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Monocyte-Induced Regulatory T Cell Differentiation

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ABSTRACT

Immediately after birth, thousands of foreign antigens challenge the newborn immune system. Many of the invaders are harmless, such as food, pollen, and beneficial bacteria. Newborns have a tolerant immune system that keeps them from developing inflammation or allergies to these new antigens. *In utero,* this immunoregulatory tendency is important for establishing tolerance to self and maternal antigens. Multiple processes contribute to fetal tolerance, including clonal deletion, anergy, changes in antigen presenting cells (APCs), and the generation of regulatory T cells (Tregs). However, the mechanism(s) of fetal Treg differentiation and the specific APCs required are unknown.

Our lab has previously shown that many $CD4^+$ and $CD8^+$ T cells from umbilical cord blood (UCB) differentiate into Forkhead box P3 $(Foxp3)^+$ Tregs after T cell receptor (TCR) stimulation *ex vivo*. Depleting $CD14^+$ monocytes from UCB abrogates Treg generation, while purified $CD14^+CD36^{hi}$ monocytes are sufficient to induce Treg differentiation from naïve T cells. The function of monocytes in protecting against bacterial infection, maintaining blood vessel integrity and promoting tissue repair are well known. However, their immunoregulatory properties have largely gone unrecognized. The goal of this dissertation is to identify the mechanisms monocytes use to induce Treg generation and describe how this process is impaired in adult blood or during disease states.

In this work, I demonstrate that monocytes induce Treg differentiation by providing three critical signals to naïve T cells: membrane-bound transforming growth factor beta (TGF-β), retinoic acid and Notch ligands. $CD14^{+}CD36^{hi}$ monocytes are the only UCB cells capable of presenting all three molecules to T cells, highlighting their importance for immune homeostasis. Ligand binding to the CD36 receptor can impair Treg generation and skew T cells to produce effector cytokines, such as IL-4. Pathogenically elevated levels of CD36 ligands, such as oxidized low-density lipoprotein (ox-LDL) during atherosclerosis or beta-amyloid during Alzheimer's, may aggravate inflammation by impairing Treg generation.

IL-4 potently blocks Treg generation from UCB, more than other inflammatory or effector cytokines. IL-4 has several reported mechanisms of inhibiting Foxp3 expression in T cells. We found that IL-4 also differentially regulates the mediators of TGF-β signaling, Smad2 and Smad3. Downregulation of Smad3 by IL-4 correlates with impaired Treg generation and knockdown of Smad3 alone is sufficient to decrease Foxp3 expressing cells.

Together, these results demonstrate that $CD14^{+}CD36^{hi}$ monocytes are an important immunoregulatory cell, capable of simultaneously producing multiple signals required for Treg differentiation. The impairment of monocyte-induced Treg generation by CD36 ligands may be an unrecognized cause of inflammation during diseases such as atherosclerosis. Furthermore, enhanced IL-4 production by T cells in the presence of ox-LDL may be one mechanism of impairing Treg differentiation.

CHAPTER ONE

INTRODUCTION

Immune Tolerance

The field of immune tolerance was pioneered in the late 1940s and early 1950s. In 1945, Ray D. Owen described that anastomoses between the fetal blood supply of dizygotic cattle twins can result in the exchange of blood cells and hematopoietic precursors *in utero.* Adult cows subsequently maintained blood cells from their twin (1). These genetically mosaic cows could also be generated in twin calves originating from different fathers. These experiments demonstrated that the cows could tolerate genetically disparate cells obtained *in utero*.

In 1951, Medawar and colleagues found that cattle did not reject skin transplants from their dizygotic twin, including those of the opposite gender (2). This was in contrast to allogeneic human and rabbit skin grafts, which failed due to robust immune responses (2). However, cows did reject transplants from non-twin siblings. These findings supported the idea that fetuses can generate long-term tolerance to antigens they encounter *in utero.*

To rigorously test this hypothesis, Medawar and colleagues injected mouse embryos with cell suspensions of homogenized testis, kidney and spleen from donors of a different mouse strain (3). Eight weeks after birth, the mice received a skin transplant from the donor strain. A substantial percentage of the recipient mice tolerated the skin

grafts, while maintaining the ability to reject skin grafts from a third, unrelated donor. These data demonstrated that fetuses induce antigen-specific immune tolerance to antigens encountered *in utero*.

Suppressor and Regulatory T Cells

After the seminal experiments by Owen and Medawar, interest in immune tolerance steadily grew (4). Early studies tried to identify the cells that mediated immune tolerance by using different methods of inducing tolerance to a particular antigen (5). A paper in 1971 by Gershon and Kondo described what they termed, "infectious immunological tolerance" (6). They showed T cells from mice tolerized to sheep red blood cells could dominantly suppress T-dependent antibody production when co-transferred with non-tolerized T cells. At the time, the authors speculated this dominant suppression was due to a factor the tolerized T cells produced or stimulated other cells to produce. They named this immunosuppressive factor IgY, since it suppressed immunoglobulin production (6, 7).

In the following year, Gershon and colleagues published a second paper that demonstrated thymocytes could also suppress the proliferation of sensitized T cells when co-transferred into a lethally irradiated host and challenged with antigen (8). In this paper, the authors considered the possibility that there was a unique subset of "suppressor T cells." However, they did not isolate or identify this population further.

By the mid 1970s, it was still unclear whether a distinct subset of T cells existed with suppressive function or whether the same population that provided B cell help could also suppress. In 1976, Herzenburg and colleagues used complement based depletion of

Ly-1^+ (CD5) or Ly-2^+ (CD8) cells and found that suppressive activity was lost with Ly-2^+ depletion (9). In contrast, it was known that helper T cells expressed Ly-1, but lacked Ly-2 (10, 11). These data suggested suppressor T cells were distinct from helper T cells, and were found within the $CD8^+$ subset.

Despite these findings, skepticism in a distinct suppressor T cell population grew in the 1980s for a number of reasons(5). There were no known markers to distinguish suppressor cells from effector T cells. Clinicians lacked evidence that a loss of suppressor T cells contributed to human disease. A final blow to the suppressor T cell field was the supposed discovery of the I-J molecule that mediated suppressor T cell function and was encoded in the MHC locus (12–14). Later studies demonstrated the MHC locus contained no such gene (15).

It had been noted since 1969 that thymectomy in neonatal mice precipitated autoimmune disease (16). The combination of non-lethal irradiation and thymectomy of adult rats also induced autoimmunity (17). Researchers hypothesized that neonatal thymectomy or adult thymectomy with radiation depleted a suppressive population of T cells. In agreement with this, later studies showed that adding back total thymocytes or only $CD4^+CD8^-$ thymocytes could prevent autoimmunity in these animals, suggesting the thymus gave rise to an immunosuppressive $CD4^+$ T cell (17, 18).

Researchers sought to identify this immunosuppressive population by transferring various $CD4^+$ subsets into animals lacking T cells. Using this method, they found murine $CD4^+$ T cells expressing high levels of CD5 or low levels of CD45B suppressed autoimmunity, while $CD45B^{hi}CD4^+$ and $CD5^{lo}CD4^+$ T cells exacerbated it (19–21).

Similarly, in rats, suppressive T cells were contained within the $CD45C^{low}CD4^{+}$ or $RT6.1^{+}$ T cells (22, 23). These data suggested that a population of autoreactive T cells escape thymic negative selection, but are normally prevented from initiating autoimmunity by a subgroup of $CD4^{+}$ T cells with suppressive function (24).

To find a more definitive marker for immunoregulatory T cells, Sakaguchi and colleagues searched for a surface protein whose expression correlated with high levels of CD5 and low levels of CD45B, consistent with previous studies of suppressive cells (24). In this way, they identified the IL-2 receptor alpha chain, CD25, as expressed on $CD5^{hi}$ $CD45B^{-}$ T cells. Approximately 10% of $CD4^{+}$ T cells, but only a minor percentage of $CD8^{+}$ and non-T cells, expressed CD25. To test whether $CD25^{+}$ T cells were suppressive and prevented autoimmunity, the authors transferred CD25 depleted cells from the spleens and lymph nodes to T cell deficient recipients. CD25 depleted cells, but not total lymphocytes, induced multi-organ autoimmunity, involving the gastrointestinal tract, ovaries, thyroid gland, salivary glands, adrenal gland and pancreas. These results could be recapitulated by injecting purified $CD4^{+}CD25^{-}$ cells. In contrast, co-transfer of enriched $CD4^{+}CD25^{+}$ prevented autoimmunity, definitively demonstrating that $CD4^{+}CD25^{+}$ cells are immunosuppressive and required for immune homeostasis. Of note, co-transfer of $CD8^{+}$ lymphocytes also protected against autoimmunity, but to a lesser extent than $CD4^{+}CD25^{+}$ cells. Because of the previous skepticism toward suppressor T cells, these newly identified $CD4^{+}CD25^{+}$ T cells were termed “regulatory T cells” (Tregs).

A few years later, several groups discovered that mutations in the transcription factor Foxp3 causes the fatal multi-organ autoimmune disease in humans with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX; also known as x-linked autoimmunity-allergic dysregulation syndrome, XLAAD) (25–28). Mutations in Foxp3 also lead to a similar lymphoproliferative disease in scurfy mice (29, 30).

The symptoms of scurfy mice and IPEX patients resembled the experimentally induced autoimmunity caused by depletion of $CD4^+CD25^+$ Tregs, leading to the hypothesis that Foxp3 was expressed by Tregs and that a loss of Tregs caused these diseases. Indeed, a series of publications in 2003 demonstrated that Foxp3 is specifically expressed on murine $CD4^+CD25^+$ Tregs and that Tregs are lost in scurfy mice (31–33). Adoptive transfer of $CD4^+CD25^+$ Tregs into Foxp3 null mice rescued them from autoimmunity (33). Furthermore, overexpression of Foxp3 in $CD4^+CD25^-$ cells induced suppressive function (32). Later studies demonstrated that CD4 specific knockout of Foxp3 is sufficient to induce widespread autoimmunity (34). Together, these data demonstrated that the lymphoproliferative disease in mice and humans lacking Foxp3 is due to a loss of $CD4^+CD25^+$ Tregs.

Foxp3 is necessary for the development of Tregs and mediates their suppressive activity. In mice, Foxp3 is specifically expressed in T cells with regulatory function. However, in humans, Foxp3 is expressed transiently in all activated T cells, but is only stably maintained in Tregs (35).

Peripherally-Induced Regulatory T Cells

Early on, $CD4^+CD25^+$ T cells with suppressive function were found not only in blood and secondary lymphoid organs, but also in the thymus (36). This led to the conclusion that $CD4^+CD25^+Foxp3^+$ regulatory T cells are generated in the thymus and these cells were termed thymic-derived Tregs (tTregs), or more commonly, naturally arising Tregs (nTregs) (37). Later, researchers found that Foxp3 expression could also be induced on peripheral naïve $CD4^+$ T cells by TGF-β along with TCR stimulation, costimulation, and IL-2 (38–41). These induced Tregs suppressed T cell proliferation *in vitro* and *in vivo*, similar to nTregs. Peripherally induced Tregs are termed pTregs when induced *in vivo* or iTregs when induced *in vitro* (37). Beyond the minimum requirement for TCR stimulation, costimulation, IL-2 and TGF-β, multiple signals can enhance induced $Foxp3^+$ Treg generation. Among these are IL-10, Notch and retinoic acid (42–54).

$CD8^+$ Regulatory T Cells

$CD8^+$ T cells with regulatory function have been recognized since the early 1970s (9). However, their phenotype, function, and generation during steady state are still ambiguous. Numerous phenotypes have been used to identify these cells, including $CD8^+CD122^+$, $CD8^+CD28^-$, $CD8^+Foxp3^+$, $CD8^+CD103^+$, $CD8^+LAG\text{-}3^+CTLA\text{-}4^+$, $CD8^+IL\text{-}10^+CCR7^+CD45RO^+$, $CD8^+CD45RC^{low}$, $CD8^+CD122^+PD\text{-}1^+$ and $CD8^+CD11c^{high}$ [reviewed in (55)]. Whether these markers identify distinct subsets needs to be examined more carefully. However, it is clear that more than one population of $CD8^+$ Tregs exists.

$CD8^+$ Tregs can be generated in the thymus or in the periphery (55, 56). Like their $CD4^+$ counterpart, $CD8^+$ Tregs cells can be induced by APCs *in vitro* or *in vivo* (57). However, other cells that do not express MHCII may also induce $CD8^+$ Tregs. For example, TGF-β2-expressing corneal endothelial cells or B7-1/B7-2 expressing pigmented epithelial cells induce $CD8^+$ suppressive cells in the eye (58–60). A growing body of literature describes a different group of $CD8^+$ Tregs that are induced upon stimulation by the non-classical MHCI, Qa-1 (called HLA-E in humans) (55, 57). $CD8^+$ cells recognize peptides presented on Qa-1 via their inhibitory receptor complex NKG2/CD94 (61). Alternatively, the TCR can recognize Qa-1/peptide complexes and stimulate $CD8^+$ Tregs (62). Therefore, while $CD4^+$ Treg induction requires MHCII-restricted antigen presentation by an APC, antigens presented from a variety of cell types can induce $CD8^+$ Tregs.

