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Determining the Genetic and Phenotypic Profile of Cord Blood Derived T Regulatory Cells and the Effect of Calcitriol on Immune Suppression

Anya Nikolai-Yogerst

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LOYOLA UNIVERSITY CHICAGO

DETERMINING THE GENETIC AND PHENOTYPIC PROFILE OF
CORD BLOOD DERIVED T REGULATORY CELLS AND
THE EFFECT OF CALCITRIOL ON IMMUNE SUPPRESSION

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY
ANYA M. NIKOLAI-YOGERST
CHICAGO, ILLINOIS
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ABSTRACT

Recent outbreaks in vertically transmitted viruses, such as Zika virus and HIV, has rejuvenated interest in fetal and neonatal immune tolerance. Babies are susceptible to vertically transmitted viruses because fetal and neonatal immune systems are considered to be immune privileged and will tolerate antigens presented to them by their mother. Thus, understanding the mechanisms of this tolerance is important to design effective treatments for infected mothers to protect their offspring. Here, I define a novel Treg, a type of T cell that mediates tolerance, named bidirectional T cells (BiT), found in human umbilical cord blood (UCB). These cells are induced by monocytes that provide transforming growth factor beta (TGF-β), a necessary molecule for Treg induction. BiT express classical Treg markers, such as Foxp3 and CD25, but also produce proinflammatory cytokines such as GM-CSF and IL-8. This profile suggests that BiT are capable of suppressing and activating the immune system. These differences suggest that BiTs may function differently from in vitro induced Tregs (iTregs), peripheral in vivo induced Tregs (pTregs) and thymus derived Tregs (tTregs).

Our lab has been able to show that physiological levels of calcitriol, the active form of vitamin D, is able to upregulate the induction of BiT. Furthermore, we also discovered that calcitriol upregulates NRP-1, a receptor and activator of TGF-β, on monocytes. We decided to further determine the effect of calcitriol on T cells and monocytes to better understand its role in the immune suppression. We found that
calcitriol promoted expression of molecules that turn off the immune system, CTLA-4 and PD-L1, on T cells. The NRP-1 expression on these monocytes was upregulated, which lead to increased migration of cells. The migration towards VEGF signifies what happens after tissue damage or during the growth of new blood vessels. In summary, we describe a novel Treg (BiT), and found that calcitriol promoted immune suppression by upregulating BiT, molecules that turn off the immune system, and IFN-β impeded the migration of cells to areas that require growth.
CHAPTER ONE: REVIEW OF THE LITERATURE

T Cell Subsets

T lymphocytes, or T cells, develop in the thymus and play key roles in adaptive immunological responses. T cells are activated through the stimulation of their T cell receptor (TCR) with peptide presented on a major histocompatibility complex (MHC), along with co-stimulation of CD28 by binding with CD80 or CD86 (Bjorkman et al., 1987; Haskins et al., 1983; Jenkins, Ashwell, & Schwartz, 1988; Lenschow et al., 1994; Madrenas, Chau, Smith, Bluestone, & Germain, 1997; Mueller, Jenkins, & Schwartz, 1989). Further, cytokines aid in stimulation and directing the function and differentiation of these T cells. There are two main lineages of T cells, defined by the composition of their TCR: αβ (alpha beta) and γδ (gamma delta) (Brenner et al., 1987; Meuer et al., 1983). The primary focus of this research is on αβ T cells, which divide into two subtypes, CD4 T cells and CD8 T cells. CD4 and CD8 are T cell co-receptors, which aid in TCR stimulation. They bind MHC class II (MHC II) and MHC class I (MHC I), respectively (Bjorkman et al., 1987; Madrenas et al., 1997). CD8 T cells are also known as cytotoxic T cells because upon activation they release perforin and granzymes, which lead to apoptosis of neighboring cells (Kisielow et al., 1975; Krähenbühl et al., 1988; Peters et al., 1991). On the other hand, CD4 T cells, also known as helper T cells, have multiple subsets.
CD4 T Helper Subsets and their Functions

CD4 T helper (Th) cells are divided into seven different subsets. Each subset is defined by the cytokines they produce and at least one key upregulated transcription factor. The existence of two distinct subtypes of CD4 T cells, Th1 and Th2 cells, were discovered in 1986 (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986). This study found T cell clones split into two distinct groups that produced different cytokine profiles upon stimulation, where Th1 produced IL-2 and IFN-γ while Th2 produced BSF-1, now known as IL-4 (Hsieh et al., 1993; Le Gros, Ben-Sasson, Seder, Finkelman, & Paul, 1990; Mosmann et al., 1986; Swain, Weinberg, English, & Huston, 1990). In the following years, the search began to find what factors lead to the clonal diversity of T cells. IL-12 was not discovered until 3 years after the discovery of Th1 cells, and it took until 1993 to discover that Th1 cells are induced by IL-12 (Hsieh et al., 1993; Kobayashi et al., 1989). Since IL-4 was already a known factor, the discovery that Th2 cells are induced by IL-4 came within four years of their initial discovery (Hsieh et al., 1993; Le Gros et al., 1990; Mosmann et al., 1986; Romagnani, 1999; Swain et al., 1990). At the time, reagents did not exist to determine which other factors were produced by Th1 and Th2 cells. However, it is now known that Th1 cells express the transcription factor T-bet and produce IFN-γ and TNF-α, while Th2 cells express GATA3 and produce IL-4, IL-5, IL-10, and IL-13 (Romagnani, 1999; Szabo et al., 2000; W. Zheng & Flavell, 1997). Th1 cells generate immune responses against infection caused by intracellular parasites, like bacteria and viruses. In contrast, Th2 cells are more prevalent in allergic responses, stimulate B cells to produce antibodies, and provide protection against helminths and
extracellular parasites (Mosmann et al., 1986; Romagnani, 1999).

Another more recently discovered Th subset is Th9. These cells express the transcription factors GATA-3 and IRF4, produce IL-9, are induced by the presence of IL-4, and also play a role in allergic response like Th2 cells, but also play a role in tissue inflammation (Dardalhon et al., 2008; Schmitt et al., 1994). However, in addition to IL-4, transforming growth factor-beta (TGF-β) is also necessary to induce these cells and for the expression of the transcription factors IRF-4 and FoxO1 (Dardalhon et al., 2008; Schmitt et al., 1994; Staudt et al., 2010; Veldhoen et al., 2008). The necessity and function of these cells outside of allergic disease are still under scrutiny, due to conflicting experimental results and since other cell types can produce IL-9 (Kaplan, 2013).

Three Th subsets all require the presence of IL-6 for their differentiation. These are Th22, Tfh, and Th17. Th22 is similar to Th9 and Th2 in the sense that these cells also play a role in allergic responses and tissue inflammation, but they express the aryl hydrocarbon receptor (AhR) as a key transcription factor and require TNF-α in addition to IL-6 for induction (Duhen, Geiger, Jarrossay, Lanzavecchia, & Sallusto, 2009; Trifari, Kaplan, Tran, Crellin, & Spits, 2009). These cells produce IL-22, TNF-α, and IL-13 (Duhen et al., 2009; Trifari et al., 2009). T follicular helper cells (Tfh) are located in germinal centers due to CXCR5 expression where they perform B cell help (Breitfeld et al., 2000; C. H. Kim et al., 2001; Schaefer et al., 2000). IL-6 and IL-21 are necessary for T cells to differentiate into Tfh and this leads to the upregulation of the transcription factor BCL-6 and subsequent production of IL-21, which further aids in germinal center
formation and B cell help (Bauquet et al., 2009; Chtanova et al., 2004; Eto et al., 2011; Johnston et al., 2009; Linterman et al., 2010; R. I. Nurieva et al., 2008; R. I. Nurieva et al., 2009; Vogelzang et al., 2008). The final subset that requires IL-6 for induction is Th17 (Zhou et al., 2007). These cells also require TGF-β and their dominant transcription factor is RORγT (Annunziato et al., 2007; Mangan et al., 2006; R. Nurieva et al., 2007). These cells play a role in tissue inflammation, autoimmunity, and clearance of extracellular pathogens (Annunziato et al., 2007; Langrish et al., 2005). They do this through the production of IL-17 and IL-22 (Annunziato et al., 2007; S. C. Liang et al., 2006; R. Nurieva et al., 2007; Yao et al., 1995).

The final type of Th cell are T regulatory cells (Tregs). Tregs only require the presence of TGF-β and IL-2 which then induces the transcription factor Foxp3, which is necessary for suppression (Bettelli et al., 2006; Fontenot, Gavin, & Rudensky, 2003; S. Fu et al., 2004; S. G. Zheng, Wang, Wang, Gray, & Horwitz, 2007). In fact, TGF-β signaling is necessary for Foxp3 transcription. Mice deficient in TGF-β1 develop a lethal lymphoproliferative autoimmune syndrome (Shull et al. 1992). These cells are unique in the fact that they suppress immune responses and promote immunological tolerance rather than promote inflammation or clearance of pathogens (Fontenot et al., 2003; Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995). These cells can further produce IL-10 and TGF-β, leading to more immune suppression (Asseman, Fowler, & Powrie, 2000). Lastly, these cells are also necessary for immunological tolerance, which is when cells determine if an antigen is self or non-self (Asseman et al., 2000; Sakaguchi et al., 1995). This dissertation focuses primarily on immune suppression and Tregs.
T Regulatory Cell Subsets

There are four main types of Tregs: tTregs, pTreg, iTreg, and Tr1 (Abbas et al., 2013). The first type of Treg to be defined were thymic Tregs (tTregs). Thymectomy and non-lethal irradiation were found to precede the development of autoimmune disease (Nishizuka & Sakakura, 1969; Penhale, Farmer, McKenna, & Irvine, 1973). This suggested that the thymus produces a suppressive cell. A few years later, studies showed that adding back total thymocytes or specifically CD4+CD8- from the thymus could prevent the onset of autoimmunity in thymectomized mice, suggesting the thymus gives rise to an immunosuppressive CD4+ T cell (Penhale et al., 1973; Sakaguchi, Takahashi, & Nishizuka, 1982). This finding suggested that Tregs are naturally generated in the thymus, and thus these Tregs were termed thymic Tregs (tTregs) (Itoh et al., 1999). Peripheral Tregs (pTregs) develop from naïve CD4 T cells in the periphery, rather than the thymus (Curotto de Lafaille, Lino, Kutchukhidze, & Lafaille, 2004; Kretschmer et al., 2005). To better understand Treg biology and in an effort to understand how to culture Tregs for therapeutic use, researchers sought to understand how to induce Tregs in a laboratory setting. Naïve CD4 T cells in vitro TCR stimulated in combination with recombinant TGF-β and IL-2 induces Tregs that can suppress to the same capacity as tTregs and pTregs both in vitro and in vivo (W. Chen et al., 2003; Davidson, DiPaolo, Andersson, & Shevach, 2007; Fantini et al., 2004; S. G. Zheng et al., 2007). The final type of Treg are type 1 Tregs (Tr1). These are induced in the periphery via IL-10 and produce high levels of IL-10 in comparison to other Treg subtypes (Gagliani et al., 2013; Groux et al., 1997). This Treg subtype is unique in the
fact that it does not express Foxp3, the key suppressive transcription factor of other
Treg subtypes. Tr1 also has unique markers, CD49b and Lag-3 on their surface to
distinguish them from other Treg subtypes which makes them easier to study (Gagliani

For years, the field has attempted to find differences in surface marker and
transcription factor expression to distinguish tTreg, pTreg, and iTreg subsets from each
other. The first marker to be discovered for Tregs was CD25. Suppressive cells from
thymocytes were found to express CD5 and low levels of CD45B, so the hunt was on to
find a specific marker for this subset. This study uncovered the IL-2 receptor alpha
chain, CD25. From here, the authors adoptively transferred CD25 depleted
spleenocytes to T cell-deficient recipients. The recipients that received CD25 depleted
cells, but not those that received total spleenocytes, suffered from severe autoimmunity.
In contrast, co-transfer of enriched CD4+CD25+ T cells prevented the development of
autoimmunity, definitively demonstrating that CD4+CD25+ cells are immunosuppressive
and required for immune homeostasis (Sakaguchi et al., 1995). Now, it is known that all
three types express CTLA-4, GITR, PD-1, and CD25 (Curotto de Lafaille et al., 2004;
Shevach & Thornton, 2014). There have been suggestions that the transcription factor
Helios and a secondary TGF-β receptor, neuropilin-1 (NRP-1), distinguish tTregs from
other Treg subtypes (Curotto de Lafaille et al., 2004; X. Lin et al., 2013; Shevach &
Thornton, 2014). However, in humans, NRP-1 is no longer considered a marker for
Tregs and Helios can be expressed in other Treg subsets (Milpied et al., 2009).
The expression of the master transcription factor Foxp3 is shared among tTregs,
pTregs, and iTregs. In 2003, three groups defined this transcription factor as the functional driver of suppression in Tregs (Fontenot et al., 2003; Hori, Nomura, & Sakaguchi, 2003; Khattri, Cox, Yasayko, & Ramsdell, 2003). These groups found that mice without the presence of Foxp3, also known as scurfy mice, succumb to severe lymphoproliferative diseases and autoimmunity (Fontenot et al., 2003; Khattri et al., 2003). Scurfy mice have a missense mutation in foxp3, however it was discovered that addition of CD4+CD25+Foxp3+ T cells or retroviral foxp3 gene transfer into these mice rescued them from disease (Fontenot et al., 2003; Hori et al., 2003). A follow-up study using a CD4 specific knockout of Foxp3 was sufficient to induce autoimmunity (Fontenot et al., 2005). In humans, immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome occurs when Foxp3 is mutated (Bennett et al., 2001). This disease leads to rapid formation of autoimmunity, and if not treated (typically with a bone marrow transplant), the patient will die (Bennett et al., 2001). From these studies, Foxp3 is essential and non-redundant for stabilizing the CD4+CD25+ Treg lineage and controlling Treg development and function (Gavin et al. 2007; Fontenot et al. 2003).

While tTregs, pTregs, and iTregs express Foxp3 in order to be functionally suppressive, there is evidence that Foxp3 is differentially regulated on the epigenetic level via variances in methylation (Kitagawa, Ohkura, & Sakaguchi, 2013; Lal et al., 2009; Morikawa et al., 2014). These variances are still the only true marker to distinguish between different Treg subsets (Minskaia et al., 2018). Epigenetic regulation by methylation occurs at the 5' -cytosine of CpG dinucleotides (Morikawa et al., 2014). Accessible CpG dinucleotides are called CpG islands, and methylation at these sites
leads to downregulation of transcription by preventing binding of transcription factors near these sites (Kitagawa et al., 2013; Minskaia et al., 2018). Foxp3 has three conserved non-coding sequences (CNSs) (Y. Zheng et al., 2010). Induction of Foxp3 is controlled by TGF-β signaling activating the transcription factor Smad3. Smad3 binds to the Foxp3 locus on the CNS1 region where it positively regulates the transcription of Foxp3 (Tone et al. 2008). tTregs are defined as having DNA hypomethylation on Foxp3 CNS2 (Morikawa et al., 2014; Someya et al., 2017; Y. Zheng et al., 2010). The CNS2 aids in stability of Foxp3 expression (Someya et al., 2017; Y. Zheng et al., 2010). In contrast, iTregs and pTregs have increased CNS2 methylation, and rather demethylation of CNS1 (Y. Zheng et al., 2010). CNS1 aids in iTreg and pTreg generation, while CNS3 controls Foxp3 expression in precursor T cells (Y. Zheng et al., 2010). CNS1 is dispensable for tTreg induction, however T cells from mice lacking CNS1 were unable to generate pTregs/iTregs and instead had excessive Th2 mediated inflammation. This demonstrated the importance of the CNS1 region for the generation of pTregs and iTregs (Josefowicz et al., 2012). In mice, Foxp3 is expressed only by T cells with regulatory function. However, in humans, Foxp3 is transiently expressed in all activated T cells, but is only stably maintained in Tregs due to hypomethylation of CNS2 (Gavin et al., 2006).

Taken together, there are multiple important Treg subsets in humans that are functionally suppressive. All but one subset express Foxp3, and the epigenetic regulation of Foxp3 is different depending on the type of Treg. However, besides this methylation, it is not well understood what the genetic differences are between different
types of Tregs and what markers can be used to distinguish between them.

