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Phenotypic Analysis of Murine Gut Associated Lymphocytes

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LIST OF ABBREVIATIONS

AIRE	Autoimmune regulator
APC	Antigen presenting cells
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNS	Conserved non-coding
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
ED17.5	Embryonic day 17.5
Foxp3	Forkhead box P3
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocytes
IFN	Interferon
IKZF	Ikaros zinc finger
IL	Interleukin
iTreg	*In vitro* induced Tregs
LPL	Lamina propria lymphocytes
MHC	Major histocompatibility complex
N-terminal	Amino terminal
pTreg	Periphery Treg

TCR	T cell receptor
TGF-β	Transforming growth factor β
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
tTreg	Thymic Tregs
UCB	Umbilical cord blood

CHAPTER ONE

REVIEW OF LITERATURE

T Cell Development

T cells, along with other mature immune cells, develop from hematopoietic stem cells (HSCs) that reside in the bone marrow. Weissman et al. were the first group to identify these bone marrow-derived progenitor cells, the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). CMP is the precursor for the development of all myeloid lineages, which include megakaryocyte-erythrocyte progenitors, or granulocyte-macrophage progenitors[1]. On the other hand, CLPs give rise to all lymphoid cells, including T cells, B cells, and natural killer (NK) cells[2]. Both the common myeloid and the common lymphoid progenitors go on to further differentiate into specific subsets of cells, forming the mature immune system.

The development of T cells specifically takes place within the thymus[3,4], where double negative ($CD4^-$ $CD8^-$) precursor cells receive signals from stromal cells to differentiate[5]. Rearrangement of the T cell receptor (TCR) occurs in an orderly fashion early in the differentiation pathway, leading to a diverse repertoire of loci expressing either TCR$\gamma\delta$ or TCR$\alpha\beta$ with distinct variable regions[6]. The ability for T cells to continue to generate within the thymus depends on the influx of stem cells from the bone marrow via blood, as stem cells in the thymus have limited self-renewing ability[7,8].

It was determined that the earliest progenitor cell can give rise to T, B, NK, myeloid/granulocytic, and dendritic cells, and is dependent on the microenvironment in which

they are found. These cells are called thymic multipotent precursors (TMPs) and mark the branching point between T and B cell lineage[9,10]. Upon entering the thymus TMPs, which have high levels of Notch receptors on their surface, must receive signals from Notch1 in order to commit to the T cell lineage. At this point, the TMPs lose B cell potential and instead differentiate into developing T cells[10,11].

γδ T Cell Development

Initially it was unclear how these thymic precursors differentiated into TCRαβ and TCRγδ lineages. Further research determined that the commitment to either αβ or γδ T cell lineage occurs at the double negative ($CD4^-$ $CD8^-$) stage in the thymus[12]. Although the exact mechanisms are not fully understood yet, it has been found that SOX13[13] (high-mobility group transcription factor), as well as Id3 (Inhibitor of DNA binding 3)[14], promotes the development of γδ T cells while also opposing commitment to the αβ lineage. The strength of the TCR signal is another factor that contributes to lineage determination, where strong signals promote γδ commitment, while weak signals promote αβ commitment[15].

γδ T cell development involves the rearrangement of specific γ and δ gene segments which appear in waves during ontogeny[16,17]. Once γδ's leave the thymus, most will localize to non-lymphoid tissues which is based on the Vγ chains they express[18,19]. While Vγ influences where in the body γδ's go, any functional differences between each subset of γδ's has not been well studied. T cells with distinct combinations of Vγ and Vδ appear in 1 of 4 waves during development. The first wave in the murine model occurs at embryonic day (ED) 14.5, in which Vγ5 and Vδ1 is seen[20]. These T cells localize to the skin epidermis after development. The next wave peaks around ED 16, when Vγ6 is seen and later localizes to the uterus, lungs, and tongue[21]. In the third wave (ED 18), Vγ7 appears, although the exact timepoint is still unclear as

of now[23]. Vγ7 cells localized in gut mucosal tissues. The final wave (ED 20), is when Vγ1 and Vγ4 appear and are later found in the periphery, including blood, spleen, and lymph nodes[24,25]. When working with fetal mice, as was done in my thesis, it is important to understand this developmental order and the presence of γδ's in the developing embryo.

αβ T Cell Development

There is a lot more known about αβ T cell development than γδ T cell development, providing us with a better understanding of the developmental process. During the double negative (DN) stage within the thymus, TCRβ, TCRγ, or TCRδ are rearranged, committing the precursor cell to either the αβ or γδ lineage[6,26]. If TCRβ chain is successfully rearranged, then the pre-TCR complex is formed[27,28], which consists of the rearranged TCRβ chain and the pre-TCRα chain[29]. Signaling from the pre-TCR complex leads to many events that assist in the further development of TCRαβ T cells, including a burst of proliferation with an upregulation of CD4 and CD8 coreceptors. Along with this, the rearrangement of the TCRα gene is initiated, while the TCRγ gene is silenced[30]. Once the TCRα gene has been rearranged, the thymocytes will become double positive (DP), expressing both CD4 and CD8 on their surface[31].

Upon entering the double positive stage, the thymocytes will undergo positive and negative selection. Positive selection occurs in the thymus cortex, where thymocytes that express a TCR which recognizes self-MHC molecules are targeted[32-34]. This process is mediated by cortical thymic epithelial cells, or cTECs, which will express a self-peptide in the context of MHC class I or MHC class II. The determination of whether a thymocyte will survive, relies on the strength of the TCR signal with the cTECs. If the TCR signaling is weak, then the survival of that thymocyte is promoted. However, if there is strong TCR signaling, then the cell will undergo activation induced apoptosis, which is the function of negative selection[35]. As implicated, the

goal of negative selection is to remove any self-recognizing thymocytes. This occurs in the medulla and is mediated by medullary thymic epithelial cells (mTECs) and dendritic cells (DCs).

Once surviving thymocytes have undergone positive selection, those that recognize major histocompatibility complex (MHC), a group of genes encoding cell-surface proteins, class I will become $CD4^-$ $CD8^+$ single positive (SP) cells[36]. Thymocytes that recognize MHC class II transition into $CD4^+$ $CD8^-$ SP cells. Single positive thymocytes continue into their next stage of development in which they undergo functional maturation. This involves the upregulation of CD62L, S1P1, and CCR7, all of which help to facilitate the movement of these thymocytes into circulation[37,38]. T cells that have left the thymus are then referred to as recent thymic emigrants, and further continue their maturation within the periphery, eventually resulting in mature naïve $CD4^+$ and $CD8^+$ T cells[39,40]. Besides γδ and αβ T cells, another important subset of T cells exists, regulatory T cells.

Tregs

Treg Discovery

Regulatory T cells, or Tregs, are critical in maintaining immune homeostasis throughout the body with their immunosuppressive effects. They function to prevent unwarranted and excessive inflammation of tissues, as well as promote an adequate amount of self-tolerance. The concept that the thymus generated some type of suppressor T cell was first introduced in the late 1960's and early 1970's when it was observed that following thymectomy of 3-day old neonatal mice, the ovaries were subsequently destroyed, along with other tissues, leading to the idea of an autoimmune-type disease as the cause[41]. Shortly after this, it was discovered that a subset of T cells were found to dampen immune responses and was termed "suppressor T cells"[42].

The exploration of these cells was halted until the 1980's, when studies into the role self-tolerance played in autoimmune disease development led to the finding of T cells that had autoimmune-suppressive activity[43]. With this finding, subsequent studies were performed, most notable being a complete thymectomy followed by sublethal irradiation which resulted in autoimmune thyroiditis and type I diabetes in adult rats[44,45]. Upon transfer of syngeneic T cells into these rats, they were found to be protected from both thyroiditis and diabetes[46,47]. These studies confirmed the existence of a group of T cells in the thymus that prevents autoimmune disease.

In the 1990's, suppressive $CD4^+$ T cells were found to express the IL-2 receptor α-chain (CD25)[48]. Upon depletion of these $CD4^+$ $CD25^+$ T cells, mice were observed to have spontaneous development of autoimmune diseases, as well as an increased immune response to foreign antigens. However, it was shown that if the $CD4^+$ $CD25^+$ T cells were reconstituted soon after depletion, the mice did not develop any autoimmune diseases, and had normal response to foreign antigens[48]. 3 days after birth, $CD4^+$ $CD25^+$ T cells are detected in the periphery of neonates, which increases rapidly until they reach adult-like levels around 2 weeks after birth[49]. However, if a neonatal thymectomy is performed around this 3-day mark, the presence of these cells in the periphery is eliminated and autoimmune diseases are observed. The autoimmune disease formation can be prevented however if $CD4^+$ $CD25^+$ T cells are transferred back into the mice post-thymectomy[49].

Types of Tregs

There are 3 different types of Tregs, thymic Tregs (tTregs), peripheral Tregs (pTregs), and *in vitro*-induced Tregs (iTregs)[50]. As the name implies, thymic Treg generation occurs within the thymus when thymocytes bind MHC-self peptides strongly and is mediated by

mTECs that express Aire, an autoimmune regulator[51]. tTregs are important in maintaining self-tolerance and assists in identifying any self-reactive T cells that have avoided negative selection. pTregs are generated in the periphery when naïve $CD4^+$ T cells are stimulated with cognate antigens in the presence of transforming growth factor-β (TGF-β), retinoic acid, and IL-2[52,53]. These cells have been found to accumulate in areas that are exposed frequently to foreign antigens, such as in the intestines, and help to control the immune response at these sites[54,55]. iTregs can be induced from $CD4^+$ T cells after TCR activation, and are useful in studying Tregs.

Mechanisms of Tregs

When the immune system becomes activated against a pathogen, it produces an inflammatory response which can damage local tissues. These damaged tissues will then produce anti-inflammatory cytokines, such as TGF-β, which induces Tregs at the site[56,57]. For Tregs to have suppressive functions, they must first be TCR-activated in the presence of IL-2[58]. Once activated, they are then able to suppress other conventional-type T cells even without antigen exposure, known as bystander suppression[59].

There are multiple mechanisms in which Tregs function to maintain homeostasis. At a site of inflammation, Tregs bind with high affinity to IL-2 which inhibits IL-2 availability, consequently limiting $CD8^+$ T cell activation and proliferation[60]. This dampens the cytotoxic activity, promoting a decrease in the inflammatory response. Along with this, Tregs express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which is a coinhibitory molecule that modifies antigen presenting cell (APC) activity[61,62,63]. The expression of CTLA-4 downregulates CD80 and CD86 which are found on APCs, such as dendritic cells, suppressing the proliferation of conventional T cells[64,65]. Tregs also produce immunosuppressive cytokines themselves, including TGF-β and IL-10[66,67], as well as effector T cell transcription factors, like T-bet[68].

These mechanisms show the many ways in which Tregs can cause suppressive effects during pathogen exposure and help to maintain a state of homeostasis. This is especially important in sites that are constantly being exposed to antigens, like in the intestine. However, immediately after birth, neonates do not have these protective cells, and so a gap in knowledge exists in what kind, if any, cells are offering protection.

Foxp3

Forkhead box P3, or Foxp3, is a transcription factor that functions as the master regulator of Tregs. Foxp3 was first discovered in 2001, when its single mutation in the X-chromosome was linked to the cause of spontaneous and severe autoimmunity in Scurfy mice[69]. Around the same time, it was discovered that a mutation in the human version of Foxp3 caused a similar disease termed IPEX (immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome)[70,71]. The effects this mutation had rendered similar to that of mice deficient in Tregs, leading to the hypothesis that Foxp3 is essential for the development and proper function of Tregs. Studies on the relationship between Foxp3 and Tregs eventually showed that it was a crucial factor in their generation and overall function[72,73,74].

Along with the promoter at the Foxp3 gene locus, 3 conserved noncoding DNA sequences (CNS) have been identified[75]. These are termed CNS1, CNS2, and CNS3, all of which contribute to the differentiation of tTregs and pTregs. CNS1 is essential for both iTreg and pTreg development, containing binding sites for different transcription factors, including Smad2 and Smad3[76]. Both Smad2 and Smad3 are necessary for the TGF-β-mediated induction of Foxp3 in pTregs. The Smad family of proteins are essential in signal transduction for TGF-β receptors. When CNS2 is demethylated, Foxp3 will bind to it and through a positive feedback loop, will enhance its expression[75]. This occurs after demethylation of CpG sites on CNS2, further

contributing to stable Foxp3 expression in tTregs[77,78]. The CNS3 region is important in tTreg and pTreg differentiation and helps control the expression of Foxp3. It contains a binding site for c-Rel, a NFkB family member, which after binding promotes the transcription of Foxp3 by forming a Foxp3-speicfic enhanceosome[79,80].

More recently, a new site, CNS0, was discovered 8.5kb upstream of the transcription start site (TSS)[81]. It was identified to contain the binding sites for Satb1, a global genome organizer. Satb1 works to induce transcriptional and epigenetic regulation through the formation of novel nuclear architecture[82]. While this area is still being studied, it is hypothesized that the binding of Satb1 to the CNS0 region may play an essential role in commitment to the Treg lineage. While the structure has been well studied, and the importance of Foxp3 in Tregs is apparent, work done in my thesis invokes a challenge on this current knowledge. With Foxp3 appearing in neonatal intestines immediately after birth, it seems that it may play a regulatory role in the developing immune system within γδ T cells, a topic that has not yet been addressed.

γδ T Cells

Along with Tregs, γδ T cells appear to also be important in mucosal sites, such as in the intestines. Our current knowledge, however, on their specific mechanisms and functions is lacking. γδ T cells were first discovered in the mid-1980's[83-85], after the accidental discovery of a third TCR chain in 1984, the γ chain.[86]. γδ T cells are found highly concentrated in mucosal sites, which is the opposite of αβ T cells, which are found mainly in secondary lymphoid organs. Of these mucosal sites, γδ's have been identified in the epidermis[87], in the lung, tongue, and uterus[22], as well as in the intestinal epithelium[88,89]. Their location in the body has been shown to dictate which Vγ/Vδ chains of the TCR are present within each subset[18,19]. Along with a diverse

TCR, γδ T cells express CD3 on their surface. What makes γδ T cells unique is their ability to recognize molecules in both the classical MHC molecule-type way, as well as independently[90-95].