During experimentally induced colitis, transfer of either $CD4^+CD45RB^{low}$ Tregs or $CD8^+CD122^+$ Tregs ameliorates disease. However, transfer of both $CD4^+$ and $CD8^+$ Tregs provides synergistic protection (63). These results are consistent with the hypothesis that $CD4^+$ and $CD8^+$ Tregs recognize distinct targets and have non-redundant roles in maintaining homeostasis and resolving inflammation. However, more studies are needed to understand the division of labor between $CD4^+$ and $CD8^+$ Tregs.

$CD8^+$ Tregs mediate immune suppression through both contact-dependent and independent mechanisms. They can secrete immunosuppressive factors, such as IL-10, indoleamine 2,3 dioxygenase, and TGF-β (55). $CD8^+CD28^-$ Tregs inhibit DC maturation and upregulate inhibitory receptors, impairing the ability of DCs to activate effector T

cells (64–66). CD8 Tregs can also kill effector T cells through FasL-Fas induced apoptosis or perforin-mediated cytolysis (55). During animal models of multiple sclerosis, it is thought that $CD8^+$ Tregs kill pathogenic T cells through recognition of their autoreactive TCRs (67).

In summary, numerous CD8 Treg populations have been described with varied mechanisms of antigen recognition and suppression. Although not well understood, $CD8^+$ Tregs play a distinct role in maintaining immune homeostasis and tolerance.

Mechanisms of Neonatal Tolerance

Since Owen's discovery of fetal tolerance in calves, researchers have sought to understand how fetal tolerance is established. It is increasingly clear that multiple mechanisms play a role. Early studies showed fetal antigen specific T cells undergo clonal deletion in response to foreign cells experimentally injected into mouse fetuses (68, 69). Some clones escape deletion, but subsequently develop anergy, their function restored with exogenous IL-2 (69).

More recent studies suggest fetuses generate $CD4^+CD25^+$ Tregs to promote tolerance to antigens encountered *in utero*. The fetus has an increased percentage of Tregs, however, their frequency decreases to adult levels by birth (70, 71). An exception to this is in pre-term newborns that have increased Tregs at the time of birth that persist at elevated levels past the first year of life (70). Although Tregs are not found at higher percentages in UCB than adult PMBCs, they expand more readily from UCB (72). In addition, upon stimulation by immature DCs, a higher percentage of $CD4^+CD25^-$ T cells from UCB differentiate into suppressive $CD4^+CD25^+$CTLA-4^+ cells than from adult

PBMCs (73). In agreement with this, other studies showed UCB naïve $CD4^+$ T cells have an intrinsic propensity to differentiate into Tregs compared to adult naïve $CD4^+$ T cells (74, 75). This may be due to increased expression of PD-1 on UCB T cells, diminishing the strength of CD28 costimulation and promoting Treg differentiation (74, 76, 77). From these data, UCB has been considered a source of Tregs and UCB T cells thought to readily differentiate into Tregs. This is likely important for establishing self-tolerance *in utero*, since the majority of antigens encountered in this setting are self-antigens.

In addition to self-antigens, human fetuses encounter maternal DNA and cells that cross the placental barrier and establish microchimerism that can be maintained into adulthood (78–81). Because of this fetal exposure, individuals are less likely to develop antibodies against non-inherited maternal antigens after blood transfusions than to other foreign human leukocyte antigens (HLAs) (82). Similarly, bone marrow and solid transplants mismatched for maternal HLAs are better tolerated than those mismatched for paternal HLAs (83, 84).

As early as 1977, Oldstone and colleagues found T cells from newborns inhibit maternal T cell proliferation (85). Consistent with this, Mold and colleagues demonstrated that human fetuses generate Tregs specific to maternal antigens (81). Like humans, mice establish maternal microchimerism through cells transferred in the placenta or breast milk and this leads to Treg induction and increased tolerance to grafts containing maternal antigens (86–89). Together, these data suggest that Treg induction to antigens encountered *in utero* is a critical mechanism of both human and murine fetal

tolerance. However, little is known about the mechanism of peripheral Treg induction in the fetus.

Some studies suggest that UCB APCs contribute to Treg differentiation (74, 90). APCs from human UCB and neonatal mice express decreased MHCII and costimulatory molecules compared to adults, with reduced capacity to activate T cells (91–93). Encabo and colleagues demonstrated that the immature phenotype of UCB DCs correlates with an increased ability to induce $CD4^+$ Tregs (90). Although these studies suggest UCB APCs are important for establishing fetal tolerance, the requirement of precise APC populations and their mechanism of inducing Tregs are largely unknown.

Monocyte Subsets

Metchnikoff described monocytes as a part of the mononuclear phagocytic system in the early 1900s (94). Early studies identified monocytes from other blood cells by their morphology and ability to phagocytize particles (95). In 1971, Yam and Crosby reported that monocytes stain with nonspecific esterases, and this staining combined with phagocytic assays was adopted by a number of researchers to define monocytes (96). Later, monoclonal antibodies were developed that specifically bound monocytic cells, many of which targeted various epitopes of CD14 (97, 98).

Studies in the 1970's and 1980's revealed monocytes were a heterogeneous population. Two to three groups of peripheral blood monocytes were identified based on size, cytoplasmic staining and nuclear morphology (99–102). These studies classified monocytes into small and large monocytes, with a third, intermediate group. Functionally, the monocyte subsets were distinct. Large monocytes expressed Fc

receptors and could induce antibody-dependent cellular cytotoxicity (ADCC), whereas small monocytes lacked these properties (101). Large monocytes had greater migration toward serum chemoattractants and produced more colony-stimulating factor (CSF) than small monocytes (102, 103). Large monocytes also produced higher levels of superoxide anions and myeloperoxidase needed for bactericidal activity (103).

The development of multi-color flow cytometry allowed the distinction of small and large monocytes by surface antigen expression. Two consecutive papers in the 1980s by Passlick, Flieger, and Ziegler-Heitbrock demonstrated two populations of $CD14^{+}$ monocytes exist: $CD14^{hi}$ and $CD14^{lo}$. $CD14^{hi}$ monocytes were larger, lacked the FcγRIII CD16 and were the most abundant subset. The smaller $CD14^{lo}$ monocytes expressed CD16 and accounted for only 13% of blood monocytes (98, 104). Compared to $CD14^{hi}$ monocytes, $CD14^{lo}$ monocytes had higher MHCII expression, but were less phagocytic of opsonized RBCs. Today it is accepted that three primary subsets of human blood monocytes exist: $CD14^{hi}CD16^{-}$, $CD14^{hi}CD16^{+}$, and $CD14^{lo}CD16^{+}$ monocytes (105).

Three populations of mouse monocytes analogous to humans were identified in the early 2000s based on expression of Cx_3Cr1, Ly6C, CCR2 and CD45 (106–109). Cx_3Cr1^{lo} $Ly6C^{hi}$ $CCR2^{hi}$ $CD45^{lo}$ murine monocytes resemble human $CD14^{hi}$ $CD16^{-}$ cells (110, 111). These two groups in mouse and man are often referred to as "inflammatory monocytes," but a recent consortium recommended "classical monocytes" as a better term (105, 111). Cx_3Cr1^{hi} $Ly6C^{lo}$ $CCR2^{lo}$ $CD45^{hi}$ murine monocytes share many properties with human $CD14^{+}$ $CD16^{+}$ monocytes and both are termed "non-classical monocytes", also referred to as "patrolling" or "alternative" monocytes (111). Finally,

mouse Ly6C^{med} CD45^{hi} and human CD14^{hi}CD16^{+} cells are referred to as intermediate monocytes.

Monocytes differentiate from a succession of precursors in the bone marrow. There has been debate over whether classical, non-classical and intermediate monocytes represent distinct lineages, or whether their phenotypes reflect different maturation stages. Several studies showed classical monocytes can differentiate into non-classical monocytes (108, 112). For example, Ly6C^{hi} monocytes are the first to populate the blood after liposome depletion, followed by Ly6C^{lo} cells. Furthermore fluorescently labeled Ly6C^{hi} monocytes convert to Ly6C^{lo} cells *in vivo* (108). A seminal paper in 2011 by Hanna and colleagues demonstrated that the transcription factor Nr4a1 (Nur77) was required for Ly6C^{lo} non-classical monocyte survival in the bone marrow (113). Nr4a1$^{-/-}$ mice selectively lost Ly6C^{lo} monocytes in the periphery, while maintaining Ly6C^{hi} monocytes. The study also demonstrated that non-classical monocytes could arise directly in the bone marrow. Nr4a1$^{-/-}$ mice became a tool to specifically delete non-classical monocytes for functional studies. Human CD14^{lo}CD16^{+} monocytes also express Nr4a1 more highly than other monocyte subsets, suggesting a conserved role of this transcription factor (114).

Because mouse and human monocytes do not share the same surface markers, a study was conducted to examine the similarities between monocyte subsets in the two species. They found non-classical monocytes from mice and humans shared 63 genes that were upregulated compared to classical monocytes (111). However, the authors also identified 33 genes that were oppositely expressed between mouse and human non-

classical monocytes. These data suggested human $CD14^{+}CD16^{+}$ and mouse $Ly6C^{lo}$ Cx_3Cr1^{hi} cells were analogous populations, but not identical.

Classical monocytes in mice and man express the adhesion molecules CD62L and CCR2 (107). In contrast, non-classical monocytes lack CCR2 and CD62L, but express higher levels of CX_3CR1. Human $CD14^{+}CD16^{+}$ monocytes also express higher levels of CCR5, which recognizes MIP1α (RANTES) (115). In murine studies, classical monocytes are mainly found in the blood and spleen at steady state, but can home to sites of inflammation through the interaction of CCR2 with CCL2 (MCP-1) in inflamed tissues (107, 116). In contrast, non-classical monocytes were found in a variety of murine tissues in the absence of inflammation through the interaction of Cx_3Cr1 with its ligand, fractalkine, on endothelial cells (107).

Cros et al. compared the function of different monocyte subsets from human peripheral blood (110). Unstimulated $CD14^{+}CD16^{-}$ cells were highly phagocytic and produced the highest levels of ROS, myeloperoxidase and lysozyme. $CD14^{+}CD16^{+}$ intermediate monocytes produced low levels of these molecules, but maintained the ability to phagocytose latex beads. Unstimulated $CD14^{lo}CD16^{+}$ monocytes had very little phagocytic ability or production of the aforementioned molecules. Upon stimulation by lipopolysaccharide (LPS) or viral ligands, $CD14^{+}CD16^{-}$ produced IL-8, IL-6, and CCL2. $CD14^{+}CD16^{+}$ cells produced the highest levels of TNF-α and IL-1β in response to LPS, and also produced TNF-α in response to some viral ligands. In contrast, $CD14^{lo}CD16^{+}$ monocytes did not respond to LPS challenge. However, they upregulated TNF-α, IL-1β, and CCL3 when challenged with viruses, toll-like receptor (TLR) 7 and TLR8 ligands.

Together, these data suggest monocyte subsets have distinct functions during steady state and infection.

Monocyte Function in the Vasculature

In 2007, Auffray and colleagues used intravital confocal microscopy to study the movement of Cx_3Cr1^{hi} (non-classical) and CX_3Cr1^{lo} (classical) monocytes during steady state and inflammation (117). $CX3Cr1^{lo}$ classical monocytes primarily circulated in blood until they encountered inflammatory signals, upon which they rolled along the blood vessel walls in the direction of the blood flow. However, Cx_3Cr1^{hi} (non-classical) monocytes displayed a peculiar "crawling" motion along the blood vessel walls in the absence of any inflammation. Their movement occurred in multiple patterns and directions, regardless of the blood flow, distinct from the characteristic "rolling" of immune cells prior to diapedesis. Crawling was completely abolished by an antibody against the integrin LFA-1, and partially blocked in CX_3Cr1 deficient mice. Upon sterile inflammation, tissue damage, or infection, Cx_3Cr1^{hi} non-classical monocytes rapidly migrated to the site of inflammation. They were the first cells to migrate to the peritoneal cavity after *L. monocytogenes* challenge, and were the earliest producers of TNF-α. However, upon recruitment of other inflammatory cells, CX_3Cr1^{hi} monocytes downregulated TNF-α and upregulated genes required for tissue remodeling. These studies revealed a novel role for non-classical monocytes as "patrolling" cells that monitor blood vessel walls during steady state, act as first responders at the site of inflammation and infection, and promote tissue repair.

Since this initial discovery, Ly6C^{lo}Cx$_3$Cr1^{hi}CCR2^{-} monocytes have been found patrolling the vessel walls in a variety of organs, including the dermis, mesentery, brain, kidney, lung, heart, cremaster muscle and liver (117–123). In mice, it is estimated that one-third of non-classical monocytes adhere to vessel walls at any one time (124). Human CD14^{lo}CD16^{+} also display patrolling behavior in an LFA-1-dependent manner when transferred into a lymphopenic *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mouse (110). Human CD14^{lo}CD16^{+} monocytes are demarginalized during exercise, due to a release of catecholamines, and are also increased in the blood during infection (125, 126).