**Immune Checkpoint Molecule Expression and Regulation**

The immune system has natural mechanisms besides Tregs to regulate T cell responses, which are referred to as checkpoint molecules (Chikuma, 2017). Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) inhibits T cell co-stimulation, and thus, inhibits activation of T cells, because it has a roughly 10X higher affinity than CD28 to bind co-stimulatory molecules CD80 and CD86 (Krummel & Allison, 1995; Linsley et al., 1994; van der Merwe, Bodian, Daenke, Linsley, & Davis, 1997; Walunas et al., 1994). CTLA-4 was later reported to have both cell-intrinsic and cell-extrinsic effects (Jain, Nguyen, Chambers, & Kang, 2010). This means that while cells that express CTLA-4 undergo anergy after contact with CD80 or CD86 (intrinsic) it has also been observed that these cells are able to extrinsically suppress neighboring cells (Jain et al., 2010; Wang et al., 2012). CTLA-4 was discovered when mice lacking it had T cells with high reactivity to self-tissues, uncontrolled proliferation, and died due to tissue infiltration within three weeks (Brunet et al., 1987; Waterhouse et al., 1995). Due to the loss of CTLA-4 and Foxp3 leading to similar phenotypes, there was an interest in studying whether these genes were part of a common pathway (Walker, 2013). However, it was found that they were not regulated in the same pathway, since mice lacking one would have an increase of the other (Walker, 2013). But there is supporting evidence that while Tregs lacking CTLA-4 can be suppressive, their suppressive function is enhanced when they co-express Foxp3 and CTLA-4 (Walker, 2013). More studies need to be further conducted on this topic because study and method variations have led to a
plethora of conflicting results on the importance and function of CTLA-4 on Tregs.

Another key checkpoint molecule is programmed death-1 (PD-1) (Ishida, Agata, Shibahara, & Honjo, 1992). This receptor binds to programmed death ligand-1 and -2 (PD-L1 and PD-L2, respectively) on B, T, myeloid, and dendritic cells. Activation of this signaling pathway leads to anergy or cell death of cells expressing PD-1 (Dermani, Samadi, Rahmani, Kohlan, & Najafi, 2019). Similar to CTLA-4, there is evidence of the PD-1/PD-L1 axis having a functional effect on Tregs. In an umbilical cord blood study, it was found that using monoclonal antibodies to block PD-1/PD-L1 signaling resulted in a significant reduction in the Foxp3+ T cell population, suggesting a role in Treg induction (de Roock et al., 2011). In agreement with this, in an APC PDL1-/- model, these mice were unable to generate pTregs, further demonstrating an important role in PD-1/PD-L1 signaling in Treg induction (Francisco et al., 2009). This study continued further to demonstrate that PD-L1 is able to enhance Foxp3 expression due to inhibiting the Akt/mTOR signaling cascades, which is known to inhibit Treg induction (Francisco et al., 2009).

For T cells, PD-1 expression is induced upon TCR stimulation (Chikuma et al., 2009). This expression is upregulated on activated T cells but reduces over time in the absence of TCR stimulation (Chikuma et al., 2009; S. Simon & Labarriere, 2017; Youngblood et al., 2011). PD-1 is considered to be the first marker of T cell activation, which is induced within 24 hours of initial antigen exposure (Chikuma et al., 2009). Mutation of the NFAT binding sight in the PD-1 promoter led to loss of promoter activity, suggesting the transcription factor NFAT directly regulates PD-1 transcription; NFAT is
downstream of TCR and calcium signaling events (Oestreich, Yoon, Ahmed, & Boss, 2008). In contrast to its receptor, PD-L1 is expressed constitutively on T cells and is further upregulated after TCR stimulation (Sharpe, Wherry, Ahmed, & Freeman, 2007). PD-L1 expression is also regulated by multiple pathways and factors, such as hypoxia, epigenetic modifications, AP-1, and tumor suppressors (Shen et al., 2019; Sun, Mezzadra, & Schumacher, 2018).

Similar to PD-1, CTLA-4 is not expressed on the surface of resting or naïve T cells but is induced after TCR stimulation (Lindsten et al., 1993). When CTLA-4 is not on the surface of T cells, it is often found stored in lysosomes, endosomes, or intracellular vesicles in memory T cells (Iida et al., 2000; Linsley et al., 1996). Intracellular calcium has been found to promote CTLA-4 surface expression and after TCR stimulation cell surface CTLA-4 was found to localize towards the site of activation (Linsley et al., 1996). It was later found that lysosomal vesicles release their stores of CTLA-4 in memory T cells towards the surface of cells allowing for increased expression post-activation (Iida et al., 2000). However, CTLA-4 expression is not upregulated on the surface of naïve T cells until after 48-72 hours, and the peak in mRNA expression occurs between 24-48 hours post-activation (Jago, Yates, Câmara, Lechner, & Lombardi, 2004; Krummel & Allison, 1995; Lindsten et al., 1993; Linsley et al., 1992; Metzler, Burkhart, & Wraith, 1999; Walunas et al., 1994). CTLA-4 is regulated by rapid membrane recycling via the clathrin-dependent internalization pathway by binding to AP-2 via a YVKM motif ((Bradshaw et al., 1997; Chuang et al., 1997; Nakaseko et al., 1999; Perkins et al., 1996; Schneider & Rudd, 2014; Shiratori et al.,
1997; Y. Zhang & Allison, 1997). This pathway allows for either recycling or degradation of CTLA-4 in lysosomes which allows for control of total CTLA-4 levels (Chuang et al., 1997; Iida et al., 2000; Shiratori et al., 1997). Akin to PD-1, NFAT has also found to play a role in positively regulating CTLA-4 specifically in Tregs and CD4 T cells, rather than CD8 T cells (Chan et al., 2014; Gibson et al., 2007; Takahashi et al., 2000; Y. Wu et al., 2006). Taken together, understanding the regulation and expression of these molecules is important for determining the suppressive capability of the cells expressing them.

**Fetal/Neonatal Tolerance versus Adult Immune Tolerance**

**The Discovery of Fetal Tolerance**

Immunological tolerance is the state of unresponsiveness towards an antigen. Tolerance allows for the immune system to not attack “self” or host antigens. It is important to understand the mechanisms of tolerance, as failure of tolerance to host tissues can result in autoimmunity and tolerance to foreign antigens varies from person to person. Fetal tolerance was first discovered in 1945 by Ray D. Owen (Owen, 1945). In his study, he found that blood flow shared in the fetus between dizygotic cattle twins results in exchange of blood cells and hematopoietic cells in utero. As these calves grew into adulthood, they maintained blood cells from their twin. In fact, Owen found that twins from different bulls (fathers) also had identical blood patterns (Owen, 1945). In response to this study, Medawar and colleagues tested the extent of tolerance from these mosaic cattle (Anderson,Billingham, Lampkin, & Medawar, 1951). Allogeneic skin grafts were known to be rejected in humans and rabbits due to robust immune responses to non-self-tissues (Anderson et al., 1951). However, they found that
dizygotic twins, regardless of gender, did not reject the skin grafts from each other (Anderson et al., 1951; Holick et al., 1980). Two years later, a follow-up study in mice was performed. In this experiment, they demonstrated that CBA mice (strain A) injected in utero with homogenized tissues from a second strain of mice (strain B) did not reject a skin graft from strain B eight weeks post-birth (Billingham, Brent, & Medawar, 1953). However, strain A mice still had the ability to reject the skin graft from a third, unrelated strain of mice (strain C) (Billingham et al., 1953). The observations from these three seminal studies demonstrated that exposure to foreign antigens in utero generate long-term antigen-specific tolerance.

While these experiments demonstrated that fetal tolerance exists, the mechanism for how it is established is still not well understood. In recent years, studies have found that fetuses generate Tregs that promote tolerance to in utero exposed antigens. Umbilical cord blood (UCB) studies and pre-mature birth studies have demonstrated an increased percentage of Tregs in comparison to adults (Dirix, Vermeulen, & Mascart, 2013; Takahata et al., 2004). While the basal percentage of Tregs in UCB is not different from adult blood, we and others have described that UCB has a higher propensity to expand and differentiate into Tregs (C. C. Chang et al., 2005; de Roock et al., 2011; J. Lee et al., 2020; Torelli et al., 2012). In agreement with discoveries made by Owens, Billingham, and Medawar, human fetuses encounter maternal DNA that crosses the placental barrier. Studies have found that tolerance to these antigens persists into adulthood and promotes tolerogenic Tregs in utero (Maloney et al., 1999; Mold et al., 2008). These data suggest that fetal tolerance is in
part due to an environment that leads to a higher propensity for Treg induction. These Tregs aid both in establishing self-tolerance and tolerance to maternal allo-antigens. More needs to be done to understand why there are more Tregs in UCB and if there are any genetic differences between these Tregs and those found in adults.

**Tolerance and Aging**

Above, I explain the history of fetal tolerance and how tolerance in early life can impact patterns in lifelong tolerance. However, in this section, it is important to discuss the differences in tolerance and Tregs as humans age. As neonates, T cells have a higher propensity to develop into Tregs to promote tolerance to self and maternal antigens. pTregs make up 3% of the total CD4+ repertoire at birth, in which they have been found to persist in the body for an extended period of time where they assist in promoting tolerance and an anti-inflammatory immune environment (Burlingham et al., 1998; Mackroth et al., 2011; A. K. Simon, Hollander, & McMichael, 2015; Takahata et al., 2004). As the adaptive immune system develops after birth in response to antigen stimulation and exposure to their own gut microbiota, Treg cell numbers decline while other Th subsets increase to match the proportion of naïve T cells (Arismendi, Kallás, Santos, Carneiro-Sampaio, & Kayser, 2012; Santner-Nanan et al., 2008; Shearer et al., 2003). This change is rather rapid, where evidence shows that cord blood, peripheral blood post-birth, 1 week, 4 weeks, and 12 weeks post-birth all have unique immunological signatures; in fact, the Treg population falls drastically in the first 36 months of age (Olin et al., 2018; Shearer et al., 2003; Zhao et al., 2007).

As humans age, there is a reduction in the function and size of the thymus, this
process is known as thymic involution (Boehm & Swann, 2013). The thymus is largest at birth through the first five years of life and then atrophy begins. This results in a decline in thymopoeisis, the production of T cells, for both Tregs and naïve T cells emigrating from the thymus. Thiault and colleagues sought to determine whether Tregs or other T cell populations were more affected by thymic involution and observed that kinetics of reduction in Treg versus conventional T cells (Tconv) are different (Thiault et al., 2015). Treg emigration declines at a significantly faster rate than Tconv (Thiault et al., 2015). The field now hypothesizes that with age, Tregs display an effector/memory phenotype and circulate back to the thymus where they then compete for available IL-2, thus inhibiting the production of more Tregs while allowing for continued production of Tconv cells (Arismendi et al., 2012; Darrigues, van Meerwijk, & Romagnoli, 2018; Santner-Nanan et al., 2008; Thiault et al., 2015). The reduction in the pTreg repertoire has also been reported in the elderly (Darrigues et al., 2018; Jagger, Shimojima, Goronzy, & Weyand, 2014). This decline in the Treg population and function has been a suggested cause for the increase in prevalence of autoimmunity, inflammation, and infection in the elderly, along with a reduction in naïve T cell population leading to a decreased repertoire that can be primed against new antigens (Elyahu et al., 2019; Fessler, Ficjan, Dufneter, & Dejaco, 2013; Fujimaki et al., 2008; Jagger et al., 2014; Santner-Nanan et al., 2008). These examples demonstrate a stark difference between the available Treg populations in adolescence and adulthood in comparison to fetal and neonatal stages. However, it is not well understood at the gene level if there are differences in the Treg repertoires at different age points.
Organ Development and Immune/Inflammatory Response

Effect of Infections and Inflammation on Organ Development

Fetal/neonatal tolerance is essential for survival. TORCH infections (Toxoplasma gondii, other (such as HIV, Listeria monocytogenes, and parvovirus), Rubella, Cytomegalovirus (CMV), and Herpesviruses (HSV)) demonstrate when tolerance is overcome and inflammation occurs to combat the infection (Arora, Sadovsky, Dermody, & Coyne, 2017). TORCH infections are infections capable of breaking the maternal-placental barrier that activate an immune response that can lead to abnormal placental development, miscarriages, fetal demise, neural tube defects, abnormal brain development, pre-term labor, and abnormal lung development (Arora et al., 2017; Yockey & Iwasaki, 2018). Varying immune responses can activate based on the type of the infection, leading to the production of cytokines which cause different birth complications (Yockey & Iwasaki, 2018).

The organ most often impacted by infections during development is the brain (Cordeiro, Tsimis, & Burd, 2015; Phillips et al., 2016; Silasi et al., 2015; Yockey & Iwasaki, 2018; Yockey et al., 2018). Neurulation begins three weeks into gestation and brain development continues through the first five months of gestation. After that, myelination begins and continues into adulthood. There have been numerous studies demonstrating that viral, bacterial, and fungal infections during pregnancy lead to brain deformities, seizures, psychiatric disorders (such as bipolar disorder and schizophrenia), neurodevelopmental delays, autism, cognitive impairment, and mental retardation (Cordeiro et al., 2015; Phillips et al., 2016; Silasi et al., 2015; Yockey et al.,
Currently, there are no treatments or preventative measures against these infections.

Of interest, viral vertical transmission is detrimental to the developing fetus. Viral infections cause an upregulation in inflammatory cytokine expression of IL-6, TNF-α, IL-1β, IFN-γ, and IFN-α/β (Yockey & Iwasaki, 2018). Mouse and rat studies have demonstrated that in utero parvovirus infection leads to a 90-fold increase of IFN-β expression; trophoblast cells respond to type 1 IFNs by producing IFN-stimulated genes which results in impaired differentiation, reduced developmental capacity, and fetal growth (Baines et al., 2020; Rostovsky & Davis, 2015). Furthermore, it was found that type 1 interferons (IFN-α/β) were responsible for growth restriction, microcephaly, spontaneous abortions, and fetal demise after Zika virus infection (Yockey et al., 2018). Supporting the evidence of IFN-β negatively affecting fetal and placental sufficiency, a case study of a woman taking IFN-β to control her multiple sclerosis (MS) during her pregnancy lead to severe fetal growth restriction, pre-term birth, respiratory distress, and severe pre-eclampsia (Salahudheen & Begam, 2016). Neural stem cells are capable of responding to viral infection and are a known source of IFN-β (J. Y. Lin, Kuo, & Huang, 2019). Taken together, these studies highlight that the anti-viral immune response is detrimental to fetus development. However, the IFN anti-viral response is important for combating infection, and without the IFN response against the virus there is increased maternal and fetal disease severity (Racicot et al., 2017). These infections highlight the need for a better understanding of how infections are able to cross the maternal-placental barrier and how to develop vaccinations that work against them in
uterine, so the immune system does not activate and lead to detrimental health outcomes.

Besides the direct effects on the inhibition of fetal development, IFN-β is also a potent inhibitor of angiogenesis, the sprouting of new blood vessels. Type I IFNs are produced by multiple tissues upon viral and bacterial infections and play essential roles in eliciting an anti-viral and anti-microbial immune response (Perry, Chen, Zheng, Tang, & Cheng, 2005). IFN-β, a type I IFN, has previously been shown to inhibit angiogenesis by upregulating nitric oxide and reducing growth and chemotactic factor production for monocytes and endothelial cells (Hallene et al., 2006; K. J. Kim et al., 1993; Takano, Ishikawa, Matsuda, Yamamoto, & Matsumura, 2014; Yang et al., 2003; Zeiher, Fisslthaler, Schray-Utz, & Busse, 1995; H. Zheng et al., 2011).

Vascular endothelial growth factor (VEGF) plays a pivotal role in angiogenesis by acting as a chemotactic factor for endothelial cells and monocytes (Heil et al., 2000; Hong, Ryu, & Han, 2005). It also promotes an increase in permeability of blood vessels and cell adhesion at the site of angiogenesis (Heil et al., 2000; Hong et al., 2005; P. Lee et al., 2002; Zeiher et al., 1995). Angiogenesis is particularly important during placental and fetal development because proper blood flow is necessary for both the health of the pregnancy and for the fetus’ development (Mayhew, Charnock-Jones, & Kaufmann, 2004; Reynolds et al., 2006). This blood flow aids in nutrient transfer from mother to fetus (Mayhew et al., 2004; Reynolds et al., 2006). There is evidence that when utero-placental blood flow and placental blood flow is restricted there is decreased fetal growth, vascularity, and an increased probability for compromised pregnancies
The role of angiogenic factors on organogenesis demonstrate these factors are able to signal to organs to instruct patterning and morphogenesis during formation (Crivellato, 2011). For example, VEGF signaling plays a necessary role in heart development, neural stem cell proliferation, kidney vasculogenesis, glomerulogenesis, and tubulogenesis, physiological and pathological lung formation, bone, and neural development (Crivellato, 2011). Together, these data demonstrate that angiogenesis is necessary for proper development in the placenta and fetus. IFN-β is a potent inhibitor of angiogenesis, suggesting that this cytokine can impede both brain and fetal development during viral infection. Inflammation resulting from responding to infection can be detrimental to the fetus/neonate developmentally, where tolerance allows for proper growth.

