestinal CD8+TCRγδ+NKG2A + intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. *J Clin Invest*. 2008;118(1):281-293. doi:10.1172/JCI30989

202. Cash HL, Whitham C V., Behrendt CL, Hooper L V. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science (80-)*. 2006;313(5790):1126-1130. doi:10.1126/science.1127119

203. Fahrer AM, Konigshofer Y, Kerr EM, et al. Attributes of γδ intraepithelial lymphocytes as suggested by their transcriptional profile. *Proc Natl Acad Sci U S A*. 2001;98(18):10261-10266. doi:10.1073/pnas.171320798

204. McMenamin C, Oliver J, Girn B, et al. Regulation of T-cell sensitization at epithelial surfaces in the respiratory tract: Suppression of IgE responses to inhaled antigens by CD3+ TcRα-/β- lymphocytes (putative γ/δ T cells). *Immunology*. 1991;74(2):234-239. /pmc/articles/PMC1384598/?report=abstract. Accessed December 14, 2020.

205. McMenamin C, Pimm C, McKersey M, Holt PG. Regulation of IgE responses to inhaled antigen in mice by antigen-specific γδ T cells. *Science (80-)*. 1994;265(5180):1869-1871. doi:10.1126/science.7916481

206. Mengel J, Cardillo F, Aroeira LS, Williams O, Russo M, Vaz NM. Anti-γδ T cell antibody blocks the induction and maintenance of oral tolerance to ovalbumin in mice. *Immunol Lett*. 1995;48(2):97-102. doi:10.1016/0165-2478(95)02451-4

207. Ke Y, Pearce K, Lake JP, Ziegler HK, Kapp JA. Gamma delta T lymphocytes regulate the induction and maintenance of oral tolerance. *J Immunol*. 1997;158(8).

208. Kapp JA, Kapp LM, Mckenna KC, Lake JP. γδ T-cell clones from intestinal intraepithelial lymphocytes inhibit development of CTL responses ex vivo. *Immunology*. 2004;111(2):155-164. doi:10.1111/j.0019-2805.2003.01793.x

209. Locke NR, Stankovic S, Funda DP, Harrison LC. TCRγδ Intraepithelial Lymphocytes Are Required for Self-Tolerance. *J Immunol*. 2006;176(11):6553-6559. doi:10.4049/jimmunol.176.11.6553

210. Ferrick DA, Schrenzei MD, Mulvaniat T, Hsieh B, Ferlin WG, Lepper H. Differential production of interferon-γ and interleukin-4 in response to Th1- and Th2-stimulating pathogens by γδ T cells in vivo. *Nature*. 1995;373(6511):255-257. doi:10.1038/373255a0

211. Hsieh B, Schrenzel MD, Mulvania T, Lepper HD, DiMolfetto-Landon L, Ferrick DA. In vivo cytokine production in murine listeriosis: Evidence for immunoregulation by γδ+ T cells. *J Immunol*. 1996;156(1):232-237. https://pubmed-ncbi-nlm-nih-gov.archer.luhs.org/8598467/. Accessed December 14, 2020.

212. Wen L, Barber DF, Pao W, Wong FS, Owen MJ, Hayday A. Primary gamma delta cell clones can be defined phenotypically and functionally as Th1/Th2 cells and illustrate the association of CD4 with Th2 differentiation. *J Immunol*. 1998.

213. Ebert LM, Meuter S, Moser B. Homing and Function of Human Skin γδ T Cells and NK Cells: Relevance for Tumor Surveillance. *J Immunol*. 2006;176(7):4331-4336. doi:10.4049/jimmunol.176.7.4331

214. Kapp JA, Kapp LM, Mckenna KC, Lake JP. γδ T-cell clones from intestinal intraepithelial lymphocytes inhibit development of CTL responses ex vivo. *Immunology*. 2004;111(2):155-164. doi:10.1111/j.0019-2805.2003.01793.x

215. Duhindan N, Farley AJ, Humphreys S, Parker C, Rossiter B, Brooks CG. Patterns of lymphokine secretion amongst mouse γδ T cell clones. *Eur J Immunol*. 1997;27(7):1704-1712. doi:10.1002/eji.1830270717

216. Asigbetse KE, Eigenmann PA, Frossard CP. Intestinal lamina propria TcRγδ+ lymphocytes selectively express IL-10 and IL-17. *J Investig Allergol Clin Immunol*. 2010.

217. Straus DB, Weiss A. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell*. 1992;70(4):585-593. doi:10.1016/0092-8674(92)90428-F

218. Veillette A, Bookman M, Horak E, Bolen J. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell*. 1988;55(2):301-308. doi:10.1016/0092-8674(88)90053-0

219. Wange RL, Malek SN, Desiderio S, Samelson LE. Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor zeta and CD3 epsilon from activated Jurkat T cells. *J Biol Chem*. 1993;268(26):19797-19801. http://www.ncbi.nlm.nih.gov/pubmed/8366117. Accessed April 20, 2020.

220. Chan AC, Iwashima M, Turck CW, Weiss A. ZAP-70: A 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. *Cell*. 1992;71(4):649-662. doi:10.1016/0092-8674(92)90598-7

221. Iwashima M, Irving BA, Van Oers NSC, Chan AC, Weiss A. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *J Immunol*. 2014;193(9):4279-4282. doi:10.1126/science.7509083

222. Neumeister EN, Zhu Y, Richard S, Terhorst C, Chan AC, Shaw AS. Binding of ZAP-70 to phosphorylated T-cell receptor zeta and eta enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. *Mol Cell Biol*. 1995;15(6):3171-3178. doi:10.1128/mcb.15.6.3171

223. Wardenburg JB, Fu C, Jackman JK, et al. Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. *J Biol Chem*. 1996;271(33):19641-19644. doi:10.1074/jbc.271.33.19641

224. Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP, Samelson LE. LAT: The ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell*. 1998;92(1):83-92. doi:10.1016/S0092-8674(00)80901-0

225. Zhang, W. Irvin, BJ. Trible, RP. Abraham, RT. Samelson L. Functional analysis of LAT in TCR-mediated signaling pathways using a LAT-deficient Jurkat cell line. - PubMed - NCBI. *Int Immunol*. 1999;11(6):943-950. doi:10.1093/intimm/11.6.943

226. Yablonski D, Kuhne MR, Kadlecek T, Weiss A. Uncoupling of nonreceptor tyrosine kinases from PLC-γ1 in an SLP-76- deficient T cell. *Science (80-)*. 1998;281(5375):413-416. doi:10.1126/science.281.5375.413

227. Zhang W, Trible RP, Zhu M, Liu SK, McGlade CJ, Samelson LE. Association of Grb2, Gads, and phospholipase C-γ1 with phosphorylated LAT tyrosine residues: Effect of LAT tyrosine mutations on T cell antigen receptor-mediated signaling. *J Biol Chem*. 2000;275(30):23355-23361. doi:10.1074/jbc.M000404200

228. Finco TS, Kadlecek T, Zhang W, Samelson LE, Weiss A. LAT is required for TCR-mediated activation of PLCγ1 and the Ras pathway. *Immunity*. 1998;9(5):617-626. doi:10.1016/S1074-7613(00)80659-7

229. Koretzky GA. The role of Grb2-associated proteins in T-cell activation. *Immunol Today*. 1997;18(8):401-406. doi:10.1016/S0167-5699(97)01088-8

230. Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature*. 1993;363(6424):45-51. doi:10.1038/363045a0

231. Nishibe S, Wahl MI, Hernández-Sotomayor SMT, Tonks NK, Rhee SG, Carpenter G. Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. *Science (80-)*. 1990;250(4985):1253-1256. doi:10.1126/science.1700866

232. Putney JW, Poggioli J, Weiss SJ. Receptor regulation of calcium release and calcium permeability in parotid gland cells. *Philos Trans R Soc Lond B Biol Sci*. 1981;296(1080):37-45. doi:10.1098/rstb.1981.0169

233. Bird GSJ, Rossier MF, Hughes AR, Shears SB, Armstrong DL, Putney JW. Activation of Ca2+ entry into acinar cells by a non-phosphorylatable inositol trisphosphate. *Nature*. 1991;352(6331):162-165. doi:10.1038/352162a0

234. Serafini AT, Lewis RS, Clipstone NA, et al. Isolation of mutant T lymphocytes with defects in capacitative calcium entry. *Immunity*. 1995;3(2):239-250. doi:10.1016/1074-7613(95)90093-4

235. Crabtree GR, Olson EN. NFAT signaling: Choreographing the social lives of cells. *Cell*. 2002;109(2 SUPPL. 1). doi:10.1016/S0092-8674(02)00699-2

236. Ebinu JO, Stang SL, Teixeira C, et al. RasGRP links T-cell receptor signaling to Ras. *Blood*. 2000;95(10):3199-3203. doi:10.1182/blood.v95.10.3199.010k37_3199_3203

237. Sun Z, Arendt CW, Ellmeier W, et al. PKC-θ is required for TCR-induced NF-κB activation in mature but not immature T lymphocytes. *Nature*. 2000;404(6776):402-407. doi:10.1038/35006090

238. Owen RD. Immunogenetic Consequences of Vascular Anastomoses Between Bovine Twins. *Science (80-)*. 1945;102(2651):400-401. doi:10.1126/science.102.2651.400

239. Billingham RE, Lampkin GH, Medawar PB, Williams HL. Tolerance to homografts, twin diagnosis, and the freemartin condition in cattle. *Heredity (Edinb)*. 1952;6(2):201-212. doi:10.1038/hdy.1952.20

240. Anderson D, Billingham RE, Lampkin GH, Medawar PB. The use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. *Heredity (Edinb)*. 1951;5(3):379-397. doi:10.1038/hdy.1951.38

241. Billingham DRE, Brent L, Medawar PPB. "Actively acquired tolerance" of foreign cells. *Transplantation*. 2003;76(10):1409-1412. doi:10.1097/01.TP.0000102675.72061.88

242. Tiegs SL, Russell DM, Nemazee D. Receptor editing in self-reactive bone marrow B cells. *J Exp Med*. 1993;177(4):1009-1020. doi:10.1084/jem.177.4.1009

243. Pike BL, Boyd AW, Nossal GJV. Clonal anergy: the universally anergic B lymphocyte. *Proc Natl Acad Sci U S A*. 1982;79(6 I):2013-2017. doi:10.1073/pnas.79.6.2013

244. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med*. 1987;165(2):302-319. doi:10.1084/jem.165.2.302

245. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: Endogenous activators of dendritic cells. *Nat Med*. 1999;5(11):1249-1255. doi:10.1038/15200

246. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol*. 2007;7(8):610-621. doi:10.1038/nri2132

247. Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, Roncarolo MG. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood*. 2005;105(3):1162-1169. doi:10.1182/blood-2004-03-1211

248. Ochi H, Abraham M, Ishikawa H, et al. Oral CD3-specific antibody suppresses autoimmune encephalomyelitis by inducing CD4+ CD25- LAP+ T cells. *Nat Med*. 2006;12(6):627-635. doi:10.1038/nm1408

249. Oida T, Zhang X, Goto M, et al. CD4+CD25- T cells that express latency-associated peptide on the surface suppress CD4+CD45RBhigh-induced colitis by a TGF-beta-dependent mechanism. *J Immunol*. 2003;170(5):2516-2522. doi:10.4049/jimmunol.170.5.2516

250. Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*. 1997;389(6652):737-742. doi:10.1038/39614

251. Passerini L, Di Nunzio S, Gregori S, et al. Functional type 1 regulatory T cells develop regardless of FOXP3 mutations in patients with IPEX syndrome. *Eur J Immunol*. 2011;41(4):1120-1131. doi:10.1002/eji.201040909

252. Tree TIM, Lawson J, Edwards H, et al. Naturally arising human CD4 T-cells that recognize islet autoantigens and secrete interleukin-10 regulate proinflammatory T-cell responses via linked suppression. *Diabetes*. 2010;59(6):1451-1460. doi:10.2337/db09-0503

253. Gianfrani C, Levings MK, Sartirana C, et al. Gliadin-Specific Type 1 Regulatory T Cells from the Intestinal Mucosa of Treated Celiac Patients Inhibit Pathogenic T Cells. *J Immunol*. 2006;177(6):4178-4186. doi:10.4049/jimmunol.177.6.4178

254. Organization WH. ICD-10 : international statistical classification of diseases and related health problems : tenth revision. 2004:Spanish version, 1st edition published by PAHO as.