Patrolling monocytes may maintain blood vessel homeostasis by clearing dead cells and debris (110, 118, 120). In the kidney vasculature, Ly6C^{lo} non-classical monocytes respond to TLR7 ligands by recruiting neutrophils, which mount an immune response that damages the endothelial cells (120). Ly6C^{lo} monocytes subsequently phagocytize the dead endothelial cells. In the lung, Ly6C^{lo} monocytes survey the blood-air barrier, phagocytize metastatic tumor cells and recruit NK cells to kill the tumor (121, 127). Mice with global or myeloid-specific deletion of Nr4a1that lack Ly6C^{lo} monocytes have increased lung metastasis in several tumor models (127). Ly6C^{lo} monocytes have also been observed patrolling cranial veins when beta-amyloid is present, but not in the absence of beta-amyloid (118). In a model of Alzheimer's disease, mice lethally irradiated and reconstituted with Nr4a1$^{-/-}$ bone marrow had a greater number of beta-amyloid deposits in the hippocampus and cortex than mice reconstituted with wild-type bone marrow. Together, these studies demonstrate non-classical monocytes can survey the vessel walls and clear debris to maintain vessel homeostasis.

Monocyte Subsets During Atherosclerosis

Both Ly6C^{hi} and Ly6C^{lo} monocytes reside in murine atherosclerotic lesions, but Ly6C^{hi} are more abundant (128). Soehnlein and colleagues suggest Ly6C^{hi} classical monocytes contribute to the generation of atherosclerotic lesions (129). The role of non-classical monocytes during atherosclerosis is still unclear. Nr4a1$^{-/-}$ Apolipoprotein E (ApoE)$^{-/-}$ mice lacking Ly6C^{lo} monocytes have increased atherosclerotic plaques, lipid uptake and macrophage deposition in the aortic root than mice deficient for ApoE only (114). Atherosclerosis is also more severe in low-density lipoprotein receptor deficient (Ldlr$^{-/-}$) mice lacking Nr4a1 in bone marrow cells (130). These results suggest a protective role of Ly6C^{lo} cells during atherosclerosis. However, mice lacking either Cx_3Cr1 or CD36, which are expressed more highly on Ly6C^{lo} monocytes compared to Ly6C^{hi}, have less severe atherosclerosis (111, 131–134). It is unclear whether the decreased disease severity in these mice is due to the function of Cx_3Cr1 and CD36 on Ly6C^{lo} cells. Alternatively, the observed phenotypes may be due to a loss of these molecules on Ly6C^{hi} cells, even though expressed at lower levels. Indeed Ly6C^{hi}, but not Ly6C^{lo}, monocytes use Cx_3Cr1 to migrate to atherosclerotic plaques, despite lower levels of the receptor on Ly6C^{hi} cells (128). Therefore, more studies need to be done to define the roles of each monocyte subset during atherosclerosis in mice.

In humans, a variety of studies have linked CD16 expressing monocytes with atherosclerosis and severe cardiovascular disease (135). CD14^{+}CD16^{+} monocytes are increased in patients with coronary artery disease, along with an increase in serum TNF-α levels (136). Both soluble and membrane-bound CD16 is elevated in patients with

coronary artery disease (137). However, it is unclear whether this increased risk is due to $CD14^{lo}CD16^{+}$ non-classical monocytes or $CD14^{hi}CD16^{+}$ intermediate monocytes (135). In two studies of patients with chronic kidney disease, elevated $CD14^{+}CD16^{+}$ intermediate monocytes was a predictor of cardiovascular events (138, 139). Together, the literature suggests CD16 expressing monocytes may contribute to atherosclerosis in humans. The role of $CD16^{-}$ classical monocytes during atherosclerosis requires further studies.

CD36

CD36 is a highly conserved class B scavenger receptor with homologs found in animals as low as the fly, worm and sponge (140, 141). The protein has a large extracellular domain, with short N-terminal and C-terminal cytoplasmic tails. Many cells express CD36 including microvascular endothelial cells, cardiac muscle, skeletal muscle, adipocytes, mammary glad cells and keratinocytes (141). Among hematopoietic cells, monocytes, macrophages, dendritic cells and platelets express CD36. A number of ligands bind CD36 including thrombospondin-1 (TSP-1), oxidized-LDL (ox-LDL), fibrillar beta-amyloid, fatty acids, collagen, and a growth hormone releasing peptide called hexarelin (142–149). It also binds components of photoreceptor outer segments, gram-positive cell walls, apoptotic cells, and red blood cells infected with *Plasmodium falciparum* (150–156).

CD36 ligand binding usually triggers endocytosis and activation of intracellular signaling events, despite its short cytoplasmic tails (141). The function of CD36 depends on the identity of its ligand. TSP-1 inhibits angiogenesis when bound to CD36 (157).

This function may be redundant with other CD36 ligands, as oxidized LDL can also inhibit endothelial cell differentiation, growth, migration and angiogenesis (157–159).

TSP-1 has a number of other CD36-dependent roles. It activates latent-TGF-β (160–163) and inhibits inflammatory cytokine production in the presence of bacterial ligands and apoptotic cells (164, 165). In other contexts, the TSP-1/CD36 interaction can initiate an inflammatory program, such as TLR4 activation and TNF-α production (166).

CD36 acts as a fatty acid translocase for long-chain fatty acids (147, 167). Loss of CD36 in mice leads to decreased fatty acid uptake in adipocytes, skeletal and cardiac muscle, with an increased dependence on glucose (168–170). Similarly, CD36 deficient humans have decreased long chain fatty acid uptake by the heart (171–173).

As a scavenger receptor, CD36 binds a number of conserved motifs to mediate macrophage clearance of apoptotic cells, pathogens and modified lipids, such as ox-LDL. CD36 is also required for macrophage recognition of some TLR2/6 ligands, suggesting a role as a TLR coreceptor (152, 153). Mice deficient for CD36 have decreased ability to phagocytize and clear *Staphylococcus aureus*, leading to increased mortality during infection (152, 153).

In the context of atherosclerosis, CD36 mediates uptake of ox-LDL by macrophages to initiate foam cell formation, the characteristic cell residing in atherosclerotic plaques (174). CD36 deficiency in mice and humans impairs macrophage uptake of ox-LDL (168, 175). Furthermore, in mouse models of atherosclerosis, loss of CD36 leads to decreased atherosclerotic plaques in the aortic tree and aortic sinus, suggesting CD36 contributes to disease (132–134). Moore et al. contested this idea

because of data showing that CD36 deficient mice have increased aortic sinus lesions (176). However, Fabbraio and colleagues suggest that measuring the total lesion area in the aortic tree is a better measure of the extent of atherosclerosis (141). Using this readout, CD36 knockout mice consistently have more severe cardiovascular disease. In mice, CD36 is expressed more highly on $Ly6C^{lo}$ non-classical monocytes than $Ly6C^{hi}$. However, in humans, it is higher on $CD14^{hi}CD16^{-}$ classical monocytes (111).

The summary of the literature suggests CD36 has many functions in many contexts. On the surface of monocytes, CD36 could limit inflammation by triggering apoptotic cell clearance or TGF-β activation. In contrast, CD36 could mediate ox-LDL uptake, foam cell formation and atherosclerosis progression. *In vivo,* CD36 plays numerous roles in maintaining steady state and during disease. Its function on monocyte subsets likely depends on the disease context and the species studied.

CHAPTER TWO

MATERIALS AND METHODS

Antibodies

Antibodies used for flow cytometry were: anti-phospho-SMAD2/3 and IL-2 (BD Biosciences; San Jose, CA), anti-LTBP1 and DLL3 (R&D Systems; Minneapolis, MN), anti-CD103 (eBioscience; San Diego, CA) and anti-TSP1 (Beckman Coulter; Brea, CA). Anti-CD3, CD4, CD8, CD14, CD25, CD28, CD36, LAP, GARP, Nrp1, HLA-A2, Helios, Foxp3, IFN-γ, IL4, IL-17a, IL-10, IL-5, IL-13, and IL-9 antibodies were from Biolegend (San Diego, CA). Functional grade antibodies for cell culture, anti-CD3 (OKT3) and anti-CD28 (CD28.2), were from Biolegend. Western blot antibodies for Notch-1 (D1E11), Notch-2 (D76A6), cleaved Notch-1 (D3B8), Smad2 (D43B4) and Smad3 (C67H9) were from Cell Signaling Technology (Danvers, MA). Anti-β-actin was from Sigma-Aldrich (St. Louis, MO).

Chemicals, Peptides and Recombinant Proteins

The following reagents were used: human IL-2, IL-4, IL-5, IFN-γ, TNF-α, IL-12, IL-6 and IL-1β (PeproTech; Rocky Hill, NJ) and human TGF-β (R&D systems); a TGF-β receptor I kinase inhibitor, SB431542 (Sigma-Aldrich); an RAR antagonist LE135 (Tocris; Bristol, UK); an RAR agonist AM580 (Tocris); a gamma secretase inhibitor, DAPT (Sigma-Aldrich); low-density lipoprotein (LDL) (Kalen Biomedical; Montgomery Village, MD); and high oxidized LDL (ox-LDL) (Kalen Biomedical). The peptides

LSKL, GGWSHW, cyclic CSVTCG, and Ova (257-264) were purchased from AnaSpec (Fremont, CA). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Carlsbad, CA).

Mononuclear Cell Isolation and Cell Purification

UCB was collected into citrate phosphate dextrose solution. Neonatal and infant samples (ages 7 days -24 months) were collected from healthy donors in EDTA. Adult PBMCs from healthy donors were collected in heparin or buffered sodium citrate. Mononuclear cells were enriched by density dependent centrifugation using Lymphocyte Separation Medium (Corning Cellgro, Tewksbury, MA). In some samples, RBCs were lysed with ACK lysis buffer (Gibco, NY). For UCB, a second isolation with Lymphocyte Separation Medium was performed after ACK lysis. Total T cells (negative selection), $CD4^+$ T cells (negative selection), naïve $CD4^+$ T cells (negative selection), $CD8^+$ T cells (positive selection) and $CD14^+$ monocytes (positive selection) were enriched from mononuclear cells using BD IMag Enrichment Sets (BD Biosciences) or EasySep enrichment kits (STEMCELL Technologies; Vancouver Canada). IMag kits were used for depleting $CD14^+$ cells. CD3 depleted, $CD14^+CD36^{hi}$, $CD14^+CD36^{lo}$, $CD14^-CD36^+$ and $CD14^-CD36^-$ cells were isolated by cell sorting on FACS Aria (BD Biosciences).

Treg Induction Culture and Plate-Bound Stimulation

Total UCB mononuclear cells were stimulated with human IL-2 (10 ng/ml; >100 U/ml) and anti-CD3 (0.2 μg/ml). Medium was changed every 2-3 days, maintaining IL-2 concentrations. Treg percentages were analyzed after 12-15 days of culture, unless otherwise specified. For purified co-cultures, T cells were cultured with monocytes or

irradiated THP-1 cells (3000rad) at a 1:3 ratio with α-CD3 and IL-2. For plate-bound stimulation, $CD4^+$ T cells isolated by immunomagnetic sorting were plated on untreated tissue culture plates that were coated with α-CD3 and α-CD28 (5 μg/ml each), washed and blocked in 10% FBS.

Where indicated SB431542 (10mM), LE135 (5mM), AM580 (1 or 100 nM), DAPT (40mM), LDL (50mg/ml), ox-LDL (50mg/ml), IL-4, IL-5, IFN-γ, TNF-α, IL-12, IL-6, IL-1β, LSKL, GGWSHW, cyclic CSVTCG, and Ova (257-264) peptide were added once at the beginning of cultures at the listed concentrations. Where indicated, the percent inhibition of Treg differentiation was calculated as $[(\%Treg_{control} - \%Treg_{treated})/ \%Treg_{control}]$.

Suppression Assay

Tregs were generated by stimulating UCB with anti-CD3 and IL-2. Foxp3 expression was confirmed by flow cytometry and $CD4^+$ and $CD8^+$ cells were separated by immunomagnetic enrichment. Unstimulated naïve $CD4^+$ T cells were enriched from allogeneic adult PBMCs, labeled with 5μM CFSE and used as responder cells. CD3 depleted cells sorted from the same PBMC donor and irradiated at 3000 rad were used as APCs. Responder cells were stimulated with anti-CD3 (0.2 μg/ml) and APCs at a 1:1 ratio, in the presence or absence of the indicated ratio of Tregs. The percent of proliferating cells was determined after 5 days of culture as the percent of cells with diluted CFSE using flow cytometry.