γδ T cells are among the first T cells to develop within the thymus of mice, appearing as thymocytes around embryonic day (ED) 14.5[96]. At this point in development, the frequency of γδ T cells is much higher than αβ T cells, the opposite of which is true in a mature immune system. The function, and potential importance of the increased γδ's is a gap in knowledge that needs to be further explored. Due to fetal murine studies, we do know that the frequency of γδ thymocytes peaks around ED16.5, followed by a gradual decrease throughout development and into the 1st week post-birth, where the amount of γδ's is about 0.3-0.5%, similar to adult levels[96]. The reason for this sudden decrease remains to be found, however. γδ's are first detected in humans around gestational week 6-9, where they have been found in the liver and in the developing gut[97].

The cytokines produced by γδ T cells appears to be variable and dependent on their localization within the body. Most of the work regarding the cytokine profile of γδ's has been completed utilizing clones, due to the limited amounts of γδ cells in the body. To assess the cytokine production post-activation, a PMA/ionomycin stimulation is commonly used. From this type of work, it has been found that γδ T cells in the epidermis of humans will produce IFN-γ and TNF 24 hours post-stimulation[98]. γδ's from murine lamina propria lymphocytes (LPL), which are found in the intestines, produce IL-10 and IL-17 after PMA/ionomycin stimulation[99]. Clones from murine spleens show high IFN-γ and TNF expression[100], while γδ's from intraepithelial lymphocytes (IEL) express IL-10[101]. Overall, γδ T cells are able to produce cytokines of both a Th1 phenotype, like IFN-γ, as well as of a Th2 phenotype, like IL-4, IL-5, and IL-10[102-104]. The multifunctionality of these cells leaves the question of whether or not there

are different functional subsets of γδ T cells, which our single cell RNA sequencing data aims to address.

γδ T cells in the Gut

A majority of γδ T cells in the gut express the CD8αα homodimer on their surface alongside the TCRγδ[105]. A gap in knowledge arises regarding the development of IEL γδ's, as there has been evidence that supports their development both within the thymus as well as thymus independent. It has been proposed that γδ IEL's are of fetal origin, as studies have shown that transferring cells from the fetal (ED 15-16) liver, generates γδ T cells in the intraepithelial layer[106]. Of interest, studies have shown that the Vγ7 chain is detected in the thymus around ED13, while it is seen even earlier, at ED11, in the fetal gut and liver of mice[23]. These data is showing that γδ precursors appear to be present in the gut prior to the thymus. Other studies have been conducted that support the idea that γδ IELs arise independent of the thymus. In one, athymic mice still had γδ T cells present in the intraepithelial layer, just with decreased levels[106].

Not only is the development of γδ IEL's unclear, but their function is not fully understood. Many studies use TCRδ$^{-/-}$ mice, which have no γδ T cells, in order to observe any functional changes that may occur without γδ's. In one of these studies, it was found that in the TCRδ$^{-/-}$ mice there was a reduction in the proliferation of epithelial cells in both the small intestine and in the colon[107], confirming the important role that γδ IELs play in the maintenance of homeostasis within the gut. Along with the decreased proliferation in the gut, TCRδ$^{-/-}$ mice are found to have increased gut permeability as the tight junctional complexes in the intestines are decreased[108]. The increased intestinal permeability correlates with an observed increased susceptibility to the development of spontaneous colitis in older mice[109]. Interestingly though,

the transfer of γδ IEL ameliorated the colitis, with a decrease in IFN-γ and an increase in TGF-β production[109]. It has also been found that there is a correlation between mice that lack γδ T cells and increased IFN-γ production in the IELs of inflammatory bowel disease (IBD) mouse models[110].

There are multiple disease models that are used to understand the function of γδ T cells in the intestines, one of the most popular being the dextran sulfate sodium (DSS)-induced colitis model system. In DSS-treated mice, large amounts of γδ T cells were observed at sites of epithelial damage and were found to express keratinocyte growth factor (KGF), an intestinal epithelial cell mitogen[111]. This observation led to the idea that γδ's may be important in upholding the integrity of intestinal epithelial sites that become damaged. Patients with celiac disease have also provided potential insight into the role of γδ T cells. Those that were on a gluten free diet and were not experiencing any symptoms of active celiac disease, were found to have an increased frequency of $CD8^+$ γδ IELs that expressed NKG2A, an inhibitory NK receptor as well as TGF-β[112]. These data suggest the potential for γδ IELs to function in a regulatory role in the intestines of celiac patients.

γδ T cells & Tolerance

One of the first studies that identified a potential role of γδ T cells in tolerance induction looked at OVA exposure in respiratory mucosa[113,114]. It was shown that upon exposure, splenic $CD8^+$ γδ T cells became activated, and suppressed the OVA-specific IgE antibody response. Along this line, it was also seen that the depletion of γδ's *in vivo* and *in vitro* led to the production of anti-OVA antibodies as well as proliferation of OVA-specific T cells[115].

Other studies using a NOD mouse model of spontaneous type I diabetes showed that $CD8\alpha\alpha^+$ $TCR\gamma\delta^+$ IELs are required for self-tolerance. In this model, the transfer of $CD8\alpha\alpha^+$

TCR$\gamma\delta^+$ IELs into 3-day old NOD mice that had undergone a recent thymectomy, prevented the development of diabetes[116]. Along with this, it was shown that even if there was an induction of Tregs through oral insulin to euthymic mice, CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IELs were still required for diabetes to be prevented[116].

Immune Tolerance

Tolerance is extremely important in maintaining a homeostatic environment within the body. It is defined by mechanisms that impair the immune response to self-antigens, while still providing an appropriate and protective response against foreign antigens[117]. Immunological tolerance was first introduced in 1945 by Ray Owen who conducted studies in cattle. He observed that dizygotic twin cattle shared red blood cells (RBCs), noting two distinct phenotypes of RBCs, that persisted into adulthood[118]. The absence of an immune response to dizygotic red blood cells led to the proposal that foreign cells acquired before birth generated immunological tolerance later in life to those same cells. Along with the tolerance to each other's RBCs, most of the cattle were also tolerant to a skin allograft from their dizygotic twin[119,120].

Billingham, Brent, and Medawar were another group that was instrumental in progressing the knowledge on tolerance. In 1953, they demonstrated that the injection of allogeneic tissue into mice while *in utero* led to the establishment of tolerance to the tissue later in life, when a skin allograft from the same strain was introduced[121]. However, mice that were injected after birth failed to acquire the same immunological tolerance[121], leading to the idea that there is a small window in which tolerance can be established during which alloantigens are exposed.

Types of Tolerance

There are two types of tolerance, central and peripheral tolerance. Central tolerance is the first line of defense against self-reactive T and B cells, taking place in the thymus and bone

marrow where lymphocytes are first generated. If a lymphocyte is deemed to have strong autoreactivity, it will undergo either clonal deletion[35] or clonal diversion[122]. In clonal diversion, T cells that express a self-reactive TCR will differentiate into tTregs, which leave the thymus and enter the periphery where they function to suppress any autoreactive T cells that may have escaped negative selection[122].

Peripheral tolerance occurs in the periphery, when lymphocytes that are autoreactive have escaped the previously described protections in the thymus. When this occurs, there are a few mechanisms in place to prevent further issues. One of these includes inducing a state of anergy within the autoreactive cells, in which cells are alive and functional, but do not respond to antigen stimulation. This concept was first introduced by Nossal in the 1980's, after the discovery of mature, autoreactive B cells within circulation that had no response to antigen stimulation[123]. This phenomenon was also seen in T cells that remained unresponsive to antigen stimulation when activated in the absence of CD28, a costimulatory molecule, or CTLA-4, a coinhibitory molecule[124].

Antigen presenting cells (APCs) also play an important role in the maintenance of peripheral tolerance. Dendritic cells (DC) specifically contain the ability to deliver coinhibitory signals, or an inadequate amount of costimulatory signals, which promote a tolerogenic response in T cells, limiting their activation and subsequent proliferation[125]. However there does not exist a singular subset of DCs that assists in peripheral tolerance, but instead it has been theorized that this function may be induced when the DCs are found in the presence of certain anti-inflammatory cytokines[126].

Another type of regulatory t cell, termed type 1 Tregs or Tr1, has been identified as playing a role in peripheral tolerance[127]. These Tr1 cells, unlike conventional Tregs, do not

express Foxp3 and have low levels of CD25[128]. Studies done in IPEX patients, a disease caused by a Foxp3 mutation, showed that Tr1 cells with suppressive capabilities can be isolated from peripheral blood, confirming that Foxp3 is not required for their induction or function[129]. While tolerance is quite well understood, the tolerogenic state of neonates leaves some room for questions. With our work showing Foxp3 expression in neonatal IEL γδ's, the question of whether these cells are taking on a tolerogenic role arises.

Perinatal Immunity

Looking further at this time in development, known as the perinatal period, it consists of both fetal and neonatal stages of life. In humans, this period begins at gestational week 22 and ends 1 week after birth[130], while in mice it begins at embryonic day 9 and lasts 7 days after birth[131]. It was initially thought that the neonatal immune system had an impaired response when compared to a mature immune system, however the more work that has been done in this field has shown that the neonatal immune system is actually very active but has a higher propensity towards existing in a state of tolerance, as is needed for proper development[132,133]. For a fetus to exist *in utero*, it must adopt a highly tolerogenic state as it exists in a semi-allogeneic environment[134]. While not fully understood, this state appears to be reached by the generation of Tregs, which help to block the fetal immune response during development. After birth, newborns are bombarded with a variety of commensal microbes, pathogens, and environmental antigens, which they must quickly learn how to distinguish between[135]. This is essential to prevent an overactive immune response and excessive inflammation that could be detrimental to the neonate's health.

The tolerogenic state that newborns find themselves in also renders them more susceptible to pathogens, which can be fatal. Most newborn deaths are caused by diseases that

are preventable upon vaccination, however since the neonatal immune system is highly tolerant, the efficacy of vaccines is significantly reduced[136,137]. Maternal antibodies have been observed to transfer to the fetus trans-placentally, offering the susceptible newborn some protection from pathogens for the first 4-6 months of life[138].

Many studies have investigated the impact growing up in a farming community plays on tolerance and immune responses later in life. From these studies, it has been observed that tolerance likely develops *in utero*, since umbilical cord blood (UCB) from fetuses with maternal exposure to farming have been found to have increased Tregs as well as an increase in suppressive capabilities[139,140]. Not only does it appear that immunosuppressive functions are increased, but there also is a decrease in IL-5 and IL-13, both of which are associated with allergic reaction[140]. Exposure during childhood to a farming environment has also been correlated with increased protection against allergic diseases later in life[141,142].

In neonatal murine models, there is an increased amount of IL-4 production in T cells in response to TCR stimulation when compared to adult T cells[143]. The Th2-like response in neonates declines within 5 days after birth and is then comparable to adult levels[144]. In human UCB, naïve T cells show more of a Th1 response, with a decrease in IFN-γ when compared to adult naïve T cells in normal conditions[145]. Upon TCR stimulation, UCB secretes increased IL-10, an anti-inflammatory cytokine, whereas adult T cells do not[146].

Both human and mice neonates have been shown to have a decrease in $CD8^+$ T cell responses to infection. Neonatal mice appear to have a deficient $CD8^+$ T cell response against influenza, herpes simplex virus (HSV), and respiratory syncytial virus (RSV)[147-149], similar to human infants, who also show a severely decreased response of $CD8^+$ T cells to RSV, HSV and influenza[150]. Neonatal $CD8^+$ T cells that are present appear to have a decrease in granzyme B,

IFN-γ, and IL-2 than in adult $CD8^+$ T cells[151]. Naïve $CD4^+$ T cells from UCB[152] and from fetal lymphoid organs[153] have a much higher frequency than adult naïve cells to differentiate into $Foxp3^+$ Tregs after antigen receptor stimulation.

Helios

When searching for the master regulator of T cell development, the transcription factor Ikaros, encoded by the *Ikzf1* gene, was first cloned[154]. 4 other members of the Ikaros family were identified shortly after, including Helios (*Ikzf2*), Aiolos (*Ikzf3*), Eos (*Ikzf4*), and Pegasus (*Ikzf5*). Ikaros are zinc-finger transcription factors that are characterized by 2 conserved zinc-finger motifs[155]. The first motif is the amino terminal (N-terminal), which contains DNA binding domains, while the second motif is the carboxyl terminal (C-terminal) which contains domains for interactions with the other Ikaros proteins, or transcriptional regulators[156,157].

Identified initially as a dimerization partner of Ikaros, Helios was first cloned in 1998[158], when it was found at centromeric heterochromatin regions within the T cell nucleus in complex with Ikaros. Helios was noted to be of T cell lineage, and was found in all fetal hematopoietic sites, earliest lymphoid progenitors, and in adult HSCs[158]. My thesis attempts to elucidate further understanding of where exactly Helios is arising from. Our current knowledge points towards HSCs as its source, which is why we chose to explore this connection further.

The group that first identified Helios performed a northern blot analysis in order to track its expression in the developing fetus. What they found was that Helios was first detected in the blood islands of the yolk sac at embryonic day (ED) 8. At ED11, Helios was expressed in the fetal liver, followed by detection of it within the epithelial lining of the gut at ED13. On ED16, Helios was found near the thymus, trachea, and epithelial linings of the esophagus. At ED17, the fetal liver no longer expresses it, but Helios is instead found in multiple epithelial tissues,

including the lungs, skin, mouth, and salivary glands. Helios mRNA was detected in both $CD4^-$ $CD8^-$ thymocytes as well as $CD4^+$ $CD8^+$ thymocytes but declines as these subsets transition into single positive thymocytes[158-160].

Helios was thought of as a tumor suppressor during initial analysis in tumor cell lines since human T cell leukemia, human T cell lymphoma, and T cell acute lymphoblastic leukemia cell lines all overexpressed a short isoform of Helios that lacked 3 out of the 4 N-terminal domains[161,162]. Upon further analysis of this Helios isoform, it was found that any association with Ikaros, Aiolos, or Helios of full length, the DNA-binding activity was inhibited[163]. $Helios^{-/-}$ mice have significant perinatal fatality, so studies with total knockout mice are unable to be done[182].

While the mechanisms behind Helios are not fully understood, many studies have implicated Helios in the function of Tregs. It has been found that $Helios^+$ Tregs appear to have an increase in suppressive capabilities when compared to $Helios^-$ Tregs[164-167]. Supporting this, Tregs that had impaired expression of Helios expressed less Foxp3 and instead had higher amounts of IFN-γ, TNF, and IL-17[168]. These Helios-deficient Tregs also failed to improve inflammatory bowel disease in $RAG^{-/-}$ mice after being transferred[168]. Helios was found to function as an IL-2 regulator, with the ability to suppress the IL-2 gene transcription in Tregs with the help of Foxp3[169]. The potential role of Helios in Tregs is strengthened by our observation that fetal and neonatal mice have high amounts of Helios in the periphery, which diminishes shortly after birth but remains high in the intestines, a site that needs to have increased tolerance.