**Angiogenesis, Monocytes, and Neuropilin-1**

The previous section highlights the importance of angiogenesis during fetal development and how angiogenesis can be disrupted when the immune system becomes inflammatory. Originally, endothelial cells were highlighted as the cell type involved in angiogenesis. However, it is now known that monocytes and macrophages also play key roles in angiogenesis (Dalton et al., 2014; Fantin et al., 2013; Gerhardt et al., 2004; P. Lee et al., 2002). Hypoxic conditions stimulate angiogenesis. This is due to the fact that myeloid cells respond to hypoxia by producing hypoxia inducible factors 1-alpha and 2-alpha (HIF-1α and HIF-2α, respectively). These factors promote transcription of genes involved in angiogenesis, such as VEGF (Büchler et al., 2003;

VEGF is the key cytokine involved in the recruitment of monocytes and is a necessary factor for angiogenesis (Carmeliet et al., 1996; Heil et al., 2000; Hong et al., 2005). Endothelial cells, macrophages, and monocyte-derived dendritic cells (mo-DCs) can all produce VEGF after HIF exposure (Dalton et al., 2014; Plaks et al., 2008). Depletion of monocytes and mo-DCs has been found to terminate pregnancies due to loss of production of VEGF and subsequent reduced angiogenesis (Plaks et al., 2008). Monocytes can express all four VEGF receptors, VEGFR1, VEGFR2, VEGFR3, and neuropilin-1 (NRP-1) (Alkharsah, 2018; Gelfand et al., 2014). VEGF acts as a chemoattractant for monocytes in two ways: (1) by directly binding VEGF receptors and (2) through stimulating endothelial cells to produce monocyte chemoattractant protein (MCP-1); both of which attract monocytes toward the endothelial tip during angiogenesis (Dejda et al., 2014; Heil et al., 2000; Hong et al., 2005). Once recruited to the tip, these monocytes produce platelet-derived growth factor (PDGF) which promotes mitotic activity in endothelial cells and vascular smooth muscle cells (Carrer et al., 2012). These monocytes also endocytose oxidized lipids, turning them into foam-cells. These foam cells stimulate smooth muscle cell migration and stimulate a change in endothelial function leading to vascular remodeling (Dalton et al., 2014; Jaipersad, Lip, Silverman, & Shantsila, 2014).

NRP-1 is a pleotropic receptor that is able to bind VEGF, SEMA3A, and TGF-β (Roy et al., 2017). The expression of NRP-1, the fourth VEGF receptor, on monocytes
has been found to be essential for angiogenesis (Dejda et al., 2014; Fantin et al., 2013; Gerhardt et al., 2004; P. Lee et al., 2002). NRP-1 knockout mice die by embryonic day 13 due to a lack of vascular development. This receptor allows for the monocytes to migrate toward VEGF where the monocytes can then aid in promoting angiogenesis (Dejda et al., 2014; Fantin et al., 2013; Gerhardt et al., 2004; P. Lee et al., 2002). The importance of angiogenesis and monocyte recruitment is well understood in the context of development. However, it is not well known what regulates the expression of NRP-1 on monocytes.

**Immune Modulation by Calcitriol**

**Calcitriol Biosynthesis**

Vitamin D is an essential vitamin for humans. Originally discovered due to an increase in rickets during the Industrial Revolution, vitamin D was first described to be helpful aiding in calcium uptake (McCollum, Simmonds, Becker, & Shipley, 1922; Mellanby, 1989). Vitamin D is a fat-soluble vitamin which is found naturally in fatty fish, beef liver, cheese, egg yolks, and fortified foods such as dairy products, orange juice, and cereal. However, the main source of vitamin D occurs from synthesis in the skin after exposure to UV radiation, such as the sun (Holick et al., 1980; Holick, MacLaughlin, & Doppelt, 1981; Holick et al., 1979).

UV-B irradiation of the skin leads 7-dehydro-cholesterol (7-DHC, also known as pro-vitamin D₃) to convert into pre-vitamin D₃, where heat then gradually converts it into vitamin D₃ (Feingold et al., 2000; Holick et al., 1980; Holick et al., 1981; Holick et al., 1979; Windaus, Schenck, & von Werder, 1936). Vitamin D₃ synthesized in the skin is
the same as what is derived from animal food sources, and we can also gain vitamin D₂ from plant sources (Askew, Bourdillon, Bruce, Jenkins, & Webster, 1930; Feingold et al., 2000). From here, vitamin D₃/₂ is transported to the liver. In the liver, enzymes CYP2R1 and CYP27A1 hydroxylate vitamin D₃/₂ into 25(OH)D (calcidiol), which is the major circulating form of vitamin D (Ichikawa et al., 1995; Thacher, Fischer, Singh, Roizen, & Levine, 2015; Usui, Noshiro, & Okuda, 1990; J. G. Zhu, Ochalek, Kaufmann, Jones, & Deluca, 2013). Here, calcidiol circulates to the kidneys or encounters macrophages which express CYP27B1. CYP27B1 further hydroxylates calcidiol into 1α,25(OH)₂D (calcitriol), which is the active metabolite of vitamin D that mediates the hormonal and immunological actions of vitamin D (Feingold et al., 2000; G. K. Fu et al., 1997; Shinki et al., 1997; St-Arnaud, Messerlian, Moir, Omdahl, & Glorieux, 1997; Takeyama et al., 1997). Calcitriol is then released into the blood for circulation (Bikle, Gee, Halloran, & Haddad, 1984; Bikle, Siiteri, Ryzen, & Haddad, 1985; Feingold et al., 2000). Typically, this transport is mediated by the vitamin D binding protein (VDBP) or albumin (Bikle et al., 1984; Bikle et al., 1985; Feingold et al., 2000). Once released from VDBP, calcitriol is able to enter cells where it can bind the vitamin D receptor (VDR) to further mediate transcription (Feingold et al., 2000).

**Calcitriol Mode of Action**

Calcitriol is the ligand for the vitamin D receptor (VDR). The VDR is located in the cytoplasm of most immune cells and is a nuclear hormone receptor (Bikle, Gee, & Pillai, 1993; Carlberg & Polly, 1998). Upon binding calcitriol, the VDR heterodimerizes with the retinoid X receptor (RXR) and the complex translocates to the nucleus (Bikle et al.,
Two exceptions to this rule are the VDR binding to the retinoic acid receptor (RAR) or the thyroid receptor (TR), rather than RXR (Schäder et al., 1993; Schäder, Müller, Nayeri, Kahlen, & Carlberg, 1994). Once in the nucleus, the VDR binds to DNA sequences on gene promoter regions known as vitamin D response elements (VDREs). The VDR also has an AF-2 domain, an area that can bind coactivators (Aranda & Pascual, 2001; Castillo, Jimenez-Lara, Tolon, & Aranda, 1999; S. Kim, Shevde, & Pike, 2005; Leo & Chen, 2000; Oda et al., 2003). Here, coactivators are able to bridge the gap between the VDR complex and the RNA polymerase transcription initiation complex (Aranda & Pascual, 2001; Castillo et al., 1999; S. Kim et al., 2005; Leo & Chen, 2000; Oda et al., 2003).

VDREs are a specific DNA-binding domain for the VDR that follow a hexameric DNA sequence. The hexameric consensus sequence is: RGKTSA (R=A or G, K=G or T, S=C or G) (Carlberg & Polly, 1998; Ramagopalan et al., 2010). The complex of VDR, RXR, a VDRE, and any coactivators are considered a molecular switch for calcitriol responsive genes (Aranda & Pascual, 2001). However, there are exceptions to this rule involving direct repeats (DR) of two consensus sequences with 3-4 intervening nucleotides (DR3-type and DR4-type, respectively) (Carlberg et al., 1993; Quack & Carlberg, 2000; Schräder, Nayeri, Kahlen, Müller, & Carlberg, 1995). The final exception occurs when there are everted repeats (ER-type) response elements with 6-9 spacing nucleotides (ER6-9, respectively) (Schräder et al., 1994; Schräder et al., 1995). With all of these exceptions, there are hundreds of potential combinations of VDREs on
a DNA promoter region allowing for calcitriol to impact the transcription of hundreds of genes.

**The Effect of Calcitriol on the Immune System**

Calcitriol is a key immunomodulatory molecule that affects the proliferation and differentiation of multiple immune cells. Our recent study demonstrated that calcitriol suppresses the expression of AhR by activated T cells and inhibits Th9 differentiation (Takami, Fujimaki, Nishimura, & Iwashima, 2015). AhR was previously found to play a role in modulating the Th1/Th2 balance towards a Th1 dominant response and is also necessary for Th22 induction (Negishi et al., 2005). Our previous data along with others have shown that calcitriol treatment of CD4 T cells, naïve, memory, in vivo, or in the context of PBMCs, leads to a reduction in Th1 associated cytokines such as IL-2, TNF-α, and IFN-γ (Alroy, Towers, & Freedman, 1995; Cantorna, Yu, & Bruce, 2008; Jeffery et al., 2009; Mahon, Wittke, Weaver, & Cantorna, 2003; Mattner et al., 2000; Panichi et al., 1998; Rausch-Fan et al., 2002; Reichel, Koeffler, Tobler, & Norman, 1987; Saggi, Federico, Balest, & Toniolo, 1989; Staeva-Vieira & Freedman, 2002). Murine models have found evidence of an increase in Th2 cytokines upon vitamin D exposure and it has been reported that calcitriol treatment also leads to an increase in Tregs (Daniel, Sartory, Zahn, Radeke, & Stein, 2008; Gorman et al., 2007; Jeffery et al., 2009; Nashold, Hoag, Goverman, & Hayes, 2001; Penna et al., 2005; Prietl et al., 2010; Sloka, Silva, Wang, & Yong, 2011). Calcitriol has also been found to inhibit Th17 differentiation and downregulate Th17 cytokine production (S. H. Chang, Chung, & Dong, 2010; Daniel et al., 2008; Moniaga, Egawa, Miyachi, & Kabashima, 2013; Tang et
al., 2009; H. Zhang, Shih, & Zhang, 2013). The skew away from Th1, Th9, Th17, Th22 along with promoting Th2 and Treg differentiation supports claims that calcitriol promotes an anti-inflammatory immune response and helps aid to combat autoimmunity.

In addition to T cells, calcitriol also plays a role on monocytes and their differentiation into macrophages. Calcitriol has been found to reduce myeloid pro-inflammatory cytokine production, while reducing inflammatory responses to IFN-γ and lipopolysaccharide (LPS) stimulation (L. Chen, Eapen, & Zosky, 2017; Helming et al., 2005; Y. Zhang et al., 2012; X. Zhu et al., 2019). This nutrient has also been found to inhibit macrophage polarization to M1 macrophages, while promoting polarization to M2 macrophages (S. Liang, Cai, Li, & Yang, 2019; Wasnik et al., 2018; X. Zhu et al., 2019). M1 macrophages are pro-inflammatory, while M2 macrophages are known to have suppressive functions and assist in wound healing (Funes, Rios, Escobar-Vera, & Kalergis, 2018). Calcitriol also inhibits the production of IL-12 by myeloid cells, which helps explain the reported reduction in Th1 cells (D’Ambrosio et al., 1998). Similar to the effect on T cells, these results further demonstrate that calcitriol skews towards an anti-inflammatory immune response.

**Calcitriol’s Effect on Tissue Development and Regeneration**

While calcitriol has multiple effects on immune cells, it also plays a role in embryogenesis and vascular regeneration. In zebrafish models, it has been found that the growth rate and length of the embryos were increased when treated with vitamin D analogs (Han et al., 2019). Cell proliferation and growth was also enhanced throughout
embryonic tissues, specifically in the liver and cardiomyocytes (Han et al., 2019). Angiotensins are another factor essential for healthy angiogenesis. One study found that calcitriol treatment could reduce the severity of symptoms from rats being treated with angiotensin II antagonists during lactation (de Almeida, Francescato, da Silva, Costa, & Coimbra, 2017). These calcitriol-supplemented rats had improved renal function and structure in comparison to those without calcitriol and showed marked reduction in inflammation (de Almeida et al., 2017). Endothelial cells after exposure to calcitriol also demonstrated enhanced proliferation and increased tubule formation, along with increased growth factor production, including VEGF (Grundmann et al., 2012).

Calcitriol has also been reported to promote vascular regeneration in a human supplement trial (Wong et al., 2014). The prevalence of angiogenic myeloid cells increased in these patients (Wong et al., 2014). In mice from the same study, supplementation of calcitriol promoted re-endothelialization in an injury and artery ligation model in a VDR dependent manner (Wong et al., 2014). In two studies, calcitriol was able to increase HIF-1α, which is known to lead to VEGF production in hypoxic conditions leading to increased angiogenesis (Büchler et al., 2003; Caponegro, Moffitt, & Tsirka, 2018; Dalton et al., 2014; Heil et al., 2000; Jiang, Zheng, & Teegarden, 2010; Krock et al., 2011; Murdoch et al., 2008; Ramakrishnan et al., 2014; Wong et al., 2014). In support of this, two other studies found that calcitriol promotes VEGF production (García-Quiroz et al., 2014; Grundmann et al., 2012). However, the opposite has been observed using cancer cell lines, where one study reported that calcitriol inhibits HIF-1α
production and yields reduced angiogenesis (Ben-Shoshan et al., 2007). This discrepancy may be due to the fact that transformed cancer cell lines were used for the later study, where the other two studies were using untransformed cells. More studies will need to investigate this further.

Lastly, calcitriol plays a positive role during gestation. IL-6, TNF-α, and IFN-γ have negative effects on fetal development (Yockey & Iwaski, 2018). Calcitriol reduces production of all three of these cytokines during gestation (Olmos-Ortiz, Avila, Durand-Carbajal, & Díaz, 2015). Taken together, calcitriol promotes embryogenesis, vascularization, angiogenesis, and suppresses inflammatory cytokine production during development.

**Calcitriol’s Effect on Combating Autoimmunity**

Experimental autoimmune encephalomyelitis (EAE) is the murine model for multiple sclerosis (MS). These diseases are characterized by paralysis, auto-antibodies against myelin basic protein, and lesions in the brain. The most common way to induce EAE is through treatment with myelin oligodendrocyte glycoprotein (MOG); disease symptoms begin roughly 9-14 days post-treatment (Bittner, Afzali, Wiendl, & Meuth, 2014; Miller & Karpus, 2007). EAE is scored on a scale of 0-5, signifying: (0) no change, (1) limp tail, (2) limp tail and weakness of the hind legs, (3) limp tail with paralysis of at least two legs, (4) limp tail with complete hind leg and partial front leg paralysis, and (5) severe paralysis or death to paralysis (Miller & Karpus, 2007). In 1991, it was discovered that calcitriol treatment prior to immunization prevented the development of EAE and the inhibited development of auto-antibodies against myelin basic protein
(Lemire & Archer, 1991). From this point on, the field has been studying to better understand how calcitriol/vitamin D have a protective effect against the development of autoimmunity. Studies have found that indeed, the VDR is necessary for the protective effects against autoimmune disorders (Mayne, Spanier, Relland, Williams, & Hayes, 2011; Meehan & DeLuca, 2002b). While the dominant amount of this work has been done with EAE/MS, calcitriol has also been found to be preventative against other autoimmune disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (Iruretagoyena, Hirigoyen, Naves, & Burgos, 2015; H. L. Zhang & Wu, 2010). Patient data has also touted remarkable success in supplemental calcitriol alleviating symptoms (Shaygannejad, Janghorbani, Ashtari, & Dehghan, 2012; Wingerchuk, Lesaux, Rice, Kremenchutzky, & Ebers, 2005).