Adult PBMC and UCB Co-Cultures

For direct co-culture, adult PBMCs and UCB were screened for expression of HLA-A2 by flow cytometry. HLA-A2 mismatched adult PBMCs and UCB were co-cultured at a 1:1 ratio. Where indicated, adult PBMCs and UCB (not screened for HLA-A2) were separated by a 0.4 μm transwell. In both cases, cells were stimulated with IL-2 (10 ng/ml; >100 U/ml) and anti-CD3 (0.2 μg/ml). Two weeks later, the percent of $Foxp3^+$cells were compared from adult and UCB T cells.

Flow Cytometry

Foxp3 staining was performed with the Foxp3 Fix/Perm Buffer Set (Biolegend). For phospho-SMAD2/3 staining, cells were fixed using Lyse/Fix Buffer (BD Biosciences) and permeabilized with Perm Buffer III (BD Biosciences). For intracellular cytokine staining, cells were restimulated with phorbol myristate acetate (PMA; 50ng/ml), ionomycin (1uM), and monensin (2uM) for 4 hours, fixed in 4% paraformaldehyde and permeabilized in a solution of 50mM NaCl, 5mM EDTA, 0.02% Sodium azide, 0.5% TritonX, pH 7.5. Prior to staining, cells were blocked in 20 μg/ml of human IgG and surface stains were performed using standard protocols. Data were collected on a FACS Canto II (BD Biosciences) or FACS LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Multiplex Cytokine Analysis

Cells were restimulated with PMA (50ng/ml) and ionomycin (1uM) for 4 hours and the supernatants harvested. Cytokine production of IL-17a, IFN-γ, TNF-α, IL-2, IL-4, IL-6 and IL-10 was determined using the human Th1/Th2/Th17 cytometric bead array kit (BD

Biosciences), according to the manufacturer's instructions, except decreasing the assay volume 5-fold.

ALDEFLUOR™ Assay

ALDH activity was measured in freshly isolated UCB mononuclear cells using the ALDEFLUOR™ Kit (STEMCELL Technologies), according to the manufacturer's instructions.

Western Blot

$CD4^+$ cells were enriched by immunomagnetic sorting and lysed in SDS sample buffer (2% SDS, 125 mM DTT, 10% glycerol, 62.5 mM Tris-HCl [pH 6.8]). Equal numbers of cells were used for polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene fluoride membranes, blocked with 5% milk and blotted with the indicated antibodies. Proteins were detected using the ECL detection reagent (GE Healthcare, Piscataway, NJ). Relative band intensities were determined using ImageJ software (National Institutes of Health).

siRNA Knockdown of Smad2 and Smad3

Allstars negative control, Smad2 gene solution and Smad3 gene solution siRNAs were purchased from Qiagen (Hilden, Germany). Enriched $CD4^+$ T cells were electroporated with 200 pmol of control or Smad2 siRNAs, or 100 pmol each of two Smad3 siRNAs using a Human T Cell Nucleofector Kit (Lonza; Basel, Switzerland). The cells were rested for approximately 5 hours and then co-cultured with irradiated THP-1 cells with IL-2 (10 ng/ml; >100 U/ml) and anti-CD3 (0.2 μg/ml).

Reverse Transcription and PCR

RNA was isolated from enriched or sorted cells using ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI) and converted to cDNA using oligo deoxythymidine and SuperScript III First-Strand Synthesis System (Life Technologies; Carlsbad, CA). PCR analysis of TSP-1 and DLL3 were performed using Ex Taq DNA polymerase (TaKaRa; Katsushika, Tokyo, Japan), with the following primers:

DLL3 forward 5'-GTCCGAGCTCGTCCGTAGA-3';

DLL3 reverse 5'-CGGACAGAATCGAGGAAGGG-3';

TSP-1 forward 5'-AACCGCATTCCAGAGTCTGG-3';

TSP-1 reverse 5'-TTCACCACGTTGTTGTCAAGGGT-3'. The conditions used for PCR were: initial denaturation at 95°C for 3 min; 30 cycles (for DLL3) or 35 cycles (for TSP-1) of denaturation at 95°C for 30 sec, annealing at 56.5°C (DLL3) or 59°C (TSP-1) for 30 sec, and extension at 72°C for 45 sec; 72°C final extension for 10 min. Real-time PCR analysis was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA), according to the manufacturer's protocol. The following primers were used:

Foxp3 forward 5'-TGGGGTAGCCATGGAAACAG-3';

Foxp3 reverse 5'-CTCATTGAGTGTCCGCTGCT-3';

Smad2 forward 5'-ATTTGCTGCTCTTCTGGCTCAGT-3';

Smad2 reverse 5'-CAGCAAGGAGTACTTGTTACCGT-3';

Smad3 forward 5'-GTCAAGAGCCTGGTCAAGAAAC-3';

Smad3 reverse 5'-CGATGGGACACCTGCAACC;

Hprt forward 5'-GAAGAGCTATTGTAATGACC-3';

and Hprt reverse 5'-GCGACCTTGACCATCTTTG. The relative expression of *Foxp3, Smad2,* and *Smad3* were quantified using the ΔΔCt method, normalizing each sample to *Hprt*.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (San Diego, CA), except Cuzick's Non-Parametric Test, which was conducted by the Clinical Research Office at Loyola. The following designation was used throughout the paper: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

CHAPTER THREE

MECHANISMS USED BY $CD14^{+}CD36^{hi}$ MONOCYTES TO INDUCE TREG DIFFERENTIATION

Introduction: Monocyte-Induced Treg Generation from UCB

Fetuses have a unique tendency to generate tolerance to antigens they encounter *in utero*, a phenomenon termed fetal tolerance. Fetal tolerance is maintained by a variety of mechanisms, including active immune suppression through Treg generation. Using UCB as a source of fetal cells, previous studies have found that Tregs can be readily differentiated or expanded from UCB (72–75). UCB T cells reportedly have an increased tendency to differentiate into Tregs compared to adult T cells and UCB APCs also support Treg differentiation. However, the mechanisms of Treg generation in the fetus and the APC population(s) required are largely unknown.

To address these questions, the Iwashima lab utilized UCB as a source of full-term fetal immune cells. In agreement with previously reports, we found unstimulated UCB has the same frequency of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs as adult PBMCs (data not shown) (70, 71). However, the majority of UCB T cells are naïve; they have not received the signals necessary for differentiation. Therefore, we asked what phenotype UCB T cells acquire when stimulated. Specifically, we hypothesized the potential for extrathymic Treg differentiation is increased in UCB compared to adult PBMCs.

Peripheral Treg generation requires TCR stimulation, costimulation, TGF-β and IL-2 (38–40). IL-2 is a general T cell growth factor that acts in an autocrine matter and is required for induced Treg differentiation (39, 41, 177). Unlike most T cells, Tregs do not produce IL-2, but rely on exogenous sources (178). Many groups have induced Tregs *in vitro* by stimulating naïve T cells with immobilized αCD3 and αCD28 in the presence of high levels of exogenous TGF-β and IL-2. Although this method efficiently generates Foxp3-expressing T cells, it is artificial. A more physiologically relevant approach is to use APCs as a source of costimulation with endogenous sources of TGF-β.

To address whether UCB has an increased propensity for Treg differentiation compared to adult PBMCs, we stimulated blood mononuclear cells with an αCD3 antibody in medium containing IL-2. A large percentage of UCB $CD4^+$ T cells (65.6%, ± 3.5) acquired the prototypic Treg markers CD25 and Foxp3, whereas few (19.5%, ± 7.3) adult $CD4^+$ cells became $CD25^+Foxp3^+$ (Fig. 1A-B). Notably, stimulated UCB gave rise to both $CD4^+$ and $CD8^+$ $Foxp3^+$ T cells. Because the phenotype of $CD8^+$ Tregs is less well characterized, we used total $CD8^+Foxp3^+$ cells (both $CD25^+$ and $CD25^-$) for statistical analysis of CD8 Tregs, unless otherwise specified.

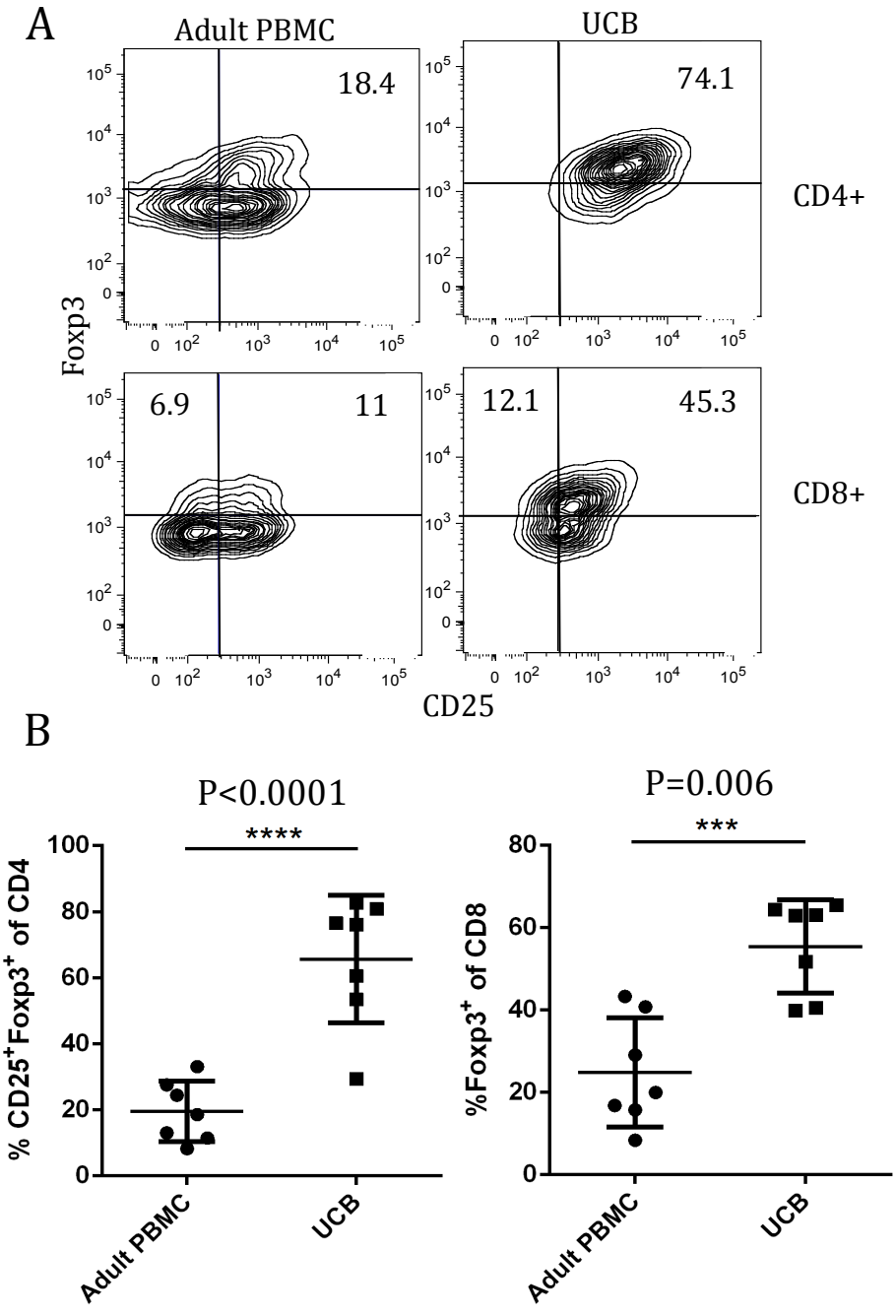


Figure 1. Induced Treg generation from UCB compared to Adult PBMCs. (A-B) Frequency of $CD4^+$ cells expressing Foxp3 and CD25 or and $CD8^+$ cells expressing Foxp3 after anti-CD3 and IL-2 stimulation of adult PBMCs or UCB. *** $p < 0.001$, **** $p < 0.0001$; n=7; 2-tailed Student *t* test.

Co-expression of CD25 and Foxp3 defines $CD4^+$ regulatory Tregs generated *in vivo* and *in vitro*. However, Treg subsets express a variety of other markers. Helios is a transcription factor thought to distinguish murine nTregs from pTregs or iTregs (37). Multiple human Treg subsets also express Helios, including nTregs and iTregs generated in the presence of APCs, but not those generated without APCs (37, 179). $CD4^+$ and $CD8^+$ cells from stimulated UCB increased the expression of Helios compared to unstimulated $CD4^+$ and $CD8^+$ cells (Fig. 2A-B), demonstrating $CD4^+$ and $CD8^+$ Tregs induced from UCB express Helios.

In our studies, stimulated UCB gives rise to both $CD4^+$ and $CD8^+$ $Foxp3^+$ cells. Although $CD8^+$ Tregs were first described in the early 1970s, they remain poorly understood. Multiple suppressive $CD8^+$ cells have been identified using various markers (55, 57). Studies using knockout mice that lack certain $CD8^+$ Treg populations demonstrate $CD8^+$ Tregs are required for normal immune homeostasis, resolution of inflammation and the generation of immune tolerance (62, 180). The non-redundant roles of $CD4^+$ and $CD8^+$ Tregs may be due to differences in the mechanisms of antigen recognition and immune suppression.