$CD8\alpha\beta^+$ $TCR\gamma\delta^+$ T Cells

Our work done in fetal and neonatal mice led to the identification of a new subset of cells within their intestines that we observed to be present in young mice but not in adult intestines.

These cells are TCRγδ$^+$ and express a CD8αβ heterodimer. There has been very limited research done on CD8αβ$^+$ TCRγδ$^+$ T cells in humans or in mice. They were first identified in the early 2000's in the IEL of athymic mice[170]. These CD8αβ$^+$ TCRγδ$^+$ T cells were observed to have cytolytic activity and were IFN-γ producing, but researchers noted that their location varied within mice and appeared to potentially be influenced by microenvironment stimuli. Other potential functions of these cells have not yet been addressed, leading us to further study them.

More recently, a paper came out in 2016 identifying CD8αβ$^+$ TCRγδ$^+$ T cells in IBD patients[171]. They observed a negative correlation between the amount of these T cells and the disease severity, noting a significant decrease in CD8αβ$^+$ TCRγδ$^+$ T cells in the mucosa of patients with active disease when compared to healthy patients. Treatment with anti-TNF appeared to increase the levels of these cells, bringing them close to what was observed in healthy persons. This paper presented a potential role of these CD8αβ$^+$ TCRγδ$^+$ T cells in maintaining gut homeostasis, and potential mucosal healing in IBD patients. While these studies offer a glimpse into the potential function of CD8αβ$^+$ γδ T cells, a large gap in knowledge is present that needs to be addressed. Understanding their origin and function, may have important implications for a possible role they may play in the developing immune system or in IBD patients.

CHAPTER TWO

MATERIALS AND METHODS

Umbilical Cord Blood

Total umbilical cord blood (UCB) was collected into blood collection bags containing a citrate phosphate dextrose solution. UCB was donated from Loyola University Medical Center donors. UCB was processed to obtain lymphocytes through density centrifugation using Lymphopure density gradient medium (Biolegend, San Diego, CA) and centrifuged at 1500rpm at 25C for 20 minutes with no brake. Red blood cells were lysed using RBC lysis buffer (Biolegend, San Diego, CA). Donors were excluded if there was evidence of any active malignancies, if any immunomodulatory medications were used, if they had uncontrolled hyper or hypothyroidism, if they had an autoimmune disorder, or if there was the presence of an active infection.

Flow Cytometry

In vitro cultured cells or *ex vivo* cells were stained. Both surface and intracellular molecules were labeled with fluorophore-conjugated antibodies following standard staining protocols. Cells were first stained with Zombie Aqua Fixable Viability dye (Livedead) for 10 minutes, then washed with FACS buffer [PBS, NaN_3, 10% FCS, deionized water]. Trustain (BioLegend, clone 93), which is used as an Fc-block, was added to cells for 10 minutes on ice in

the dark (.5ul per 10^6 cells), followed by the addition of the all antibodies for 30 minutes. Cells were then washed and fixed with True-Nuclear Transcription Factor Buffer Set (BioLegend) overnight. To stain for intracellular proteins, the 10X Perm buffer (BioLegend)was diluted to 1X and cells were washed twice before adding intracellular stain for 1 hour. Cells were then washed and filtered using nylon mesh to remove any debris. Data was collected using the FACS LSRFortessa or the FACS Canto II, both which are manufactured by BD Biosciencеses. Analysis of data was done using FlowJo software.

Antibodies

For flow cytometry, the following antibodies were used: Zombie Aqua Fixable Viability dye (Livedead), anti-mouse CD4, CD8α, CD8β, CD45, CD45.1, TCRγδ, TCRβ, Foxp3, IL-10, IFN-γ, SCA1, IL-2RB, CKIT, anti-human/mouse Helios, and anti-CD3.

Cell Marker	**Color**	**Brand**	**Titration**	**Clone**
anti-mouse CD4	FITC	Biolegend	1:200	GK1.5
anti-mouse CD8α	PE/Cy7	Biolegend	1:200	53-6.7
anti-mouse CD8β	APC	Biolegend	1:200	YTS156.7.7
anti-mouse CD45	APC/Cy7	Biolegend	1:50	30-F11
anti-mouse CD45.1	FITC	Biolegend	1:50	A20
anti-mouse TCRγδ	Percpcy5.5	Biolegend	1:200	GL3
anti-mouse TCRβ	BV711	Biolegend	1:200	H57-597
anti-mouse Foxp3	PE	Biolegend	1:50	150D
anti-mouse IL-10	APC	Biolegend	1:200	JES5-16E3
anti-mouse IFN-γ	PE	Biolegend	1:200	XMG1.2

anti-mouse IL-2Rβ	APC/Cy7	Biolegend	1:200	TM-β1
anti-mouse CKIT	FITC	Biolegend	1:200	2B8
anti-human/mouse Helios	Pacblue	Biolegend	1:2	22F6
Soluble anti-CD3		Biolegend	5ug/mL	145-2C11

Mice

C57BL/6 mice (originally received from Jackson Laboratories), RAG2 knockout mice and C57BL/6 CD45.1 mice were bred and maintained in house. Mice older than 10 days old were euthanized using CO2 and cervical spine dislocation. Mice under 10 days old were decapitated. Organs were isolated using sterile tools and immediately processed to obtain single-cell suspensions. To collect fetal samples, timed pregnancies were set up, using ED0.5 as the next day.

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

The small intestine was carefully isolated from the mouse and separated from colon. Yamamoto *et al* protocol was followed with minor modifications[172]. Payer's patches, mesentery lymph nodes, fecal matter and fat were removed from small intestine tissue. Tissues were then cut longitudinally, then into 1cm pieces which was vortexed in PBS to remove any remaining fecal waste. Tissues were then placed in 10% HBSS containing fetal calf serum, 10mM HEPES, and 1mM Dithiotthreittol (DTT, Sigma Alrich) and was incubated in 37°C warm room, rocking for 30 minutes. Tissues were vortexed and intraepithelial lymphocytes (IELs) were collected through 100μm filter. The remaining tissues were then placed in 10% HBSS containing 0.1mg/ml DNAseI (Roche, Basel, Switzerland), 2mg/ml Collagenase D (Sigma Aldrich), and 0.17U/ml Dispase (Gibco, Thermo Fisher Scientific), and cut into smaller pieces using sterile

scissors. This tissue was then placed in 37°C warm room for 30 minutes on a rocker. Lamina propria lymphocytes (LPLs) were collected through a new 100μm filter, and enzymatic digestion was stopped with 10% HBSS containing 5mM EDTA. Both IELs and LPLs were washed in 10% HBSS and then resuspended in a 40% Percoll with RPMI solution. IELs used a 70% Percoll solution for isolation, and LPLs used a 80% Percoll solution. Purified IEL/LPL were harvested from cell interphase after centrifuging for 20 minutes at 600g with no brake.

Spleen Isolation

The spleen was isolated from mice and grinded using Frosted Microscope Slides in PBS. Splenocytes were then washed, and 1ml of ACK Lysing Buffer (Thermo Fisher) was added to cells for 1 minute. Then, the cells were washed with PBS and resuspended in 2% FCS RPMI media.

Suppression Assay

The spleen from a C57BL/6 CD45.1 mouse was sterilely isolated and processed with ACK Lysing Buffer (Thermo Fisher). Splenocytes were washed with PBS and resuspended in RPMI media. Total T cells were isolated from non-T cells using the Miltenyi Biotec TCRγδ+ T Cell Isolation Kit and stained with Cell Trace Violet to track proliferation. The spleen from OT-II Transgenic mice was sterilely isolated as well and was processed to obtain splenocytes. TCRγδ+ T Cell Isolation Kit was used to purify γδ's from splenocytes and were resuspending in RPMI media. Equal amounts of CTV-labeled CD45.1 total T cells and non-T cells were mixed together in a U-bottom 96-well plate. OT-II γδ T cells were added in a dose-dependent manner (100μl of cells at $3x10^6$, $1x10^6$, and $3x10^5$ per mL). Certain conditions were stimulated with 5ug/mL anti-CD3 antibody (Clone: 145-2C11) and plate was placed in a 37°C incubator for 3

days. Cells were then harvested and labeled with anti-mouse CD45.1, CD4, CD8α, and CD8β. Samples were analyzed on FACS LSRFortessa.

Single-cell RNAseq

Intraepithelial lymphocytes (IEL) from the intestines and spleen of 2 C57BL/6 mice were sterilely isolated and processed according to the protocol previously described. Cells were diluted to 1-2 million cells in Cell Staining Buffer and stained with anti-CD45 (103154), anti-CD3 (100312), anti-mouse TotalSeq^{TM}-A CD4, TotalSeq^{TM}-A CD8α, TotalSeq^{TM}-A TCRβ chain, TotalSeq^{TM} -A TCRγδ, and TotalSeq^{TM}-A CD8β antibodies following the TotalSeq^{TM} Dual Index Protocol. TotalSeq^{TM} antibodies are conjugated with oligonucleotides which contain a barcode sequence for further single cell analysis. $CD45^{+}CD3^{+}$ cells were sorted into DMEM + 10% FCS. Sorted cells were used following 10X Genomics single-cell RNAseq protocol. Samples were sent to Northwestern University for analysis. Pathway analysis was done using Metascape, which converts gene lists into M. musculus gene IDs using GO biological processes, KEGG Pathway, Reactome Gene Sets, CORUM, TRRUST, PaGenBase, and WikiPathways. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 were grouped based on membership similarities.

Depletion Assay

The spleen from a C57BL/6 CD45.1 male mouse was sterilely isolated and processed. Cells were sorted for the following conditions: Total T cells, Total T cells depleted of $TCR\gamma\delta^{+}$ $CD8\alpha\beta^{+}$ T cells, T cells depleted of Tregs ($CD4^{+}CD25^{+}$), and T cells depleted of both $TCR\gamma\delta^{+}$ $CD8\alpha\beta^{+}$ T cells and Tregs. Equal amounts of cells from each condition was injected retro-orbitally into RAG2 knockout mice that had been irradiated 24 hours prior to injections.

Mouse weights were recorded on the day of injections as their baseline weight and every 3 days afterwards to monitor for weight loss.

Adult and Fetal HSC Transfer

Adult bone marrow and fetal liver (ED17.5) hematopoietic stem cells (HSCs) were isolated from CD45.1 mice using the EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit. CD45.1 HSCs were injected retro-orbitally into RAG2KO mice that had been irradiated 24 hours before at 2.5 Gy. 6 weeks later, peripheral blood from RAG2KO mice was collected and stained with anti-mouse CD45.1 and CD45.2 to check for HSC reconstitution. Once confirmed, mice were sacrificed and organs were harvested for analysis via flow cytometry.

CHAPTER THREE

MOLECULAR AND CELLULAR CHARACTERIZATION OF γδ IELS AND LPLS

Splenic and Intestinal Barcoded scRNAseq Analysis for Potential γδ T Cell Function

While γδ T cells were discovered nearly 40 years ago[83-85], they are still a subset of immune cells that have a lot of unanswered questions surrounding them. Part of the reason this is the case may be due to their low numbers found in adult models, especially when compared to their counterpart αβ T cells, which make up a much larger percentage of T cells. Due to this constraint, and the abundance of knowledge it would hopefully provide us with, we decided to pursue further knowledge regarding γδ T cells by performing a single cell RNA sequencing experiment utilizing a barcoded antibody-based approach. This was chosen with the intent on functionally characterizing specific clusters of cells based on their gene expression.

To prepare samples for sequencing, we first isolated lymphocytes from the gut and spleen of 2 adult C57Bl/6 mice and prepared cell suspensions for sorting. Cells were stained with CD45 and CD3 to separate out the lymphocyte population, as well as TotalSeq^{TM}-A antibodies for TCR and coreceptor identification after analysis. $CD45^{+}CD3^{+}$ live cells were sorted for and the 10X Genomics single-cell RNAseq protocol was initiated immediately after to ensure cell viability. Upon successful sample preparation, the cells were transferred to the NuSeq Core Facility at Northwestern University for sequencing and analysis.

The initial findings from this experiment, provided by the team at Northwestern University, gave us insight into how the cells were clustered from both the spleen and gut (Figure 1b) and showed distinct clusters of cells among both samples, with the gut lymphocytes characterized as group 0, group 3, and group 8 (Figure 1a). Furthermore, the results from the barcoded antibody approach allowed for a distinction between TCRαβ and TCRγδ cells within the clusters. Shown as violin plots, we see that there are 3 distinct populations of γδ T cells, groups 0, 3, and 8, (Figure 2a) which when compared to the UMAP data are all cells from the gut. This is further confirmed with analysis of the TCRβ violin plot which shows that clusters 0, 3 and 8 do not express the TCRβ chain (Figure 2b). We were able to characterize these groups of cells further with the identification of CD4, CD8α, and CD8β coreceptor expression within the γδ clusters, which each showed low CD4 and CD8β expression (Figure 3a,c). We instead saw that all 3 of the γδ clusters, group 0, 3 and 8, expressed CD8α (Figure 3b).

With this initial finding of 3 groups of γδ T cells that express CD8α, we wanted to determine what was differentiating each from one another. The genes that were found to be differentially expressed within each group from our initial data showed a potential for unique functions between the clusters. To further explore this, we decided to run a pathway analysis to identify possible functions for each group. This was done using Metascape[173] which converts gene inputs into M. musculus gene IDs using multiple ontology sources and clusters similar genes together.