255. Scheyer AF, Melis M, Trezza V, Manzoni OJJ. Consequences of Perinatal Cannabis Exposure. *Trends Neurosci*. 2019;42(12):871-884. doi:10.1016/j.tins.2019.08.010

256. Workman AD, Charvet CJ, Clancy B, Darlington R, Finlay B. Modeling transformations of neurodevelopmental sequences across mammalian species. *J Neurosci*. 2013;33(17):7368-7383. doi:10.1523/JNEUROSCI.5746-12.2013

257. Mold JE, Michaëlsson J, Burt TD, et al. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. *Science*. 2008;322(5907):1562-1565. doi:10.1126/science.1164511

258. Chang C-C, Satwani P, Oberfield N, Vlad G, Simpson LL, Cairo MS. Increased induction of allogeneic-specific cord blood CD4+CD25+ regulatory T (Treg) cells: a comparative study of naïve and antigenic-specific cord blood Treg cells. *Exp Hematol*. 2005;33(12):1508-1520. doi:10.1016/j.exphem.2005.09.002

259. Basha S, Surendran N, Pichichero M. Immune responses in neonates. *Expert Rev Clin Immunol*. 2014;10(9):1171-1184. doi:10.1586/1744666X.2014.942288

260. Liu L, Johnson HL, Cousens S, et al. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet (London, England)*. 2012;379(9832):2151-2161. doi:10.1016/S0140-6736(12)60560-1

261. Vekemans J, Ota MOC, Wang ECY, et al. T cell responses to vaccines in infants: Defective IFNγ production after oral polio vaccination. *Clin Exp Immunol*. 2002;127(3):495-498. doi:10.1046/j.1365-2249.2002.01788.x

262. Whittaker E, Goldblatt D, McIntyre P, Levy O. Neonatal immunization: Rationale, current state, and future prospects. *Front Immunol*. 2018;9(APR). doi:10.3389/fimmu.2018.00532

263. Waaijenborg S, Hahné SJM, Mollema L, et al. Waning of maternal antibodies against measles, mumps, rubella, and varicella in communities with contrasting vaccination coverage. *J Infect Dis*. 2013;208(1):10-16. doi:10.1093/infdis/jit143

264. Yu J, Liu X, Li Y, et al. Maternal exposure to farming environment protects offspring against allergic diseases by modulating the neonatal TLR-Tregs-Th axis. *Clin Transl Allergy*. 2018;8(1). doi:10.1186/s13601-018-0220-0

265. Schaub B, Liu J, Höppler S, et al. Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. *J Allergy Clin Immunol*. 2009;123(4):774-782.e5. doi:10.1016/j.jaci.2009.01.056

266. Riedler J, Braun-Fahrländer C, Eder W, et al. Exposure to farming in early life and development of asthma and allergy: A cross-sectional survey. *Lancet*. 2001;358(9288):1129-1133. doi:10.1016/S0140-6736(01)06252-3

267. Eriksson J, Ekerljung L, Lötvall J, et al. Growing up on a farm leads to lifelong protection against allergic rhinitis. *Allergy Eur J Allergy Clin Immunol*. 2010;65(11):1397-1403. doi:10.1111/j.1398-9995.2010.02397.x

268. Lo YMD, Lau TK, Chan LYS, Leung TN, Chang AMZ. Quantitative analysis of the bidirectional fetomaternal transfer of nucleated cells and plasma DNA. *Clin Chem*. 2000;46(9):1301-1309. doi:10.1093/clinchem/46.9.1301

269. Maloney S, Smith A, Furst DE, et al. Microchimerism of maternal origin persists into adult life. *J Clin Invest*. 1999;104(1):41-47. doi:10.1172/JCI6611

270. Claas FHJ, Gijbels Y, Van Der Velden-De Munck J, Van Rood JJ. Induction of B cell unresponsiveness to noninherited maternal HLA antigens during fetal life. *Science (80-)*. 1988;241(4874):1815-1817. doi:10.1126/science.3051377

271. Burlingham WJ, Grailer AP, Heisey DM, et al. The effect of tolerance to noninherited maternal HLA antigens on the survival of renal transplants from sibling donors. *N Engl J Med*. 1998;339(23):1657-1664. doi:10.1056/NEJM199812033392302

272. Wood H, Acharjee A, Pearce H, et al. Breastfeeding promotes early neonatal regulatory T-cell expansion and immune tolerance of non-inherited maternal antigens. *Allergy*. January 2021:all.14736. doi:10.1111/all.14736

273. Scharschmidt TC, Vasquez KS, Truong HA, et al. A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes. *Immunity*. 2015;43(5):1011-1021. doi:10.1016/j.immuni.2015.10.016

274. Vosters O, Lombard C, André F, Sana G, Sokal EM, Smets F. The interferon-alpha and interleukin-10 responses in neonates differ from adults, and their production remains partial throughout the first 18 months of life. *Clin Exp Immunol*. 2010;162(3):494-499. doi:10.1111/j.1365-2249.2010.04267.x

275. Chen L, Cohen AC, Lewis DB. Impaired allogeneic activation and T-helper 1 differentiation of human cord blood naive CD4 T cells. *Biol Blood Marrow Transplant*. 2006;12(2):160-171. doi:10.1016/j.bbmt.2005.10.027

276. Sarzotti M, Robbins DS, Hoffman PM. Induction of protective CTL responses in newborn mice by a murine retrovirus. *Science (80-)*. 1996;271(5256):1726-1728. doi:10.1126/science.271.5256.1726

277. Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: Turning on newborn T cells with dendritic cells. *Science (80-)*. 1996;271(5256):1723-1726. doi:10.1126/science.271.5256.1723

278. Forsthuber T, Yip HC, Lehmann P V. Induction of TH1 and TH2 immunity in neonatal mice. *Science*. 1996;271(5256):1728-1730. http://www.ncbi.nlm.nih.gov/pubmed/8596934. Accessed November 30, 2018.

279. Kay HEM, Doe J, Hockley A. Response of human foetal thymocytes to phytohaemagglutinin (PHA). *Immunology*. 1970;18(3):393.

280. Aase JM, Noren GR, Reddy D V, Geme JW. Mumps-virus infection in pregnant women and the immunologic response of their offspring. *N Engl J Med*. 1972;286(26):1379-1382. doi:10.1056/NEJM197206292862603

281. Marchant A, Goetghebuer T, Ota MO, et al. Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guérin vaccination. *J Immunol*. 1999;163(4):2249-2255. http://www.ncbi.nlm.nih.gov/pubmed/10438968. Accessed February 1, 2021.

282. Vekemans J, Amedei A, Ota MO, et al. Neonatal bacillus Calmette-Guérin vaccination induces adult-like IFN-γ production by CD4+ T lymphocytes. *Eur J Immunol*. 2001;31(5):1531-1535. doi:10.1002/1521-4141(200105)31:5<1531::AID-IMMU1531>3.0.CO;2-1

283. Malhotra I, Ouma J, Wamachi A, et al. In utero exposure to helminth and mycobacterial antigens generates cytokine responses similar to that observed in adults. *J Clin Invest*. 1997;99(7):1759-1766. doi:10.1172/JCI119340

284. Odorizzi PM, Jagannathan P, McIntyre TI, et al. In utero priming of highly functional effector T cell responses to human malaria. *Sci Transl Med*. 2018;10(463). doi:10.1126/scitranslmed.aat6176

285. McLeod R, Mack DG, Boyer K, et al. Phenotypes and functions of lymphocytes in congenital toxoplasmosis. *J Lab Clin Med*. 1990;116(5):623-635. http://www.ncbi.nlm.nih.gov/pubmed/2146348. Accessed April 23, 2020.

286. Jones AC, Miles EA, Warner JO, Colwell BM, Bryant TN, Warner JA. Fetal peripheral blood mononuclear cell proliferative responses to mitogenic and allergenic stimuli during gestation. *Pediatr Allergy Immunol*. 1996;7(3):109-116. doi:10.1111/j.1399-3038.1996.tb00117.x

287. Szépfalusi Z, Pichler J, Elsässera S, et al. Transplacental priming of the human immune system with environmental allergens can occur early in gestation. *J Allergy Clin Immunol*. 2000;106(3):530-536. doi:10.1067/mai.2000.108710

288. Jones CA, Holloway JA, Warner JO. Phenotype of fetal monocytes and B lymphocytes during the third trimester of pregnancy. *J Reprod Immunol*. 2002;56(1-2):45-60. doi:10.1016/S0165-0378(02)00022-0

289. Mellman I. Dendritic cells: master regulators of the immune response. *Cancer Immunol Res*. 2013;1(3):145-149. doi:10.1158/2326-6066.CIR-13-0102

290. Langrish CL, Buddle JC, Thrasher AJ, Goldblatt D. Neonatal dendritic cells are intrinsically biased against Th-1 immune responses. *Clin Exp Immunol*. 2002;128(1):118-123. doi:10.1046/j.1365-2249.2002.01817.x

291. Nguyen M, Leuridan E, Zhang T, et al. Acquisition of adult-like TLR4 and TLR9 responses during the first year of life. *PLoS One*. 2010;5(4). doi:10.1371/journal.pone.0010407

292. De Wit D, Olislagers V, Goriely S, et al. Blood plasmacytoid dendritic cell responses to CpG oligodeoxynucleotides are impaired in human newborns. *Blood*. 2004;103(3):1030-1032. doi:10.1182/blood-2003-04-1216

293. Maldonado-Lopez R, De ST, Michel P, et al. CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *JExpMed*. 1999;189(0022-1007 (Print)):587-592.

294. Maldonado-López R, De Smedt T, Michel P, et al. CD8α+ and CD8α- Subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J Exp Med*. 1999;189(3):587-592. doi:10.1084/jem.189.3.587

295. McGovern N, Shin A, Low G, et al. Human fetal dendritic cells promote prenatal T-cell immune suppression through arginase-2. *Nature*. 2017;546(7660):662-666. doi:10.1038/nature22795

296. Braud VM, Allan DSJ, O'Callaghan CA, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*. 1998;391(6669):795-799. doi:10.1038/35869

297. Wang Y, Xu H, Zheng X, Wei H, Sun R, Tian Z. High expression of NKG2A/CD94 and low expression of granzyme B are associated with reduced cord blood NK cell activity. *Cell Mol Immunol*. 2007;4(5):377-382.

298. Dalle JH, Menezes J, Wagner É, et al. Characterization of cord blood natural killer cells: Implications for transplantation and neonatal infections. *Pediatr Res*. 2005;57(5 I):649-655. doi:10.1203/01.PDR.0000156501.55431.20

299. Le Garff-Tavernier M, Béziat V, Decocq J, et al. Human NK cells display major phenotypic and functional changes over the life span. *Aging Cell*. 2010;9(4):527-535. doi:10.1111/j.1474-9726.2010.00584.x

300. Lau AS, Sigaroudinia M, Yeung MC, Kohl S. Interleukin-12 induces interferon-γ expression and natural killer cytotoxicity in cord blood mononuclear cells. *Pediatr Res*. 1996;39(1):150-155. doi:10.1203/00006450-199601000-00023

301. Wilson RA, Zolnai A, Rudas P, Frenyo L V. T-cell subsets in blood and lymphoid tissues obtained from fetal calves, maturing calve, and adult bovine. *Vet Immunol Immunopathol*. 1996;53(1-2):49-60. doi:10.1016/0165-2427(95)05543-6

302. Ramsburg E, Tigelaar R, Craft J, Hayday A. Age-dependent Requirement for γδ T Cells in the Primary but Not Secondary Protective Immune Response against an Intestinal Parasite. *J Exp Med*. 2003;198(9):1403-1414. doi:10.1084/jem.20030050

303. Waters WR, Harp JA. Cryptosporidium parvum infection in T-cell receptor (TCR)-alpha- and TCR-delta-deficient mice. *Infect Immun*. 1996;64(5):1854-1857. http://www.ncbi.nlm.nih.gov/pubmed/8613403. Accessed September 21, 2019.

304. Parker CM, Groh V, Band H, et al. Evidence for extrathymic changes in the T cell receptor γ/δ repertoire. *J Exp Med*. 1990;171(5):1597-1612. doi:10.1084/jem.171.5.1597

305. Dimova T, Brouwer M, Gosselin F, et al. Effector vγ9vδ2 t cells dominate the human fetal γδ t-cell repertoire. *Proc Natl Acad Sci U S A*. 2015;112(6):E556-E565. doi:10.1073/pnas.1412058112

306. Gibbons DL, Haque SFY, Silberzahn T, et al. Neonates harbour highly active γδ T cells with selective impairments in preterm infants. *Eur J Immunol*. 2009;39(7):1794-1806. doi:10.1002/eji.200939222

307. Adkins B, Hamilton K. Freshly isolated, murine neonatal T cells produce IL-4 in response to anti-CD3 stimulation. *J Immunol*. 1992;149(11):3448-3455. http://www.ncbi.nlm.nih.gov/pubmed/1431117. Accessed July 29, 2019.

308. Adkins B, Ghanei A, Hamilton K. Developmental regulation of IL-4, IL-2, and IFN-gamma production by murine peripheral T lymphocytes. *J Immunol*. 1993;151(12):6617-6626. http://www.ncbi.nlm.nih.gov/pubmed/7903095. Accessed February 9, 2020.