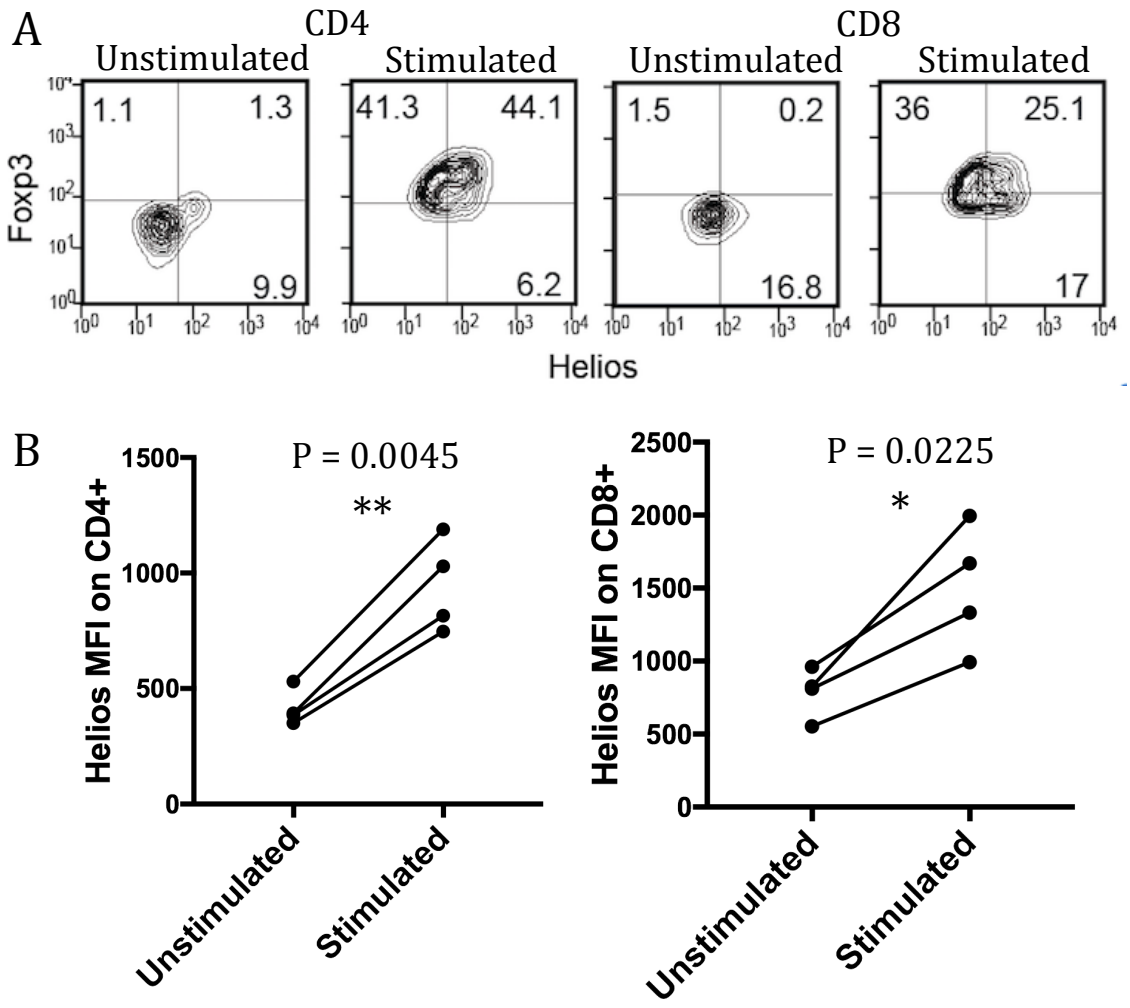


Figure 2. Helios expression on Tregs induced from UCB. Expression of Helios and Foxp3 by $CD4^+$ or $CD8^+$ gated cells from freshly isolated UCB (unstimulated) or UCB cultured for Tregs as in Figure 1 (stimulated). **(A)** Representative plot of Helios and Foxp3. **(B)** Mean fluorescence intensity (MFI) of Helios from 4 donors. * $p < 0.05$; ** $p < 0.01$; paired Student's t test; n = 4 pairs.

CD28 is a costimulatory molecule on T cells that binds the ligands CD80 and CD86 on APCs to support T cell activation during antigen presentation. One group of $CD8^+$ Tregs described previously express low levels or no CD28 (181). Similarly, the mean fluorescence intensity (MFI) of CD28 decreased on UCB $CD8^+$ T cells after stimulation compared to unstimulated $CD8^+$ T cells, while $CD4^+$ T cells upregulated CD28 expression under Treg inducing conditions (Fig. 3A-B). These data demonstrate that CD8 Tregs induced from UCB phenotypically resemble the $CD28^-$ subset of $CD8^+$ Tregs.

CD103 is the α E integrin, expressed on intestinal lymphocytes and subsets of Tregs, including some suppressive $CD8^+$ T cells (182, 183). When CD103 dimerizes with β7 integrin, it binds E-cadherin to promote cell migration to epithelial sites, including the gut and lung mucosa. A large percentage of induced $CD8^+CD25^+Foxp3^+$ cells (49.8% ± 24.26), but only a small fraction of $CD4^+CD25^+Foxp3^+$ Tregs (7.50% ± 6.46), induced from UCB expressed CD103 (Fig. 4A, B). $CD8^+CD103^+$ T cells migrate into non-lymphoid sites and remain as tissue residential T cells, mainly with cytotoxic function(184). Previous studies of $CD8^+CD103^+$ Tregs showed they lack Foxp3(183). Our data reveal a novel subset of $CD8^+$ Tregs that co-express Foxp3 and CD103. These data suggest $CD8^+Foxp3^+$ cells induced from UCB may distribute into different target tissues than UCB-derived $CD4^+$ Tregs, which lack CD103 expression.

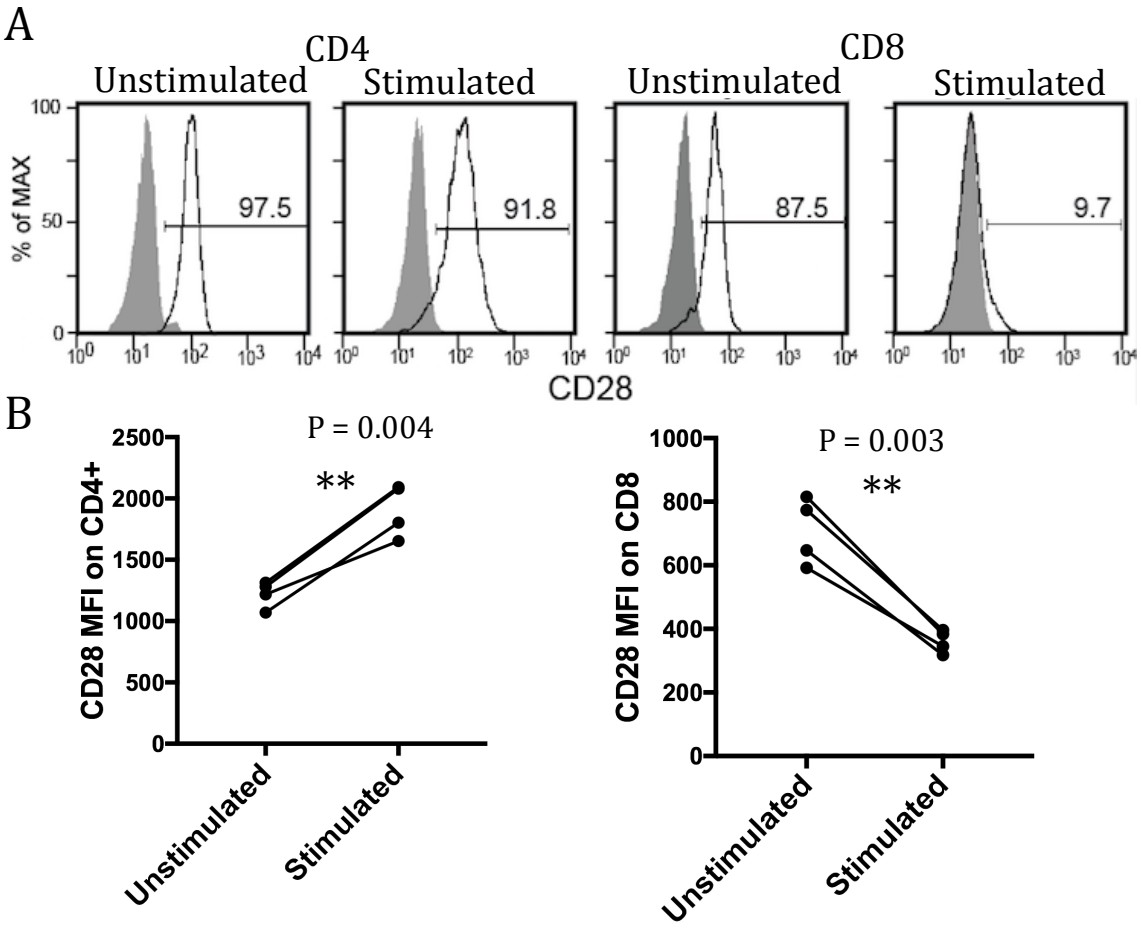


Figure 3. CD28 expression on Tregs induced from UCB. (A-B) Expression of CD28 by $CD4^+$ or $CD8^+$ gated cells from freshly isolated UCB (unstimulated) or UCB cultured for Tregs as in Figure 1 (stimulated). (A) Representative plot of CD28 (open histogram) compared to isotype control (shaded histogram). **(B)** Mean fluorescence intensity (MFI) of CD28 from 4 donors. ** indicates $p < 0.01$; paired Student's t test; n = 4 pairs.

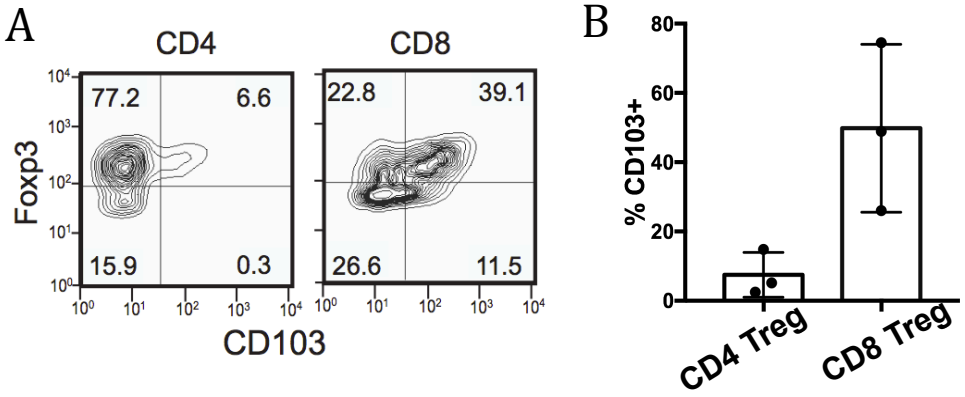


Figure 4. CD103 expression on Tregs induced from UCB. Expression of CD103 and Foxp3 by gated $CD4^+$ or $CD8^+$ Tregs ($CD25^+Foxp3^+$) from UCB cultured as in Figure 1. **(A)** Representative plot of CD103 and Foxp3. **(B)** Summarized data from 3 donors.

Activated human T cells can transiently express CD25 and Foxp3. To ensure $CD25^+Foxp3^+$ T cells induced from UCB are functional Tregs, we examined their ability to suppress the proliferation of allogeneic naïve $CD4^+$ T cells activated in the presence of APCs with anti-CD3. Responder naïve $CD4^+$ T cells were labeled with CFSE and the amount of proliferation was measured as the frequency of cells with diluted CFSE. In the absence of Tregs, a substantial portion of responder T cells proliferated upon T cell stimulation (Fig. 5). However, their proliferation dramatically decreased in the presence of $CD4^+$ Tregs or $CD8^+$ Tregs induced from UCB. These data demonstrate induced $CD4^+$ and $CD8^+$ $Foxp3^+$ cells from UCB suppress naïve T cell proliferation. Together, the phenotypic and functional analyses demonstrate that αCD3 and IL-2 stimulation of UCB induces $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$ cells that are phenotypically and functionally Tregs.