Th1 and Th17 cells have been reported as two key Th subsets that are responsible for autoimmune disease when they gain the ability to react to self-proteins. Similar to in vitro and mouse data, calcitriol was found to reduce Th1 development in the context of EAE due to decreased IL-12 production by monocytic cells, leading to diminished production of IFN-γ and IL-2 in the system (Mattner et al., 2000). IL-17A production from Th17 cells has also been known to exacerbate EAE, and in IL-17A knockout mice, EAE clinical score is significantly reduced (Komiyama et al., 2006). Calcitriol in the context of EAE reduced Th17 differentiation and IL-17A production at the transcriptional level in a VDR-dependent manner (J. H. Chang, Cha, Lee, Seo, & Kweon, 2010; Joshi et al., 2011). The effect of calcitriol on CD8 T cells has been found to be insignificant in the context of autoimmunity (Meehan & DeLuca, 2002a).
While calcitriol treatment causes a reduction in inflammation by suppressing Th1 and Th17 cells, it has also been found in a VDR-specific manner to induce Helios+Foxp3+ T cells in the central nervous system (CNS) (Nashold, Nelson, Brown, & Hayes, 2013; Spanier, Nashold, Mayne, Nelson, & Hayes, 2015). These Tregs then suppress inflammatory responses in the central nervous system to combat disease (Nashold et al., 2013; Spanier et al., 2015). In another study, calcitriol was unable to inhibit EAE in mice with disrupted IL-10 signaling, suggesting the importance of IL-10 production by Tregs (Spach, Nashold, Dittel, & Hayes, 2006). Lastly, calcitriol supplementation also increases CTLA-4 expression on T cells which can lead to increased suppression of T cell responses (Spanier, Nashold, Nelson, Praska, & Hayes, 2020).

Myeloid cells are also affected by calcitriol in EAE. Calcitriol has been reported to reduce toll-like-receptor (TLR) expression in monocytes, most significantly TLR8 and its adaptor protein, MyD88 (B. Li et al., 2013). This reduction lead to reduced inflammatory cytokine production (TNF-a and IL-1β) by monocytes (B. Li et al., 2013). Another study found that chemokine production and monocyte recruitment were inhibited after calcitriol treatment, thus reducing infiltration of inflammatory monocytes and macrophages into the CNS (de Oliveira et al., 2020; Nashold, Miller, & Hayes, 2000; Pedersen, Nashold, Spach, & Hayes, 2007). Overall, these results demonstrate that calcitriol promotes an anti-inflammatory immune response in the context of autoimmunity by reducing inflammation from Th1, Th17, monocytes, and macrophages while promoting Treg induction, CTLA-4 expression, and IL-10 production.
Purpose of Dissertation

To study neonatal immune tolerance, our lab collects umbilical cord blood (UCB) at birth and we investigate the response of T cells against antigen receptor stimulation. Here, I define a new type of Foxp3+ Treg, named bidirectional T cells (BiT) derived from UCB. BiTs are induced by monocytes that present transforming growth factor-beta (TGF-β), a necessary molecule for Treg induction. BiTs express classical Treg markers but also express other T cell markers and pro-inflammatory cytokines. This profile suggests that BiTs are capable of both suppressing and activating the immune system. Based on these differences, we hypothesized that BiTs are genetically distinct from in vitro induced Tregs (iTregs) and thymus derived Tregs (tTregs). To test this, we performed RNA-Seq on these different Treg populations and compared differentially expressed genes.

In an effort to determine if any factors could promote or hinder BiT inductions, I investigated the effects of calcitriol and IFN-β. These nutrients were chosen because vitamin D is known to promote an immunosuppressive environment and aids in healthy fetal development, while IFN-β has an opposite effect. I have demonstrated that physiological levels of calcitriol increase BiT induction and expression of NRP-1 by monocytes. Conversely, IFN-β treatment significantly reduces BiT induction and NRP-1 expression. NRP-1 is a surface receptor capable of binding multiple ligands, one of which is TGF-β which is a necessary molecule for Treg induction. Thus, we hypothesized that calcitriol promotes BiT development due to an increase in TGF-β signaling and availability from the NRP-1 upregulation on monocytes.
Our lab previously reported that calcitriol inhibits Th1 and Th9 differentiation. However, the function and phenotype of T cells that develop under these conditions were not assessed. The literature suggests that calcitriol promotes a suppressive environment, however the mechanism for suppression by these T cells is unknown. To assess the direct effect of calcitriol on T cells, we determined the phenotypic and functional changes of T cells stimulated in calcitriol sufficient conditions. Our previous work demonstrated that calcitriol reduces T cell expression of the aryl hydrocarbon receptor (AhR), a transcription factor that functions as a co-factor for TGF-β signaling. Thus, I hypothesized that calcitriol also has a direct effect on T cells that aid in BiT induction. My dissertation research aimed to investigate how BiTs differ from previously defined Tregs and understand the effect of calcitriol on monocytes and T cells to enhance BiT induction.
CHAPTER TWO: MATERIALS AND METHODS

Cell Preparation and Reagents

**BiT Cultures**

Total umbilical cord blood mononuclear cells from 3 donors were stimulated with recombinant animal-free human IL-2 (10 ng/ml; Peprotech, Rocky Hill, NJ) and soluble anti-CD3 (0.2 μg/ml) in RMPI 1640 (GE Healthcare Hyclone, Chicago, IL) supplemented with 10% fetal calf serum (Gemini Bio-Products, West Sacramento, CA). Cells were split 1:1 every 2-3 days with fresh IL-2 media. CD4+CD8-CD25+Foxp3+ status was analyzed after 13 days of culture. CD4+ samples with greater than 80% CD25+FOXP3+ status were harvested on day 14 and sorted for CD4+CD25+ cells using a FACS Aria (BD Biosciences, San Jose, CA).

**iTreg and Effector T Cell Cultures**

1.3x10^5 naïve CD4 T cells from UCB were plated in a 96 well U-button plate in 200μl of media supplemented with 10 ng/ml of recombinant human IL-2 and TGF-β (Peprotech) and immunocult activators following manufacturer’s instructions (Stem Cell Technologies). For effector T cells, the same culture was used but without TGF-β. Cells were split as necessary and harvested on day 7. Purity was assessed by labeling for CD4, CD25, and Foxp3. Donors with greater than 80% purity were subject to cell sorting (BD FACS Aria) for CD4+CD25+ cells for iTreg and CD4+CD25- for effectors.
**tTreg Cultures**

Adult PBMCs were harvested and subject to the human Treg isolation kit (Stem Cell Technologies). These cells were cultured with recombinant human IL-2 (10 ng/ml; Peprotech) in RMPI 1640 (GE Healthcare Hyclone, Chicago, IL) supplemented with 10% fetal calf serum (Gemini Bio-Products, West Sacramento, CA) and Immunocult activators following manufacturer’s instructions (Stem Cell Technologies). Cells were split 1:1 every 2-3 days with fresh IL-2 media. CD4⁺CD25⁺CD127⁻Foxp3⁺ status was analyzed after 13 days of culture. CD4⁺ samples with greater than 80% CD25⁺FOXP3⁺ status were harvested on day 14 and sorted for CD4⁺CD25⁺ cells using a FACS Aria (BD Biosciences, San Jose, CA).

**Calcitriol-Treated T Cell Cultures**

Naïve CD4 T cells were harvested from adult human PBMCs. Blood collection was performed with IRB approval (Loyola University Chicago (IRB# 203678081012)). Adult human PBMCs were collected from de-identified donors that met our collection criteria (exclusion criteria: evidence of active malignancies, use of medications that affect the immune system (such as glucocorticoids and immunosuppressants), uncontrolled hyper- or hypothyroidism, presence of an autoimmune disease, and/or presence of an active infection). Naïve CD4 T cells were obtained via negative selection using an EasySep™ Human Naïve CD4 T Cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada) or a MojoSort™ Human CD4 Naïve T cell Isolation Kit (BioLegend, San Diego, CA). All samples maintained at or above a 90% purity rating
(not shown). Cells were treated once at the time of stimulation with calcitriol (10 nM; Sigma-Aldrich, St. Louis, MO) and 10 ng/ml IL-2 (Peprotech, Rocky Hill, NJ) and plated on non-treated CytoOne 48 well plates (USA Scientific, Ocala, FL) that were coated with anti-CD3 (OKT3; 5ug/ml; BioLegend) and anti-CD28 (28.2; 5ug/ml; BioLegend) for cell stimulation. Control cells were treated with dimethyl sulfoxate (DMSO) since calcitriol was reconstituted in DMSO.

**Monocyte Cultures**

CD14+ monocytes were enriched from mononuclear cells using an EasySep enrichment kit (Stem Cell Technologies). All samples maintained at or above a 90% purity rating (not shown). CD14+ monocytes were cultured in the presence or absence of 10 ng/ml IFN-β (Peprotech) and RPMI media supplemented with 10% fetal calf serum (GE Life Sciences and Gemini Bio).

**Transwell Migration Assay**

Cells were pretreated overnight with or without 10 ng/ml IFN-β. The following day, cells were washed and resuspended in 5x10⁵/200ul. 200μl of cells were added to an 8-micron pore 24-well transwell plate (Corning). At the bottom of the well, 800μl of regular culture media or 50 ng/ml recombinant VEGF-165A supplemented media was plated (BioLegend). The assay sat overnight in a CO₂ incubator at 37°C. The next day, transwells were removed and cells at the bottom were collected and counted. Fold change is the calculation of the number of cells that migrated towards VEGF divided by the number of cells that migrated in the absence of VEGF.
Phenotype Analysis of BiT and Calcitriol Treated T cells

Suppression Assay

4 days prior to the suppression assay, naïve CD4 T cells were isolated, treated, and stimulated as described above. On day 4 post-treatment, cells were checked for expression of PD-L1, CTLA-4, CD4, and Foxp3. An allogeneic donor was collected, naïve CD4 T cells were stained with CFSE (BioLegend) and used as responder cells. Remaining PBMCs were sorted for size gated cells that were CD3 negative on a BD FACS Aria to collect APCs (BD BioSciences). APCs were irradiated at 3000 rad. tTregs, calcitriol-treated and DMSO-treated T cells were stained with cell trace violet (CTV; BioLegend). Responder cells were stimulated with anti-CD3 (0.2 μg/ml) and cultured with APCs at a 1:1 ratio (50,000 cells each) in the presence or absence of the respective CTV labeled suppressor populations in a dose-dependent manner. The percent proliferation of responders and suppressors was determined after 6 days of coculture via percent diluted CFSE and CTV, respectively, using flow cytometry.

Cytokine Expression

Cell supernatants were harvested 5 days post-treatment. Supernatants were analyzed for expression of T cell cytokines using the LEGENDplex Human Th cytokine panel (BioLegend) according to manufacturer’s instructions on a BD FACS CANTO II Flow cytometer (BD Biosciences).
Generation of a NRP1 Knockout THP-1 cell line using CRISPR/Cas9 Genome Editing

Generation of a NRP1 knockout THP-1 cell line was achieved by the transduction of LentiCRISPRv2 (Addgene 52961), encoding CRISPR guide RNA (gRNA) against human NRP-1. Guide sequences were generated using the CRISPR design tool (www.crispr.mit.edu). The sequence for the CRISPR gRNA targeting NRP-1 was: 5'-GTTGCGAAAAGCGCCGGCCG-3'. This oligonucleotide was annealed and cloned into a modified LentiCRISPRv2 (puromycin resistance was replaced by mCherry expression). Lentivirus was prepared by transfecting equal amounts of vesicular stomatitis virus G, psPAX2 (11348; Didier Trono, NIH AIDS Reagent Program), and LentiCRISPRv2 (containing the guide RNA of interest) into HEK293T cells. The viral supernatant was harvested 48 hours post-transfection, filtered through 0.45-μm filters (Millipore), and applied to THP-1 cells. 48 hours post-transduction, cells were sorted for mCherry expression via a BD FACS Aria cell sorter with 1 cell per 96 well, and single-cell clones were generated (BD Biosciences).

TGF-β Bioassay

MFB-F11 cells (TGFβ-1 deficient MEFs transfected with a SMAD-binding element promoter fused to secrete an embryonic alkaline phosphatase) were described previously and used as reported (Tesseur, Zou, Berber, Zhang, & Wyss-Coray, 2006). Briefly, culture supernatants of transduced THP-1 lines were collected after 24 hours and added to MFB-F11 cells. After 48 hours, these cumulative supernatants were
treated with PNPP in alkaline conditions. If active TGF-β was present in the culture, then color change was assessed via a plate reader at 450nm. As a control, THP-1 supernatants were first acid-treated prior to addition to MFB-F11 cells to cleave latent-TGF-β and demonstrate total TGF-β in the culture.

**Western Blot Analysis**

Equal numbers of cells (1.0x10^6 cells/100µl) were lysed in SDS sample buffer (2% SDS, 125mM DTT, 10% glycerol, 62.5mM Tris-HCl (pH 6.8)) and proteins were subjected to Western blot analysis using the following antibodies: Histone-H3 (BioLegend); SOS1 (Cell Signaling Technologies); SGK1 (Santa Cruz Biotechnologies); and THEMIS (Santa Cruz Biotechnologies). Signals were detected with the ECL system (GE Healthcare, Piscataway, NJ). The relative intensity of each band was determined by ImageJ software (National Institutes of Health) after normalization using Histone-H3 as the control.

**Flow Cytometry**

For BiT studies: cells were harvested after 14 days (7 days for iTregs) and surface labeled with anti-CD3, anti-CD8, anti-CD4, anti-CD25, anti-CD96, and anti-CD33, and intracellularly labeled with anti-Foxp3 (BioLegend). Cells were analyzed on a BD FACS CANTO II Flow cytometer (BD Biosciences). t-SNE analysis was performed using FlowJo’s built-in t-SNE and concatenation packages. Data analysis was performed using FlowJo (FlowJo, LLC, Ashland, OR). For calcitriol studies: cells 3 to 5 days post-treatment were harvested and surface labeled with anti-PD-L1, anti-CTLA4, anti-CD4, anti-CD25, and anti-PD-1, and intracellularly labeled with anti-Foxp3 and anti-CTLA4 (BioLegend and
BD Biosciences, San Jose, CA). Cells were analyzed on a BD FACS CANTO II Flow cytometer (BD Biosciences). Data analysis was performed using FlowJo (FlowJo, LLC, Ashland, OR). Surface CTLA-4 labeling was performed on ice, when labeling for surface and intracellular CTLA-4, a different clone with a different fluorophore conjugated to it was utilized after fixing and permeabilizing (True Nuclear Kit, BioLegend) the cells for intracellular CTLA-4.

**CTLA-4 Promoter Analysis for VDRE**

The CTLA-4 promoter was assessed using the Eukaryotic Promoter Database (EPD) and the PROMO database for potential VDR binding sites (Cavin Périer, Junier, & Bucher, 1998; Farré et al., 2003; Messeguer et al., 2002).

**Genotype Analysis of BiT**

**RNA Isolation**

Naïve CD4 T cells were collected as described above. Unstimulated cells were isolated for RNA on day 0 to establish a baseline level of naïve cell expression. For calcitriol studies, cells were treated and expanded for 4 days as described above. RNA was isolated using a RNeasy isolation kit (Qiagen, Germantown, MD). For RNAseq analysis, cells were isolated on day 7 (iTregs) or on day 14 (BiT, tTregs, effector T cells).

**RNASeq Analysis of T Cell Subsets**

Quality of samples were checked using FastQC (version 0.72). To improve sequence quality, samples were cleaned by Trimmomatic (version 0.36.5) to remove
any low-quality bases and the Illuminaclip function was used to clip Illumina adaptor sequences. The following parameters were used: LEADING: 28; TRAILING: 28; MINLEN: 30. The dataset was then aligned to the human Hg19 reference genome using RNA STAR (version 2.6.0b-1). Gene counts were then obtained using featureCounts (version 1.6.3). Data was normalized and differentially expressed genes were determined using DESeq2 (version 2.11.40.2), afterwards the dataset was filtered (using Filter version 1.1.0) to show genes with significant difference (p<0.05) and to extract genes with greater than log 2-fold difference.

qPCR

RNA was isolated with RNeasy kit from cells at stated times pre- and post-stimulation. Isolated RNA was reverse-transcribed with RT2 First Strand Kit. The qPCR reactions were performed using RT2 Kit (Qiagen). The qPCR reaction is 1) 95 °C for 10 min, 2) 95 °C for 15 s, 3) 60 °C for 1 min, and a repeat of steps 2 and 3 for 40 cycles.