309. Jacks RD, Keller TJ, Nelson A, Nishimura MI, White P, Iwashima M. Cell intrinsic characteristics of human cord blood naïve CD4T cells. *Immunol Lett*. 2018;193:51-57. doi:10.1016/j.imlet.2017.11.011

310. Rainsford E, Reen DJ. Interleukin 10, produced in abundance by human newborn T cells, may be the regulator of increased tolerance associated with cord blood stem cell transplantation. *Br J Haematol*. 2002;116(3):702-709. doi:10.1046/j.0007-1048.2001.03321.x

311. Adkins B. T-cell function in newborn mice and humans. *Immunol Today*. 1999;20(7):330-335. doi:10.1016/S0167-5699(99)01473-5

312. Mccarron MJ, Reen DJ. Neonatal CD8+ T-cell differentiation is dependent on interleukin-12. *Hum Immunol*. 2010;71(12):1172-1179. doi:10.1016/j.humimm.2010.09.004

313. Fernandez MA, Evans IAC, Hassan EH, Carbone FR, Jones CA. Neonatal CD8+ T cells are slow to develop into lytic effectors after HSV infection in vivo. *Eur J Immunol*. 2008;38(1):102-113. doi:10.1002/eji.200636945

314. You D, Ripple M, Balakrishna S, et al. Inchoate CD8+ T cell responses in neonatal mice permit influenza-induced persistent pulmonary dysfunction. *J Immunol*. 2008;181(5):3486-3494. doi:10.4049/jimmunol.181.5.3486

315. Welliver TP, Garofalo RP, Hosakote Y, et al. Severe Human Lower Respiratory Tract Illness Caused by Respiratory Syncytial Virus and Influenza Virus Is Characterized by the Absence of Pulmonary Cytotoxic Lymphocyte Responses. *J Infect Dis*. 2007;195(8):1126-1136. doi:10.1086/512615

316. Galindo-Albarrán AO, López-Portales OH, Gutiérrez-Reyna DY, et al. CD8+ T Cells from Human Neonates Are Biased toward an Innate Immune Response. *Cell Rep*. 2016;17(8):2151-2160. doi:10.1016/j.celrep.2016.10.056

317. Smith NL, Wissink E, Wang J, et al. Rapid proliferation and differentiation impairs the development of memory CD8+ T cells in early life. *J Immunol*. 2014;193(1):177-184. doi:10.4049/jimmunol.1400553

318. Reynaldi A, Smith NL, Schlub TE, Venturi V, Rudd BD, Davenport MP. Modeling the dynamics of neonatal CD8+ T-cell responses. *Immunol Cell Biol*. 2016;94(9):838-848. doi:10.1038/icb.2016.47

319. Xavier-da-Silva MM, Moreira-Filho CA, Suzuki E, Patricio F, Coutinho A, Carneiro-Sampaio M. Fetal-onset IPEX: report of two families and review of literature. *Clin Immunol*. 2015;156(2):131-140. doi:10.1016/j.clim.2014.12.007

320. Takahata Y, Nomura A, Takada H, et al. CD25+CD4+ T cells in human cord blood: An immunoregulatory subset with naive phenotype and specific expression of forkhead box p3 (Foxp3) gene. *Exp Hematol*. 2004;32(7):622-629. doi:10.1016/j.exphem.2004.03.012

321. Darrasse-Jèze G, Marodon G, Salomon BL, Catala M, Klatzmann D. Ontogeny of CD4+CD25+ regulatory/suppressor T cells in human fetuses. *Blood*. 2005;105(12):4715-4721. doi:10.1182/blood-2004-10-4051

322. Byrne JA, Stankovic AK, Cooper MD. A novel subpopulation of primed T cells in the human fetus. *J Immunol*. 1994;152(6):3098-3106. http://www.ncbi.nlm.nih.gov/pubmed/8144905. Accessed April 22, 2020.

323. Mold JE, Venkatasubrahmanyam S, Burt TD, et al. Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans. *Science*. 2010;330(6011):1695-1699. doi:10.1126/science.1196509

324. Bronevetsky Y, Burt TD, McCune JM. Lin28b Regulates Fetal Regulatory T Cell Differentiation through Modulation of TGF-β Signaling. *J Immunol*. 2016;197(11):4344-4350. doi:10.4049/jimmunol.1601070

325. Lee JH, Ulrich B, Cho J, Park J, Kim CH. Progesterone Promotes Differentiation of Human Cord Blood Fetal T Cells into T Regulatory Cells but Suppresses Their Differentiation into Th17 Cells. *J Immunol*. 2011;187(4):1778-1787. doi:10.4049/jimmunol.1003919

326. Arck P, Hansen PJ, Jericevic BM, Piccinni MP, Szekeres-Bartho J. Progesterone during pregnancy: Endocrine-immune cross talk in Mammalian Species and the role of stress. *Am J Reprod Immunol*. 2007. doi:10.1111/j.1600-0897.2007.00512.x

327. Ng MSF, Roth TL, Mendoza VF, Marson A, Burt TD. Helios enhances the preferential differentiation of human fetal CD4 + naïve T cells into regulatory T cells. *Sci Immunol*. 2019;4(41):eaav5947. doi:10.1126/sciimmunol.aav5947

328. Wang G, Miyahara Y, Guo Z, Khattar M, Stepkowski SM, Chen W. "Default" Generation of Neonatal Regulatory T Cells. *J Immunol*. 2010;185(1):71-78. doi:10.4049/jimmunol.0903806

329. Levy O, Coughlin M, Cronstein BN, Roy RM, Desai A, Wessels MR. The adenosine system selectively inhibits TLR-mediated TNF-alpha production in the human newborn. *J Immunol*. 2006;177(3):1956-1966. doi:10.4049/jimmunol.177.3.1956

330. Rincón M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J Exp Med*. 1997;185(3):461-469. doi:10.1084/jem.185.3.461

331. Nupponen I, Turunen R, Nevalainen T, et al. Extracellular release of bactericidal/permeability-increasing protein in newborn infants. *Pediatr Res*. 2002;51(6):670-674. doi:10.1203/00006450-200206000-00002

332. Strunk T, Doherty D, Richmond P, et al. Reduced levels of antimicrobial proteins and peptides in human cord blood plasma. *Arch Dis Child Fetal Neonatal Ed*. 2009;94(3):F230-1. doi:10.1136/adc.2008.143438

333. Adkins B. Peripheral CD4 + Lymphocytes Derived from Fetal versus Adult Thymic Precursors Differ Phenotypically and Functionally. *J Immunol*. 2003. doi:10.4049/jimmunol.171.10.5157

334. Douagi I, Andre I, Ferraz JC, Cumano A. Characterization of T cell precursor activity in the murine fetal thymus: Evidence for an input of T cell precursors between days 12 and 14 of gestation. *Eur J Immunol*. 2000;30(8):2201-2210. doi:10.1002/1521-4141(2000)30:8<2201::AID-IMMU2201>3.0.CO;2-2

335. Owen JJ, Ritter MA. Tissue interaction in the development of thymus lymphocytes. *J Exp Med*. 1969. doi:10.1084/jem.129.2.431

336. Itoi M, Kawamoto H, Katsura Y, Amagai T. Two distinct steps of immigration of hematopoietic progenitors into the early thymus anlage. *Int Immunol*. 2001;13(9):1203-1211. doi:10.1093/intimm/13.9.1203

337. Jotereau F, Heuze F, Salomon-Vie V, Gascan H. Cell kinetics in the fetal mouse thymus: precursor cell input, proliferation, and emigration. *J Immunol*. 1987;138(4).

338. Adkins B. Peripheral CD4+ lymphocytes derived from fetal versus adult thymic precursors differ phenotypically and functionally. *J Immunol*. 2003;171(10):5157-5164. doi:10.4049/jimmunol.171.10.5157

339. Adkins B, Hamilton K. Developmental ages of the thymic epithelium and of the T cell precursors together determine the proportions of peripheral CD4+ cells. *J Immunol*. 1994;153(12):5359-5365. http://www.ncbi.nlm.nih.gov/pubmed/7989742.

340. Cuddihy AR, Ge S, Zhu J, et al. VEGF-mediated cross-talk within the neonatal murine thymus. *Blood*. 2009;113(12):2723-2731. doi:10.1182/blood-2008-06-162040

341. Cowan JE, Malin J, Zhao Y, et al. Myc controls a distinct transcriptional program in fetal thymic epithelial cells that determines thymus growth. *Nat Commun*. 2019;10(1). doi:10.1038/s41467-019-13465-y

342. Signorelli K, Benoist C, Mathis D. Why is clonal deletion of neonatal thymocytes defective? *Eur J Immunol*. 1992;22(10):2487-2493. doi:10.1002/eji.1830221004

343. Yang S, Fujikado N, Kolodin D, Benoist C, Mathis D. Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance. *Science (80-).* 2015;348(6234):589-594. doi:10.1126/science.aaa7017

344. Smith H, Chen IM, Kubo R, Tung KSK. Neonatal thymectomy results in a repertoire enriched in T cells deleted in adult thymus. *Science (80-).* 1989;245(4919):749-752. doi:10.1126/science.2788921

345. Schneider R, Lees RK, Pedrazzini T, Zinkernagel RM, Hengartner H, Robson MacDonald H. Postnatal disappearance of self-reactive (V(β6+)) cells from the thymus of Mlsa mice. Implications for T cell development and autoimmunity. *J Exp Med*. 1989;169(6):2149-2158. doi:10.1084/jem.169.6.2149

346. Dong M, Artusa P, Kelly SA, et al. Alterations in the Thymic Selection Threshold Skew the Self-Reactivity of the TCR Repertoire in Neonates. *J Immunol*. 2017;199(3):965-973. doi:10.4049/jimmunol.1602137

347. Persaud SP, Parker CR, Lo WL, Weber KS, Allen PM. Intrinsic CD4 + T cell sensitivity and response to a pathogen are set and sustained by avidity for thymic and peripheral complexes of self peptide and MHC. *Nat Immunol*. 2014;15(3):266-274. doi:10.1038/ni.2822

348. Mandl JN, Monteiro JP, Vrisekoop N, Germain RN. T Cell-Positive Selection Uses Self-Ligand Binding Strength to Optimize Repertoire Recognition of Foreign Antigens. *Immunity*. 2013;38(2):263-274. doi:10.1016/j.immuni.2012.09.011

349. Deshpande NR, Parrish HL, Kuhns MS. Self-recognition drives the preferential accumulation of promiscuous CD4+ t-cells in aged mice. *Elife*. 2015;4(JULY 2015):1-12. doi:10.7554/eLife.05949

350. Azzam HS, Grinberg A, Lui K, Shen H, Shores EW, Love PE. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J Exp Med*. 1998;188(12):2301-2311. doi:10.1084/jem.188.12.2301

351. Herzenberg LA, Herzenberg LA. Toward a layered immune system. *Cell*. 1989;59(6):953-954. doi:10.1016/0092-8674(89)90748-4

352. Kantor AB, Stall AM, Adams S, Herzenberg LA, Herzenberg LA. Differential development of progenitor activity for three B-cell lineages. *Proc Natl Acad Sci U S A*. 1992;89(8):3320-3324. doi:10.1073/pnas.89.8.3320

353. Havran WL, Allison JP. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature*. 1988;335(6189):443-445. doi:10.1038/335443a0

354. Guidos CJ, Weissman IL, Adkins B. Developmental potential of CD4-8- thymocytes. Peripheral progeny include mature CD4-8- T cells bearing αβ T cell receptor. *J Immunol*. 1989;142(11):3773-3780.

355. Adkins B. Developmental regulation of the intrathymic T cell precursor population. *J Immunol*. 1991;146(5):1387-1393.