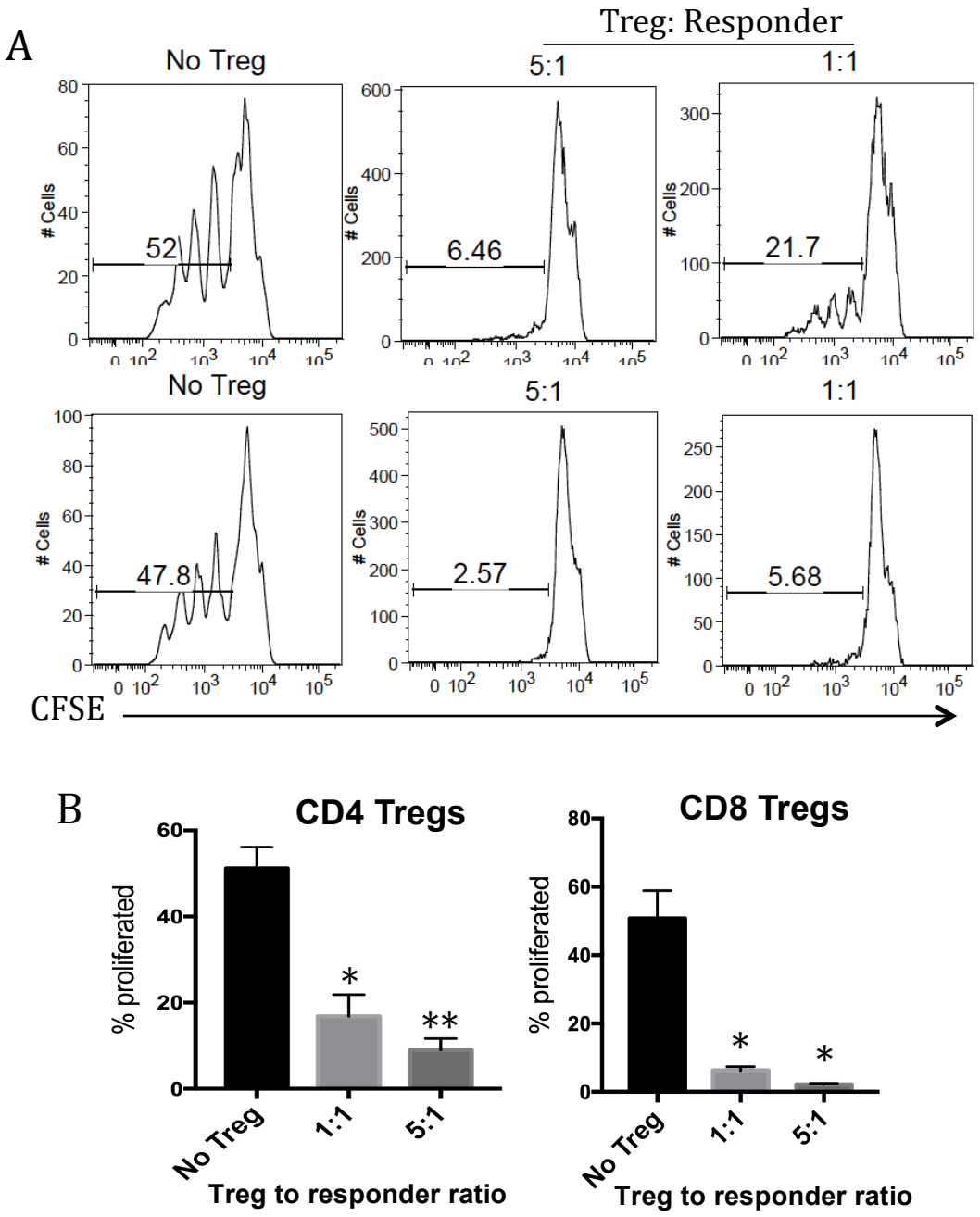


Figure 5. Suppression of T cell proliferation by Tregs induced from UCB. (A-B) *In vitro* suppression of T cell proliferation by $CD4^+$ and $CD8^+$ UCB Tregs cultured as in Figure 1 and used in a standard suppression assay. CFSE-labeled, allogeneic adult naïve $CD4^+$ T cells were used as responder cells. (A) Representative CFSE histograms of gated responder cells in the presence or absence of CD4 Tregs (top) or CD8 Tregs (bottom). (B) Summarized data from 3 Treg donors. * $p < 0.05$; ** $p < 0.01$, one-way ANOVA with Dunnett's multiple comparisons test; n=3.

To determine whether the high efficiency of Treg generation from UCB was due to an intrinsic property of UCB T cells or to the contribution of UCB APCs, we depleted various APC subsets. As previously observed, anti-CD3 and IL-2 stimulation of total UCB efficiently induced $CD4^+CD25^+Foxp3^+$ cells (68.08% ± 12.08 of $CD4^+$ cells), however monocyte depletion decreased the frequency of $CD4^+$ cells expressing CD25 and Foxp3 (41.33% ± 17.55) (Fig. 6A-B). Monocyte depletion also decreased the frequency of Foxp3 expression on $CD8^+$ cells (60.11% ± 6.38 in total UCB; 38.35% ± 9.98 in CD14 depleted). These data demonstrate monocytes contribute to UCB Treg differentiation. The generation of Tregs in the absence of monocytes could be due to incomplete monocyte depletion or due to a different population(s) also capable of inducing Tregs.

We next asked whether monocytes are sufficient to induce Treg differentiation from UCB T cells. Enriched $CD14^+$ monocytes were capable of inducing Foxp3 expression from purified $CD4^+$ and $CD8^+$ T cells (data not shown). UCB monocytes are a heterogeneous population and segregate into 2 populations based on CD14 and CD36 expression: $CD14^+CD36^{lo}$ and $CD14^+CD36^{hi}$ (Fig. 7A). To determine which population induces Tregs, we cultured purified $CD14^+CD36^{lo}$ or $CD14^+CD36^{hi}$ monocytes with naïve T cells. Purified $CD14^+CD36^{hi}$ monocytes efficiently induced Foxp3 expression from autologous naïve $CD4^+$ T cells (Fig. 7B). However, $CD14^+CD36^{lo}$ and $CD14^-$ cells did not efficiently induce Foxp3 expression. These data demonstrate that $CD14^+CD36^{hi}$ monocytes provide the necessary signals to induce Treg differentiation from naïve $CD4^+$ T cells in the absence of other UCB cells.

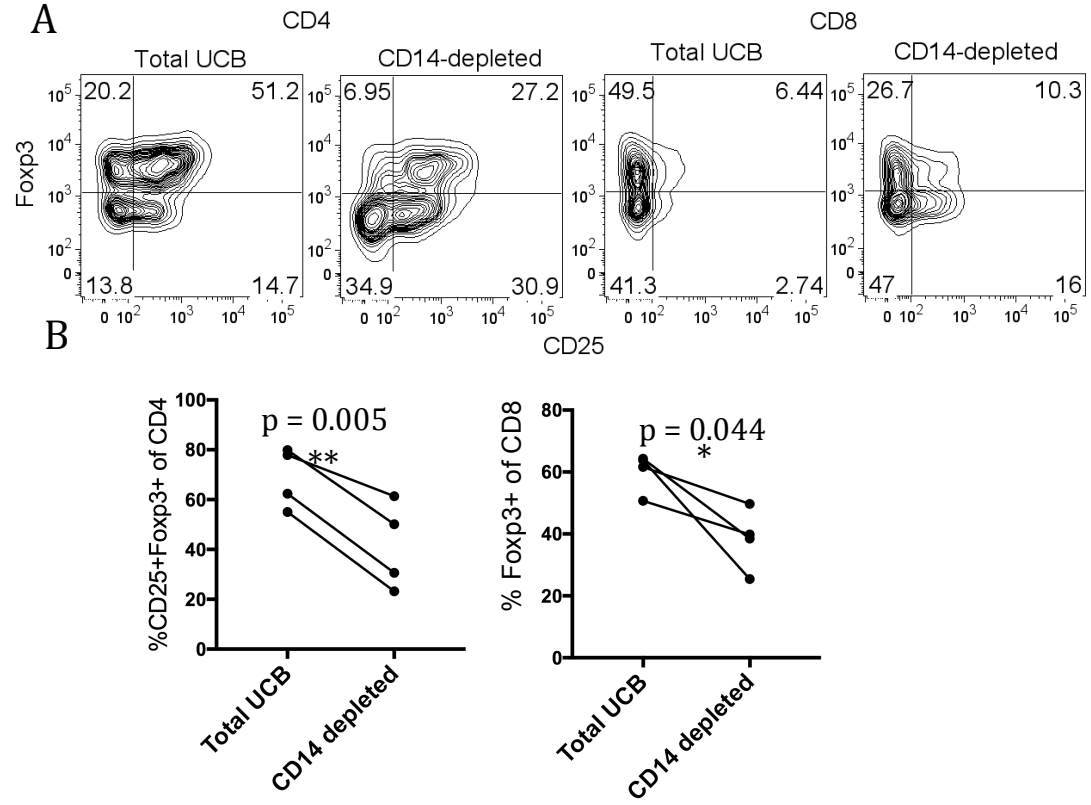


Figure 6. Treg differentiation in monocyte-depleted UCB. (A-B) The expression of CD25 and Foxp3 on gated $CD4^+$ or $CD8^+$ T cells from total UCB or CD14 depleted UCB stimulated with anti-CD3 and IL-2. (A) A representative plot and (B) summarized data from 4 donors are shown. Statistical significance was determined using the paired Student *t* test, * $p < 0.05$; ** $p < 0.01$.

Monocytes are commonly classified by expression of CD14 and the Fc receptor, CD16 (105). Human classical monocytes express high levels of CD14 and lack CD16. Non-classical monocytes express CD16 and lower levels of CD14. The majority of $CD14^+CD36^{hi}$ monocytes express low levels of CD16, consistent with human classical monocytes (data not shown).

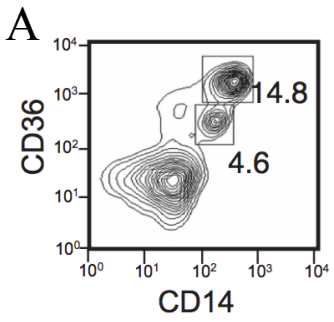
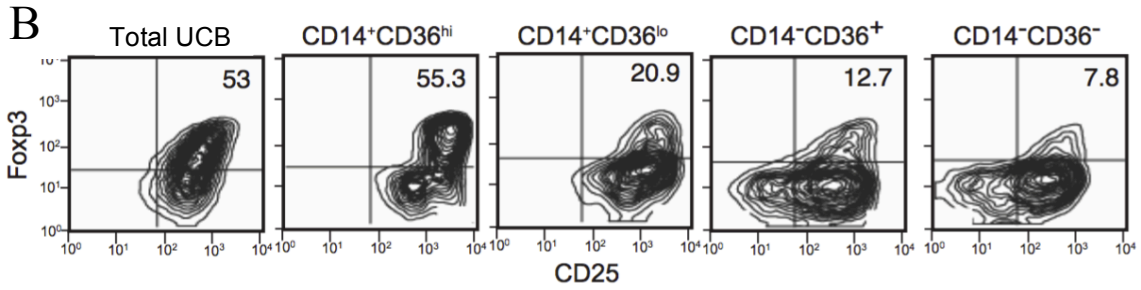


Figure 7. Treg differentiation by CD14^{+}CD36^{hi} monocytes. (A) Expression of CD14 and CD36 among freshly isolated total UCB mononuclear cells. (**B**) Expression of CD25 and Foxp3 by gated CD4^{+} T cells from total UCB cultures or UCB naïve CD4^{+} T cells co-cultured with purified CD14^{+}CD36^{hi}, CD14^{+}CD36^{lo}, CD14^{-}CD36^{+}, or CD14^{-}CD36^{-} cells. All cultures were stimulated with anti-CD3 and IL-2. Data represent at least 3 independent experiments.



Finally, we examined whether monocytic cell lines maintain the ability to induce Treg differentiation. THP-1 cells are a human monocytic leukemia cell line. Irradiated THP-1 cells induced Foxp3 expression on a large percentage of CD4 (74.4% ± 24.05) and CD8 (45.95% ± 15.2) T cells when co-cultured with UCB T cells in the presence of anti-CD3 and IL-2 (Fig. 8A-B). Treg induction by THP-1 cells was as efficient as in total UCB cultures (74.5% ± 16.06 of CD4; 48.85% ± 17.18 of CD8). Together, these data demonstrate that both primary and transformed human monocytes efficiently induce Treg differentiation from stimulated T cells.