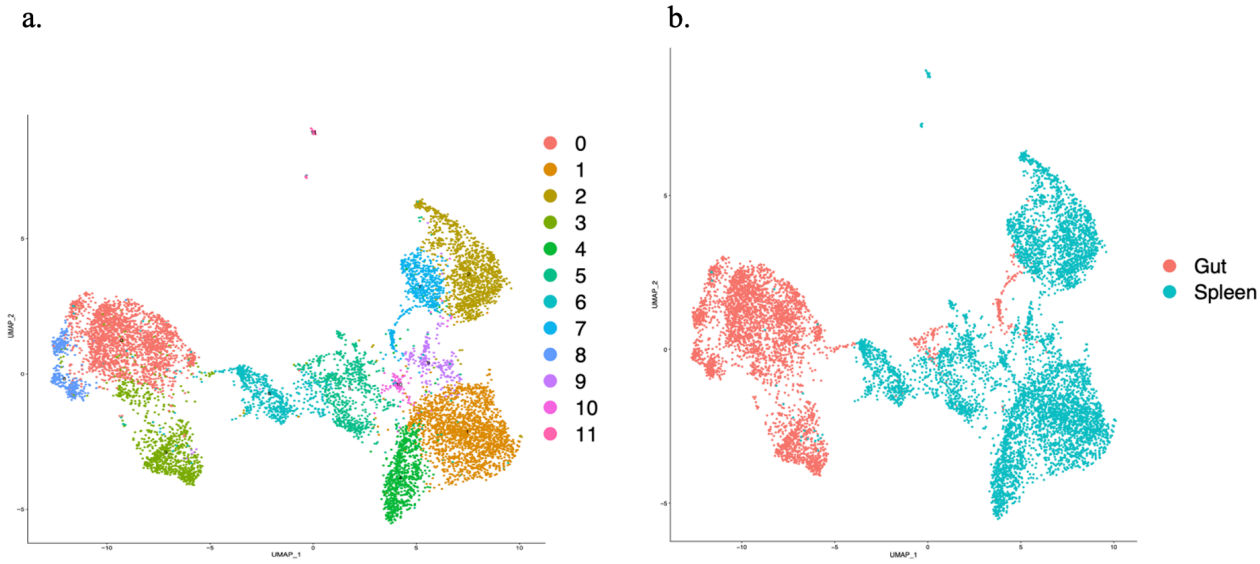

Figure 1. Single Cell RNAseq UMAP Clustering. Analysis via single cell RNAseq of 2 gut and 2 spleen samples from C57Bl/6 mice prepared using 10X Genomics protocol. (a) Clustering of individual cell subsets, identified by colors on right hand side. (b) Clustering of total gut and spleen cells.

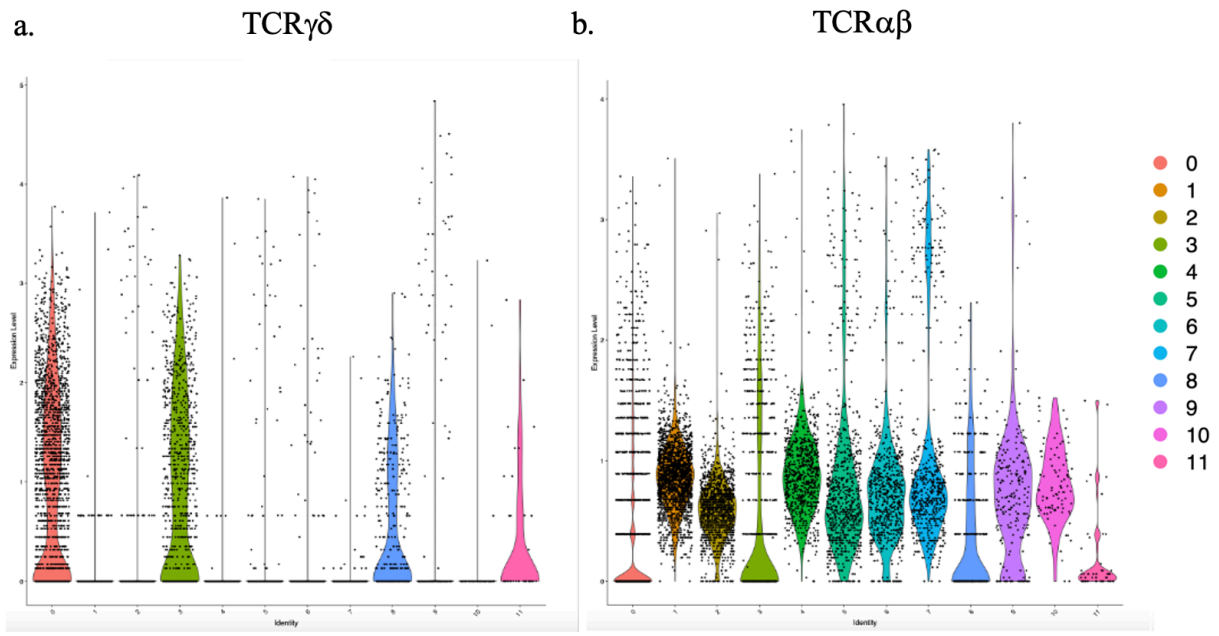

Figure 2. Violin Plots of TCRγδ and TCRαβ Cell Clusters. Data from scRNAseq experiment was analyzed using barcoded antibodies that were added during sample preparation. Expression level is measured on y-axis, with cluster ID on x-axis. (a) Cell clusters positive for TCRγδ (Groups 0, 3, 8). (b) Cell clusters positive for TCRαβ (Groups 1-2, 4-7, 9-11).

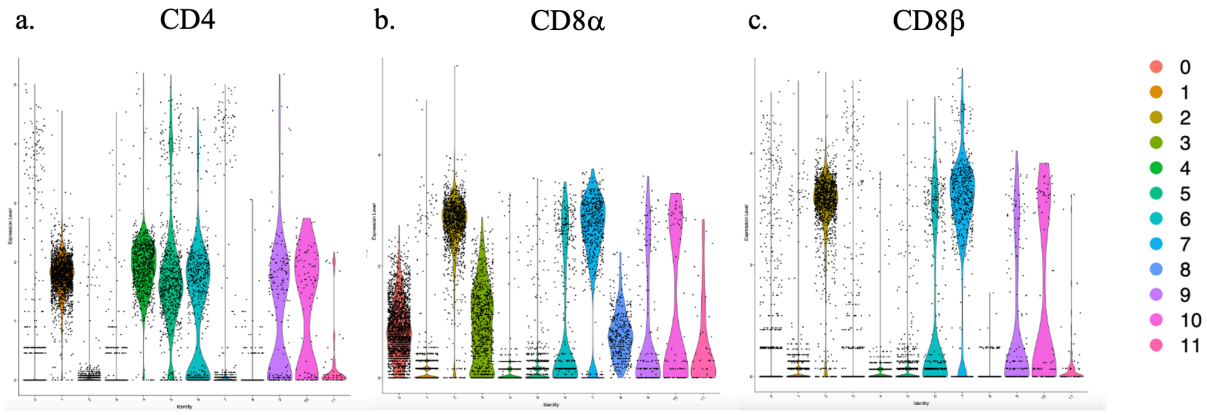

Figure 3. Violin Plots of CD4, CD8α and CD8β Cell Clusters. Data from scRNAseq experiment was analyzed using barcoded antibodies that were added during sample preparation. Expression level is measured on y-axis, with cluster ID on x-axis. (a) Cell clusters positive for CD4 (Groups 1, 4-6, 9-10). (b) Cell clusters positive for CD8α (Groups 0, 2, 3, 6-8). Cell clusters positive for CD8β (Groups 2, 6, 7, 9, 10).

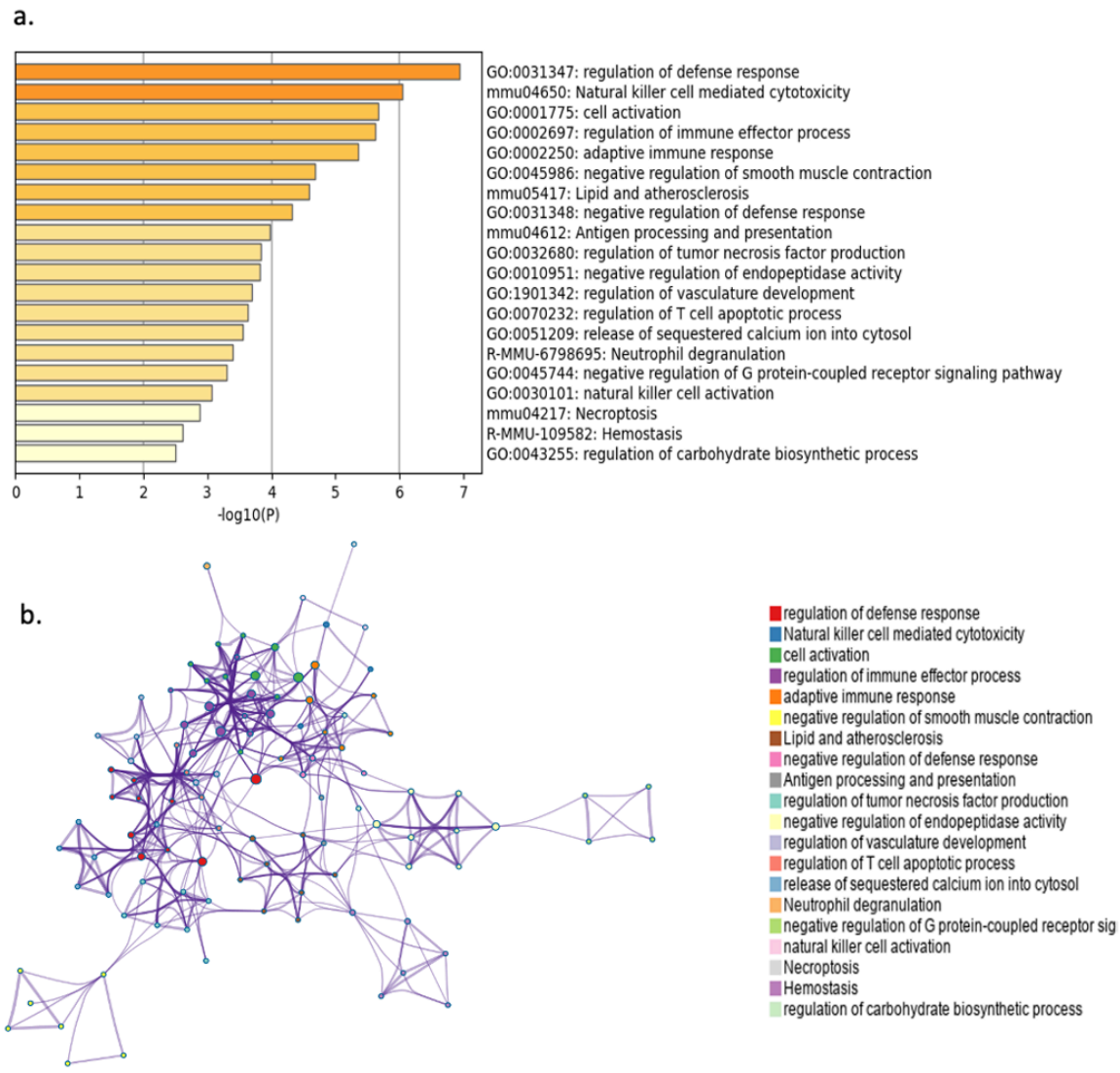

Figure 4. Pathway Analysis Results, Cluster 0. Pathway analysis using differential gene sets from single cell RNAseq experiment performed using Metascape software. Cluster 0 is TCR$\gamma\delta^{+}$ CD8α^{+}. (a) Enriched ontology clusters, terms listed to the right of bar graph. (b) Network plot of enriched ontology terms, color coded with terms on right hand side.

The first group of γδ's that was analyzed was cluster 0. Using the Metascape software, we produced an analysis of its enriched ontology clusters. These were hierarchically clustered by the most significant gene populations and the functional terms that best describe each group was mapped out (Figure 4a). It appears that this group of gut γδ T cells have an increased propensity

towards regulating the defense response within the gut. This could potentially mean that this group of cells is performing a function like that of Tregs, in which it may be limiting the immune response in the intestines. As a mucosal site that is constantly bombarded with environmental antigens, these cells may be playing a crucial role in deciphering when an immune response is necessary. To visualize the relationship between the enriched terms, a network plot was generated using Metascape software. From this network, the cells within this cluster that appear to regulate the defense response in the gut, are also closely related to those that regulate the immune effector process (Figure 4b).

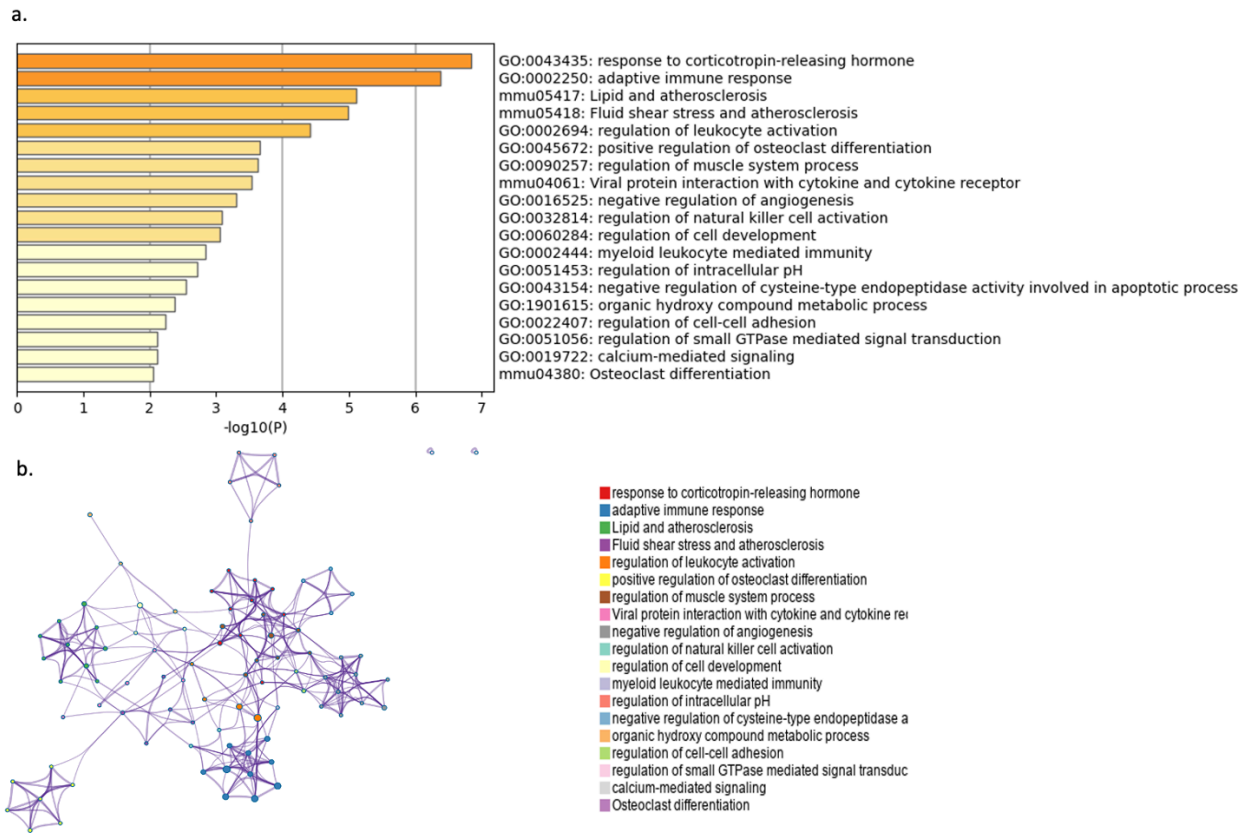

Figure 5. Pathway Analysis Results, Cluster 3. Pathway analysis using differential gene sets from single cell RNAseq experiment performed using Metascape software. Cluster 3 is TCRγδ^{+} CD8α^{+}. (a) Enriched ontology clusters, terms listed to the right of bar graph. (b) Network plot of enriched ontology terms, color coded with terms on right hand side.