Bisulfite Sequencing

Only male donors were used as all female cells carry a methylated allele from one of the X chromosomes. Cells were isolated and cultured as described above, only donors with >80% purity were used. DNA isolation was conducted using DNeasy Blood and Tissue Kit (Qiagen) and bisulfite conversion was conducted following the manufacturer’s protocol (Single-step EZ DNA methylation-Direct Kit, Zymo Research).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad
software, CA). One-way ANOVA with Tukey’s posttest for multiple comparisons were used to compare changes in expression between multiple groups. When comparing only two groups, student’s t-tests were performed.
CHAPTER THREE: RESULTS

SECTION 1: Characterization of Bidirectional T regulatory cells (BiTs) from Umbilical Cord Blood versus iTregs and tTregs

BiTs areSuppressive but Express Inflammatory Cytokines

Previous work from our lab (generated by Alex Nelson, M.S.) uncovered striking differences between BiT and tTregs from adult peripheral blood (APB) (J. Lee et al., 2020). On initial harvest, UCB shows 1.2% Tregs, where adult blood has 2.2% Tregs defined by Foxp3+CD4+ cells (Figure 1A). After BiT induction cultures in vitro, we saw that 74.1% of UCB CD4 T cells differentiate into Tregs (BiT) while only 18.4% of adult blood CD4 T cells differentiated into Tregs (Figure 1B/C). These BiT cells are also functionally suppressive, as tested by a standard suppression assay (Figure 1D). Our lab has found that these Tregs are not an expansion from previously existing CD4+CD25+ cells, but rather induction of new cells. This has been tested by depletion of CD4+CD25+ cells (pre-existing Tregs, purity >90%) and then performing a BiT induction culture (Figure 1E). These data demonstrated that between total UCB and UCB depleted of CD4+CD25+ cells both induced ~56% BiT. Foxp3 expression is also stable in BiT, as demonstrated by in vitro expansion for two months, where Tregs from APB lose their Foxp3 expression after three weeks (Figure 1F).

The lab next questioned whether BiT were different from tTregs in APB and assessed this by cytokine expression after restimulation on day 14. Nelson found that
BiT expressed GM-CSF in 60% of cells, and IL-8 was expressed in about 30% of cells, while APB Tregs did not express these cytokines (Figure 2).

These data suggest that BiT induced from UCB are phenotypically different from tTregs found in APB. We were especially interested in the differences in cytokine expression and the prolonged stability of Foxp3, as tTregs are known to be key immune suppressors but the production of GM-CSF and IL-8 by BiT may indicate the ability to promote an inflammatory response, particularly in innate immune cells. Interestingly,
BiTs did not produce IL-10, a cytokine produced by iTregs (data not shown). Based on these data, we hypothesized that BiTs are genetically distinct from tTregs and iTregs and sought to determine their genetic profile through methylation studies and RNA-sequencing.

**Figure 2. Intracellular Cytokine Production by BiT from UCB and tTregs from APB.**
GM-CSF (left) and IL-8 (right) production by BiT and tTregs after re-stimulation on Day 14 with PMA and ionomycin (+) or no stimulation (-). Data represent the average of 3-7 donor samples, *p<.05, **p<.005, ***p<.0005, and ****p<.0001. Figure adapted from (J. Lee et al., 2020), work done by Alex Nelson, M.S.

**BiTs are Methylated on Foxp3 CNS2**

Foxp3+ Tregs can be defined based on the methylation status of the CNS2 region of the foxp3 promoter, where tTregs are hypomethylated on this region, and effector T cells, pTregs, and iTregs are hypermethylated on this region. Since BiTs can be induced from naïve CD4 T cells from UCB, we hypothesized their CNS2 region would be methylated, similar to iTregs and unlike tTregs. To do this, we isolated DNA from day 14 BiTs, iTregs, expanded adult tTregs, and effector T cells from UCB male donors. We chose male donors since males only have one X chromosome, where Foxp3 is located. This allows for avoiding detection of a silenced X chromosome in
females. Yi Wei Lim, a member of our lab, then performed bisulfite sequencing, which converts de-methylated cytosine to uracil, where methylated cytosines remain unchanged. The Foxp3 CNS2 region has fourteen CpG islands, so we assessed the level of methylation on these islands. We observed that BiTs had similar methylation to iTregs and effector T cells, rather than tTregs (Figure 3). Variations in tTreg levels are most likely due to the presence of pTregs in the culture because the Tregs were collected from peripheral blood, not the thymus. These data suggest that BiTs are more akin to induced forms of Tregs, rather than naturally arising tTregs since they do not have a stabilized CNS2 from de-methylation.

Figure 3. Foxp3 in BiT are Methylated at the CNS2 Site.
Demethylation status at Foxp3 CNS2 CpG sites in BiT, iTreg, tTreg, and UCB effector cells. Data represent the average of 3-4 donor samples, error bars represent standard deviation, *p<.05 Figure from (J. Lee et al., 2020), work done by Yi Wei Lim.

RNA- Sequencing and qPCR of Differentially Expressed Genes of BiTs, in Comparison to tTregs and iTregs

While BiTs seem to be more like effector T cells, pTregs, or iTregs, we know they have a differing cytokine profile and maintain prolonged Foxp3 expression (Figures 1
and 2). With the ability to produce GM-CSF and IL-8 but not IL-10, this suggests a genetically distinct subset of Treg. To determine the genetic profile of BiT, we isolated RNA from day 14 BiTs (>80% pure CD4+CD25+Foxp3+ population), along with effector T cells, adult tTregs, and iTregs. We performed RNA-seq and determined differentially expressed genes (DEGs), defined as p<0.05 and greater than a log 2-fold difference. From this, we found there are 2016 DEGs between effector T cells, BiT, tTregs, and iTregs.

Next, we sought to determine whether BiTs expressed genes specific to any previously defined T helper subsets, which could help us better characterize these cell types. To achieve this, we analyzed the expression of Th subset specific genes across effectors, iTregs, tTregs, and BiT (Figure 4). These data indicate that BiT did not fit uniformly under any of the previously defined T helper subsets, but rather tended to upregulate 30-40% of genes from each subset (Figure 4).

Since BiT did not fit into a previously defined subset, we sought to determine potential markers for BiT. From the DEG list, we highlighted four genes of interest. These genes were highly upregulated by BiTs and have known roles in immune function. The first gene, THEMIS (thymocyte expressed molecule involved in selection) is known to play a role in positive T cell selection in late thymocyte development (Lesourne et al., 2009). This gene is necessary for lineage commitment and maturation of T cells (Lesourne et al., 2009). THEMIS acts as an adaptor molecule which regulates TCR signaling and p-ERK by binding Grb2 and LAT (Lesourne et al., 2009). The next gene, SOS1 (son of sevenless 1), impacts signal transduction in early thymocyte
development (Kortum et al., 2011). SOS1 is a guanine exchange factor (GEF) for Ras which in turn activates Ras (Hillig et al., 2019; Innocenti et al., 2002). In effector T cells, Ras is able to activate RasGRP1, however our lab has found the opposite in tTregs, where TGF-β suppresses RasGRP1 and there is reduced SOS1 in tTregs in comparison to effector T cells (Takami, Cunha, Motohashi, Nakayama, & Iwashima, 2018). Since we know that BiTs are likely not tTregs, this gene could be a good marker since it expresses a gene with typically low expression by Tregs. A third gene that piqued our interest was SGK1 (serum- and glucocorticoid-regulated kinase 1), which is known to be critical for development and function of Th17 cells and restrains the function and development of Treg cells (C. Wu et al., 2018). SGK1 is a Ser/Thr kinase that regulates stress response and inhibits IL23-R (C. Wu et al., 2018). This gene is intriguing since Th17 and Tregs are both induced by TGF-β, but Th17 is only induced if IL-6 is also present. SGK1 is important for this axis; however, we have never detected the presence of Th17 cytokines in BiTs, suggesting further control of SGK1.

The final gene of interest is CD33 (SIGLEC 3). We were particularly interested in this gene because the protein resides on the surface of cells which would assist in the detection of BiTs. However, CD33 is traditionally expressed by myeloid cells, not lymphocytes. Interestingly, CD33 has ITIM and ITSM, similar to PD-1’s cytoplasmic domain, and we know that PD-1 signaling has been known to aid in iTreg and pTreg differentiation. If this marker is found on BiT, it could be uniquely expressed, since it is typically known to be myeloid restricted. If any of these genes of interest are a marker for BiT, then we expect to see high expression in BiT but not other Treg populations.
Figure 4. BiT are not a Previously Defined Th Subset.
Heatmaps of Th subset specific genes comparing Teff, iTreg, tTreg, and BiT; n=3. Blue signifies downregulation and red signifies gene upregulation. RTE: Recent thymic emigrant.
To confirm our RNA-seq results, we performed qPCR on tTreg, iTreg, and BiT donors for these genes of interest. THEMIS, SOS1, and SGK1 all showed significant increases in mRNA expression in comparison to tTregs and iTregs, suggesting promise as BiT specific genes (Figure 5A-C). We were unable to detect CD33 mRNA expression, which we believe correlates to the small number of counts in the RNA-seq data (less than 300 reads, data not shown). But these mRNA results demonstrate that THEMIS, SGK1, or SOS1 could be promising markers for BiTs.

**Figure 5. mRNA Expression of BiT Upregulated Genes.**
Fold change in mRNA expression for (A) THEMIS, (B) SOS1, and (C) SGK1 in tTreg, iTreg, and BiT. Data is normalized to respective 18SrRNA as a housekeeping control and then fold change is calculated by dividing each sample by the average of the tTreg donor values. Data represent the average of 3 donor samples, error bars represent standard deviation. *p<.05, and **p<.005.

**The Function of BiT-Upregulated Genes**

While we were unable to detect mRNA levels of CD33, we sought to detect surface protein expression via flow cytometry. BiTs showed a significant increase of CD33 expression on Foxp3+CD25+CD4+CD3+ cells in comparison to tTregs and iTregs (Figure 6A/B). In order to better visualize this, we next performed t-SNE analysis of BiTs, tTregs, and iTregs for CD3, CD4, Foxp3, CD25, and CD33 (Figure 7).
Concatenated data of 2-3 samples per cell type demonstrated that there is a distinct separation between BiT, tTreg, and iTreg populations, with more overlap between tTregs and iTregs than BiT (Figure 7A). We next sought to compare the prevalence of CD33 as a marker for these donors. Our data signify a robust Foxp3+CD33+ population in BiT that is sparse in tTregs and iTregs (Figure 7B). These data suggest that BiT uniquely have a population of CD33+Foxp3+CD25+CD4+ cells and that BiT is different from iTreg and tTregs.

To this end, our lab has been unable to find a marker for BiT to determine whether BiT are a pre-existing cell type. With the discovery of CD33 on BiT with limited expression on iTreg and tTreg populations, we sought to elucidate whether CD33+Foxp3+ cells exist in fresh UCB versus APB. To test this, we isolated fresh PBMCs from APB and UCB and labeled for CD3, CD4, CD8, Foxp3, and CD33. Of interest, we found that in fresh UCB that there is a pre-existing subset of CD33+ Tregs that does not exist in APB (Figure 8). This could suggest that the CD33+ population is expanded in in vitro culture, since before our lab previously expanded CD25-depleted cells. Further work will need to be performed to determine the growth of this population.

In congruence with our qPCR data, we were next able to detect the expression of SGK1, THEMIS, and SOS1 by western blot (Figure 9). There was a slight, 1.5-fold increase in expression of SOS1 in BiT. THEMIS expression was heightened 2-fold by BiTs in comparison to tTregs from adults. However, iTregs also elevated THEMIS expression, greater than BiTs, which makes this protein less viable as a marker for BiT. Lastly, we observed an interesting phenomenon with SGK1 expression. While tTregs
and iTregs expressed SGK1, BiT donors expressed a smeared pattern of SGK1. A smeared pattern suggests either ubiquitination or sumoylation. Both processes are post-translational modifications. Ubiquitination flags proteins with ubiquitin for degradation (N. Zheng & Shabek, 2017). Sumoylation is a similar process in which small polypeptides are enzymatically attached to target proteins, modifying them by masking binding sites or adding surfaces to the protein (Geiss-Friedlander & Melchior, 2007). This can alter localization, activity, and stability of the flagged protein (Geiss-Friedlander & Melchior, 2007). Both modifications are reversible, however, they achieve the same outcome where the protein that is marked often loses functionality (Geiss-Friedlander & Melchior, 2007; N. Zheng & Shabek, 2017). This suggests that SGK1 while upregulated by BiT, is negatively controlled by either of these processes.

Figure 6. CD33 Surface Expression in BiT versus APB Tregs.
Representative figure of CD33 expression in tTregs, iTregs, and BiT (A) and quantified (B). Isotype control is a BiT donor stained with respective isotype antibodies. Data represent the average of 2-4 donor samples, *p<.05, **p<.005, and ***p<.0005.
Figure 7. t-SNE Plots Demonstrate BiT as Different from tTregs and iTregs.

t-SNE plots of (A) BiT (blue), iTreg (orange), and tTreg (green) concatenated donors; n=2-3, and (B) iTreg (left), tTreg (center), and BiT (right) donors highlighting CD3+CD4+CD25+ populations expressing Foxp3+CD33+ (blue), Foxp3+CD33- (orange), or Foxp3- (green); n=2-3. Data collected via flow cytometry labeling of CD3, CD4, CD25, Foxp3, CD96, and CD33.
Calcitriol Promotes Treg/BiT Induction

Our data, along with others, demonstrated that a one-time treatment with calcitriol upregulated Foxp3+CD25+CD4+ T cells. However, in BiT induction cultures, our lab uses total PBMCs. We questioned from this whether calcitriol’s effect on BiT/Treg induction was a direct effect on T cells or if another cell type, such as
monocytes, were involved. To address this, we first assessed the function of calcitriol on UCB monocytes and later tested the function of calcitriol as a differentiating factor for naïve CD4 T cells.

**SECTION 2: The Effect of Calcitriol on Monocytes**

**Monocytes are Necessary for BiT Induction**

Our lab previously found that BiT induction was enhanced in the presence of other UCB cells, rather than just stimulation of T cells alone. To determine the population responsible for the enhancement in induction, our lab depleted various immune cell populations and ran BiT induction cultures. To this end, our lab found that depletion of CD14+ monocytes resulted in a significant decrease in Foxp3+CD25+ expression, identifying that monocytes are important for BiT induction (Figure 10A/B).

Along with this, it was found that endogenous TGF-β from monocytes was important for BiT induction cultures. Depletion of CD14+ monocytes lead to decreased Smad2/3 activation in the T cells, which are transcription factors known to be activated during TGF-β signaling (Figure 10C/D). These data suggest that monocytes are essential for BiT induction and are a key TGF-β producer. In concurrence with this, our lab also has found that monocytes from both UCB and APB are able to induce Foxp3 and CD25 expression in T cells in a similar manner (data not shown). Together, these data indicate that monocytes are a key player in BiT/Treg induction.
Figure 9. Protein Expression of SGK-1, THEMIS, and SOS1.
(A) Western blot analysis of SGK1, THEMIS1, SOS1, and Histone-H3 by BiT (left three lanes), tTreg (center three lanes), and iTreg (right three lanes). Numbers on the left depict kDa weight. Numbers under each lane represent normalization to their respective Histone-H3 control and then normalized to the average of all tTreg donors. (B) Quantification of part (A). Band intensity calculated by ImageJ software, *p<.05, **p<.005, and ***p<.0005.

Calcitriol Promotes NRP-1 on Monocytes

My data has demonstrated that BiT induction cultures with one-time treatment of
calcitriol at culture start leads to increased BiT induction (Figure 11 A/C). In correlation, I have demonstrated that UCB monocytes express increased NRP-1 expression after treatment with calcitriol (Figure 11 A/B). These data show that calcitriol upregulates NRP-1 on monocytes, and that NRP-1 expression on day 1 correlated with increased BiT induction on day 14 (Figure 11D).

Figure 10. Monocytes are Necessary for BiT Induction.
Total UCB or CD14 monocyte-depleted UCB were cultured in the presence of anti-CD3 and IL-2. For (A/B) cells were harvested after 14 days. For (C/D) cells were harvested after 2 days. (A/B) Foxp3 and CD25 expression of BiT induced from total UCB (left) or CD14 depleted UCB (right). (C/D) pSmad2/3 levels in CD4+ cells 48 hours after BiT induction culture start in the presence (left) or absence (right) of CD14+ monocytes, *p<.05, **p<.005. Figure adapted from (J. Lee et al., 2020).
Figure 11. Calcitriol Promotes NRP-1 Expression on Monocytes.

(A) BiT induction after 10nM calcitriol or DMSO treatment labeled for Foxp3 and CD25 expression on Day 14 (left) and NRP-1 and CD36 on Day 1 CD14+ monocytes (right). (B) NRP-1 expression on CD14+CD36+ monocytes from UCB 24 hours after calcitriol or DMSO treatment. (C) Quantification of calcitriol-mediated BiT induction compared to DMSO control. Data represent 4 donor samples, *p<.05 (D) Linear regression of Day 1 NRP-1+ monocyte expression compared to Day 14 BiT induction measured by CD4+Foxp3+CD25+ cells.