356. Wang J, Wissink EM, Watson NB, Smith NL, Grimson A, Rudd BD. Fetal and adult progenitors give rise to unique populations of CD8+ T cells. *Blood*. 2016;128(26):3073-3082. doi:10.1182/blood-2016-06-725366

357. Smith NL, Patel RK, Reynaldi A, et al. Developmental Origin Governs CD8 + T Cell Fate Decisions during Infection. *Cell*. 2018;174(1):117-130.e14. doi:10.1016/j.cell.2018.05.029

358. Ikuta K, Kina T, MacNeil I, et al. A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell*. 1990;62(5):863-874. doi:10.1016/0092-8674(90)90262-D

359. White GP, Watt PM, Holt BJ, Holt PG. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells. *J Immunol*. 2002;168(6):2820-2827. doi:10.4049/jimmunol.168.6.2820

360. Zens KD, Chen JK, Guyer RS, et al. Reduced generation of lung tissue-resident memory T cells during infancy. *J Exp Med*. 2017;214(10):2915-2932. doi:10.1084/jem.20170521

361. Intlekofer AM, Takemoto N, Kao C, et al. Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. *J Exp Med*. 2007;204(9):2015-2021. doi:10.1084/jem.20070841

362. Georgopoulos K, Moore D, Derfler B. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science*. 1992;258(5083):808-812. doi:10.1126/SCIENCE.1439790

363. Molnar A, Georgopoulos K. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. *Mol Cell Biol*. 1994;14(12):8292-8303. doi:10.1128/MCB.14.12.8292-8303.1994

364. Molnár A, Georgopoulos K. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. *Mol Cell Biol*. 1994;14(12):8292-8303. doi:10.1128/mcb.14.12.8292

365. Sun L, Liu A, Georgopoulos K. Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. *EMBO J*. 1996;15(19):5358-5369. doi:10.1002/j.1460-2075.1996.tb00920.x

366. Basta J, Rauchman M. The nucleosome remodeling and deacetylase complex in development and disease. *Transl Res*. 2015;165(1):36-47. doi:10.1016/j.trsl.2014.05.003

367. Perdomo J, Holmes M, Chong B, Crossley M. Eos and pegasus, two members of the Ikaros family of proteins with distinct DNA binding activities. *J Biol Chem*. 2000;275(49):38347-38354. doi:10.1074/JBC.M005457200

368. Kelley CM, Ikeda T, Koipally J, et al. Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors. *Curr Biol*. 1998;8(9):508-S1. doi:10.1016/S0960-9822(98)70202-7

369. Sridharan R, Smale ST. Predominant interaction of both Ikaros and Helios with the NuRD complex in immature thymocytes. *J Biol Chem*. 2007;282(41):30227-30238. doi:10.1074/jbc.M702541200

370. Georgopoulos K, Bigby M, Wang JH, et al. The ikaros gene is required for the development of all lymphoid lineages. *Cell*. 1994;79(1):143-156. doi:10.1016/0092-8674(94)90407-3

371. Morgan B, Sun L, Avitahl N, et al. Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiatisn. *EMBO J*. 1997;16(8):2004-2013. doi:10.1093/emboj/16.8.2004

372. Thornton AM, Korty PE, Tran DQ, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol*. 2010;184(7):3433-3441. doi:10.4049/jimmunol.0904028

373. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios Expression Is a Marker of T Cell Activation and Proliferation. Molina-Paris C, ed. *PLoS One*. 2011;6(8):e24226. doi:10.1371/journal.pone.0024226

374. Serre K, Bénézech C, Desanti G, et al. Helios is associated with CD4 T cells differentiating to T helper 2 and follicular helper T cells in vivo independently of Foxp3 expression. *PLoS One*. 2011;6(6):e20731. doi:10.1371/journal.pone.0020731

375. Gottschalk R a, Corse E, Allison JP. Expression of Helios in Peripherally Induced Foxp3+ Regulatory T Cells. *J Immunol*. 2012;188(3):976-980. doi:10.4049/jimmunol.1102964

376. Kim HJ, Barnitz RA, Kreslavsky T, et al. Stable inhibitory activity of regulatory T cells requires the transcription factor Helios. *Science (80-)*. 2015;350(6258):334-339. doi:10.1126/science.aad0616.Stable

377. Peters C, Oberg HH, Kabelitz D, Wesch D. Phenotype and regulation of immunosuppressive Vδ2-expressing γδ T cells. *Cell Mol Life Sci*. 2014;71(10):1943-1960. doi:10.1007/s00018-013-1467-1

378. Nakase K, Ishimaru F, Fujii K, et al. Overexpression of novel short isoforms of Helios in a patient with T-cell acute lymphoblastic leukemia. *Exp Hematol*. 2002;30(4):313-317. doi:10.1016/S0301-472X(01)00796-2

379. Fujii K, Ishimaru F, Nakase K, et al. Over-expression of short isoforms of Helios in patients with adult T-cell leukaemia/lymphoma. *Br J Haematol*. 2003;120(6):986-989. doi:10.1046/j.1365-2141.2003.04216.x

380. Tabayashi T, Ishimaru F, Takata M, et al. Characterization of the short isoform of Helios overexpressed in patients with T-cell malignancies. *Cancer Sci*. 2007;98(2):182-188. doi:10.1111/j.1349-7006.2006.00372.x

381. Zhang Z, Swindle CS, Bates JT, Ko R, Cotta C V, Klug CA. Expression of a non-DNA-binding isoform of Helios induces T-cell lymphoma in mice. 2007;109:2190-2197. doi:10.1182/blood

382. Getnet D, Grosso JF, Goldberg M V., et al. A role for the transcription factor Helios in human CD4+CD25+ regulatory T cells. *Mol Immunol*. 2010;47(7-8):1595-1600. doi:10.1016/j.molimm.2010.02.001

383. Cai Q, Dierich A, Oulad-Abdelghani M, Chan S, Kastner P. Helios Deficiency Has Minimal Impact on T Cell Development and Function. *J Immunol*. 2009;183(4):2303-2311. doi:10.4049/jimmunol.0901407

384. Takatori H, Kawashima H, Matsuki A, et al. Helios enhances treg cell function in cooperation with FoxP3. *Arthritis Rheumatol*. 2015;67(6):1491-1502. doi:10.1002/art.39091

385. Sebastian M, Lopez-Ocasio M, Metidji A, Rieder SA, Shevach EM, Thornton AM. Helios Controls a Limited Subset of Regulatory T Cell Functions. *J Immunol*. 2016;196(1):144-155. doi:10.4049/jimmunol.1501704

386. Chougnet C, Hildeman D. Helios-controller of Treg stability and function. *Transl Cancer Res*. 2016;5(Suppl 2):S338-S341. doi:10.21037/tcr.2016.07.37

387. Sugita K, Hanakawa S, Honda T, et al. Generation of Helios reporter mice and an evaluation of the suppressive capacity of Helios+ regulatory T cells in vitro. *Exp Dermatol*. 2015;24(7):554-556. doi:10.1111/exd.12711

388. Baine I, Basu S, Ames R, Sellers RS, Macian F. Helios Induces Epigenetic Silencing of Il2 Gene Expression in Regulatory T Cells . *J Immunol*. 2013;190(3):1008-1016. doi:10.4049/jimmunol.1200792

389. Rudra D, Deroos P, Chaudhry A, et al. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol*. 2012;13(10):1010-1019. doi:10.1038/ni.2402

390. Seng A, Krausz KL, Pei D, et al. Coexpression of FOXP3 and a Helios isoform enhances the effectiveness of human engineered regulatory T cells. *Blood Adv*. 2020;4(7):1325-1339. doi:10.1182/bloodadvances.2019000965

391. Hill JA, Feuerer M, Tash K, et al. Foxp3 Transcription-Factor-Dependent and -Independent Regulation of the Regulatory T Cell Transcriptional Signature. *Immunity*. 2007;27(5):786-800. doi:10.1016/j.immuni.2007.09.010

392. Molnár A, Wu P, Largespada DA, et al. The Ikaros gene encodes a family of lymphocyte-restricted zinc finger DNA binding proteins, highly conserved in human and mouse. *J Immunol*. 1996;156(2).

393. Kirstetter P, Thomas M, Dierich A, Kastner P, Chan S. Ikaros is critical for B cell differentiation and function. *Eur J Immunol*. 2002;32(3):720-730. doi:10.1002/1521-4141(200203)32:3<720::aid-immu720>3.0.co;2-p

394. Dumortier A, Kirstetter P, Kastner P, Chan S. Ikaros regulates neutrophil differentiation. *Blood*. 2003;101(6):2219-2226. doi:10.1182/BLOOD-2002-05-1336

395. O'Neill D, Schoetz S, Lopez R, et al. An ikaros-containing chromatin-remodeling complex in adult-type erythroid cells. *Mol Cell Biol*. 2000;20(20):7572-7582. doi:10.1128/MCB.20.20.7572-7582.2000

396. Koipally J, Renold A, Kim J, Georgopoulos K. Repression by Ikaros and Aiolos is mediated through histone deacetylase complexes. *EMBO J*. 1999;18(11):3090-3100. doi:10.1093/EMBOJ/18.11.3090

397. Wang J-H, Nichogiannopoulou A, Wu L, et al. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity*. 1996;5(6):537-549. doi:10.1016/S1074-7613(00)80269-1

398. Quirion MR, Gregory GD, Umetsu SE, Winandy S, Brown MA. Cutting Edge: Ikaros Is a Regulator of Th2 Cell Differentiation. *J Immunol*. 2009;182(2):741-745. doi:10.4049/JIMMUNOL.182.2.741

399. Thomas RM, Chen C, Chunder N, et al. Ikaros Silences T-bet Expression and Interferon-γ Production during T Helper 2 Differentiation. *J Biol Chem*. 2010;285(4):2545. doi:10.1074/JBC.M109.038794

400. Umetsu SE, Winandy S. Ikaros Is a Regulator of Il10 Expression in CD4 + T Cells. *J Immunol*. 2009;183(9):5518-5525. doi:10.4049/jimmunol.0901284

401. Clambey ET, Collins B, Young MH, et al. The Ikaros Transcription Factor Regulates Responsiveness to IL-12 and Expression of IL-2 Receptor Alpha in Mature, Activated CD8 T Cells. *PLoS One*. 2013;8(2):e57435. doi:10.1371/JOURNAL.PONE.0057435

402. O'Brien S, Thomas RM, Wertheim GB, Zhang F, Shen H, Wells AD. Ikaros imposes a barrier to CD8+ T cell differentiation by restricting autocrine IL-2 production. *J Immunol*. 2014;192(11):5118. doi:10.4049/JIMMUNOL.1301992

403. Morgan B, Sun L, Avitahl N, et al. Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation. *EMBO J*. 1997;16(8):2004-2013. doi:10.1093/EMBOJ/16.8.2004

404. Wang JH, Avitahl N, Cariappa A, et al. Aiolos Regulates B Cell Activation and Maturation to Effector State. *Immunity*. 1998;9(4):543-553. doi:10.1016/S1074-7613(00)80637-8

405. Quintana FJ, Jin H, Burns EJ, et al. Aiolos promotes TH17 differentiation by directly silencing Il2 expression. *Nat Immunol*. 2012;13(8):770. doi:10.1038/NI.2363

406. Ridley ML, Fleskens V, Roberts CA, et al. IKZF3/Aiolos Is Associated with but Not Sufficient for the Expression of IL-10 by CD4 + T Cells . *J Immunol*. 2020;204(11):2940-2948. doi:10.4049/jimmunol.1901283

407. Honma Y, Kiyosawa H, Mori T, et al. Eos: a novel member of the Ikaros gene family expressed predominantly in the developing nervous system. *FEBS Lett*. 1999;447(1):76-80. doi:10.1016/S0014-5793(99)00265-3

408. Liu S-Q, Jiang S, Li C, Zhang B, Li Q-J. miR-17-92 cluster targets phosphatase and tensin homology and Ikaros Family Zinc Finger 4 to promote TH17-mediated inflammation. *J Biol Chem*. 2014;289(18):12446-12456. doi:10.1074/JBC.M114.550723

409. Pan F, Yu H, Dang E V, et al. Eos mediates Foxp3-dependent gene silencing in CD4+ regulatory T cells. *Science*. 2009;325(5944):1142-1146. doi:10.1126/SCIENCE.1176077

410. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol*. 2010;10(3):170-181. doi:10.1038/nri2711

411. Ding L, Linsley PS, Huang LY, Germain RN, Shevach EM. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J Immunol*. 1993;151(3):1224-1234. http://www.ncbi.nlm.nih.gov/pubmed/7687627. Accessed March 23, 2020.

412. Tomioka H, Shimizu T, Maw WW, Ogasawara K. Roles of tumour necrosis factor-alpha (TNF-alpha), transforming growth factor-beta (TGF-beta), and IL-10 in the modulation of intercellular adhesion molecule-1 (ICAM-1) expression by macrophages during mycobacterial infection. *Clin Exp Immunol*. 2000;122(3):335-342. doi:10.1046/j.1365-2249.2000.01393.x

413. Waal Malefyt R De, Haanen J, Spits H, et al. Interleukin 10 (il-10) and viral il-10 strongly reduce antigen-specific human t cell proliferation by diminishing the antigen-presenting capacity of monocytes via dowm'egulation of class h major histocompatibility complex expression. *J Exp Med*. 1991;174(4):915-924. doi:10.1084/jem.174.4.915

414. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. INTERLEUKIN -10 AND THE INTERLEUKIN -10 RECEPTOR. *Annu Rev Immunol*. 2001;19(1):683-765. doi:10.1146/annurev.immunol.19.1.683

415. Ding L, Shevach EM. IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. *J Immunol*. 1992;148(10):3133-3139. http://www.ncbi.nlm.nih.gov/pubmed/1578140. Accessed May 12, 2020.

416. Abrams J, Figdor CG, De Waal Malefyt R, Bennett B, De Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. *J Exp Med*. 1991;174(5):1209-1220. doi:10.1084/jem.174.5.1209

417. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol*. 1991;147(11):3815-3822. http://www.ncbi.nlm.nih.gov/pubmed/1940369. Accessed May 12, 2020.

418. Gruber MF, Williams CC, Gerrard TL. Macrophage-colony-stimulating factor expression by anti-CD45 stimulated human monocytes is transcriptionally up-regulated by IL-1 beta and inhibited by IL-4 and IL-10. *J Immunol*. 1994;152(3):1354-1361. http://www.ncbi.nlm.nih.gov/pubmed/8301137. Accessed May 12, 2020.

419. D'andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL-10) Inhibits human lymphocyte interferon γ-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med*. 1993;178(3):1041-1048. doi:10.1084/jem.178.3.1041

420. Fiorentino DF, Zlotnik A, Vieira P, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol*. 1991;146(10):3444-3451. http://www.ncbi.nlm.nih.gov/pubmed/1827484. Accessed March 23, 2020.