Following the same procedure, the genes expressed in cluster 3, another subset of γδ's that express CD8α alongside TCRγδ, were analyzed. From the enriched ontology clusters, it appears that this group of cells may be important in the adaptive immune response, as well as in regulating leukocyte activation (Figure 5a). This subset of γδ T cells appears to be potentially acting on pathogens and promoting activation of the immune system when necessary, opposite of the first group of γδ's. With the help of a network plot to visualize any relationship between these two functions, it does appear that the genes within both ontology groups are related (Figure 5b). As this kind of response is typically carried out by CD4^{+} T cells, it is important to note again

that this cluster of cells, along with the other two, do not appear to be expressing CD4 (Figure 3a).

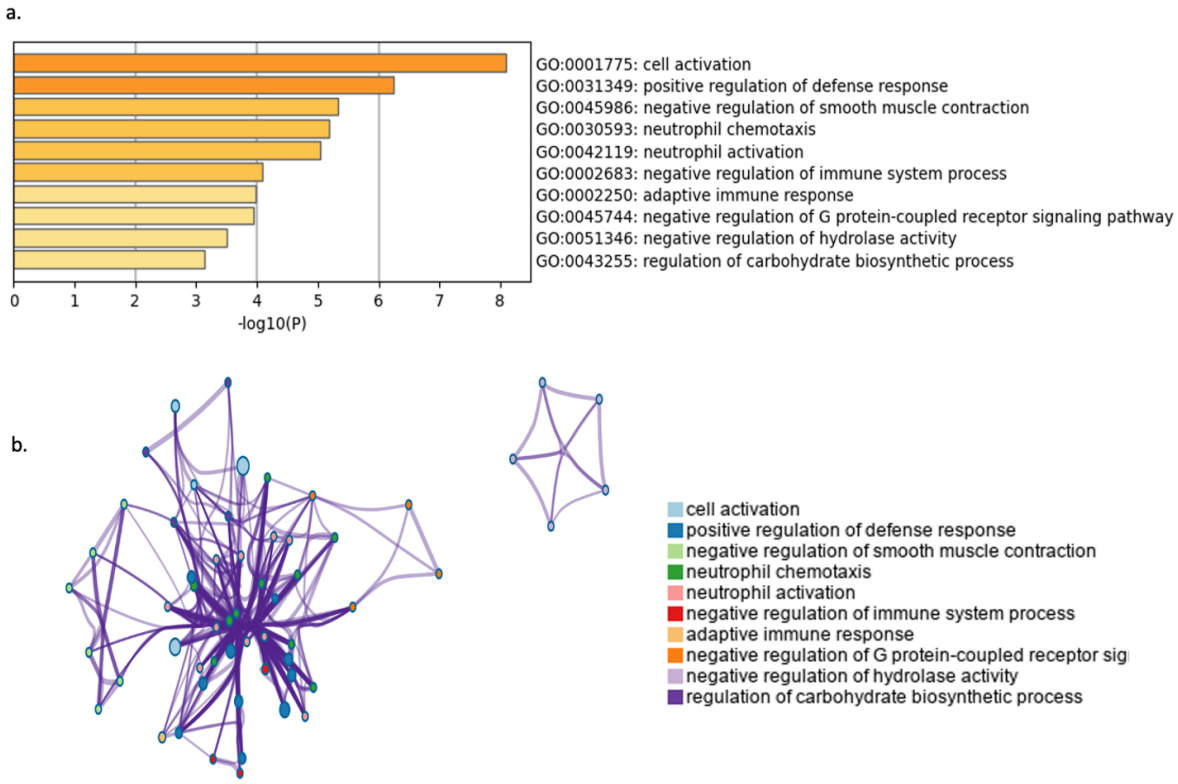

Figure 6. Pathway Analysis Results, Cluster 8. Pathway analysis using differential gene sets from single cell RNAseq experiment performed using Metascape software. Cluster 8 is TCRγδ^{+} CD8α^{+}. (a) Enriched ontology clusters, terms listed to the right of bar graph. (b) Network plot of enriched ontology terms, color coded with terms on right hand side.

Lastly, cluster 8 was analyzed to gain insight into their potential role in the gut. The enriched ontology from the genes expressed by this group of cells shows that they may be playing a similar role to that of the γδ cells in cluster 3. These cells are enriched for genes that are important for cell activation, as well as genes that help to increase the immune defense response when needed, noted as positive regulation (Figure 6a). Along with these functions, γδ's in cluster 8 appear to interact with neutrophils, their activation and movement towards an

indicated site. The network plot for this group does not appear to provide any valuable contribution towards the functional relationships of these cells (Figure 6b).

While each group of γδ T cells in the gut appear to be similar, with each expressing CD8α alongside TCRγδ, the subset of cells typically seen in the intestines, this data provides evidence for our hypothesis that there may be different subtypes of γδ T cells working together within the gut, and potentially other mucosal sites, to maintain a state of homeostasis. One group may be involved in regulating the defense response, so that the gut is not constantly inflamed, while another group is prepared for when the immune response needs to be activated to protect the host, and lastly a third group appears to also be activating cells but seems to be geared more towards recruitment of other immune cells, such as neutrophils, to aid in the defense. The differential genes within each group were tested for protein expression to confirm their presence within the gut, however at this time we have not found any significant gene expression within the gut.

Discovery of a New Foxp3^{+} Subset of γδ T Cells

The perinatal immune system, which involves both fetal and neonatal stages[130,131] of life, is a highly tolerogenic state that the developing fetus and newborn must remain in to survive. After birth, the neonate is bombarded with an array of new antigens, some commensals and some pathogenic, which they must quickly learn how to distinguish[138]. The mechanism behind this aspect of development is what we decided to seek out, specifically in relation to the gut environment, where exposure to new environmental antigens is abundant.

Initially, we sought to compare the intestinal phenotypes between three different ages of mice: fetal mice (embryonic day 17.5/18.5), neonatal mice (5 days old), and adult mice (6-8 weeks old). We isolated the small intestine and spleens from each age group, pooling together

the whole fetal guts to obtain adequate cell numbers. After separating intraepithelial lymphocytes (IEL) from lamina propria lymphocytes (LPL), cells were stained with antibodies and analyzed using flow cytometry. We discovered that a subset of γδ T cells that expressed the heterodimer CD8αβ within the fetal and neonatal intestines. This subset was absent in the adult intestines (Figure 7a-c). Exploring this subset further, we looked at the intracellular expression of Helios and Foxp3. We found that within the neonatal intestines, there was a small portion of $CD8\alpha\beta^{+}$ $TCR\gamma\delta^{+}$ cells that expressed Foxp3 (Figure 8). Foxp3 is an important transcription factor for regulatory T cells (Tregs), acting as a key player in their development and as a master regulator in their function[72,73,74]. Due to this knowledge, we hypothesized that these intestinal $Foxp3^{+}$ $CD8\alpha\beta^{+}$ $TCR\gamma\delta^{+}$ T cells in neonates are acting in a manner like that of Tregs, providing immunosuppressive effects immediately after birth. Since the cells are present in fetal intestines but absent of Foxp3, it is possible that they may play a role in development but once exposed to the outside environment transition into a regulatory role to help maintain homeostasis after birth.

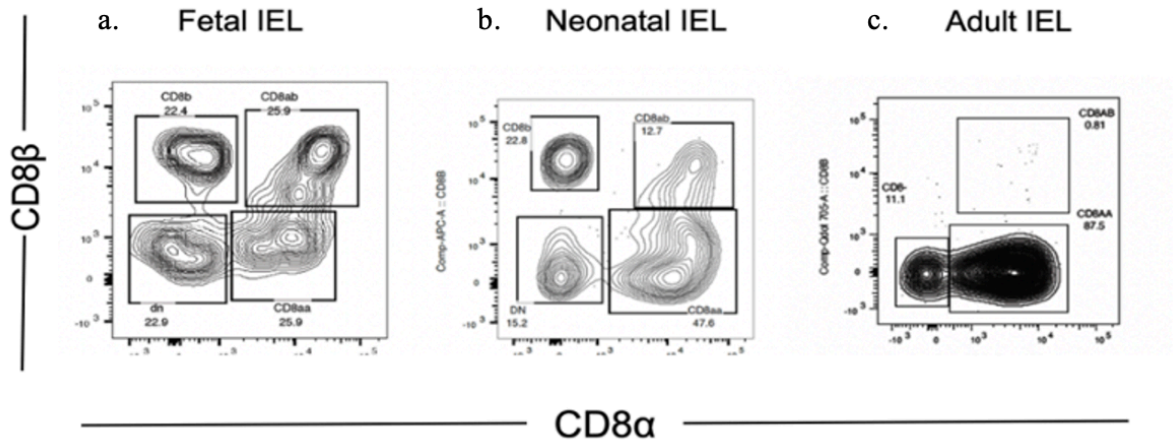

Figure 7. Fetal, Neonatal, and Adult IEL CD8 Expression. Intraepithelial lymphocytes isolated from (a) fetal (ED 17.5/18.5), (b) neonatal (5 day old), and (c) adult (4-6 weeks) C57Bl/6 mice were processed and stained with antibodies for analysis via flow cytometry. Gating in all panels shows CD8α and CD8β expression in live cells previously gated for $CD45^+$ $CD3^+$ $TCR\gamma\delta^+$ cells. N=6 (adult), N=3 (pooled neonate litter), N=3 (pooled fetal litter)

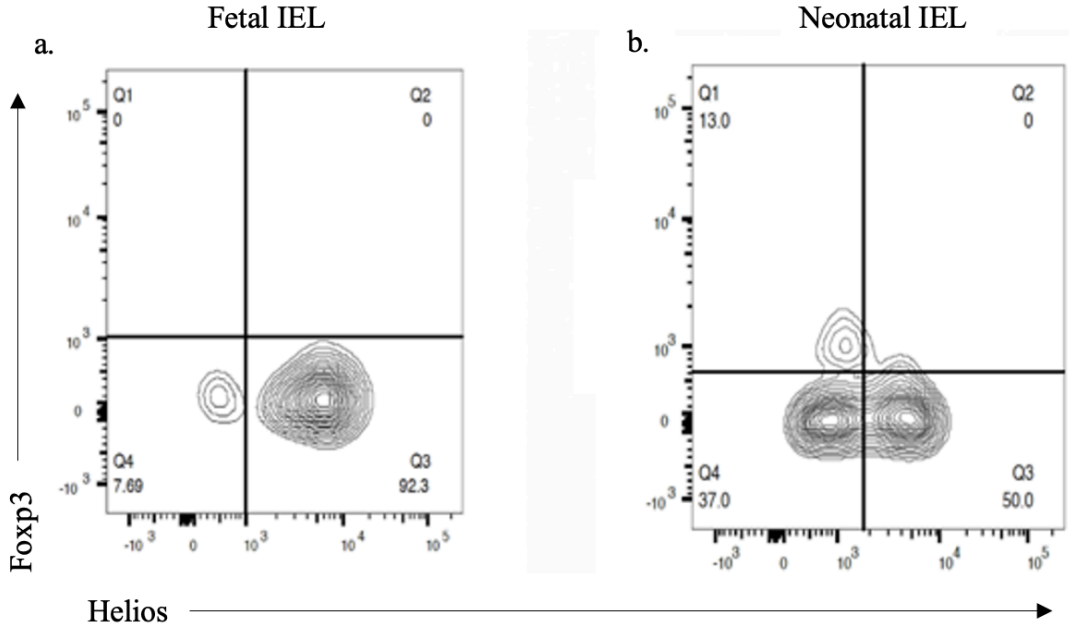

Figure 8. Foxp3 and Helios Expression in $TCR\gamma\delta^+$ $CD8\alpha\beta^+$ Fetal and Neonatal IELs. Intraepithelial lymphocytes isolated from (a) fetal (ED 17.5/18.5) and (b) neonatal (5 day old) C57Bl/6 mice were processed and stained with antibodies for analysis via flow cytometry. Gating in all panels shows Foxp3 and Helios expression in live cells previously gated for $CD45^+$ $CD3^+$ $TCR\gamma\delta^+$ $CD8\alpha\beta^+$ cells. N=3 (pooled fetal liver), N=3 (pooled neonatal liver)