Since our lab demonstrated that monocytes are essential for BiT induction, I hypothesized that this upregulation of NRP-1 on UCB monocytes is a key factor for the increase in BiT induction after calcitriol treatment. NRP-1 upregulation also correlates with the finding that UCB monocytes were key in presenting TGF-β to T cells, as NRP-1 is able to bind both latent and active TGF-β (Glinka & Prud'homme, 2008; Glinka, Stoilova, Mohammed, & Prud'homme, 2011). Furthermore, Stephanie Chapman, M.S.,
a previous student in the lab also found donors that had sufficient levels of vitamin D in their serum had elevated NRP-1 on their monocytes in comparison to those that were deficient, along with a positive correlation between Treg and NRP-1 frequency (data not shown). Together, these data demonstrate that the effects we see with calcitriol in vitro are relevant to in vivo data.

**NRP-1 Monocyte Effect on BiT Induction**

The previous section demonstrates a correlation between NRP-1 on monocytes and BiT induction. We sought to confirm whether there is a direct effect of NRP-1 function on BiT induction without calcitriol being present in the culture, which could skew results. To answer this, I designed a CRISPR-Cas9 gene editing system to knock out NRP-1. I found that THP-1, a human AML monocytic cell line, had constitutive expression of NRP-1 (Figure 12A). After transduction, I generated a NRP-1 knockout cell line from single clones (Figure 12B). Following cloning, I then performed a BiT induction culture using THP-1 in place of monocytes with total CD3 T cells and IL-2 for 14 days. I hypothesized that cells cultured with NRP-1 knockouts would have reduced BiT induction compared to the vector control. However, we saw no significant change in BiT induction regardless of NRP-1 status (Figure 12C).

In order to discern whether day 14 was too late of a timepoint, we decided to detect p-SMAD2/3 expression after 48 hours. Previous data suggested that p-SMAD2/3 expression is upregulated in the first few days of culture by monocytes (Figure 10C/D). However, we also observed no difference in p-SMAD2/3 expression of cells cultured with NRP1-/- THP-1 versus vector control (data not shown).
Figure 12. NRP-1 Knockout in THP-1 did not change BiT induction.
(A) NRP-1 expression on THP-1 (light grey) compared to isotype control (dark grey). (B) The purity of NRP-1 knockout THP-1 line (right) in comparison to vector control (left). The y-axis shows expression of the mCherry surrogate marker for the CRISPR/Cas9 expression vector with NRP-1 expression on the x-axis. (C) BiT induction with vector control THP-1 (left) or NRP-1 knockout THP-1 (right) in place of monocytes after 14 days. Total T cells were enriched from UCB and then co-cultured at a 4:1 ratio with respective irradiated THP-1 population with IL-2 and anti-CD3.

Lastly, our lab previously demonstrated that monocytes were a key TGF-β producer in BiT induction cultures. We hypothesized that NRP1-/- THP-1 would have reduced active TGF-β in the culture supernatants due to the fact that NRP-1 is known to
have the ability to bind latent TGF-β and aid in activating TGF-β. Indeed, we found that both knockout lines had reduced active TGF-β in the culture supernatants in comparison to the vector control (Figure 13). However, there was no difference in total TGF-β in the culture system, which may explain why we failed to observe any significant difference in BiT induction or p-SMAD2/3 activation (Figure 12 and 13).

**Figure 13. TGF-β Production by Transduced THP-1 Lines.**

Culture supernatants of transduced THP-1 lines were collected after 24 hours and added to MFB-F11 cells. After 48 hours, these cumulative supernatants were treated with PNPP in alkaline conditions. If active TGF-β was present in the culture, then color change was assessed via a plate reader at 450nm. As a control, THP-1 supernatants were first acid-treated prior to addition to MFB-F11 cells to cleave latent-TGF-β and demonstrate total TGF-β in the culture. Data points depict two biological replicates per cell line, *p<.05.

**IFN-β Effect on Monocyte NRP-1 and VEGF Chemotaxis**

Calcitriol increases BiT induction and expression of NRP-1 by monocytes. Conversely, IFN-β treatment significantly reduces Treg induction (Srivastava, Koch,
Pepper, & Campbell, 2014). Thus, I hypothesized that IFN-β would also reduce monocytic NRP-1. To address this, I assessed NRP-1 expression on both purified monocytes and monocytes in total cord blood cultures in vitro overnight in the presence or absence of IFN-β. We purified nucleated cell fractions from UCB, then treated cells with IFN-β. When monocytes were cultured in the presence of IFN-β, both as total PBMCs and enriched for CD14+ cells enriched and as total PBMCs, we observed a significant reduction in the level and frequency of NRP-1 expression by CD14+ monocytes demonstrating that IFN-β directly works on monocytes (Figure 14).

While we failed to demonstrate NRP-1 as a necessary factor for BiT induction, NRP-1 can bind multiple ligands. Another such ligand is vascular endothelial growth factor (VEGF). VEGF plays a pivotal role in organogenesis in embryos and angiogenesis in adults. VEGF promotes migration of monocytes which supports angiogenesis by producing growth factors. Since NRP-1 is a co-receptor for VEGF, which induces chemotaxis of monocytes, we next tested if IFN-β reduced monocyte chemotaxis towards VEGF. To address this, we performed a monocyte transwell migration assay to detect VEGF-induced migration of untreated monocytes in comparison to IFN-β treated monocytes to the bottom of the well overnight. As hypothesized, IFN-β treatment significantly reduced the migration of VEGF-exposed monocytes, suggesting that monocyte’s chemotactic response to VEGF is reduced by IFN-β (Figure 15A).
Figure 14. IFN-β Reduces NRP-1 Expression on Monocytes.
Purified UCB monocytes cultured in the presence or absence of 10ng/ml IFN-β overnight. (A) Representative figure depicting monocyte expression of NRP-1 cultured with other UCB cells in the presence or absence of 10ng/ml IFN-β overnight. (B) Quantification of NRP-1 percent change and MFI shift gated on monocytes (CD14+), ****p<0.0005 and **p<0.005, respectively. (C) Representative Figure of NRP-1 MFI shift on purified monocytes. (D) Quantification of NRP-1 percent change and MFI on purified monocytes, **p<0.005 and ***p<0.001, respectively. Data represent 4-5 donors. Isotype controls represent IFN-β treated monocytes labeled with isotype antibodies.
Figure 15. Reduced NRP-1 Resulted in Decreased Migration towards VEGF for IFN-β Treated, NRP1-/- cells, and anti-NRP-1 Treated Cells.

(A) Transwell migration fold change for IFN-β treated cells in the presence or absence of VEGF. Cell numbers are normalized to their respective non-VEGF control normalized to 1. **p<0.005. (B) Transwell migration fold change induced by VEGF. Relative number of the migrated cells with VEGF for each cell line is shown (number of THP-1 cells that migrated without VEGF are normalized to 1); * p<0.05 and **p<0.0001. (C) Transwell migration fold change induced by VEGF after primary monocytes were incubated with anti-NRP-1 neutralizing antibody or isotype control, number of monocytes migrated are normalized to respective isotype control, with isotype normalized to 1; *p<.05, **p<.005, ***p<.0005, and ****p<.0001.

If reduced monocyte chemotaxis in response to VEGF is caused by the reduction of NRP-1 expression, then monocytes that do not express NRP-1 would fail to respond to VEGF. To test this hypothesis, we utilized the NRP-1 knockout THP-1 cell lines
described above. As observed for untreated primary monocytes, VEGF induced chemotaxis of THP-1 cells transduced with Cas9 alone. In contrast, both NRP-1 knockout clones had significantly reduced migration in response to VEGF, similar to IFN-β treated monocytes (Figure 15B), demonstrating that expression of NRP-1 plays a critical role in VEGF-induced chemotaxis of monocytes. Finally, to demonstrate the role of NRP-1 on VEGF chemotaxis for primary monocytes, I repeated the transwell assay after pre-incubating primary monocytes with an anti-NRP-1 neutralizing antibody. As expected, blocking NRP-1 lead to reduced chemotaxis towards VEGF in a dose-dependent trend (Figure 15C). Together, these data demonstrate that IFN-β has a direct effect on reducing monocyte chemotaxis toward a VEGF enriched environment via reduction of NRP-1.

**SECTION 3: The Effect of Calcitriol on T cells**

**Calcitriol Promotes a Unique Cytokine Profile**

It is well appreciated that calcitriol is a key immunomodulatory molecule that has pleiotropic effects on immune cells, as discussed in Chapter 1. However, the effect of calcitriol on naïve CD4 T cell differentiation is not well defined. Our previous data along with others have shown that calcitriol treatment of CD4 T cells leads to a reduction in Th1 and Th9 cytokines and an upregulation in Th2 cytokines, while other reports show an increase in Treg induction (Figure 26C)(Cantorna et al., 2008; Mahon et al., 2003; Mattner et al., 2000; Nashold et al., 2001; Rausch-Fan et al., 2002; Sloka et al., 2011; Staeva-Vieira & Freedman, 2002; Takami et al., 2015). Therefore, we sought to
determine the cytokine profile of calcitriol-treated T cells. To do this, we treated naïve CD4 T cells isolated from human PBMCs with plate-bound anti-CD3/anti-CD28 stimulation in the presence of IL-2 for five days and then measured culture supernatants for T helper cytokines using a cytometric bead array (Figure 16). Coinciding with other studies concluding an increase in Th2 cells, we observed a significant increase in IL-13 production (Figure 16A). However, we observed no detectable levels of IL-4 and no significant change in IL-5 production compared to control (data not shown and Figure 16C). Instead, we report a striking elevation in IL-6 production (Figure 16B). In agreement with others, we also observed a decrease in IFN-γ and IL-22 production (Figure 16C). However, in contrast to the literature, we detected no significant changes in TNF-α or IL-10 (data not shown & 16C) (Di Rosa, Malaguarnera, Nicoletti, & Malaguarnera, 2011; Joshi et al., 2011). To our surprise, we also found a significant increase in macrophage colony stimulating factor (M-CSF) after calcitriol treatment (Figure 16D).

Our previous findings indicate that calcitriol inhibits AhR signaling which is important for T cell differentiation into Th9 cells and is also known to skew towards a Th1 dominant immune response (Negishi et al., 2005; Takami et al., 2015). However, when we tested whether AhR inhibition was necessary for this cytokine profile, utilizing a chemical antagonist specific to AhR (CH223191), we did not see any significant changes in cytokine production in comparison to control except for IFN-γ reduction (Figure 17). This suggests that other transcription factor(s) are important for the phenotype induced by calcitriol, and not directly from inhibition of AhR.
Figure 16. Calcitriol Promotes IL-13 and IL-6 Production by Human Naïve CD4 T Cells.
The effect of calcitriol on (A) IL-13, (B) IL-6, (C) IFN-γ, IL-5, IL-10, IL-22 and (D) M-CSF. Human naïve CD4 T cells enriched from adult PBMCs were stimulated under neutral conditions with IL-2 in the presence (squares) or absence (DMSO; circles) of 10nM calcitriol. Secreted cytokine concentrations were determined by cytometric bead array. Data represent the average of 7-9 donor samples, error bars represent standard deviation. *p<.05, **p<.005, and ***p<.0005.

Calcitriol Promotes CTLA-4 and PD-L1 Expression on T Cells

With the evidence that vitamin D is protective against autoimmunity and helps
alleviate symptoms, we hypothesized that calcitriol will upregulate checkpoint molecules to reduce inflammation. In line with our hypothesis, recent papers found that total CTLA-4 expression was upregulated after calcitriol treatment (Jeffery et al., 2009; Jeffery et al., 2015; Kickler, Ni Choileain, Williams, Richards, & Astier, 2012; Spanier et al., 2020).

To determine if our model produced results consistent with the literature, we treated naïve CD4 T cells with or without (DMSO) calcitriol in the presence of IL-2 on plate-bound anti-CD3 and anti-CD28 stimulation for 3-5 days and then labeled for extracellular and intracellular CTLA-4. We observed that calcitriol treatment upregulated surface CTLA-4 MFI and expression on CD4 T cells in comparison to the DMSO control (Figure 18A). The percent of cells expressing CTLA-4 on their surface was consistently higher than DMSO treated cells over time, where DMSO treated T cells began to lose expression of surface CTLA-4 after day 4 while calcitriol-treated T cells maintained heightened expression (Figure 18B). This observation is unique in the fact that the literature only detected total CTLA-4 instead of distinguishing between surface and intracellular expression. In congruence with the literature, we found total CTLA-4 to be upregulated in T cells after calcitriol treatment (Figure 18C), in which all cells were positive for CTLA-4 expression, with a majority of cells expressing both surface and intracellular CTLA-4. Further, MFI of both intracellular and extracellular CTLA-4 was elevated after calcitriol treatment (Figure 18D). Here, we demonstrate that CTLA-4 expression is drastically enhanced extracellularly after calcitriol treatment, while also maintaining elevated intracellular CTLA-4 expression in comparison to DMSO-treated control cells. To determine whether this upregulation of CTLA-4 was due to a change in
transcription, we performed qPCR. We also observed that CTLA-4 mRNA was elevated after calcitriol treatment (Figure 18E). Together, these data demonstrate that calcitriol has a direct effect on CTLA-4 upregulation, both at the mRNA and protein levels in naïve CD4 T cells which leads to increased CTLA-4 surface expression on these cells.

Figure 17. Inhibition of AhR does not Control Calcitriol-Treated Cell Cytokine Profile.
Calcitriol and AhR inhibitor (CH223191) effect on (A) IL-13, (B) IL-6, (C) IFN-γ and (D) TNF-α cytokine expression by naïve CD4 T cells stimulated by plate-bound anti-CD3 and anti-CD28 with IL-2 for 5 days in the presence of DMSO (circles), calcitriol (squares), or CH223191 (triangles). Data represent 3 donor samples, *p<.05 and **p<.005.
Figure 18. The Effect of Calcitriol on CTLA-4 Expression.
Surface CTLA-4 expression day 4 post-treatment on unstimulated, DMSO, and calcitriol-treated CD4 T cells, isotype control are calcitriol-treated cells labeled with respective isotype antibodies. (A) representative donor and (B) quantification on days 3-5, n=4. (C) CTLA-4 extra- and intracellular co-labeling. (D) MFI expression, n=4-5. (E) Relative CTLA-4 mRNA expression of unstimulated or stimulated cells with or without calcitriol treatment normalized to 18SrRNA (housekeeping) and then to the respective Day 0 control, n=3. Human naïve CD4 T cells from adult PBMCs were stimulated under neutral conditions with IL-2 in the presence or absence of calcitriol and labeled for flow cytometry days 3-5 post-treatment or isolated for RNA day 4 post-treatment. Data represent the average of 3-5 donor samples, error bars represent standard deviation. *p<.05, **p<.005, and ***p<.0005.
Since it is known that calcitriol aids in preventing autoimmunity and we observed an increase in CTLA-4 expression, we hypothesized that calcitriol would also upregulate the PD-1/PD-L1 axis on CD4 T cells. To test this, we used the same culturing method as in Figure 18. First, we measured PD-1 expression after harvesting cells on day 4 post-treatment and performed flow cytometry for surface expression of PD-1 and observed no significant difference in expression on CD4 T cells (Figure 19B, right). Next, we looked at a ligand for PD-1, PD-L1. In contrast to PD-1, we observed an increase in PD-L1 MFI and expression on the surface of CD4 T cells after calcitriol treatment on both 3- and 5-days post-treatment (Figure 19A/B). A recent report suggests that T cells have the ability to co-express multiple immune checkpoint molecules, and it is already known that both CTLA-4 and PD-L1 signaling is important for Treg function so we hypothesized that both pathways would be upregulated after calcitriol treatment (Baitsch et al., 2012). To determine if calcitriol treatment co-upregulates PD-L1 and CTLA-4 expression on human CD4 T cells, after 4 days we labeled cells for surface expression of PD-L1 and CTLA-4. We found that calcitriol treatment lead to a robust double positive population of both PD-L1 and CTLA-4, with few cells expressing only one checkpoint molecule in comparison to DMSO treated T cells (Figure 19C/D). These data demonstrate that calcitriol upregulates both CTLA-4 and PD-L1 expression on the surface of naïve CD4 T cells in comparison to DMSO-treated controls.
Figure 19. The Effect of Calcitriol on PD-L1 Expression.
Representative donor showing PD-L1 expression on unstimulated cells, DMSO treated and calcitriol-treated CD4 T cells after 5 days. (B) Quantification on day 5 for both mean fluorescence intensity (MFI) and percent positive cells. (C) Representative donor and (D) quantification displaying double positive phenotype for PD-L1 and CTLA-4 4 days after calcitriol treatment. Human naïve CD4 T cells from adult PBMCs were stimulated under neutral conditions with IL-2 in the presence or absence of calcitriol and labeled for flow cytometry days 3-5 post-treatment. Data represent the average of 3 or more donor samples, error bars represent standard deviation. *p<.05, **p<.005, and ***p<.0005.
Intrinsic and Extrinsic Suppression of the Immune System via CTLA-4

CTLA-4 has the ability to suppress in both an intrinsic and extrinsic manner (Bour-Jordan et al., 2011). Intrinsically, CTLA-4 competes with CD28 co-stimulation and leads to inhibition of IL-2 production, proliferation, and promotes cellular anergy. Extrinsic suppression occurs when CTLA-4 is able to suppress neighboring cells that do not express CTLA-4. One mechanism for this is transendocytosis, where CTLA-4 is able to remove CD80 and CD86 from the surface APCs and internalize them into the host cell (Qureshi et al., 2011). This reduces the bio-available pool of CD80 and CD86 to stimulate neighboring cells. Previous papers have demonstrated that calcitriol-treated T cells have the ability to suppress responder T cells in a suppression assay in a CTLA-4 dependent manner (Jeffery et al., 2009; Kickler et al., 2012). Further, one paper has demonstrated that calcitriol-treated T cells also have the ability to perform transendocytosis (Jeffery et al., 2015). However, it has yet to be elucidated whether CTLA-4 is acting intrinsically on the calcitriol-treated T cells. To address this, we expanded calcitriol-treated T cells for 4 days before co-culturing with naïve responder T cells and antigen presenting cells (APCs) for 6 days. To determine the proliferation of both responders and calcitriol-treated cells, we stained responders with CFSE and calcitriol-treated T cells with CTV. Indeed, we observed that calcitriol-treated T cells had potent suppressive capability over responder T cells in a dose dependent manner (Figure 20A/B). Further, we found that CTLA-4 did intrinsically suppress proliferation of calcitriol-treated T cells in comparison to DMSO control cells (Figure 20C/D). We demonstrate here that calcitriol-treated T cells proliferated at a slower rate than DMSO treated cells.
To determine if this reduced proliferation was CTLA-4 dependent, we repeated the assay using a neutralizing antibody against CTLA-4. We found calcitriol-treated T cell proliferation is restored after treatment with anti-CTLA-4 (Figure 20C/D). For both responders and calcitriol-treated cells, anti-PD-L1 had only a marginal impact on suppression of proliferation (data not shown). These data demonstrate that calcitriol promotes cells to have a suppressive capacity, while also upregulating functional CTLA-4 that intrinsically suppresses the proliferation of cells that are treated with calcitriol, where the increase of PD-L1 does not have an effect on this model.