421. de Waal Malefyt R, Yssel H, de Vries JE. Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. *J Immunol*. 1993;150(11):4754-4765. http://www.ncbi.nlm.nih.gov/pubmed/7684412. Accessed May 12, 2020.

422. Taga K, Mostowski H, Tosato G. Human interleukin-10 can directly inhibit T-cell growth. *Blood*. 1993;81(11):2964-2971. doi:10.1182/blood.v81.11.2964.bloodjournal81112964

423. Schandené L, Alonso-Vega C, Willems F, et al. B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. *J Immunol*. 1994;152(9):4368-4374. http://www.ncbi.nlm.nih.gov/pubmed/7512591. Accessed May 12, 2020.

424. Zhu L, Shi T, Zhong C, Wang Y, Chang M, Liu X. IL-10 and IL-10 Receptor Mutations in Very Early Onset Inflammatory Bowel Disease. *Gastroenterol Res*. 2017;10(2):65-69. doi:10.14740/gr740w

425. Glocker EO, Kotlarz D, Boztug K, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med*. 2009;361(21):2033-2045. doi:10.1056/NEJMoa0907206

426. Kühn R, Löhler J, Rennick D, Rajewsky K, Müller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993;75(2):263-274. doi:10.1016/0092-8674(93)80068-p

427. Berg DJ, Davidson N, Kühn R, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest*. 1996;98(4):1010-1020. doi:10.1172/JCI118861

428. Appendix 9. Alcohol - 2015-2020 Dietary Guidelines | health.gov. https://health.gov/our-work/food-nutrition/2015-2020-dietary-guidelines/guidelines/appendix-9/. Accessed May 5, 2020.

429. Drinking Levels Defined | National Institute on Alcohol Abuse and Alcoholism (NIAAA). https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/moderate-binge-drinking. Accessed May 5, 2020.

430. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science (80-)*. 1998;282(5396):2085-2088. doi:10.1126/science.282.5396.2085

431. Zarember KA, Godowski PJ. Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their Products, and Cytokines. *J Immunol*. 2002;169(2):1136-1136. doi:10.4049/jimmunol.169.2.1136

432. Brightbill HD, Libraty DH, Krutzik SR, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science (80-)*. 1999;285(5428):732-736. doi:10.1126/science.285.5428.732

433. Jones BW, Means TK, Heldwein KA, et al. Different Toll-like receptor agonists induce distinct macrophage responses. *J Leukoc Biol*. 2001;69(6):1036-1044. doi:10.1189/jlb.1106655

434. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila toll protein signals activation of adaptive immunity. *Nature*. 1997;388(6640):394-397. doi:10.1038/41131

435. Muralidharan S, Ambade A, Fulham MA, Deshpande J, Catalano D, Mandrekar P. Moderate alcohol induces stress proteins HSF1 and hsp70 and inhibits proinflammatory cytokines resulting in endotoxin tolerance. *J Immunol*. 2014;193(4):1975-1987. doi:10.4049/jimmunol.1303468

436. Mandrekar P, Bala S, Catalano D, Kodys K, Szabo G. The Opposite Effects of Acute and Chronic Alcohol on Lipopolysaccharide-Induced Inflammation Are Linked to IRAK-M in Human Monocytes. *J Immunol*. 2009;183(2):1320-1327. doi:10.4049/jimmunol.0803206

437. Pang M, Bala S, Kodys K, Catalano D, Szabo G. Inhibition of TLR8- and TLR4-induced Type I IFN induction by alcohol is different from its effects on inflammatory cytokine production in monocytes. *BMC Immunol*. 2011;12. doi:10.1186/1471-2172-12-55

438. Pruett SB, Zheng Q, Fan R, Matthews K, Schwab C. Ethanol suppresses cytokine responses induced through Toll-like receptors as well as innate resistance to Escherichia coli in a mouse model for binge drinking. *Alcohol*. 2004;33(2):147-155. doi:10.1016/j.alcohol.2004.08.001

439. Lau AH, Abe M, Thomson AW. Ethanol affects the generation, cosignaling molecule expression, and function of plasmacytoid and myeloid dendritic cell subsets in vitro and in vivo. *J Leukoc Biol*. 2006;79(5):941-953. doi:10.1189/jlb.0905517

440. Maraslioglu M, Oppermann E, Blattner C, et al. Chronic ethanol feeding modulates inflammatory mediators, activation of nuclear factor- B, and responsiveness to endotoxin in murine kupffer cells and circulating leukocytes. *Mediators Inflamm*. 2014;2014. doi:10.1155/2014/808695

441. McClain CJ, Cohen DA. Increased tumor necrosis factor production by monocytes in alcoholic hepatitis. *Hepatology*. 1989;9(3):349-351. doi:10.1002/hep.1840090302

442. Boé DM, Richens TR, Horstmann SA, et al. Acute and chronic alcohol exposure impair the phagocytosis of apoptotic cells and enhance the pulmonary inflammatory response. *Alcohol Clin Exp Res*. 2010;34(10):1723-1732. doi:10.1111/j.1530-0277.2010.01259.x

443. Romeo J, Wärnberg J, Díaz LE, González-Gross M, Marcos A. Effects of moderate beer consumption on first-line immunity of healthy adults. *J Physiol Biochem*. 2007;63(2):153-159. doi:10.1007/BF03168226

444. Romeo J, Wärnberg J, Nova E, Díaz LE, González-Gross M, Marcos A. Changes in the immune system after moderate beer consumption. *Ann Nutr Metab*. 2007;51(4):359-366. doi:10.1159/000107679

445. Cohen S, Tyrrell DA, Russell MA, Jarvis MJ, Smith AP. Smoking, alcohol consumption, and susceptibility to the common cold. *Am J Public Health*. 1993;83(9):1277-1283. doi:10.2105/ajph.83.9.1277

446. Mendenhall CL, Theus SA, Roselle GA, Grossman CJ, Rouster SD. Biphasic in vivo immune function after low- versus high-dose alcohol consumption. *Alcohol*. 1997;14(3):255-260. doi:10.1016/S0741-8329(96)00150-4

447. Messaoudi I, Asquith M, Engelmann F, et al. Moderate alcohol consumption enhances vaccine-induced responses in rhesus macaques. *Vaccine*. 2013;32(1):54-61. doi:10.1016/j.vaccine.2013.10.076

448. Takkouche B, Regueira-Méndez C, García-Closas R, Figueiras A, Gestal-Otero JJ, Hernán MA. Intake of wine, beer, and spirits and the risk of clinical common cold. *Am J Epidemiol*. 2002;155(9):853-858. doi:10.1093/aje/155.9.853

449. Ouchi E, Niu K, Kobayashi Y, et al. Frequent alcohol drinking is associated with lower prevalence of self-reported common cold: A retrospective study. *BMC Public Health*. 2012;12(1). doi:10.1186/1471-2458-12-987

450. Mili F, Flanders WD, Boring JR, Annest JL, DeStefano F. The associations of alcohol drinking and drinking cessation to measures of the immune system in middle-aged men. *Alcohol Clin Exp Res*. 1992;16(4):688-694. doi:10.1111/j.1530-0277.1992.tb00662.x

451. Gonzalez-Quintela A, Alende R, Gude F, et al. Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. *Clin Exp Immunol*. 2008;151(1):42-50. doi:10.1111/j.1365-2249.2007.03545.x

452. Mühlbauer E, Karsten U, Rottmann M, Rommelspacher H. Impaired immunoglobulin M production by incubation of hybridoma cells with ethanol. *Alcohol*. 2001;24(3):179-187. doi:10.1016/S0741-8329(01)00152-5

453. Gheorghiu M, Bâra C, Pasarica D, et al. Ethanol-induced dysfunction of hepatocytes and leukocytes in patients without liver failure. *Roum Arch Microbiol Immunol*. 2004;63(1-2):5-33.

454. Percival SS, Sims CA. Wine modifies the effects of alcohol on immune cells of mice. *J Nutr*. 2000;130(5):1091-1094. doi:10.1093/jn/130.5.1091

455. Boyadjieva NI, Dokur M, Advis JP, Meadows GG, Sarkar DK. Beta-endorphin modulation of lymphocyte proliferation: effects of ethanol. *Alcohol Clin Exp Res*. 2002;26(11):1719-17127. doi:10.1097/01.ALC.0000036925.42090.9D

456. Cook RT, Waldschmidt TJ, Ballas ZK, et al. Fine T-cell subsets in alcoholics as determined by the expression of L-selectin, leukocyte common antigen, and beta-integrin. *Alcohol Clin Exp Res*. 1994;18(1):71-80. http://www.ncbi.nlm.nih.gov/pubmed/7515214. Accessed November 29, 2018.

457. Cook RT, Ballas ZK, Waldschmidt TJ, Vandersteen D, LaBrecque DR, Cook BL. Modulation of T-Cell Adhesion Markers, and the CD45R and CD57 Antigens in Human Alcoholics. *Alcohol Clin Exp Res*. 1995;19(3):555-563. doi:10.1111/j.1530-0277.1995.tb01548.x

458. Song K, Coleman RA, Zhu X, et al. Chronic ethanol consumption by mice results in activated splenic T cells. *J Leukoc Biol*. 2002;72(6):1109-1116. doi:10.1189/JLB.72.6.1109

459. Zhang H, Meadows GG. Chronic alcohol consumption in mice increases the proportion of peripheral memory T cells by homeostatic proliferation. *J Leukoc Biol*. 2005;78(5):1070-1080. doi:10.1189/jlb.0605317

460. Cho BK, Rao VP, Ge Q, Eisen HN, Chen J. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *J Exp Med*. 2000;192(4):549-556. doi:10.1084/jem.192.4.549

461. Thakur L, Kojicic M, Thakur SJ, et al. Alcohol consumption and development of acute respiratory distress syndrome: A population-based study. *Int J Environ Res Public Health*. 2009;6(9):2426-2435. doi:10.3390/ijerph6092426

462. O'Brien JM, Lu B, Ali NA, et al. Alcohol dependence is independently associated with sepsis, septic shock, and hospital mortality among adult intensive care unit patients. *Crit Care Med*. 2007;35(2):345-350. doi:10.1097/01.CCM.0000254340.91644.B2

463. Saitz R, Ghali WA, Moskowitz MA. The impact of alcohol-related diagnoses on pneumonia outcomes. *Arch Intern Med*. 1997;157(13):1446-1452. http://www.ncbi.nlm.nih.gov/pubmed/9224223. Accessed May 4, 2020.

464. Hudolin V. Tuberculosis and alcoholism. *Ann N Y Acad Sci*. 1975;252(1):353-364. doi:10.1111/j.1749-6632.1975.tb19179.x

465. Schiff ER, Ozden N. Hepatitis C and alcohol. *Alcohol Res Heal*. 2003;27(3):232-239. doi:10.1097/00004836-200303000-00012

466. Baum MK, Rafie C, Lai S, Sales S, Page JB, Campa A. Alcohol use accelerates HIV disease progression. *AIDS Res Hum Retroviruses*. 2010;26(5):511-518. doi:10.1089/aid.2009.0211

467. Meyerholz DK, Edsen-Moore M, McGill J, Coleman RA, Cook RT, Legge KL. Chronic Alcohol Consumption Increases the Severity of Murine Influenza Virus Infections. *J Immunol*. 2008;181(1):641-648. doi:10.4049/jimmunol.181.1.641

468. Gurung P, Young BM, Coleman RA, et al. Chronic ethanol induces inhibition of antigen-specific CD8+ but not CD4+ immunodominant T cell responses following Listeria monocytogenes inoculation. *J Leukoc Biol*. 2008;85(1):34-43. doi:10.1189/jlb.0208101

469. Cook RT, Garvey MJ, Booth BM, Goeken JA, Stewart B, Noel M. Activated CD-8 cells and HLA DR expression in alcoholics without overt liver disease. *J Clin Immunol*. 1991;11(5):246-253. doi:10.1007/BF00918182

470. Schirren CA, Jung MC, Zachoval R, et al. Analysis of T cell activation pathways in patients with liver cirrhosis, impaired delayed hypersensitivity and other T cell-dependent functions. *Clin Exp Immunol*. 1997;108(1):144-150. doi:10.1046/j.1365-2249.1997.d01-985.x

471. Devière J, Denys C, Schandene L, et al. Decreased proliferative activity associated with activation markers in patients with alcoholic liver cirrhosis. *Clin Exp Immunol*. 1988;72(3):377-382. http://www.ncbi.nlm.nih.gov/pubmed/3262458. Accessed May 11, 2020.

472. Yeh M, Chang MP, Norman DC. Effects of exogenous cytokines on the ethanol-mediated suppression of murine thymocyte proliferation. *Int J Immunopharmacol*. 1996;18(3):219-226. doi:10.1016/0192-0561(96)82091-1

473. Spinozzi F, Agea E, Bassotti G, et al. *Ethanol Specific Impairment of T-Lymphocyte Activation Is Caused by a Transitory Block in Signal-Transduction Pathways*. Vol 105.; 1993. https://www.gastrojournal.org/article/0016-5085(93)90156-7/pdf. Accessed January 22, 2019.