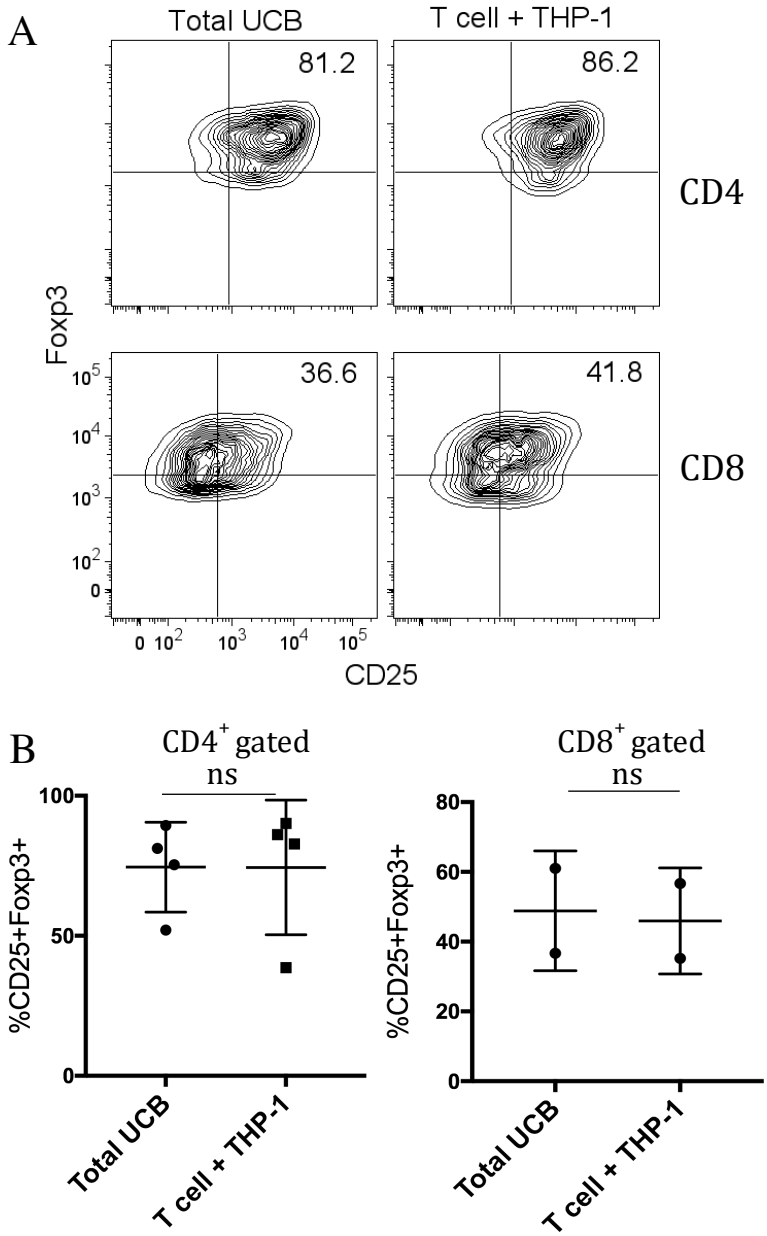


Figure 8. Treg differentiation by THP-1 cells. (**A-B**) Total UCB cells stimulated as in Figure 1 or UCB T cells from the same donor stimulated with irradiated THP-1 cells in the presence of anti-CD3 and IL-2. After co-culture, Foxp3 expression was assessed on $CD4^+$ T cells and $CD8^+$ T cells. (A) Representative plot and (B) data from 3 donors are depicted. ns = not significant, $p > 0.05$; paired Student *t* test.

The Role of TGF-β in $CD14^{+}CD36^{hi}$ Monocyte-Induced Treg Generation

Tregs can be induced *in vitro* by providing TCR stimulation to naïve T cells, along with high levels of active TGF-β and IL-2. TGF-β is essential for extra-thymic Treg generation(38, 40). However, Treg generation from UCB does not require exogenous TGF-β. The TGF-β receptor kinase inhibitor (SB431542) reduces the frequency of $CD25^{+}Foxp3^{+}$ expression on $CD4^{+}$ and $CD8^{+}$ T cells from stimulated UCB by approximately 50% (47.26 ± 10.05 %inhibition of $CD4^{+}$ Tregs; 55.16 ± 13.99 %inhibition of $CD8^{+}$ Tregs) (Fig. 9A). These data suggest endogenously produced TGF-β contributes to Treg generation from UCB. The incomplete loss of Tregs in the presence of the TGF-β receptor kinase inhibitor could indicate that TGF-β promotes Treg differentiation not only through canonical TGF-β signaling, but also through non-canonical pathways.

I determined when TGF-β is required during monocyte-induced Treg differentiation by adding the TGF-β receptor kinase inhibitor at various times after T cell stimulation. $CD4^{+}CD25^{+}Foxp3^{+}$ Treg induction was substantially reduced when the inhibitor was added during the first two days of T cell stimulation (Fig. 9B-C). However, adding the inhibitor at later time points either minimally decreased Treg differentiation or had no effect. These data indicate that TGF-β signaling is required within the first 24~48 hours after T cell stimulation, and that the events occurring during this period determine the differentiation pathway of the T cells in our culture conditions.

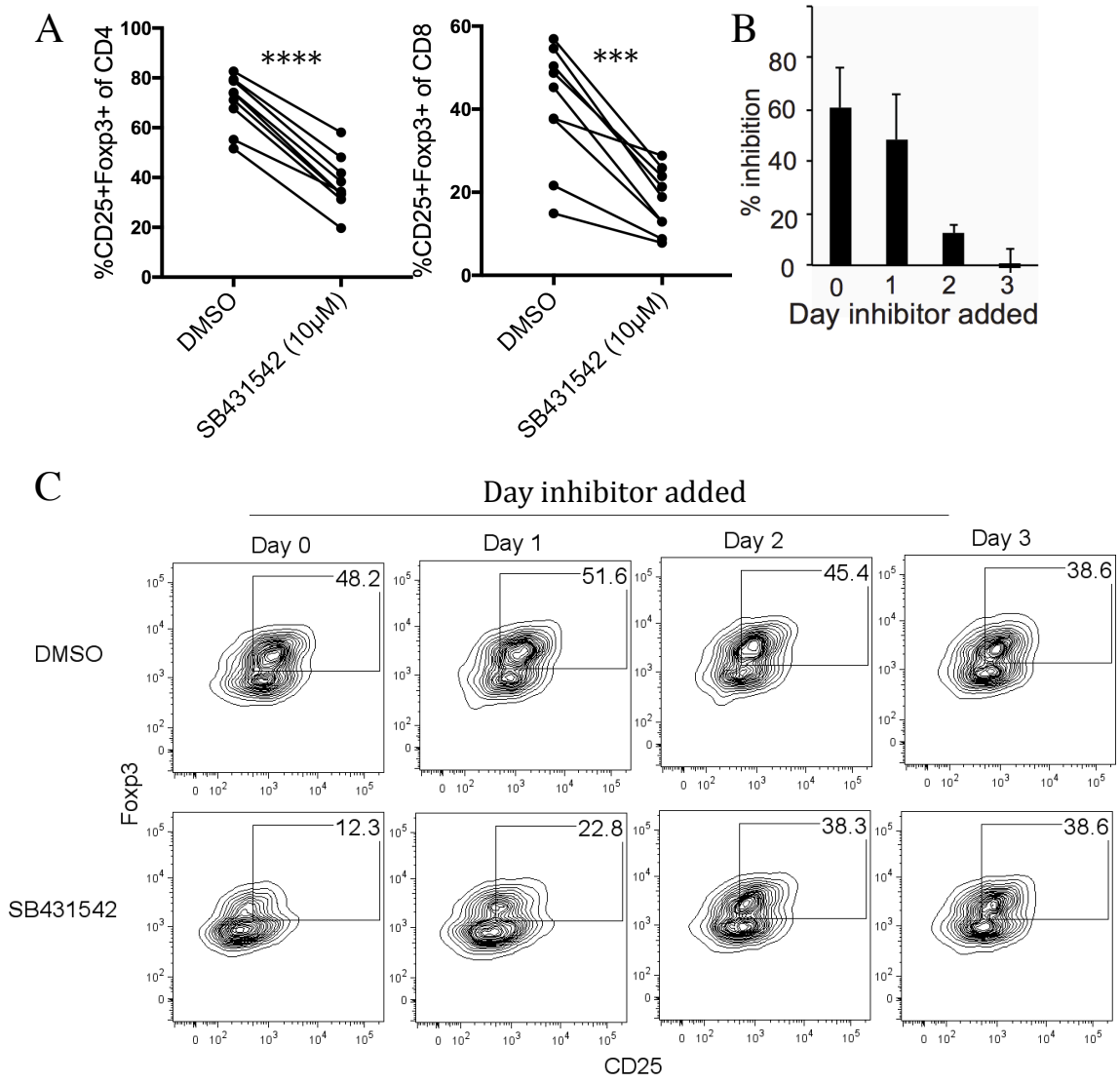


Figure 9. The role of TGF-β signaling in UCB Treg differentiation. (A) Frequency of $CD25^+$ $Foxp3^+$ expression among $CD4^+$ and $CD8^+$ gated cells from UCB after stimulation by anti-CD3 and IL-2 with the addition of a TGF-β receptor kinase inhibitor (SB431542) or the DMSO carrier control at the start of the culture. *** $p< 0.001$; **** $p< 0.0001$; paired Student *t* test; n=9. **(B-C)** Inhibition of $CD4^+$ Treg generation by SB431542 compared to the DMSO carrier control added at various times after the start of UCB stimulation as in (A). (B) Data summarized from 3 donors. % inhibition is calculate as $[(\%Tregs_{\text{DMSO treated}} - \%\text{ Treg}_{\text{SB431542 treated}})/ \%Tregs_{\text{DMSO treated}}] \times 100$. (C) Representative plot from (B) is shown.

Because monocytes induce Treg differentiation and endogenous TGF-β contributes to this process, we hypothesized $CD14^+CD36^{hi}$ monocytes produce active

TGF-β. However, when we tested the culture supernatant of $CD14^{+}CD36^{hi}$ monocytes in a TGF-β bioassay, the level of active TGF-β was below the detectable level (<1pg/ml) (data not shown). TGF-β can be secreted or presented on the cell surface (185–191). Because $CD14^{+}CD36^{hi}$ monocytes do not secrete active TGF-β, we hypothesized they present TGF-β on their cell surface.

Activation of the TGF-β receptor leads to Smad2 and Smad3 phosphorylation, dimerization with Smad4, and translocation to the nucleus to regulate target gene transcription(192) (Fig. 10A). If monocytes are the endogenous source of TGF-β for Treg differentiation, then we predict that depleting monocytes will abrogate Smad2/3 activation in T cells. To test this hypothesis, we measured Smad2/3 phosphorylation in UCB T cells stimulated in the presence or absence of monocytes. When total UCB cells were stimulated for 1 day with anti-CD3 and IL-2, we observed substantial phosphorylation of Smad2/3 (pSmad2/3) in $CD4^{+}$ and $CD8^{+}$ T cells (Fig. 10B-C). Smad2/3 was also phosphorylated in T cells cultured with enriched $CD14^{+}$ monocytes, but greatly reduced in T cells from CD14 depleted UCB cultures. These data demonstrate that $CD14^{+}$ cells are sufficient and required for the majority of TGF-β signaling in UCB T cells.

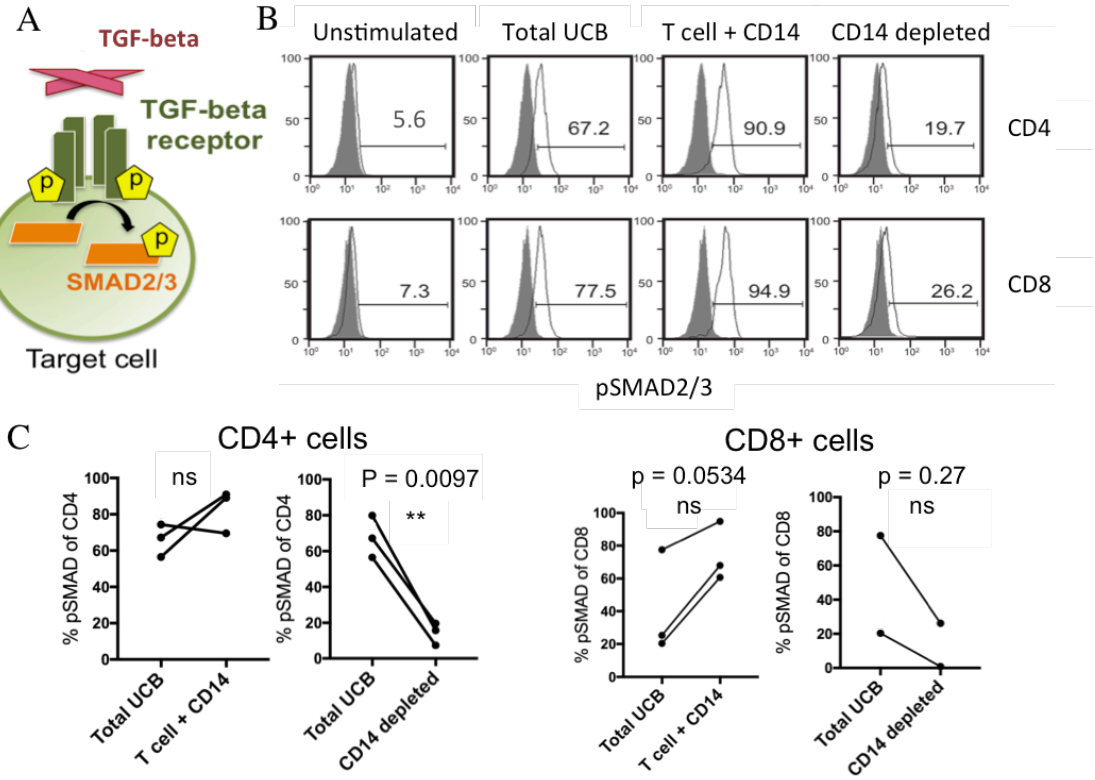


Figure 10. Monocyte-dependent Smad2/3 phosphorylation in T cells. (A) Schematic of TGF-β signaling. TGF-β receptor binding leads to downstream phosphorylation of Smad2 and Smad3 in target cells. **(B)** Smad2/3 activation in $CD4^+$and $CD8^+$ T cells from total UCB, purified T cells co-cultured with $CD14^+$ cells, or CD14 depleted UCB. Cells were stimulated for 1 day with anti-CD3 and IL-2, or cultured without stimulation (unstimulated). Samples stained for pSMAD2/3 (solid line) were compared to isotype control (shaded). Gates were based on the isotype control. **(C)** Summary plots from 2-3 donors are shown. Statistical analysis was done using the paired Student *t* test.