**Calcitriol-Mediated Inhibition of AhR Controls Surface CTLA-4 Expression.**

While elevated expression of total CTLA-4 has been reported after calcitriol treatment in CD4 T cells, a mechanism has yet to be determined for this upregulation. Our previous study demonstrated that calcitriol inhibits the activation and expression of AhR. AhR is a transcription factor with pleiotropic roles in T cell differentiation and expression of cytokines and surface antigens in response to environmental stimuli. We hypothesized that calcitriol-mediated inhibition of AhR leads to the increase in CTLA-4 expression. To test this, we compared CTLA-4 and PD-L1 expression after calcitriol treatment along with using an AhR agonist, pyocyanin (Figure 21). We demonstrated that the AhR agonist reduced CTLA-4 expression on the surface of CD4 T cells, while calcitriol upregulated CTLA-4 expression and that calcitriol treatment could reverse the effects of pyocyanin (Figure 21A/C). To investigate further, we attempted to knock down AhR in T cells using siRNA. However, transfection conditions lead to a lack in CTLA-4 surface expression and thus we could not measure the effect of AhR loss.
We further hypothesized that calcitriol has a direct effect on CTLA-4 transcription. It is known that the turnover rate of CTLA-4 protein traditionally is linked to the rate of transcription, thus suggesting that the observed heightened transcription and protein expression of CTLA-4 (Figure 18 A/C/E) could be a direct effect from calcitriol (Valk, Rudd, & Schneider, 2008). To begin to answer this question, we analyzed the CTLA-4 promoter for VDRE binding sites. We found seven potential VDRE on the CTLA-4 promoter upstream of the transcription start site, suggesting that calcitriol may directly control CTLA-4 transcription (Figure 22). Further assays will need to be performed to determine the functionality of these VDRE.
Figure 20. Calcitriol-Treated Cells Suppress Responder T cells and are Intrinsically Suppressed by CTLA-4.
Representative sample of suppression of proliferation of responder T cells (CFSE) by calcitriol treated cells (grey) or DMSO-treated Cells (white) (A) and (B) quantified at 1 “suppressor” to 8 responder ratio between three donors. (C/D) Proliferation of calcitriol-treated or DMSO-treated cells (CTV) in the context of a suppression assay treated with anti-CTLA-4 or the respective isotype, p<.05.
Figure 21. Calcitriol Overcomes AhR Agonist, Pyocyanin, effect on CTLA-4 and PD-L1.
Quantification of (A) surface CTLA-4 percentage and (B) surface PD-L1 percentage on T cells after treatment with DMSO, calcitriol, pyocyanin (AhR inhibitor), and co-treatment of calcitriol and pyocyanin. (C) Representative donor flow plot depicting co-expression of PD-L1 and CTLA-4. Human naïve CD4 T cells from adult PBMCs were stimulated under neutral conditions with IL-2 in the presence or absence of calcitriol and labeled for flow cytometry 4 days post-treatment. Data represent the average of 2-3 donor samples, error bars represent standard deviation. *p<.05, **p<.005, and ***p<.0005
Figure 22. The Human CTLA-4 Promoter has Seven VDRE Sites. Sequence range of -1000 to 100 of the human CTLA-4 promoter. Capitalized letters are upstream of the transcription start site. Bolded sequences are VDRE segments, all with p<0.001.
CHAPTER FOUR: DISCUSSION

Summary of Data

We first sought to determine whether cord blood derived Tregs, BiTs, were genetically distinct from iTregs and tTregs. Data obtained previously in our lab demonstrated that BiTs had a unique cytokine profile in comparison to tTregs (Figure 2) (J. Lee et al., 2020). In this dissertation work, we examined the genetic profile of these cells by performing RNA-seq. We determined that indeed, BiTs are a unique subset of T cells, where they express unique markers while also expressing various markers from all T helper cell lineages (Figures 4-9). Of interest, BiTs have Foxp3 regulated similar to iTregs or pTregs, rather than tTregs, where the CNS2 promoter region is hypermethylated, suggesting a plasticity of these cells (Figure 3). We next examined BiT upregulated genes and determined that SGK1 and CD33 were potential markers for these cells, with SGK1 showing evidence of post-translational modification only in BiTs (Figures 7-9).

Previous data performed by us and others demonstrated that calcitriol promotes Treg induction (Gorman et al., 2007; Jeffery et al., 2009; Nashold et al., 2013; Penna et al., 2005; Prietl et al., 2010). We found in congruence with these results that calcitriol promoted BiT induction. However, in BiT induction cultures there are multiple cell types present. We next sought to determine whether calcitriol acted directly on T cells to promote BiT induction or if the manner was indirect through another cell type. We
decided to investigate monocytes because our lab previously found that monocytes were necessary to aid in BiT induction (Figure 10).

First, we investigated the effect on monocytes. We found that calcitriol upregulated NRP-1 expression on monocytes (Figure 11). It is known that NRP-1 is able to bind both latent and active TGF-β, while also having the role of being able to activate TGF-β. We hypothesized that this upregulation of NRP-1 would aid in BiT induction due to an increased pool of active TGF-β. However, while there was a direct correlation between NRP-1 expression and BiT induction, our knockout experiments did not yield any significant difference in BiT induction (Figure 12). However, since this was only using the THP-1 cell line, future experiments should be performed to confirm the same is true with primary monocytes. The only difference we observed was that NRP-1 had an increased effect on active TGF-β in the culture, but the total TGF-β pool was unchanged (Figure 13).

We and others have found that IFN-β decreases Treg induction and this held true for BiTs. When we looked at the effect of IFN-β on monocytes, we observed a complete loss of NRP-1 expression overnight (Figure 14). It is known that NRP-1 can respond to VEGF signaling in a chemotactic fashion (Dejda et al., 2014; P. Lee et al., 2002). Since IFN-β is known to reduce angiogenesis and VEGF is essential for angiogenesis and the recruitment of monocytes for angiogenesis, we hypothesized that the ablation of NRP-1 expression would lead to reduced monocytes chemotaxis (Hallene et al., 2006; Heil et al., 2000; Hong et al., 2005; K. J. Kim et al., 1993; Takano et al., 2014; Yang et al., 2003; Zeiher et al., 1995; H. Zheng et al., 2011). Indeed, we found a direct correlation
between NRP-1 expression, IFN-β treatment, and chemotaxis towards VEGF (Figure 15).

Finally, we decided to investigate the direct effect of calcitriol on T cells. Previous data by us and the literature stated that calcitriol promoted BiT/Treg induction, along with differentiation away from Th1 and towards a Th2 lineage (Figure 17) (Cantorna et al., 2008; Daniel et al., 2008; Jeffery et al., 2009). However, we found that upon stimulation of naïve CD4 T cells that calcitriol promoted differentiation of a unique subset of T cells. These T cells expressed suppressive markers similar to Tregs, CTLA-4 and PD-L1, however they produced cytokines such as IL-6, IL-13, and M-CSF (Figures 16-19). Even though these cells were not Tregs, they were functionally suppressive (Figure 20). The CTLA-4 expression was found to further have an intrinsic effect on suppressing proliferation of T cells (Figure 21).

In summary, we found that BiTs are a unique T cell subset that differs from previously defined Tregs. Calcitriol, which promotes BiT induction, was found to promote the expression of checkpoint molecules on the surface of naïve CD4 T cells. These checkpoint molecules, namely CTLA-4, had an intrinsic suppressive effect, thus bolstering immune suppression. Lastly, while NRP-1 was not necessary for BiT induction, we found that calcitriol upregulated NRP-1 and IFN-β downregulated NRP-1 on monocytes. This robust downregulation of NRP-1 is a novel mechanism by which NRP-1 could inhibit angiogenesis.

**BiTs are a Unique Cell Type**

Our lab previously described that BiTs upon restimulation expressed GM-CSF...
and IL-8, cytokines that stimulate macrophages and neutrophils, respectively (Figure 2) (J. Lee et al., 2020). These cytokines were not produced by Tregs harvested from APB which suggested that BiT were not classical Tregs even though they expressed Foxp3 and CD25 (Figure 2). While there are different types of Tregs, there is a lack of knowledge in the field of markers that distinguish them from each other (Curotto de Lafaille et al., 2004; Shevach & Thornton, 2014). Instead, currently the only definitive way of determining tTregs, pTregs, and iTregs from each other is the methylation status of the foxp3 promoter (Kitagawa et al., 2013; Lal et al., 2009; Minskaia et al., 2018; Morikawa et al., 2014). The CNS2 region confers stability of Foxp3 expression when it is de-methylated, a hallmark of tTregs (Morikawa et al., 2014; Someya et al., 2017; Y. Zheng et al., 2010). Our data show that BiT are more similar to iTregs and effector T cells, where the CNS2 region is methylated, suggesting plasticity in these cells. Our lab plans to further test methylation status on the CNS1 region in BiT, since iTregs and pTregs have a hypomethylated CNS1, while tTregs do not. This could further elucidate a mechanism for Foxp3 regulation in BiTs.

In order to determine whether BiTs are a unique T cell subset or if they had any distinguishing markers, we performed RNA-seq. From this, we found there are 2016 DEGs between effector T cells, BiT, tTregs, and iTregs. Our data suggests that BiT do not fall into any previously defined Th subsets, as they lack uniform upregulation of genes from any of these subsets (Figure 4). Next, we investigated whether there were any potential markers upregulated by BiT from the list of DEGs. There was one candidate gene that is known to be expressed on the surface of cells, CD33 (SIGLEC
3). CD33 has previously been recognized as a myeloid-specific marker that acts as an inhibitory receptor via its ITIM domain (Paul, Taylor, Stansbury, & McVicar, 2000). However, it has been reported that human T and NK cells can express splice isoforms of CD33 (Hernández-Caselles et al., 2006). The previous report suggests that CD33 can aid in inhibiting cytotoxic function in NK cells, however there have been no proposed functions for CD33 in T cells (Hernández-Caselles et al., 2006). Our data demonstrate that tTregs and iTregs do not express CD33, but a population of cells from BiT induction cultures do (Figures 6 and 7). Further experiments will need to be performed to determine which isoform of CD33 are expressed by these cells, if the ligand binding domain is intact, and whether it has a functional role in T cells. Our data also show a Foxp3+CD33+CD4+ population in fresh UCB that does not exist in fresh APB, further studies will have to be performed on this pre-existing population to define if these are naturally arising BiT (Figure 8).

**Novel Upregulated Genes in BiTs and their Functional Relevance on Foxp3 Expression**

Besides CD33 as a potential surface marker for BiT, we also examined three intracellular proteins: SOCS1, THEMIS, and SGK1. While BiT had upregulated SOCS1 and THEMIS on the mRNA level in comparison to tTregs and iTregs, on the protein level SOCS1 was moderately upregulated, and THEMIS was only upregulated in comparison to tTregs and expressed less than iTregs. However, we observed a striking phenotype when detecting SGK1 expression in BiTs. Unlike in iTregs and tTregs, SGK1 in BiTs were post-translationally modified as demonstrated by a smear on western blot
Figure 9). Post translational modifications, such as ubiquitination and sumoylation, add weight to their target protein, which is what leads to this smear. These modifications can alter the localization, activity, and stability of the flagged protein leading to loss of functionality or degradation of the protein (Geiss-Friedlander & Melchior, 2007; N. Zheng & Shabek, 2017). This suggests that while SGK1 is upregulated by BiT, it is also negatively controlled by potentially one of these processes. Of interest, SGK1 has been reported as a mechanism for control of the Th17-Treg axis (C. Wu et al., 2018). The mechanism involves phosphorylated SGK1 binding and sequestering Foxo1 from entering the nucleus. This downstream leads to the inhibition of Foxp3 transcription and promotes IL-23R transcription, allowing for Th17 differentiation while inhibiting Treg differentiation (C. Wu et al., 2018). However, another article defines that SGK1 can be ubiquitinated by a complex of Rictor and Cullin-1, which leads to degradation of SGK1 (Gao et al., 2010). Of note, one of the DEGs upregulated by BiT is Rictor, while Cullin1 counts are relatively equal among all samples (data not shown). Further, another member in the lab recently demonstrated that restimulating BiTs in the presence of differentiating cytokines does not differentiate BiTs into other T cell subsets, and Foxp3 expression is similar among all treatments suggesting that Foxp3 is stable in these samples regardless of CNS2 methylation status (data not shown). This also coincides with previous data that Foxp3 expression remains stable in BiT for months (Figure 1). These data suggest that post-translational modification of SGK1 in BiT could inhibit its ability to sequester Foxo1, leading to subsequent stable expression of Foxp3. However, further experiments should be performed to determine whether BiT can be induced from
already differentiated cells, such as Th1 or Th2 cells which could uncover whether naïve T cells are necessary for this phenotype.

**Implications of these Data: Foxp3 Control in BiT**

It is well appreciated that understanding tolerance in fetuses and neonates is important, as the tolerance acquired as a fetus and in early life can last a lifetime (Billingham et al., 1953; Maloney et al., 1999; Mold et al., 2008). In these data, we define BiT, a Treg unique from other previously defined Treg subsets. Our data identify CD33 as a candidate surface marker because we observed CD33 in fresh UCB and not APB on Foxp3+CD4+ T cells. Since CD33 is a surface protein, CD33 expression will allow for tracking of this subset and further phenotypic testing (Figures 6-8). Previously, without knowledge of a marker for BiT, we were unable to bring our studies into a mouse model. With CD33 in mind, our lab can continue to look for a similar marker in mice, which could allow for *in utero* and *in vivo* studies, something we have been limited from performing with human-only samples.

With the existence of a surface marker for BiT, our lab can begin to address questions pertaining to the persistence of BiT. We know that BiT were not detectable in healthy adult peripheral blood (Figure 8) in contrast to UCB and NICU blood. This suggests to us that this cell type only persists at a young age, but we have not assessed whether BiT arise during immune dysregulation or during pregnancy in mothers. However, it is known that T cells from UCB and neonatal PBMCs have a higher propensity to develop into Tregs (Figure 1) (Burlingham et al., 1998; Mackroth et al., 2011; A. K. Simon, Hollander, & McMichael, 2015; Takahata et al., 2004). This
propensity rapidly dwindles during the first 36 months of life (Olin et al., 2018; Shearer et al., 2003; Zhao et al., 2007). Preliminary experiments performed by Alex Nelson, M.S., and Christina Cunha in our lab assessed the ability to induce BiT from adult naïve T cells. Nelson found that depletion of granulocytes allowed for BiT induction, while conversely, Cunha found that adding adult T cells to a UCB culture inhibited BiT induction. Both experiments provide rationale that factors from adult immune cells inhibit BiT induction, however more experiments need to be performed to determine which factor(s) are responsible and why.