474. Ghare S, Patil M, Hote P, et al. Ethanol inhibits lipid raft-mediated TCR signaling and IL-2 expression: Potential mechanism of alcohol-induced immune suppression. *Alcohol Clin Exp Res*. 2011;35(8):1435-1444. doi:10.1111/j.1530-0277.2011.01479.x

475. Denny CH, Acero CS, Naimi TS, Kim SY. Consumption of Alcohol Beverages and Binge Drinking Among Pregnant Women Aged 18-44 Years - United States, 2015-2017. *MMWR Morb Mortal Wkly Rep*. 2019;68(16):365-368. doi:10.15585/mmwr.mm6816a1

476. Koren G, Navioz Y. Historical perspective: The original description of fetal alcohol spectrum disorder in France, 1967. *Ther Drug Monit*. 2003;25(2):131. doi:10.1097/00007691-200304000-00001

477. Lemoine P, Harousseau H, Borteyru JP, Menuet JC. Children of alcoholic parents observed anomalies: Discussion of 127 cases. *Ther Drug Monit*. 2003;25(2):132-136. doi:10.1097/00007691-200304000-00002

478. Jones KL, Smith DW, Hanson JW. The fetal alcohol syndrome: clinical delineation. *Ann N Y Acad Sci*. 1976;273(1):130-137. doi:10.1111/j.1749-6632.1976.tb52873.x

479. Jones KL, Smith DW. Recognition of the fetal alcohol syndrome in early infancy. *Lancet*. 1973;302(7836):999-1001. doi:10.1016/S0140-6736(73)91092-1

480. Jones KL, Smith DW, Ulleland CN, Streissguth AP. Pattern of malformations in offspring of alcoholic mothers. *Lancet*. 1973;301(7815/7815):1267–1271. https://www.ncbi.nlm.nih.gov/pubmed/4126070. Accessed May 1, 2020.

481. *Fetal Alcohol Syndrome - Alaska, Arizona, Colorado, and New York, 1995-1997*. Vol 51.; 2002. doi:10.1001/jama.288.1.38-jwr0703-2-1

482. May PA, Gossage JP, Kalberg WO, et al. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Dev Disabil Res Rev*. 2009;15(3):176-192. doi:10.1002/ddrr.68

483. May PA, Baete A, Russo J, et al. Prevalence and characteristics of fetal alcohol spectrum disorders. *Pediatrics*. 2014;134(5):855-866. doi:10.1542/peds.2013-3319

484. May PA, Chambers CD, Kalberg WO, et al. Prevalence of fetal alcohol spectrum disorders in 4 US communities. *JAMA - J Am Med Assoc*. 2018;319(5):474-482. doi:10.1001/jama.2017.21896

485. Wada K, Konishi K, Tamura T, Shiraki M, Iwasa S, Nagata C. Alcohol Intake During Pregnancy and Offspring's Atopic Eczema Risk. *Alcohol Clin Exp Res*. 2016;40(5):1037-1043. doi:10.1111/acer.13048

486. Zhang X, Lan N, Bach P, et al. Prenatal alcohol exposure alters the course and severity of adjuvant-induced arthritis in female rats. *Brain Behav Immun*. 2012;26(3):439-450. doi:10.1016/j.bbi.2011.11.005

487. Chen L, Nyomba BLG. Effects of prenatal alcohol exposure on glucose tolerance in the rat offspring. *Metabolism*. 2003;52(4):454-462. doi:10.1053/meta.2003.50073

488. Gauthier TW, Drews-Botsch C, Falek A, Coles C, Brown LAS. Maternal alcohol abuse and neonatal infection. *Alcohol Clin Exp Res*. 2005;29(6):1035-1043. http://www.ncbi.nlm.nih.gov/pubmed/15976530. Accessed November 11, 2018.

489. Church MW, Gerkin KP. Hearing disorders in children with fetal alcohol syndrome: Findings from case reports. *Pediatrics*. 1988;82(2):147-154. doi:10.1016/0196-0709(89)90097-5

490. Johnson S, Knight R, Marmer DJ, Steele RW. Immune Deficiency in Fetal Alcohol Syndrome. *Pediatr Res*. 1981;15:908-911. https://www.nature.com/articles/pr19812093.pdf?origin=ppub. Accessed November 11, 2018.

491. Seeler, RA., Israel, JN., Royal, JE., Kaye, CI., Rao, S., Abulaban M. Ganglioneuroblastoma and fetal hydantoin-alcohol syndromes. *Pediatrics*. 1979;63(4). https://www-ncbi-nlm-nih-gov.archer.luhs.org/pubmed/?term=Ganglioneuroblastoma+and+fetal+hydantoin-alcohol+syndromes. Accessed May 1, 2020.

492. Kinney H, Faix R, Brazy J. The fetal alcohol syndrome and neuroblastoma. *Pediatrics*. 1980;66(1):130-132.

493. Khan A, Bader JL, Hoy GR, Sinks LF. Hepatoblastoma in child with fetal alcohol syndrome. *Lancet*. 1979;313(8131):1403-1404. doi:10.1016/S0140-6736(79)92035-X

494. Hornstein L, Crowe C, Gruppo R. Adrenal carcinoma in child with history of fetal alcohol syndrome. *Lancet*. 1977;310(8051):1292-1293. doi:10.1016/S0140-6736(77)92706-4

495. Ewald SJ, Walden SM. Flow cytometric and histological analysis of mouse thymus in fetal alcohol syndrome. *J Leukoc Biol*. 1988;44(5):434-440. doi:10.1002/jlb.44.5.434

496. Ewald SJ, Frost WW. Effect of prenatal exposure to ethanol on development of the thymus. *Thymus*. 1987;9(4). http://www.ncbi.nlm.nih.gov/pubmed/3499687. Accessed November 11, 2018.

497. Clausing P, Ali SF, Taylor LD, Newport GD, Rybak S, Paule MG. Central and peripheral neurochemical alterations and immune effects of prenatal ethanol exposure in rats. *Int J Dev Neurosci*. 1996;14(4):461-469. doi:10.1016/0736-5748(96)00001-9

498. Redei E, Clark WR, McGivern RF. Alcohol exposure in utero results in diminished T-cell function and alterations in brain corticotropin-releasing factor and ACTH content. *Alcohol Clin Exp Res*. 1989;13(3):439-443. http://www.ncbi.nlm.nih.gov/pubmed/2546466. Accessed July 10, 2018.

499. Gottesfeld Z, Ullrich SE. Prenatal alcohol exposure selectively suppresses cell-mediated but not humoral immune responsiveness. *Int J Immunopharmacol*. 1995;17(3):247-254. doi:10.1016/0192-0561(94)00099-a

500. Gottesfeld Z, Christie R, Felten DL, LeGrue SJ. Prenatal ethanol exposure alters immune capacity and noradrenergic synaptic transmission in lymphoid organs of the adult mouse. *Neuroscience*. 1990;35(1):185-194. doi:10.1016/0306-4522(90)90133-O

501. Jerrells TR, Weinberg J. Influence of ethanol consumption on immune competence of adult animals exposed to ethanol in utero. *Alcohol Clin Exp Res*. 1998;22(2):391-400. http://www.ncbi.nlm.nih.gov/pubmed/9581645. Accessed November 29, 2018.

502. Norman DC, Chang MP, Castle SC, Van Zuylen JE, Taylor AN. Diminished proliferative response of con A-blast cells to interleukin 2 in adult rats exposed to ethanol in utero. *Alcohol Clin Exp Res*. 1989;13(1):69-72. http://www.ncbi.nlm.nih.gov/pubmed/2646980. Accessed November 29, 2018.

503. Oleson DR, Magee RM, Donahoe RM, Falek A, Coles CD. Immunity and prenatal alcohol exposure. A pilot study in human adolescents. *Adv Exp Med Biol*. 1998;437:255-264. http://www.ncbi.nlm.nih.gov/pubmed/9666278. Accessed April 14, 2019.

504. McGill J, Meyerholz DK, Edsen-Moore M, et al. Fetal exposure to ethanol has long-term effects on the severity of influenza virus infections. *J Immunol*. 2009;182(12):7803-7808. doi:10.4049/jimmunol.0803881

505. Ahluwalia B, Wesley B, Adeyiga O, Smith DM, Da-Silva A, Rajguru S. Alcohol modulates cytokine secretion and synthesis in human fetus: an in vivo and in vitro study. *Alcohol*. 2000;21(3):207-213. doi:10.1016/S0741-8329(00)00076-8

506. Terasaki LS, Schwarz JM. Effects of Moderate Prenatal Alcohol Exposure during Early Gestation in Rats on Inflammation across the Maternal-Fetal-Immune Interface and Later-Life Immune Function in the Offspring. *J Neuroimmune Pharmacol*. 2016;11(4):680-692. doi:10.1007/s11481-016-9691-8

507. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. *Proceedings Biol Sci*. 2015;282(1821):20143085. doi:10.1098/rspb.2014.3085

508. Wilson CB, Westall J, Johnston L, Lewis DB, Dower SK, Alpert AR. Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. *J Clin Invest*. 1986;77(3):860-867. doi:10.1172/JCI112383

509. De Wit D, Tonon S, Olislagers V, et al. Impaired responses to toll-like receptor 4 and toll-like receptor 3 ligands in human cord blood. *J Autoimmun*. 2003;21(3):277-281. http://www.ncbi.nlm.nih.gov/pubmed/14599853. Accessed September 16, 2019.

510. Crespo M, Martinez DG, Cerissi A, et al. Neonatal T-cell maturation and homing receptor responses to Toll-like receptor ligands differ from those of adult naive T cells: relationship to prematurity. *Pediatr Res*. 2012;71(2):136-143. doi:10.1038/pr.2011.26

511. Burt TD. Fetal regulatory T cells and peripheral immune tolerance in utero: implications for development and disease. *Am J Reprod Immunol*. 2013;69(4):346-358. doi:10.1111/aji.12083

512. Lee JG, Jaeger KE, Seki Y, et al. Human CD36hi monocytes induce Foxp3+ CD25+ T cells with regulatory functions from CD4 and CD8 subsets. *Immunology*. 2021. doi:10.1111/imm.13316

513. Yamamoto M, Seki Y, Iwai K, et al. Ontogeny and localization of the cells produce IL-2 in healthy animals. *Cytokine*. 2013;61(3):831-841. doi:10.1016/j.cyto.2012.11.026

514. PrabhuDas M, Adkins B, Gans H, et al. Challenges in infant immunity: implications for responses to infection and vaccines. *Nat Immunol*. 2011;12(3):189-194. doi:10.1038/ni0311-189

515. Kollmann TR, Kampmann B, Mazmanian SK, Marchant A, Levy O. Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny. *Immunity*. 2017;46(3):350-363. doi:10.1016/j.immuni.2017.03.009

516. Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev*. 2011;241(1):260-268. doi:10.1111/j.1600-065X.2011.01018.x

517. Beck R, Lam-Po-Tang PRL. Comparison of cord blood and adult blood lymphocyte normal ranges: a possible explanation for decreased severity of graft versus host disease after cord blood transplantation. *Immunol Cell Biol*. 1994;72(5):440-444. doi:10.1038/icb.1994.65

518. D'Arena G, Musto P, Cascavilla N, et al. Flow cytometric characterization of human umbilical cord blood lymphocytes: Immunophenotypic features. *Haematologica*. 1998;83(3):197-203.

519. De Vries E, De Bruin-Versteeg S, Comans-Bitter MM, et al. Longitudinal survey of lymphocyte subpopulations in the first year of life. *Pediatr Res*. 2000;47(4):528-537. doi:10.1203/00006450-200004000-00019

520. Kmieciak M, Gowda M, Graham L, et al. Human T cells express CD25 and Foxp3 upon activation and exhibit effector/memory phenotypes without any regulatory/suppressor function. *J Transl Med*. 2009;7. doi:10.1186/1479-5876-7-89

521. Wing K, Larsson P, Sandström K, Lundin SB, Suri-Payer E, Rudin A. CD4+CD25+ FOXP3+ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. *Immunology*. 2005;115(4):516-525. doi:10.1111/j.1365-2567.2005.02186.x

522. R.Walker M, Kasprowicz DJ, Gersuk VH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25– T cells. *J Clin Invest*. 2003;112(9):1437-1443. doi:10.1172/jci19441

523. Hahn SA, Bellinghausen I, Trinschek B, Becker C. Translating Treg Therapy in Humanized Mice. *Front Immunol*. 2015;6:623. doi:10.3389/fimmu.2015.00623

524. Schroeder MA, DiPersio JF. Mouse models of graft-versus-host disease: advances and limitations. *Dis Model Mech*. 2011;4(3):318-333. doi:10.1242/dmm.006668

525. Qian X, Wang K, Wang X, Zheng SG, Lu L. Generation of human regulatory T cells de novo with suppressive function prevent xenogeneic graft versus host disease. *Int Immunopharmacol*. 2011;11(5):630-637. doi:10.1016/j.intimp.2010.11.036

526. Parmar S, Liu X, Tung SS, et al. Third-party umbilical cord blood-derived regulatory T cells prevent xenogenic graft-versus-host disease. *Cytotherapy*. 2014;16(1):90-100. doi:10.1016/j.jcyt.2013.07.009

527. Saito Y, Ellegast JM, Rafiei A, et al. Peripheral blood CD34+ cells efficiently engraft human cytokine knock-in mice. *Blood*. 2016;128(14):1829-1833. doi:10.1182/blood-2015-10-676452

528. Billerbeck E, Barry WT, Mu K, Dorner M, Rice CM, Ploss A. Development of human CD4+FoxP3+ regulatory T cells in human stem cell factor-, granulocyte-macrophage colony-stimulating factor-, and interleukin-3-expressing NOD-SCID IL2R null humanized mice. *Blood*. 2011;117(11):3076-3086. doi:10.1182/blood-2010-08-301507

529. Cooke KR, Kobzik L, Martin TR, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. *Blood*. 1996;88(8):3230-3239. http://www.ncbi.nlm.nih.gov/pubmed/8963063. Accessed November 3, 2018.