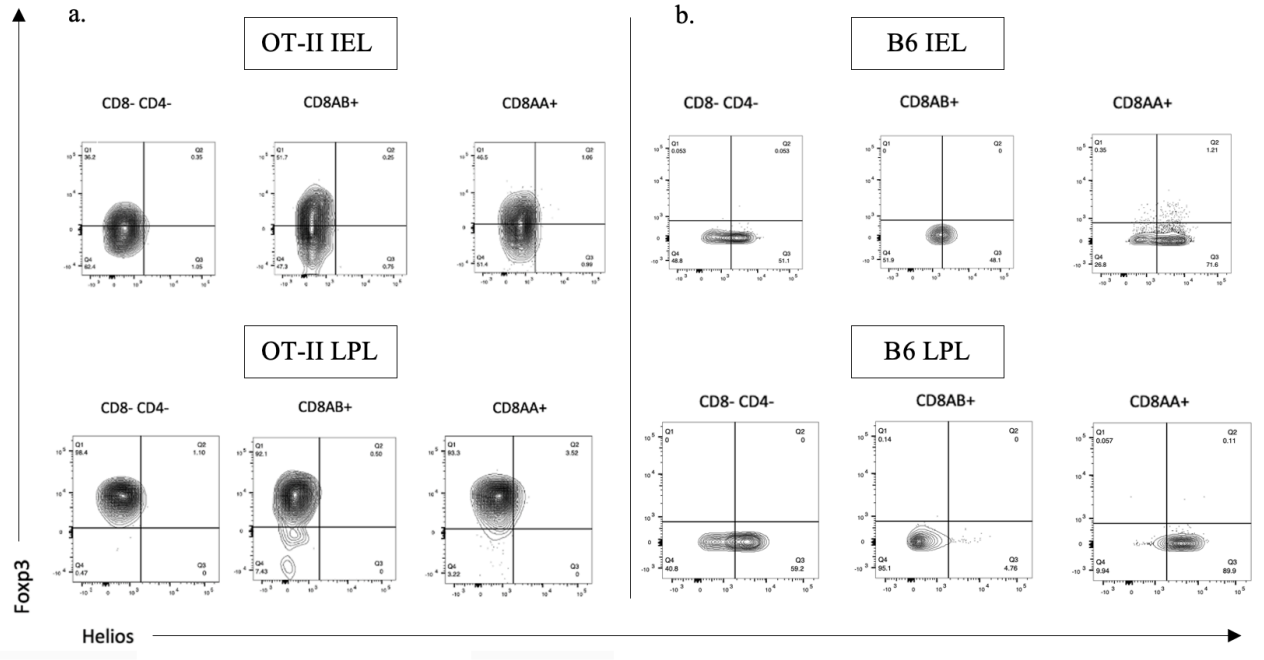

Figure 9. Foxp3 and Helios Expression in OT-II Intestines. Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) isolated from (a) adult OT-II transgenic mice and (b) adult C57Bl/6 mice were processed and stained with antibodies for analysis via flow cytometry. Gating in all panels shows Foxp3 and Helios expression in each subset of live cells previously gated for $CD45^+$ $CD3^+$ $TCR\gamma\delta^+$ cells. N=2 (OT-II), N=3 (B6)

$Foxp3^+$ γδ T Cells Suppress T Cell Proliferation

Since $Foxp3^+$ $CD8\alpha\beta^+$ $TCR\gamma\delta^+$ T cells appeared to be a small percentage of cells, we decided to pursue a potential function for $Foxp3^+$ γδ T cells instead. γδ T cells have not been reported to have Foxp3 expression except when induced *in vitro* with TGF-β and IL-15[174,175]. We hypothesized that since the γδ T cells express Foxp3, they would have immunosuppressive functions like Tregs. To test this, we utilized OT-II transgenic mice which we had previously found to express high levels of Foxp3 in $TCR\gamma\delta^+$ IELs and LPLs (Figure 9). If our hypothesis is correct, we expect to see a decrease in proliferation of T cells due to the immunosuppressive effects that the γδ T cells would produce.

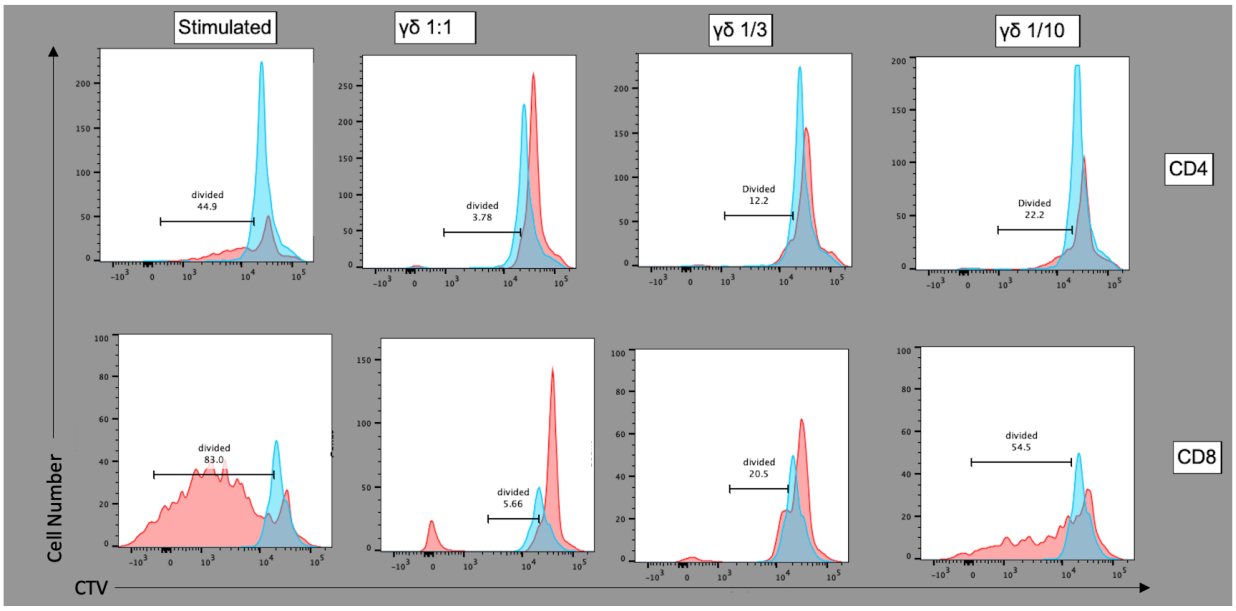

Figure 10. OT-II Splenic γδ T Cells Suppress T Cell Proliferation. CD45.1$^+$ C57Bl/6 T cells were separated from APCs by magnetic separation. γδ T cells were sterilely isolated from OT-II spleens and added to wells containing combined T cells and APCs at different ratios (1:1, 1:3, 1:10). Cells were stimulated with anti-CD3 in a 96-well plate and incubated at 37°C for 3 days. CTV expression is shown on the x-axis, and cell number is shown on the y-axis. Both CD4$^+$ and CD8$^+$ T cells are shown above. Stimulated samples represent CD45.1 T cells and APCs that were stimulated with anti-CD3 but did not receive any OT-II γδ's. Gating shows percentage of cells divided in each condition. N=1

Magnetic separation was used to isolate γδ T cells from the spleen of an OT-II mouse. To track proliferation of T cells, total T cells were isolated from a CD45.1 C57Bl/6 spleen and stained with cell trace violet. Total T cells were combined with non-labeled CD45.1 APC's, followed by the addition of 3 differing amounts of OT-II γδ T cells (1:1, 1:3, 1:10) from the spleen. We decided to test if addition of γδ T cells in these culture suppress T cell proliferation. Cells were then stimulated with anti-CD3 antibodies and incubated at 37°C for 3 days. Once adequate proliferation was observed, cells were harvested and stained for flow cytometry analysis.

After stimulation with anti-CD3 antibodies for 3 days, we observed that the CD45.1 CTV-labeled T cells and non-labeled APC's with the highest concentration of spleen γδ T cells (1:1) had a substantial decrease in proliferation among T cells (Figure 10). The suppression of T cells is seen in both $CD4^+$ and $CD8^+$ cells. In $CD4^+$ T cells, a 1:1 ratio of OT-II γδ T cells decreases the proliferation percentage from 44.9% to 3.78%. An even larger suppressive effect is seen in $CD8^+$ T cells, in which proliferation decreases from 83% to 5.66%. When less γδ T cells from the OT-II spleen were added to the splenocytes there was a decreased effect on suppressing proliferation of T cells, so it appears that the amount of suppression is dependent on the number of γδ T cells added. Similar data was observed in the γδ T cells isolated from OT-II intestines. Together these data support our hypothesis that $Foxp3^+$ γδ T cells have suppressive effects.

CHAPTER FOUR

DEVELOPMENTAL CONTROL OF HELIOS EXPRESSION BY GUT-ASSOCIATED T CELLS

Helios Expression by Fetal, Neonatal, and Adult IELs

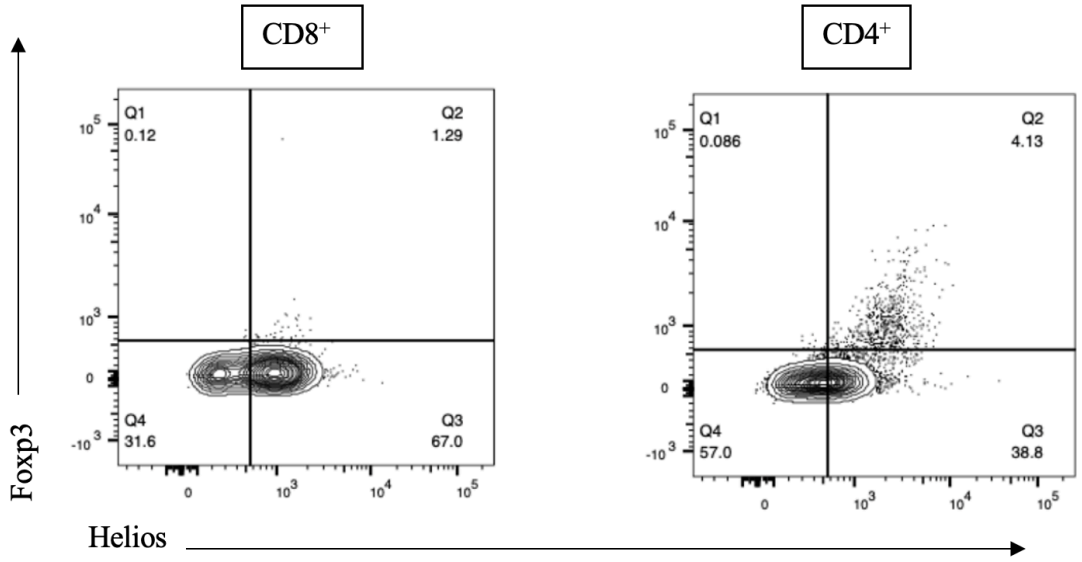

Figure 11. Helios and Foxp3 Expression in Umbilical Cord Blood. Total UCB was collected into blood collection bags containing a citrate phosphate dextrose solution. Lymphocytes were obtained via density centrifugation with Lymphopure medium. Cells were processed and stained with antibodies for analysis via flow cytometry. Gating in all panels shows Foxp3 and Helios expression in $CD8^+$ and $CD4^+$ subsets of live cells previously gated for $CD45^+$ $CD3^+$ cells. N=3

With the knowledge that the perinatal immune system takes on a highly tolerogenic state to allow for proper development, our lab previously studied potential transcription factors that may contribute to this state. From this, we discovered that Helios, which is important in the maintenance of Treg stability and suppression[176-179] was highly expressed in umbilical cord

blood (UCB) (Figure 11). Along with its appearance in human perinatal cells, we also observed high levels of Helios in murine intestinal γδ T cells. Due to these findings, we hypothesized that we would see higher Helios expression in fetal and neonatal intestines, due to their propensity to induce a tolerogenic state.

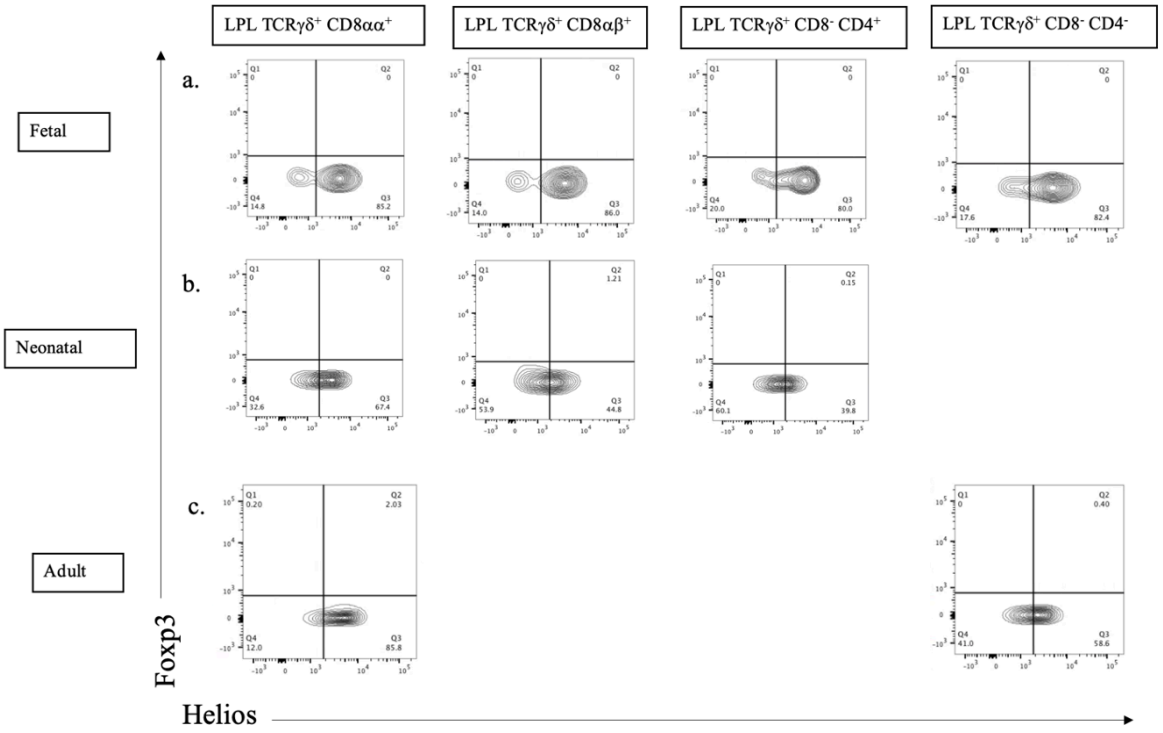

Figure 12. Helios and Foxp3 Expression in Fetal, Neonatal, and Adult IELs and LPLs. Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated from the small intestine of (a) fetal (ED17.5/18.5), (b) neonatal (5 day old), and (c) adult C57Bl/6 mice. Cells were processed and stained with antibodies for analysis via flow cytometry. Gating in all panels shows Foxp3 and Helios expression in the CD8αα$^+$ subset of live cells previously gated for CD45$^+$ CD3$^+$ TCRγδ$^+$ cells. N=3 (pooled fetal liver), N=3 (pooled neonatal liver)