Vaccination in utero and in early life has posed difficulties in the field due to suboptimal immune responses toward the vaccine, in which a tolerant and Treg-rich environment have been named as culprits (Ndure & Flanagan, 2014). Understanding how to control or manipulate this tolerance could allow for designing co-treatments with vaccinations to boost fetal and neonatal responses and thus protecting them from viral infections (Ndure & Flanagan, 2014). However, while it is well known that T cells from UCB have an increased propensity to become Tregs, the mechanism for this is not well understood (C. C. Chang et al., 2005; de Roock et al., 2011; Dirix et al., 2013; J. Lee et al., 2020; Takahata et al., 2004; Torelli et al., 2012). Our data propose a model for Foxp3 regulation in BiT involving post-translational modification of SGK1 (Figure 23). Further studies will need to be performed to determine whether the mechanism is sumoylation or ubiquitination, and whether the previously described Rictor-Cullin1 complex is present in BiT (Gao et al., 2010). Uncovering and understanding this mechanism for Foxp3 control can help uncover why T cells from UCB have a higher
propensity to become Tregs. In the context of vaccinations, manipulating this proposed system by administering a Rictor inhibitor to inhibit Foxp3 stability could lead to a heightened response to vaccines. Further in vivo testing would need to be performed to test this model.

**Figure 23. Proposed Model for Foxp3 Stability in BiT.**
Figure depicting a proposed model for Foxp3 stability in BiT. This model involves the presence of Rictor and Cullin-1 complexing and poly-ubiquitinating SGK-1. This poly-Ub sequesters SGK1 away from FoxO1, allowing for FoxO1 to translocate into the nucleus and promote transcription of Foxp3.

**NRP-1** **Contributes to a Larger Pool of Active TGF-β but is not Necessary for BiT Induction**

Our lab previously described the necessity for monocytes in BiT induction
cultures and that monocyte-derived TGF-β was important for this effect (Figure 10) (J. Lee et al., 2020). Jessica found that monocytes were essential for TGF-β production by depleting different non-T cell populations from UCB and performing BiT induction cultures. The only cultures she found that had reduced BiT induction were CD14+ monocytes, which reduced BiT induction by approximately 50%. She then looked at pSMAD2/3 induction in the presence or absence of CD14+ monocytes, which is indicative of TGF-β signaling, and found that this was reduced by roughly ~40% when there were no monocytes in the culture (Figure 10). We found that calcitriol upregulated NRP-1 expression on monocytes and that calcitriol promoted increased BiT induction (Figure 11). It has been previously established that NRP-1 is able to bind both latent and active TGF-β, and also have the functionality to activate TGF-β (Glinka & Prud'homme, 2008; Glinka et al., 2011). We hypothesized that this upregulation of NRP-1 would aid in BiT induction due to an increased pool of active TGF-β. However, while there was a direct correlation between NRP-1 expression and BiT induction, our NRP-1 knockout experiments did not yield any significant difference in BiT induction (Figure 12). The only difference we observed was that deletion of NRP-1 reduced the concentration of active TGF-β in the culture, but the total TGF-β pool was unchanged (Figure 13). These data suggest to us that since the total TGF-β pool was unchanged, the presence of NRP-1 is not a determining factor in BiT induction and further studies must be performed to determine how calcitriol promotes BiT induction in the context of monocytes.
Novel Discovery that IFN-β Controls NRP-1 Expression

Angiogenesis is important for fetal development because proper blood flow is necessary for both the health of the pregnancy and for the fetus’ development by aiding in nutrient transfer from the mother to the fetus (Mayhew et al., 2004; Reynolds et al., 2006). When this blood flow is restricted there is decreased fetal growth, vascularity, and an increased probability for compromised pregnancies (Crivellato, 2011; Mayhew et al., 2004; Reynolds et al., 2006). Angiogenic factors, such as VEGF, play a role in organogenesis by signaling to organs to instruct patterning and morphogenesis (Crivellato, 2011). With how crucial angiogenesis is for fetal development, it is important to recognize mechanisms which can bolster or restrict angiogenesis. Our studies focused primarily on effects that could impede VEGF-mediated chemotaxis of monocytes. It is accepted that VEGF plays a pivotal role in angiogenesis by acting as a chemotactic factor for endothelial cells and monocytes (Heil et al., 2000; Hong et al., 2005). One receptor known to respond to VEGF signaling in a chemotactic fashion is NRP-1 (Hallene et al., 2006; Heil et al., 2000; Hong et al., 2005; K. J. Kim et al., 1993; Takano et al., 2014; Yang et al., 2003; Zeiher et al., 1995; H. Zheng et al., 2011). Further, it is also known that IFN-β is a potent inhibitor of angiogenesis through multiple mechanisms (Hallene et al., 2006; K. J. Kim et al., 1993; Takano et al., 2014; Yang et al., 2003; Zeiher et al., 1995; H. Zheng et al., 2011). However, our data report a novel mechanism for IFN-β mediated downregulation of NRP-1 which leads to a subsequent significant reduction in chemotaxis towards a VEGF enriched environment (Figures 13, 14, and 15).
Implications of these Data: A Novel Mechanism for IFN-β Mediated Reduction in Angiogenesis

While previous reports established mechanisms by which IFN-β can reduce angiogenesis and chemotaxis toward a VEGF-enriched environment, these mechanisms were indirect (Hallene et al., 2006; K. J. Kim et al., 1993; Takano et al., 2014; Yang et al., 2003; Zeiher et al., 1995; H. Zheng et al., 2011). In contrast, we uncovered a novel mechanism by which IFN-β directly reduces NRP-1 expression on monocytes (Figure 13). We further demonstrate that this loss of NRP-1 correlates to subsequent reduced monocyte chemotaxis toward a VEGF-enriched environment (Figure 14 and 24).

Clinically, this mechanism is important to consider during fetal and neonatal development. In the context of TORCH infections resulting in IFN-β production, this could lead to the inhibition of NRP-1 expression on monocytes, resulting in reduced angiogenesis and organogenesis and subsequent developmental impairments (Crivellato, 2011; Gerhardt et al., 2004; Hallene et al., 2006; Phillips et al., 2016; Yockey et al., 2018). IFN-β can be produced by various cell types dependent on the type and location of infection, such as: fibroblasts, epithelial cells, NK cells, macrophages, and dendritic cells (Scheu, Dresing, & Locksley, 2008; Swiecki & Colonna, 2011). TORCH infections are infections capable of breaking the maternal-placental barrier that activate an immune response that can lead to abnormal development and pregnancies (Arora et al., 2017; Yockey & Iwasaki, 2018). When these infections pass the maternal-placental barrier, they can come in contact with cells in the placenta and developing fetus.
Varying immune responses can activate based on the type of the infection, leading to the production of cytokines which cause different birth complications (Yockey & Iwasaki, 2018). Trophoblast cells, which are necessary for implantation and provide nutrients to the embryo and develop to be part of the placenta, respond to type 1 IFNs by producing IFN-stimulated genes which results in impaired differentiation, reduced developmental capacity, and fetal growth (Baines et al., 2020; Rostovsky & Davis, 2015). Further, exposure to infection by glial cells in the CNS also causes robust production of IFN-β which can lead to brain developmental defects (Owens, Khoroooshi, Wlodarczyk, & Asgari, 2014; Yockey & Iwasaki, 2018). During pregnancy, VEGF expression is upregulated both in placental tissue, peripheral blood, and in the mother’s serum (Ren et al., 2014; Wheeler et al., 1999). This VEGF subsequently aids in angio- and organogenesis which is necessary for fetal development and the health of the pregnancy (Wheeler et al., 1999). Our data suggest that IFN-β produced in response to TORCH infections will lead to the reduction of monocytic NRP-1 in the fetus, thus resulting in reduced chemotaxis towards this special VEGF-enriched environment and lead to subsequent reduction in angiogenesis, organogenesis, and placental health. This model will need to be tested in vivo.

In contrast, IFN-β has recently been established as a promising treatment used to inhibit vascularization and angiogenesis in tumor microenvironments, particularly in the brain (Takano et al., 2014). In these previous studies, researchers have found that presence of monocytes and macrophages at the sites of these tumors leads to poor prognosis (Cao et al., 2001; Caponegro et al., 2018; Takano et al., 2014). Our data
suggest that IFN-β-mediated reduction of NRP-1 may lead to reduced monocyte and macrophage chemotaxis to the VEGF+ tumor microenvironment, aiding in reducing angiogenesis at the site of the tumor. These possibilities require further investigations with in vivo models.

**Figure 24. Proposed IFN-β-Mediated NRP-1 Downregulation Model.** Diagram depicting that without IFN-β, monocytes express NRP-1 and are able to migrate towards a VEGF enriched environment. In contrast, the presence of IFN-β reduces NRP-1 expression and the monocytes no longer migrate towards VEGF.

**The Suppressive Effect of Calcitriol on T Cells**

It is well known that calcitriol can upregulate Tregs, protect against autoimmunity,
and alleviate autoimmune symptoms (Jeffery et al., 2009; Lemire & Archer, 1991) (Shaygannejad et al., 2012; Wingerchuk et al., 2005). We further showed that calcitriol can also upregulate BiT induction (Figure 11). However, the mechanism by which calcitriol alters naïve T cells to promote this suppressive environment is not well understood.

In this work, we demonstrated that calcitriol promotes co-expression of surface CTLA-4 and PD-L1. Further, total CTLA-4 is increased, consistent with previous literature (Figure 18) (Jeffery et al., 2009; Jeffery et al., 2015). CTLA-4 is known to have both intrinsic and extrinsic effects, where it can suppress the cell in which it is activated on and also suppress neighboring cells by both the power of its signal and by transendocytosis (Jain et al., 2010; Wang et al., 2012). Our data show that CTLA-4 has an intrinsic effect on calcitriol-treated T cells (Figure 20). In line with testing the extrinsic effect of CTLA-4, another team reported that calcitriol treated cells do perform transendocytosis (Jeffery et al., 2015). Further, we and others reported that calcitriol-treated cells are suppressive of responder T cells, similar to tTregs (Figure 20) (Jeffery et al., 2009). However, testing whether that suppression is dependent on CTLA-4 is a difficulty in the field. In classic Treg studies, there are an abundance of conflicting reports on the functionality of CTLA-4 in suppression and there is a wide variation in culture setup and anti-CTLA-4 blocking antibody function (Walker, 2013). One such difficulty is that suppression assays take days and CTLA-4 is rapidly recycled, produced, and degraded which means that originally blocked CTLA-4 may no longer be present, and the blocking antibodies can get degraded with it. In our preliminary studies,
one donor showed CTLA-4 dependent suppression, while another did not. Further testing will have to be performed to answer whether suppression is CTLA-4 dependent.

**Calcitriol-Treated T Cells Express a Unique Cytokine Profile**

While it is known that calcitriol can promote immune suppression, it was unknown whether calcitriol is a differentiating factor for naïve CD4 T cells. Literature reports that calcitriol leads to an increased repertoire of Th2 cells and Tregs, with a resulting decrease in Th1 cells (Cantorna et al., 2008; Daniel et al., 2008; Jeffery et al., 2009). However, differentiation from naïve cells or in purified cultures had not been performed. Our data demonstrate that calcitriol does indeed reduce Th1 cytokine production (IFN-γ) and promotes Th2 cytokine production (IL-13) (Figure 16). However, there was no significant difference in IL-10 production (Treg) or IL-5 (Th2) and there was no detectable level of IL-4 production (Th2) (Figure 16 and data not shown). In contrast, there was also a significant increase in IL-6 and M-CSF production (Figure 16). M-CSF is known to shift monocytes to differentiate into the immune-suppressive M2 macrophage phenotype. This suggests that calcitriol promotes M-CSF production by T cells which could in turn boost immune suppression through M2 macrophage differentiation. Further, IL-6 is not considered as a T cell cytokine under healthy conditions, however, one group has described IL-6 producing T cells from mitogen stimulated PBMCs, that they termed Th6 (Azizi-Semrad et al., 2010; Willheim et al., 1999). These data suggest to us that calcitriol can promote Th6 differentiation, but further studies will need to be performed to determine their function. Th6 function is still unknown, because prior to this study Th6 was only found in total PBMC cultures and no
differentiating factor was known, making it impossible to isolate. With the discovery that calcitriol can promote IL-6 production from naïve CD4 T cells, more work is required elucidate their immunological role.

Implications of these Data: Calcitriol Promotes Immune Suppression Through Upregulation of CTLA-4

The evidence that calcitriol promotes checkpoint molecules CTLA-4 and PD-L1, has the functional capacity to suppress neighboring cells, and CTLA-4 has intrinsic suppressive functionality supports the correlation that calcitriol protects against autoimmunity and alleviates autoimmune severity (Figures 18-20) (Jeffery et al., 2009; Lemire & Archer, 1991) (Shaygannejad et al., 2012; Wingerchuk et al., 2005). In contrast, these data suggest that calcitriol treatment during cancer treatment could be detrimental, since calcitriol promotes CTLA-4 and PD-L1 expression, which are two signaling pathways commonly co-inhibited as a cancer therapy (Wei et al., 2019). More studies will have to be performed to better understand the role of calcitriol in the context of cancer, and whether these markers get upregulated in a tumor microenvironment in response to calcitriol.

While it has been previously reported that calcitriol can upregulate total CTLA-4, the mechanism by which this occurs nor the surface phenotype had been elucidated (Jeffery et al., 2009). Two previously established mechanisms for controlling CTLA-4 transcription are NFAT and cAMP (Gibson et al., 2007; J. Li et al., 2013). However, calcitriol is known to inhibit both NFAT and cAMP pathways, suggesting to us that there is another mechanism (Berg & Haug, 1999; Takeuchi et al., 1998). Our data show that
calcitriol’s ability to upregulate CTLA-4 is dominant over an AhR agonist, suggesting that AhR may play a role in CTLA-4 control (Figure 21 and 25). However, upon transfection with siRNA against AhR or a scrambled control, we were unable to restore surface CTLA-4 expression meaning we could not measure the effect of AhR on CTLA-4. Further studies will need to be performed, potentially using a CRISPR/Cas9 system to further elucidate this mechanism. However, calcitriol may be able to directly promote CTLA-4 transcription, determined by the presence of six VDRE binding elements upstream of the transcription start site on the CTLA-4 promoter (Figure 22 and 25). We plan to design luciferase assays testing these VDRE sites in order to test this hypothesis in the future.

**Figure 25. Proposed Model for Calcitriol and AhR Control of CTLA-4.** Diagram depicting the proposed model of AhR inhibiting and calcitriol promoting CTLA-4 transcription, leading to altered CTLA-4 on the surface of the T cell.
REFERENCES


Askew, F., Bourdillon, R., Bruce, H., Jenkins, R., & Webster, T. (1930). The Distillation of Vitamin D. *107*, 76-90.


McCollum, E., Simmonds, N., Becker, J., & Shipley, P. (1922). An experimental demonstration of the existence of a vitamin which promotes calcium deposition. 52, 293-298.


dihydroxyvitamin D3. *Int Arch Allergy Immunol, 128*(1), 33-41.
doi:10.1159/000058001

doi:10.1073/pnas.84.10.3385


doi:10.1097/00054725-199911000-00009


induced production of IL-2 and IFN-gamma and the proliferation of human peripheral blood leukocytes while enhancing the surface expression of HLA class II molecules. *J Endocrinol Invest, 12*(5), 329-335. doi:10.1007/BF03349999


Trifari, S., Kaplan, C. D., Tran, E. H., Crellin, N. K., & Spits, H. (2009). Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol, 10*(8), 864-871. doi:10.1038/ni.1770


VITA

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In 2014, Anya was accepted into the Loyola University Chicago Interdisciplinary Program in Biomedical Sciences. While at Loyola, Anya joined the Department of Microbiology and Immunology and performed her doctoral work in the laboratory of Dr. Makio Iwashima. Anya’s doctoral work focused on understanding the differences between Treg populations in umbilical cord blood and adult peripheral blood and how the effect of calcitriol on monocytes and T cells enhances immune suppression. This work was supported by the T32 Immunology Training Grant awarded to Dr. Katherine Knight and the Arthur J. Schmitt Fellowship.