530. Hannon M, Lechanteur C, Lucas S, et al. Infusion of clinical-grade enriched regulatory T cells delays experimental xenogeneic graft-versus-host disease. *Transfusion*. 2013;54(2):n/a-n/a. doi:10.1111/trf.12279

531. Ehx G, Somja J, Warnatz H-J, et al. Xenogeneic Graft-Versus-Host Disease in Humanized NSG and NSG-HLA-A2/HHD Mice. *Front Immunol*. 2018;9:1943. doi:10.3389/fimmu.2018.01943

532. Hempel L, Körholz D, Nußbaum P, Bönig H, Burdach S, Zintl F. High interleukin-10 serum levels are associated with fatal outcome in patients after bone marrow transplantation. *Bone Marrow Transplant*. 1997;20(5):365-368. doi:10.1038/sj.bmt.1700902

533. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006;203(7):1693-1700. doi:10.1084/jem.20060468

534. Frommer M, McDonald LE, Millar DS, et al. A genomic sequencing protocol that yields a positive display of 5- methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A*. 1992;89(5):1827-1831. doi:10.1073/pnas.89.5.1827

535. Robinson JT, Thorvaldsdóttir H, Winckler W, et al. Integrative genomics viewer. *Nat Biotechnol*. 2011;29(1):24-26. doi:10.1038/nbt.1754

536. Yang XO, Nurieva R, Martinez GJ, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*. 2008;29(1):44-56. doi:10.1016/j.immuni.2008.05.007

537. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood*. 2006;108(1):253-261. doi:10.1182/blood-2005-11-4567

538. Gao Y, Tang J, Chen W, et al. Inflammation negatively regulates FOXP3 and regulatory T-cell function via DBC1. *Proc Natl Acad Sci U S A*. 2015;112(25):E3246-E3254. doi:10.1073/pnas.1421463112

539. Zhang X, Sliwowska JH, Weinberg J. Prenatal alcohol exposure and fetal programming: effects on neuroendocrine and immune function. *Exp Biol Med (Maywood)*. 2005;230(6):376-388. http://www.ncbi.nlm.nih.gov/pubmed/15956767. Accessed October 26, 2018.

540. Gauthier TW. Prenatal Alcohol Exposure and the Developing Immune System. *Alcohol Res*. 2015;37(2):279-285. http://www.ncbi.nlm.nih.gov/pubmed/26695750. Accessed October 23, 2018.

541. Leonard WJ, Depper JM, Crabtree GR, et al. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. *Nature*. 1984;311(5987):626-631. doi:10.1038/311626a0

542. Sharon M, Klausner RD, Cullen BR, Chizzonite R, Leonard WJ. Novel interleukin-2 receptor subunit detected by cross-linking under high-affinity conditions. *Science (80-)*. 1986;234(4778):859-863. doi:10.1126/science.3095922

543. Takeshita T, Asao H, Ohtani K, et al. Cloning of the γ chain of the human IL-2 receptor. *Science (80-)*. 1992;257(5068):379-382. doi:10.1126/science.1631559

544. Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW. Interleukin-2 receptor α chain regulates the size and content of the peripheral lymphoid compartment. *Immunity*. 1995;3(4):521-530. doi:10.1016/1074-7613(95)90180-9

545. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005;6(11):1142-1151. doi:10.1038/ni1263

546. Adkins B, Du RQ. Newborn mice develop balanced Th1/Th2 primary effector responses in vivo but are biased to Th2 secondary responses. *J Immunol*. 1998;160(9):4217-4224. http://www.ncbi.nlm.nih.gov/pubmed/9574522. Accessed November 30, 2018.

547. Thai NL, Fu F, Qian S, et al. Cytokine mRNA profiles in mouse orthotopic liver transplantation. Graft rejection is associated with augmented TH1 function. *Transplantation*. 1995;59(2):274-281.

548. Bugeon L, Cuturi MC, Hallet MM, Paineau J, Chabannes D, Soulillou JP. Peripheral tolerance of an allograft in adult rats--characterization by low interleukin-2 and interferon-gamma mRNA levels and by strong accumulation of major histocompatibility complex transcripts in the graft. *Transplantation*. 1992;54(2):219-225. doi:10.1097/00007890-199208000-00006

549. Fisher A, Ceredig R. Gamma delta T cells expressing CD8 or CD4low appear early in murine foetal thymus development. *Int Immunol*. 1991;3(12):1323-1328. doi:10.1093/INTIMM/3.12.1323

550. Hayday A, Theodoridis E, Ramsburg E, Shires J. Intraepithelial lymphocytes: Exploring the Third Way in immunology. *Nat Immunol*. 2001;2(11):997-1003. doi:10.1038/ni1101-997

551. van Wijk F, Cheroutre H. Intestinal T cells: Facing the mucosal immune dilemma with synergy and diversity. *Semin Immunol*. 2009;21(3):130-138. doi:10.1016/j.smim.2009.03.003

552. Latthe M, Terry L, Macdonald TT. High frequency of CD8αα homodimer-bearing T cells in human fetal intestine. *Eur J Immunol*. 1994;24(7):1703-1705. doi:10.1002/eji.1830240737

553. Howie D, Spencer J, DeLord D, et al. Extrathymic T cell differentiation in the human intestine early in life. *J Immunol*. 1998;161(11):5862-5872.

554. Lin T, Matsuzaki G, Kenai H, Nomoto K. Progenies of fetal thymocytes are the major source of CD4−CD8+ αα intestinal intraepithelial lymphocytes early in ontogeny. *Eur J Immunol*. 1994;24(8):1785-1791. doi:10.1002/eji.1830240810

555. Manzano M, Abadía-Molina AC, García-Olivares E, Gil A, Rueda R. Absolute counts and distribution of lymphocyte subsets in small intestine of BALB/c mice change during weaning. *J Nutr*. 2002;132(9):2757-2762. doi:10.1093/jn/132.9.2757

556. VanKaer L, Algood HMS, Singh K, et al. CD8αα+ innate-type lymphocytes in the intestinal epithelium mediate mucosal immunity. *Immunity*. 2014;41(3):451-464. doi:10.1016/j.immuni.2014.08.010

557. Kadivar M, Petersson J, Svensson L, Marsal J. CD8αβ + γδ T Cells: A Novel T Cell Subset with a Potential Role in Inflammatory Bowel Disease . *J Immunol*. 2016;197(12):4584-4592. doi:10.4049/jimmunol.1601146

558. Ruscher R, Lee ST, Salgado OC, Breed ER, Osum SH, Hogquist KA. Intestinal CD8αα IELs derived from two distinct thymic precursors have staggered ontogeny. *J Exp Med*. 2020;217(8). doi:10.1084/JEM.20192336

559. Helgeland L, Brandtzaeg P, Rolstad B, Vaage JT. Sequential development of intraepithelial gamma delta and alpha beta T lymphocytes expressing CD8 alpha beta in neonatal rat intestine: requirement for the thymus. *Immunology*. 1997;92(4):447-456. doi:10.1046/J.1365-2567.1997.00379.X

560. de la Rosa M, Rutz S, Dorninger H, Scheffold A. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur J Immunol*. 2004;34(9):2480-2488. doi:10.1002/EJI.200425274

561. Wunderlich M, Chou F-S, Sexton C, et al. Improved multilineage human hematopoietic reconstitution and function in NSGS mice. Stoddart CA, ed. *PLoS One*. 2018;13(12):e0209034. doi:10.1371/journal.pone.0209034

562. Powell TJ, Streilein JW. Neonatal tolerance induction by class II alloantigens activates IL-4-secreting, tolerogen-responsive T cells. *J Immunol*. 1990;144(3):854-859. http://www.ncbi.nlm.nih.gov/pubmed/2136901. Accessed November 9, 2019.

563. Malhotra I, Mungai P, Muchiri E, et al. Distinct Th1- and Th2-type prenatal cytokine responses to Plasmodium falciparum erythrocyte invasion ligands. *Infect Immun*. 2005;73(6):3462-3470. doi:10.1128/IAI.73.6.3462-3470.2005

564. Kitz A, Dominguez-Villar M. Molecular mechanisms underlying Th1-like Treg generation and function. *Cell Mol Life Sci*. 2017;74(22):4059-4075. doi:10.1007/s00018-017-2569-y

565. Sefik E, Geva-Zatorsky N, Oh S, et al. Individual intestinal symbionts induce a distinct population of RORγ+ regulatory T cells. *Science (80-)*. 2015;349(6251):993-997. doi:10.1126/science.aaa9420

566. Chaudhry A, Rudra D, Treuting P, et al. CD4+ regulatory T cells control TH17 responses in a stat3-dependent manner. *Science (80-)*. 2009;326(5955):986-991. doi:10.1126/science.1172702

567. Tan TG, Mathis D, Benoist C. Singular role for T-BET+CXCR3+ regulatory T cells in protection from autoimmune diabetes. *Proc Natl Acad Sci U S A*. 2016;113(49):14103-14108. doi:10.1073/pnas.1616710113

568. Duhen T, Duhen R, Lanzavecchia A, Sallusto F, Campbell DJ. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. *Blood*. 2012;119(19):4430-4440. doi:10.1182/blood-2011-11-392324

569. Lee JG, Jaeger KE, Seki Y, et al. CD36 hi monocytes play immunoregulatory roles in human umbilical cord blood. doi:10.1101/461459

570. Blazar BR, Taylor PA, Smith S, Vallera DA. Interleukin-10 Administration Decreases Survival in Murine Recipients of Major Histocompatibility Complex Disparate Donor Bone Marrow Grafts. *Blood*. 1995;85(3):842-851. doi:10.1182/BLOOD.V85.3.842.BLOODJOURNAL853842

571. Abraham S, Choi J gi, Ye C, Manjunath N, Shankar P. IL-10 exacerbates xenogeneic GVHD by inducing massive human T cell expansion. *Clin Immunol*. 2015;156(1):58-64. doi:10.1016/j.clim.2014.11.004

572. Paczesny S, Hanauer D, Sun Y, Reddy P. New perspectives on the biology of acute GVHD. *Bone Marrow Transplant*. 2010;45(1):1-11. doi:10.1038/bmt.2009.328

573. Chen BJ, Cui X, Sempowski GD, Liu C, Chao NJ. Transfer of allogeneic CD62L-memory T cells without graft-versus-host disease. *Blood*. 2003;103(4):1534-1541. doi:10.1182/blood-2003-08-2987

574. Ferrara JLM, Krenger W. Graft-versus-host disease: The influence of type 1 and type 2 T cell cytokines. *Transfus Med Rev*. 1998;12(1):1-17. doi:10.1016/S0887-7963(98)80085-0

575. Rowbottom AW, Lepper MW, Garland RJ, et al. Interleukin-10-induced CD8 cell proliferation. *Immunology*. 1999;98(1):80-89. doi:10.1046/j.1365-2567.1999.00828.x

576. Stelmaszczyk-Emmel A, Zawadzka-Krajewska A, Szypowska A, Kulus M, Demkow U. Frequency and activation of CD4+CD25high FoxP3+ regulatory T cells in peripheral blood from children with atopic allergy. *Int Arch Allergy Immunol*. 2013;162(1):16-24. doi:10.1159/000350769

577. Linneberg A, Petersen J, Grønbæk M, Benn CS. Alcohol during pregnancy and atopic dermatitis in the offspring. *Clin Exp Allergy*. 2004;34(11):1678-1683. doi:10.1111/j.1365-2222.2004.02101.x

578. Chauhan SK, Saban DR, Lee HK, Dana R. Levels of Foxp3 in Regulatory T Cells Reflect Their Functional Status in Transplantation. *J Immunol*. 2009;182(1):148-153. doi:10.4049/jimmunol.182.1.148

579. Mayer CT, Floess S, Baru AM, Lahl K, Huehn J, Sparwasser T. CD8+Foxp3+ T cells share developmental and phenotypic features with classical CD4+Foxp3+ regulatory T cells but lack potent suppressive activity. *Eur J Immunol*. 2011;41(3):716-725. doi:10.1002/eji.201040913

580. Choi WS, Kim YM, Combs C, Frohman MA, Beaven MA. Phospholipases D1 and D2 Regulate Different Phases of Exocytosis in Mast Cells. *J Immunol*. 2002;168(11):5682-5689. doi:10.4049/jimmunol.168.11.5682

581. Frohman MA, Morris AJ. Phospholipase D structure and regulation. In: *Chemistry and Physics of Lipids*. Vol 98. ; 1999:127-140. doi:10.1016/S0009-3084(99)00025-0

582. Reid PA, Gardner SD, Williams DM, Harnett MM. The antigen receptors on mature and immature T lymphocytes are coupled to phosphatidylcholine-specific phospholipase D activation. *Immunology*. 1997;90(2):250-256. doi:10.1046/j.1365-2567.1997.00150.x

583. Stewart SJ, Cunningham GR, Strupp JA, et al. Activation of phospholipase D: A signaling system set in motion by perturbation of the T lymphocyte antigen receptor/CD3 complex. *Mol Biol Cell*. 1991;2(10):841-850. doi:10.1091/mbc.2.10.841

584. Stewart SJ, Cunningham GR, House FS. Activation of phospholipase D following perturbation of the human T lymphocyte antigen receptor/CD3 complex is dependent upon protein kinase C. *Cell Signal*. 1993;5(3):315-323. doi:10.1016/0898-6568(93)90022-E

585. Mollinedo F, Gajate C, Flores I. Involvement of phospholipase D in the activation of transcription factor AP-1 in human T lymphoid Jurkat cells. *J Immunol*. 1994;153(6):2457-2469. http://www.ncbi.nlm.nih.gov/pubmed/8077660. Accessed April 10, 2020.