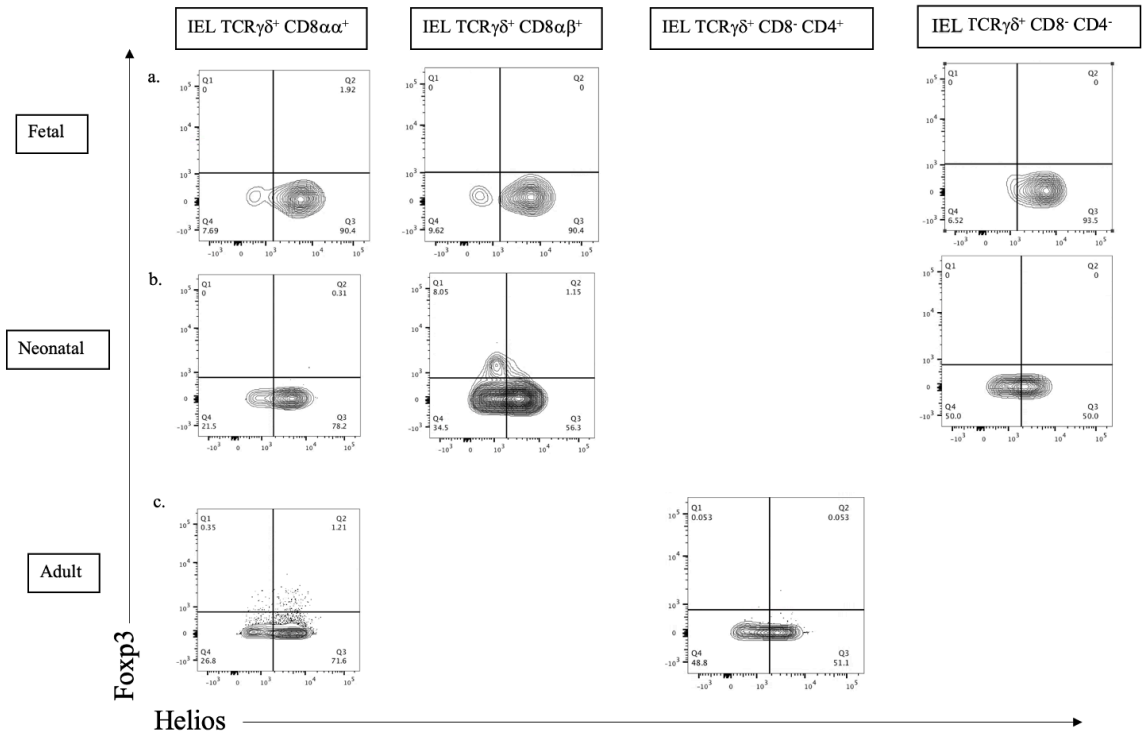

Figure 13. Helios and Foxp3 Expression in Fetal, Neonatal, and Adult LPLs. Lamina propria lymphocytes (LPLs) were isolated from the small intestine of (a) fetal (ED17.5/18.5), (b) neonatal (5 day old), and (c) adult C57Bl/6 mice. Cells were processed and stained with antibodies for analysis via flow cytometry. Gating in all panels shows Foxp3 and Helios expression in each subset of live cells previously gated for $CD45^+$ $CD3^+$ $TCR\gamma\delta^+$ cells. N=3 (pooled fetal liver), N=3 (pooled neonatal liver)

To test this, we analyzed the intestines and spleen from fetal (embryonic day 17.5/18.5), neonatal (5-day old), and adult B6 mice for Helios expression. Across all ages, Helios expression was most prominent in $CD8\alpha\alpha^+$ γδ T cells, which are the most abundant subset we see in the intestines. We observed the high Helios expression in $CD8\alpha\alpha^+$ γδ T cells from both fetal IELs and LPLs (Figure 12, 13). High Helios expression was also seen in intestinal $CD8\alpha\alpha^+$ γδ T cells of neonates, but expression at this age appears more similar to adult Helios expression than fetal (Figure 12, 13).

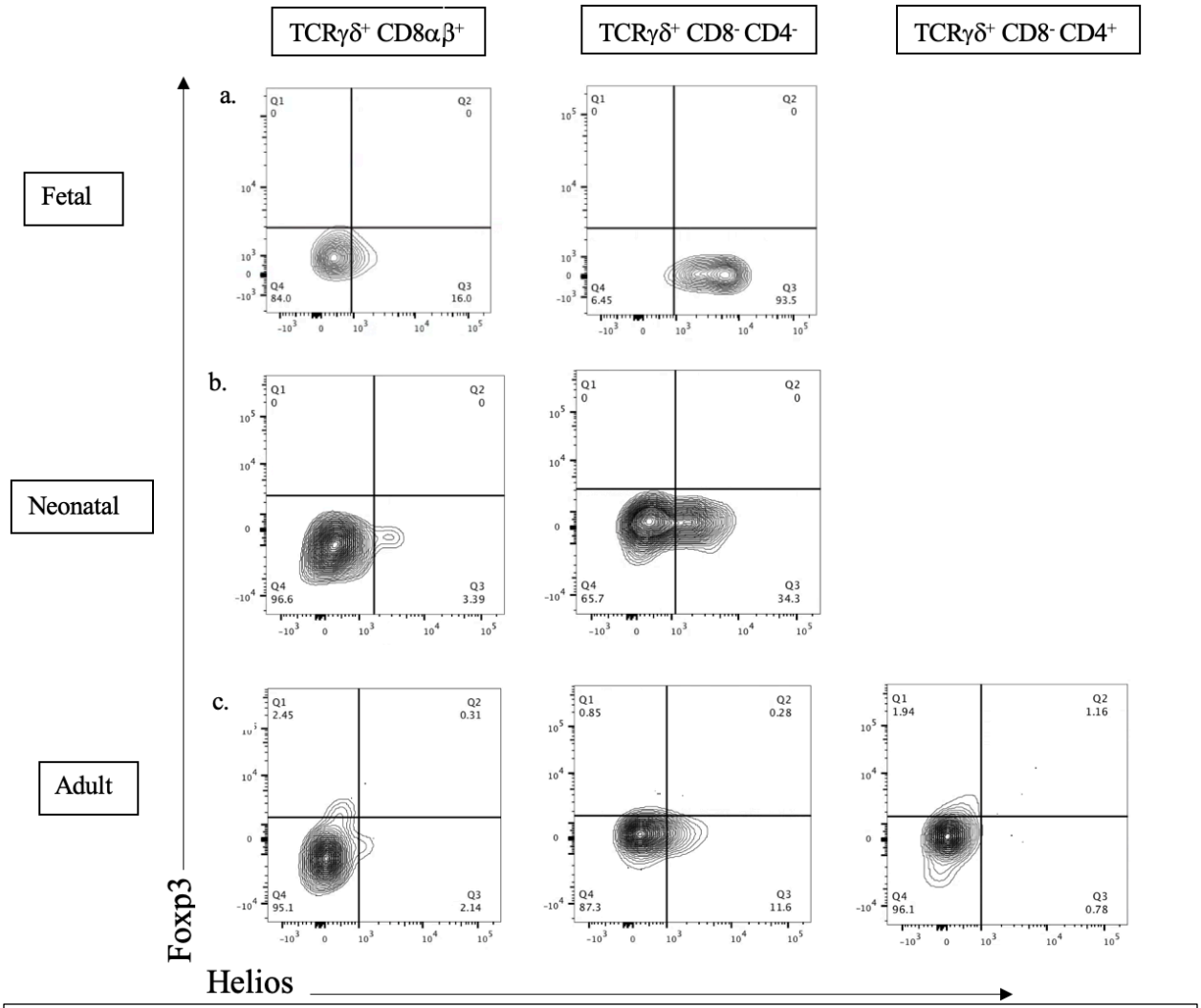

Figure 14. Helios and Foxp3 Expression in Fetal, Neonatal, and Adult Spleen. Splenocytes were isolated from the spleen of (a) fetal (ED17.5/18.5), (b) neonatal (5 day old), and (c) adult C57Bl/6 mice. Cells were processed and stained with antibodies for analysis via flow cytometry. Gating in all panels shows Foxp3 and Helios expression in subsets of live cells previously gated for $CD45^+$ $CD3^+$ $TCR\gamma\delta^+$ cells. N=3 (pooled fetal liver), N=3 (pooled neonatal liver)

While it appeared that Helios was highly expressed in intestinal γδ T cells across the 3 different age groups, we wanted to determine if this was specific only to the gut or if it was seen in the periphery. To do this, we analyzed Helios expression from fetal, neonatal, and adult spleens. We saw that Helios expression was decreased in $CD8\alpha\beta^+$ γδ T cells from each age group, but that fetal spleens had the highest expression overall (~93.5%) in $CD8^-$ $CD4^-$ γδ T cells

(Figure 14). While this subset still showed high Helios expression, it appeared that overall, Helios was decreased among splenic γδ T cells.

Overall, these data show that Helios is playing an important role in fetal development, especially within the intestines. Since the developing immune system lacks Tregs during the first few days after birth, it is possible that these Helios$^+$ γδ T cells are functioning to promote and maintain perinatal tolerance. With the decrease in expression in the spleens across all ages, it appears that peripheral T cells lose Helios expression right after birth. This contributes to the notion that Helios has an important role in the intestinal environment specifically.

Transferred Fetal and Adult HSC gives rise to Helios-expressing γδ T cells in RAG2KO Mice

The ability for Helios to potentially contain self-renewing capabilities was observed in acute myeloid leukemia, where IKZF2 was found to play an important role in driving self-renewal of leukemia stem cells and inhibiting myeloid differentiation[180]. Perinatal-derived γδ T cells, like those found at mucosal sites, have the ability to self-renew locally instead of being replaced by circulating precursors[181]. Helios is found in fetal hematopoietic sites and in adult HSCs[158], however its origin is still unknown. To address this gap in knowledge, we decided to pursue where Helios is originating from. We hypothesized that Helios$^+$ γδ T cells in the gut were coming exclusively from hematopoietic stem cells (HSCs) and that this expression was maintained by self-renewing properties. Since peripheral T cells appear to lose Helios shortly after birth, we proposed the concept that the T cells in the periphery lack this self-renewing property while intestinal γδ T cells continue to self-renew.

To test this, we set up an experiment using HSCs isolated from adult bone marrow and fetal liver of C57Bl/6 CD45.1 mice. Once processed, these cells were retro-orbitally injected into

previously irradiated RAG2KO mice, which lack T and B cells. Approximately 6-8 weeks later, we checked for adequate reconstitution of CD45.1$^+$ cells in the periphery prior to sacrifice. Upon successful reconstitution, the gut and spleen from these mice were analyzed for Helios expression. If our hypothesis was correct, then we would expect to see Helios expression only in the mice that had received HSCs from the fetal liver, and not those that had received the adult bone marrow HSCs.

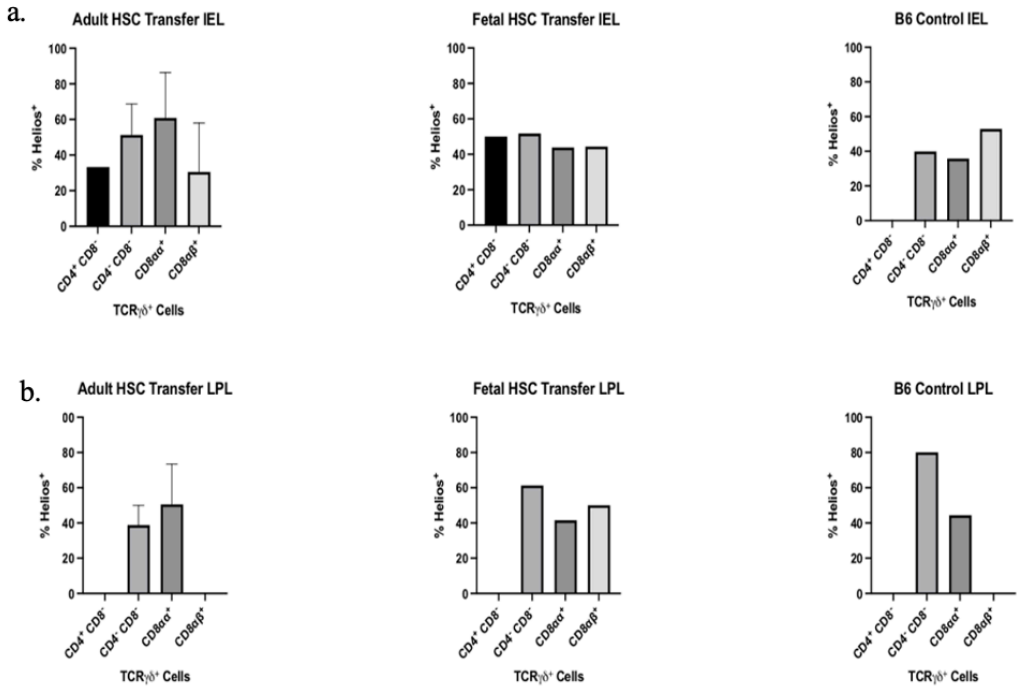

Figure 15. Helios Expression in TCRγδ$^+$ IEL & LPL Cells. Hematopoietic stem cells (HSCs) isolated from either adult bone marrow (adult HSC transfer) or fetal (ED17.5/18.5) liver (fetal HSC transfer) were retro-orbitally injected into RAG2 deficient mice. (a) intraepithelial lymphocytes (IEL) and (b) lamina propria lymphocytes (LPL) were both analyzed. Confirmation of reconstitution was done 6-8 weeks later through flow cytometry analysis confirming the presence of CD45.1$^+$ cells. Mice were then sacrificed, and their organs were analyzed for Helios expression. A C57Bl/6 mouse, pictured on the right, was used as a control. Cells in all panels are previously gated on live CD45.1$^+$ CD3$^+$ cells. N=3 (adult), N=3 (fetal), N=3 (B6)

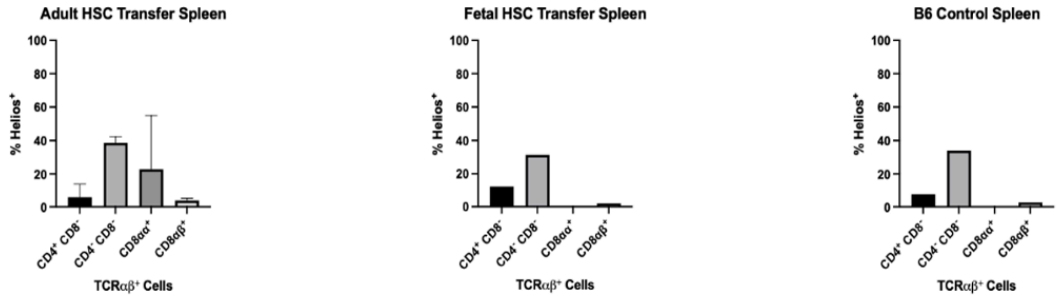

Figure 16. Helios Expression in TCRαβ$^+$ Splenocytes. Hematopoietic stem cells (HSCs) isolated from either adult bone marrow (adult HSC transfer) or fetal (ED17.5/18.5) liver (fetal HSC transfer) were retro-orbitally injected into RAG2 deficient mice. Confirmation of reconstitution was done 6-8 weeks later through flow cytometry analysis confirming the presence of CD45.1$^+$ cells. Mice were then sacrificed, and their organs were analyzed for Helios expression. A C57Bl/6 mouse, pictured on the right, was used as a control. Cells in all panels are previously gated on live CD45.1$^+$ CD3$^+$ cells. N=3 (adult), N=3 (fetal), N=3 (B6)

Our results however showed that Helios expression was observed in intestinal γδ T cells after HSC transfer from both fetal liver and adult bone marrow (Figure 15). We also observed similar levels of Helios expression in splenocytes after both fetal and adult HSC transfer (Figure 16). We initially isolated HSCs from embryonic day (ED) 17.5/18.5 fetal livers, but after observing a similar phenotype to adult HSCs, we speculated that this age may be too late in development to produce an accurate depiction of Helios expression in the fetus. Therefore, we decided to repeat the experiment with HSCs isolated from ED 15 fetal livers instead, with the hypothesis that HSCs from a less developed fetus may produce different Helios expression. We observed that in the RAG2KO mice that had received fetal liver HSCs form ED15, Helios expression was similar to both ED17.5/18.5 and adult HSCs (Figure 17).