586. Cano E, Angeles Muñoz-Fernández M, Fresno M. Regulation of interleukin-2 responses by phosphatidic acid. *Eur J Immunol*. 1992;22(7):1883-1889. doi:10.1002/eji.1830220731

587. Zhu M, Foreman DP, O'Brien SA, Jin Y, Zhang W, Brien SAO. Phospholipase D in TCR-Mediated Signaling and T Cell Activation. *J Immunol*. 2018;200(6):2165-2173. doi:10.4049/jimmunol.1701291

588. Ravichandran KS, Burakoff SJ. Evidence for differential intracellular signaling via CD4 and CD8 molecules. *J Exp Med*. 1994;179(2):727-732. doi:10.1084/JEM.179.2.727

589. Chien Y -h., Bonneville M. Gamma delta T cell receptors. *Cell Mol Life Sci C 2006 6318*. 2006;63(18):2089-2094. doi:10.1007/S00018-006-6020-Z

590. RA B, KS T, SL T, LJ W. Imaging Tolerance Induction in the Classic Medawar Neonatal Mouse Model: Active Roles of Multiple F1-Donor Cell Types. *Am J Transplant*. 2015;15(9):2346-2363. doi:10.1111/AJT.13278

591. Ross EM, Bourges D, Hogan T V., Gleeson PA, van Driel IR. Helios defines T cells being driven to tolerance in the periphery and thymus. *Eur J Immunol*. 2014;44(7):2048-2058. doi:10.1002/eji.201343999

592. Visekruna A, Hartmann S, Sillke YR, et al. Intestinal development and homeostasis require activation and apoptosis of diet-reactive T cells. *J Clin Invest*. 2019;129(5):1972-1983. doi:10.1172/JCI98929

593. Chen N, Field EH. Enhanced type 2 and diminished type 1 cytokines in neonatal tolerance. *Transplantation*. 1995;59(7):933-941. doi:10.1097/00007890-199504150-00002

594. Adkins B. Peripheral CD4 + Lymphocytes Derived from Fetal versus Adult Thymic Precursors Differ Phenotypically and Functionally . *J Immunol*. 2003;171(10):5157-5164. doi:10.4049/jimmunol.171.10.5157

595. Park SM, Cho H, Thornton AM, et al. IKZF2 Drives Leukemia Stem Cell Self-Renewal and Inhibits Myeloid Differentiation. *Cell Stem Cell*. 2019;24(1):153-165.e7. doi:10.1016/j.stem.2018.10.016

596. Fan X, Rudensky AY. Hallmarks of Tissue-Resident Lymphocytes. *Cell*. 2016;164:1198-1211. doi:10.1016/j.cell.2016.02.048

597. Azzam HS, Grinberg A, Lui K, Shen H, Shores EW, Love PE. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J Exp Med*. 1998;188(12):2301-2311. doi:10.1084/jem.188.12.2301

598. Daley SR, Hu DY, Goodnow CC. Helios marks strongly autoreactive CD4+ T cells in two major waves of thymic deletion distinguished by induction of PD-1 or NF-κB. *J Exp Med*. 2013;210(2):269-285. doi:10.1084/jem.20121458

599. Thornton AM, Lu J, Korty PE, et al. Helios + and Helios − Treg subpopulations are phenotypically and functionally distinct and express dissimilar TCR repertoires. *Eur J Immunol*. 2019;49(3):398-412. doi:10.1002/eji.201847935

600. Rocha B, Vassalli P, Guy-Grand D. The Vβ repertoire of mouse gut homodimeric α CD8+ intraepithelial T cell receptor α/β+ lymphocytes reveals a major extrathymic pathway of T cell differentiation. *J Exp Med*. 1991;173(2):483-486. doi:10.1084/jem.173.2.483

601. Rocha B, Von Boehmer H, Guy-Grand D. Selection of intraepithelial lymphocytes with CD8 α/α co-receptors by self-antigen in the murine gut. *Proc Natl Acad Sci U S A*. 1992;89(12):5336-5340. doi:10.1073/pnas.89.12.5336

602. Lin T, Yoshida H, Matsuzaki G, et al. Autospecific γδ thymocytes that escape negative selection find sanctuary in the intestine. *J Clin Invest*. 1999;104(9):1297-1305. doi:10.1172/JCI7437

603. Madakamutil LT, Christen U, Lena CJ, et al. CD8αα-Mediated Survival and Differentiation of CD8 Memory T Cell Precursors. *Science (80-)*. 2004;304(5670):590-593. doi:10.1126/science.1092316

604. Das G, Augustine MM, Das J, Bottomly K, Ray P, Ray A. An important regulatory role for CD4+CD8αα T cells in the intestinal epithelial layer in the prevention of inflammatory bowel disease. *Proc Natl Acad Sci U S A*. 2003;100(9):5324-5329. doi:10.1073/pnas.0831037100

605. Shires J, Theodoridis E, Hayday AC. Biological insights into TCRγδ+ and TCRαβ+ intraepithelial lymphocytes provided by serial analysis of gene expression (SAGE). *Immunity*. 2001;15(3):419-434. doi:10.1016/S1074-7613(01)00192-3

606. Williams CJ, Naito T, Gómez-Del Arco P, et al. The chromatin remodeler Mi-2β is required for CD4 expression and T cell development. *Immunity*. 2004;20(6):719-733. doi:10.1016/j.immuni.2004.05.005

607. Yoshida T, Hazan I, Zhang J, et al. The role of the chromatin remodeler Mi-2β in hematopoietic stem cell self-renewal and multilineage differentiation. *Genes Dev*. 2008;22(9):1174-1189. doi:10.1101/gad.1642808

608. Olias P, Etheridge RD, Zhang Y, Holtzman MJ, Sibley LD. Toxoplasma Effector Recruits the Mi-2/NuRD Complex to Repress STAT1 Transcription and Block IFN-γ-Dependent Gene Expression. *Cell Host Microbe*. 2016;20(1):72-82. doi:10.1016/j.chom.2016.06.006

609. Lu X, Kovalev GI, Chang H, et al. Inactivation of NuRD component Mta2 causes abnormal T cell activation and lupus-like autoimmune disease in mice. *J Biol Chem*. 2008;283(20):13825-13833. doi:10.1074/jbc.M801275200

610. Olin A, Henckel E, Chen Y, et al. Stereotypic Immune System Development in Newborn Children. *Cell*. 2018;174(5):1277-1292.e14. doi:10.1016/j.cell.2018.06.045

611. Bunis DG, Bronevetsky Y, Krow-Lucal E, et al. Single-Cell Mapping of Progressive Fetal-to-Adult Transition in Human Naive T Cells. *Cell Rep*. 2021;34(1):108573. doi:10.1016/j.celrep.2020.108573

612. Robinson DP, Klein SL. Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis. *Horm Behav*. 2012;62(3):263. doi:10.1016/J.YHBEH.2012.02.023

613. Polanczyk MJ, Carson BD, Subramanian S, et al. Cutting Edge: Estrogen Drives Expansion of the CD4 + CD25 + Regulatory T Cell Compartment. *J Immunol*. 2004;173(4):2227-2230. doi:10.4049/jimmunol.173.4.2227

614. Tai P, Wang J, Jin H, et al. Induction of regulatory T cells by physiological level estrogen. *J Cell Physiol*. 2008;214(2):456-464. doi:10.1002/jcp.21221

615. Conly PW, Morrison T, Sandberg DH, Cleveland WW. Concentrations of progesterone in the plasma of mothers and infants at time of birth. *Pediatr Res*. 1970;4(1):76-81. doi:10.1203/00006450-197001000-00009

616. Nagata C, Iwasa S, Shiraki M, Shimizu H. Estrogen and α-Fetoprotein Levels in Maternal and Umbilical Cord Blood Samples in Relation to Birth Weight. *Cancer Epidemiol Prev Biomarkers*. 2006;15(8):1469-1472. doi:10.1158/1055-9965.EPI-06-0158

617. Oguro H, McDonald JG, Zhao Z, Umetani M, Shaul PW, Morrison SJ. 27-Hydroxycholesterol induces hematopoietic stem cell mobilization and extramedullary hematopoiesis during pregnancy. *J Clin Invest*. 2017;127(9):3392-3401. doi:10.1172/JCI94027

618. Kang N, Tang L, Li X, et al. Identification and characterization of Foxp3+ γδ T cells in mouse and human. *Immunol Lett*. 2009;125(2):105-113. doi:10.1016/j.imlet.2009.06.005

619. Casetti R, Agrati C, Wallace M, et al. Cutting Edge: TGF-β1 and IL-15 Induce FOXP3 + γδ Regulatory T Cells in the Presence of Antigen Stimulation. *J Immunol*. 2009;183(6):3574-3577. doi:10.4049/jimmunol.0901334

620. Kouakanou L, Peters C, Sun Q, et al. Vitamin C supports conversion of human γδ T cells into FOXP3-expressing regulatory cells by epigenetic regulation. *Sci Rep*. 2020;10(1):1-13. doi:10.1038/s41598-020-63572-w

621. Umetsu SE, Winandy S. Ikaros Is a Regulator of Il10 Expression in CD4 + T Cells . *J Immunol*. 2009;183(9):5518-5525. doi:10.4049/jimmunol.0901284

622. Huber SA, Graveline D, Newell MK, Born WK, O'Brien RL. Vγ1 + T Cells Suppress and Vγ4 + T Cells Promote Susceptibility to Coxsackievirus B3-Induced Myocarditis in Mice. *J Immunol*. 2000;165(8):4174-4181. doi:10.4049/jimmunol.165.8.4174

623. Hahn Y-S, Taube C, Jin N, et al. Different Potentials of γδ T Cell Subsets in Regulating Airway Responsiveness: Vγ1 + Cells, but Not Vγ4 + Cells, Promote Airway Hyperreactivity, Th2 Cytokines, and Airway Inflammation. *J Immunol*. 2004;172(5):2894-2902. doi:10.4049/jimmunol.172.5.2894

VITA

Yi Wei Lim was born in Penang, Malaysia, on February 22, 1991, to Seong Tat Lim and Cho Hiok Iau. She attended Marquette University, Wisconsin where she earned a Bachelor of Science, *Magna cum laude,* in Biomedical Science, with a minor in Psychology, in May 2013. While at Marquette University, she earned her first research experience in the laboratory of Dr. John Mantsch. In Dr. Mantsch's lab, Yi Wei studied the role of β2-adrenergic receptors in stress-induced cocaine reinstatement. After graduation, Yi Wei joined Dr. Christopher Olsen's laboratory at Medical College of Wisconsin where she worked as a research technologist for two years. In Dr. Olsen's laboratory, Yi Wei investigated the effects of mild traumatic brain injury on voluntary alcohol intake.

Yi Wei matriculated into the Loyola University Chicago Interdisciplinary Program in Biomedical Sciences in 2015 and began her graduate education in the Biochemistry and Molecular Biology program under the mentorship of Dr. Makio Iwashima. Yi Wei's dissertation work focuses on understanding the mechanisms in which perinatal T cell contributes to immune tolerance. Her work was supported by the T32 training grant awarded to Dr. Mashkoor Choudhry by NIH NIAAA. After completion of her graduate studies, Yi Wei will continue her research endeavors as a postdoctoral research fellow at NIH NCATS in Dr. Marc Ferrer's laboratory.