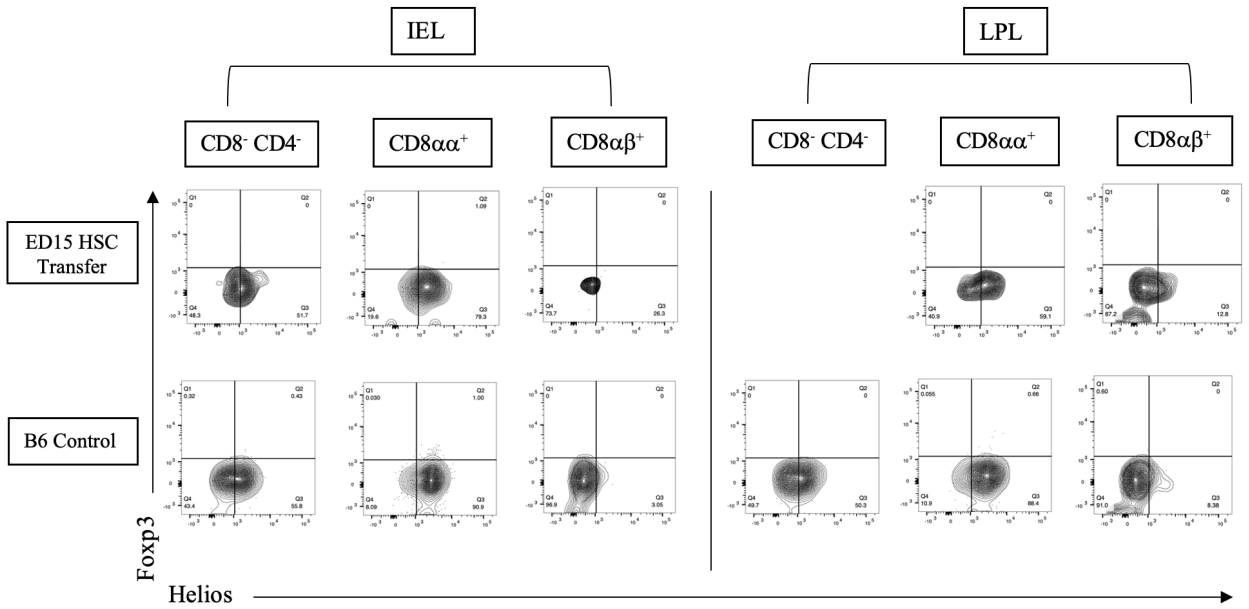

Figure 17. Gut γδ T Cell Helios Expression after ED15 HSC Transfer to RAG2KO Mice. Hematopoietic stem cells (HSCs) isolated from fetal (ED17.5/18.5) liver (fetal HSC transfer) were retro-orbitally injected into RAG2 deficient mice. Confirmation of reconstitution was done 6-8 weeks later through flow cytometry analysis confirming the presence of CD45.1$^+$ cells. Mice were then sacrificed, and their organs were analyzed for Helios expression. A C57Bl/6 mouse was used as a control. Cells in all panels are previously gated on live CD45.1$^+$ CD3$^+$ TCRγδ$^+$ cells. N=3

These data suggest that Helios expression is not determined at the stem cell level. Instead, we predict that there is either a specific subset of HSCs that become Helios$^+$, or Helios is induced by a specific environment such as in the intestines. If the latter is true, we would expect that a subset of T cells in the intestines would express Helios after localization in the tissue.

CHAPTER FIVE

DISCUSSION

Three Distinct Groups of γδ T Cells

γδ T cells are a unique subset of immune cells that have multiple functions within our bodies. With the ability to recognize both molecules associated with and without MHC[90-95], γδ T cells provide a variety of responses. They can present with both a Th1 and a Th2 phenotype[102-104], supporting the notion that these cells are functionally distinct from other T cells. With the knowledge of γδ T cells being multifunctional, we sought to expand our knowledge on this subset through a barcoded single cell RNAseq experiment. From this, we identified 3 individual subsets of TCRγδ$^+$ T cells within the intestines of C57Bl/6 mice. Each of the groups of γδ's appeared to be functionally distinct from one another, as they all were found to have unique differentially expressed genes. To explore this further, and potentially decipher functions of each subset of gut γδ T cells, we ran pathway analyses using data gathered from the scRNAseq experiment.

Our pathway analysis results showed that these 3 groups appear to have key functional differences that give insight into the role they are playing within the intestines. The first group of gut γδ T cells seem to play a role in the regulation of the immune defense response. With the expression of genes like IL-2Rβ and CD160, this group has the potential to act in a similar manner to, or in the recruitment of, regulatory T cells. Since these cells were only identified in the gut and not in the spleen, this regulation of immune response to antigens may be gut-specific

of these γδ T cells. A future direction regarding this subset of cells would be to look for similar genes to that of Tregs, which they cells appear to be acting similar too. If none, it may also be impactful to find markers specific to this cluster of γδ's in order to separate this group out for further studies.

The second group of γδ T cells that were identified appear to be important in the adaptive immune response, as well as in the regulation of lymphocytic activation. This subset of γδ's, unlike the first group, seem to be crucial in promoting activation of the immune response when necessary, and the gut is responding to foreign antigens. What is interesting however, is that typically we associated $CD4^+$ T cells with this type of response, but our data from the barcoded antibodies we included shows that this group, and neither of the other 2 γδ subsets express CD4. Instead, they all coexpress CD8α along with the TCRγδ. Since the majority of intestinal γδ's that we have observed are CD8αα, it may be possible that these different γδ subsets are all coming from $CD8\alpha\alpha^+$ γδ T cells. This group of γδ's also appears to have high expression of genes controlling the response to corticotropin-releasing hormone (CRH). CRH is a key element in the body's response to stress, as well as is present in inflammatory diseases. This function brings up the question of the role this subset of γδ T cells is playing in gut stress response, which potentially may be related to inflammatory diseases as well. If these cells were contributing to the response of CRH, it may be important to be further studied in the context of inflammatory bowel disease, since there may be dysregulation involving this subset of γδ T cells.

The third group of γδ T cells appear to be similar to the second group, in that they express genes important for cell activation. They were also found to increase the immune defense responses, with multiple genes associated with the recruitment of other immune cells, especially neutrophils. Their propensity to drive neutrophils to a site of attack supports the idea that this

group of γδ T cells are promoting the immune response upon microbial exposure. It would be important to test next if these cells produce high levels of IL-17, which promotes neutrophilic activation.

The overall functions of γδ T cells have been described by researchers previously, however, there has not been research done on functionally distinct subsets within γδ T cells. Our data provides evidence for the concept of three unique γδ T cell groups within the intestines that are potentially interacting together, but with separate goals, to maintain homeostasis of the gut environment. While the scRNAseq data and pathway analysis that was completed appear to provide new information regarding unique subsets of γδ T cells, it is important to note that our attempt at confirming the differentially expressed genes within each subset through protein analysis was not successful.

A Subset of Foxp3$^+$ γδ T Cells

The perinatal immune system is well recognized as existing in a highly tolerogenic state due to the lack of mature immune responses. We wanted to further explore the mechanisms behind how this state is achieved and maintained within the gut environment. To do this, we looked at phenotypes from fetal (ED 17.5/18.5), neonatal (5-day old), and adult (6-8 weeks) intraepithelial lymphocytes and lamina propria lymphocytes. Upon analysis, we discovered that a new subset of T cells was present within the intestines of the fetal and neonatal mice that was not present in the adult intestines. This subset coexpressed TCRγδ with the heterodimer CD8αβ. Further analysis showed that this group of CD8αβ$^+$ TCRγδ$^+$ T cells in neonatal intestines only had a small proportion of cells that were Foxp3$^+$. Since Foxp3 is an important transcription factor for Tregs, we hypothesized that this subset of cells may be playing a similar role within the fetal

and neonatal intestines prior to the development of Tregs, which do not appear until about 3 days after birth.

Since the newly identified subset of T cells, CD8αβ$^+$ TCRγδ$^+$, appeared to be a small percentage of cells, we decided to further explore a potential function for them by looking more broadly at Foxp3$^+$ γδ T cells. The only reports that have been published on Foxp3$^+$ γδ T cells were looking at γδ's that had been induced *in vitro* with TGFβ and IL-15. Due to their Foxp3 expression, we again hypothesized that these cells would have immunosuppressive effects. Some of our previous screening efforts on intestinal T cells in other mouse strains, identified that OT-II transgenic mice had high expression of Foxp3 in all subsets of γδ T cells, which we confirmed with flow cytometry. We hypothesize that this high Foxp3 expression may be due to a difference in γδ T cell development in OT-II mice. Our analysis shows that unlike wildtype B6 mice, the OT-II intestinal γδ T cells have low levels of Helios expression but significantly higher expression of Foxp3.

Using the spleen and gut from these OT-II mice, we set up a suppression assay to assess if the addition of Foxp3$^+$ γδ T cells would affect any proliferation after stimulation with anti-CD3. Analysis of this experiment showed that addition of both OT-II splenic and gut Foxp3$^+$ γδ T cells caused a substantial decrease in proliferation of CD45.1 T cells. These data support our hypothesis, showing the potential for Foxp3$^+$ γδ T cells to have immunosuppressive effects. However, these data would need to be repeated as this experiment has only been done once so far. It would also be important to check if the decrease in proliferation is due to actual immunosuppressive effects or if cytotoxicity played a factor, which could be done by looking at granzyme expression.

Fetal, Neonatal, and Adult IEL Helios Expression

The transcription factor Helios, encoded by the *IKZF2* gene, was discovered by our lab to be highly expressed in donated umbilical cord blood as well as in murine intestinal γδ T cells. Because Helios is important in Treg stability and suppression, we hypothesized that Helios may play an important role in the maintenance of perinatal tolerance before Foxp3$^+$ Tregs are present. This led us to also propose that Helios will be more expressed in fetal and neonatal intestines than in adult intestines, since Tregs are typically present in adult intestines to help maintain tolerance.

Analysis of fetal, neonatal, and adult intestines showed that overall, the fetal IELs and LPLs did indeed have the highest amount of Helios expression when compared to the other age groups. However, both neonatal and adult LPL were shown to express high levels of Helios as well, but its expression was significantly decreased in both of their IELs. To check for peripheral expression of Helios, we analyzed the spleens from each age group. Again, the highest expression of Helios was seen in the fetal spleens, but even this was significantly decreased from what we see in the fetal intestines. This led us to conclude overall that Helios is important in the development within the intestines but may play a lesser role elsewhere in the body. Helios$^+$ γδ T cells may be functioning to maintain the perinatal tolerance that is extremely important for their survival and proper development.

Fetal and Adult HSCs Promote Helios Expression

To try and further our knowledge on the origin of Helios, which would allow for a better understanding of the transcription factor, we decided to test where its expression is coming from. We hypothesized that Helios$^+$ γδ T cells in the intestines are originating from fetal HSCs and their expression is maintained by self-renewal. Since we see a decrease in Helios expression in

the periphery, we propose that these cells do not have this self-renewing property and therefore lose their Helios expression over time. To test this, we transferred HSCs from the fetal liver and adult bone marrow into RAG2KO mice. After waiting for adequate reconstitution of these cells, we analyzed their organs to compare Helios expression. If our hypothesis was correct, we would expect to see that only the mice that had received fetal liver HSCs would have Helios expression. However, our results showed the opposite of this, and we observed Helios expression after both fetal liver and adult bone marrow HSC transfer. These data tell us that Helios is not determined at the stem cell level, but that there is instead a potentially specific subset of HSCs that become positive for Helios. Another possibility for Helios expression is that it may be induced within specific environments, such as the intestines after HSCs localize in the tissues.

Conclusion

In conclusion, the findings presented in this thesis contribute new knowledge to the field of γδ T cells and the transcription factor Helios. With the highly informative data received from our barcoded single cell RNAsequencing experiment, 3 functionally unique subsets of γδ's were identified. Further research into each subset and the identification of potential markers would be an important next step. Along with this, a subset of $Foxp3^+$ γδ T cells has been identified and evidence for them playing an immunosuppressive role was shown. To strengthen this finding further, further rounds of the suppression assay would need to be completed and compared.

The research done regarding Helios expression and where it originates from contributes to our understanding of this transcription factor. Its high prevalence in fetal and neonatal intestines implies an important role for Helios in maintaining homeostasis, especially within the intestines. While we do not have a conclusive answer regarding where Helios is originating from, we were able to show that it does not originate from hematopoietic stem cells. There is still a lot

to understand about Helios and its function in the immune system, however the data presented furthers the current knowledge.

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VITA

Madison Flood was born in Oak Park, IL on December 11th, 1997. She grew up in Bolingbrook, IL, a southwest suburb of Chicago, with her loving mom and 3 siblings. She currently lives in Chicago with her boyfriend Jacob and their 3 cats. Madison attended Loyola University Chicago for both her undergraduate and graduate programs. She graduated in May 2020 with a B.S. in Molecular and Cellular Neuroscience, and a minor in bioethics. While at Loyola, she studied abroad in Rome, Italy, and is looking forward to traveling back there after graduation.

Upon entering the program in Infectious Disease and Immunology (INDIRI) in August 2020, Madison joined Dr. Makio Iwashima's lab as his work on T cells in perinatal immunology interested her. Madison's thesis focuses on the phenotypic analysis of γδ T cells and the origin of Helios expression. After graduation, Madison will be working as a medical writer for AbbVie.