TGF-β is translated as a polypeptide, which is cleaved into latency associated peptide (LAP) and the growth factor domain (Fig 11A). The two peptides then bind non-covalently to form the small latency complex (SLC). The SLC may further associate with TGF-β binding proteins (LTBPs) to form the large latency complex (LLC) (193). Upon secretion, TGF-β may be tethered to the extracellular matrix through the association of LTBP with matrix proteins. Alternatively, the SLC may be bound to the cell membrane

through interactions with transmembrane proteins, such as neuropilin-1 (Nrp-1) or Glycoprotein A Repetitions Predominant (GARP) (194–197). TGF-β must subsequently be released from LAP to signal through its receptor.

Because monocytes are required for the majority of TGF-β signaling in T cells, but do not secrete substantial levels of active TGF-β, we hypothesized that monocytes present membrane-bound TGF-β to T cells. No antibodies are available to distinguish active TGF-β from inactive TGF-β bound to LAP, therefore we assessed membrane bound TGF-β using anti-LAP antibodies. Among UCB cells, $CD14^{+}CD36^{hi}$ monocytes were the major group of cells that expressed LAP on the cell surface (Fig. 11B-C). In most UCB donors, a significant fraction of $CD14^{+}CD36^{hi}$, but not $CD14^{+}CD36^{lo}$, cells expressed surface LAP. The increase in LAP expression on $CD14^{+}CD36^{hi}$ cells was not significantly different in our sample set due to one outlier donor that did not express detectable levels of LAP on the $CD14^{+}CD36^{hi}$ cells. This could be due to poor sample quality or a normal biologic variant. If this outlier is removed from the sample set, the expression of LAP on $CD14^{+}CD36^{hi}$ cells is statistically increased over other populations.

To determine how LAP is tethered to the monocyte cell membrane, we examined LAP co-expression with several molecules known to bind LAP: Nrp-1, GARP and LTBP-1. Freshly isolated monocytes express little Nrp-1 and inconsistently express GARP (data not shown). In contrast, a large percentage of $CD14^{+}CD36^{hi}$ monocytes express LTBP-1 on the cell surface (Fig. 12A-B). A smaller percentage of $CD14^{+}CD36^{lo}$ cells also express LTBP-1, however only $CD14^{+}CD36^{hi}$ cells co-express LAP with LTBP-1 (Fig 12C-D). Very few $CD14^{-}$ cells express LTBP-1. Among $CD14^{+}CD36^{hi}$

cells, all LAP^{+} cells express LTBP-1 and the expression level of the LAP increases as LTBP-1 increases (Fig. 12C, E). These data suggest CD14^{+}CD36^{hi} monocytes present LAP on the cell surface as a complex with LTBP-1.

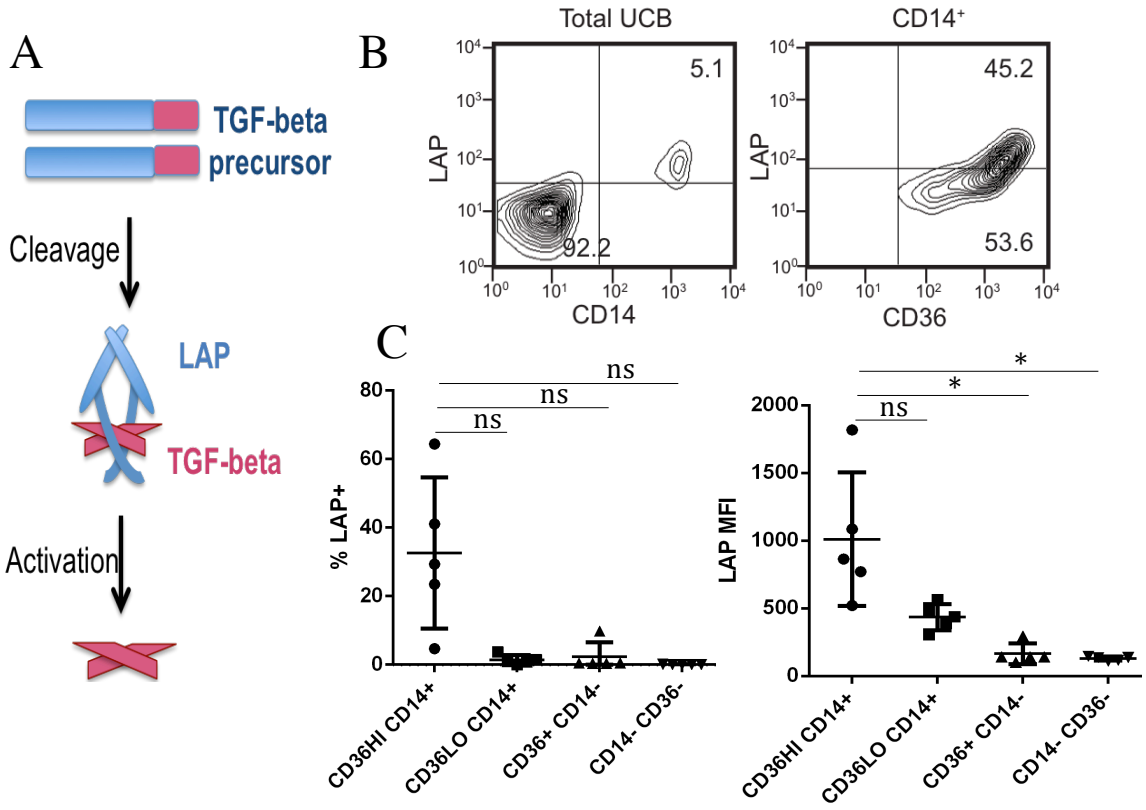


Figure 11. LAP expression by CD14^{+}CD36^{hi} monocytes. (A) Schematic of TGF-β production and activation. TGF-β is translated as a pro-protein, dimerizes and is cleaved into latency associated peptide (LAP) and the active cytokine. TGF-β non-covalently binds to LAP and must be released from LAP to bind its receptor. **(B)** LAP expression by total UCB cells (left) and CD14^{+} gated monocytes (right). **(C)** LAP frequency and MFI on mononuclear cell subsets gated by CD14/CD36 expression. ns = not significant, * $p < 0.05$, one-way ANOVA with Dunnett's multiple comparisons test, n =5.

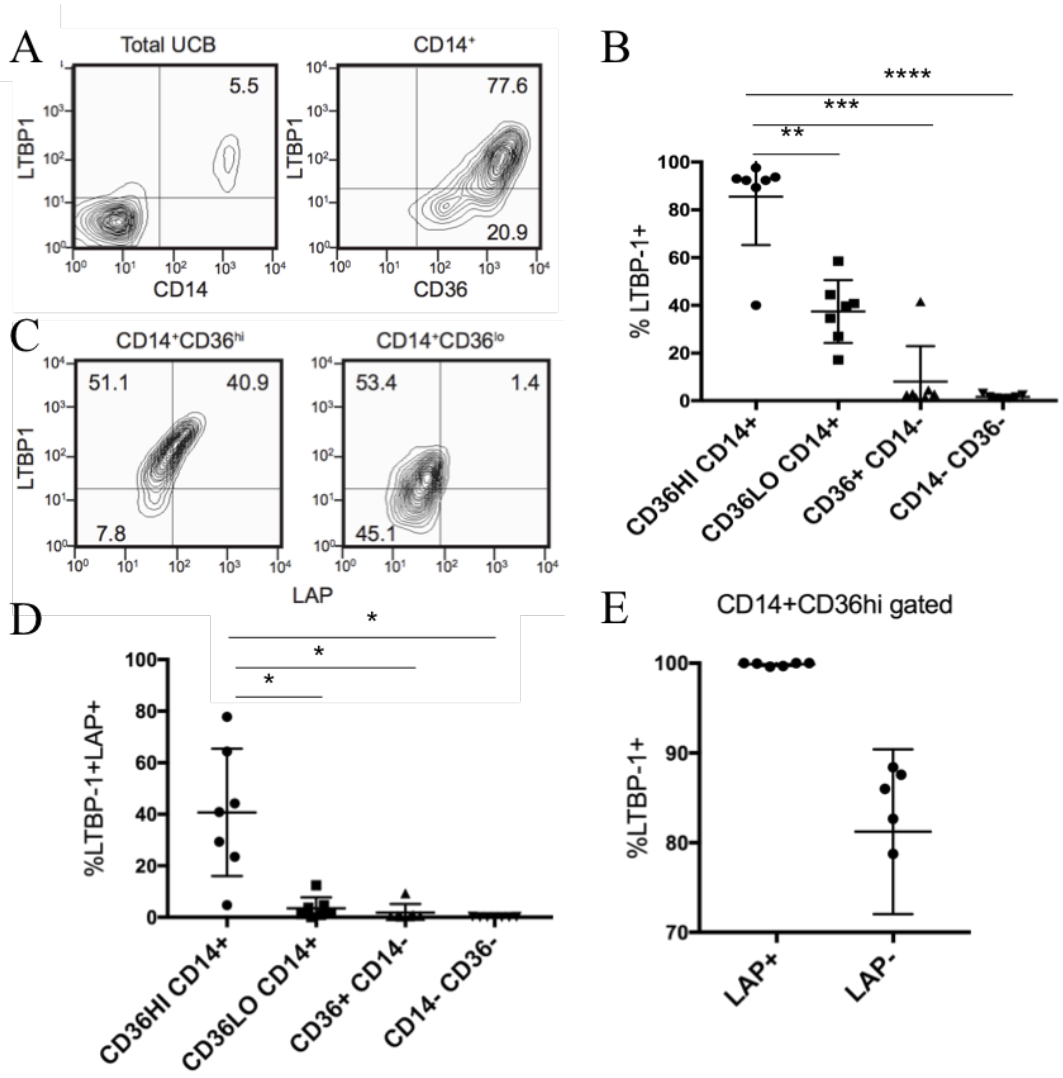


Figure 12. LAP and LTBP-1 co-expression by CD14^{+}CD36^{hi} monocytes. (A) LTBP-1 expression on total UCB (left) and gated CD14^{+} cells (right). **(B)** Summary graphs of LTBP-1 frequency among 7 donors; **(C)** Expression of LAP and LTBP1 by CD14+CD36^{hi} and CD14^{+}CD36^{lo} gated cells. **(D)** Summary graph of LTBP-1 and LAP co-expression by CD14/36 gated subsets. **(E)** LTBP-1 expression among LAP^{+} and LAP^{-} CD14^{+}CD36^{hi} monocytes. Negative/positive gates were determined by isotype control staining. * p <0.05; ** p <0.01; *** p <0.001; **** p< 0.0001; one-way ANOVA with Dunnett's multiple comparison's test.

The Role of Retinoic Acid in $CD14^{+}CD36^{hi}$ Monocyte-Induced Treg Generation

Our data leads to a model in which $CD14^{+}CD36^{hi}$ monocytes induce Treg differentiation by presenting membrane-bound TGF-β in a complex with LTBP-1. It is known that Tregs induced by exogenous TGF-β in the absence of APCs *in vitro* are not as stable as Tregs generated *in vivo* (37). Therefore, it is thought that APCs provide other signals in addition to TGF-β to promote Treg differentiation and stability.

Previous reports suggest retinoic acid, a vitamin A metabolite, supports Treg differentiation in a TGF-β dependent manner (43, 44, 54). To determine whether retinoic acid contributes to UCB Treg generation, we cultured UCB in the presence of a retinoic acid receptor (RAR) antagonist (LE135) (Fig. 13A, B). The antagonist decreased Foxp3 expression in $CD4^{+}$ and $CD8^{+}$ T cells. This effect was specific to inhibition of the RAR since Treg differentiation was restored when an RAR agonist (AM580) was added in addition to the antagonist.

Since the levels of vitamin A are not optimized in our culture media, we tested if an exogenous RAR agonist could enhance UCB Treg differentiation. The frequency of $CD8^{+}$ $Foxp3^{+}$ cells increased with the addition of the RAR agonist in a dose-dependent manner (Fig. 14). The frequency of $CD4^{+}$ $Foxp3^{+}$ cells also statistically increased with AM580 treatment, but the change was less dramatic than in $CD8^{+}$ cells due to the high frequency of $CD4^{+}$ Treg generation in the absence of an exogenous RAR agonist. Together these data demonstrate that endogenous retinoic acid contributes to UCB Treg differentiation and the addition of an exogenous RAR agonist can further enhance Treg generation.

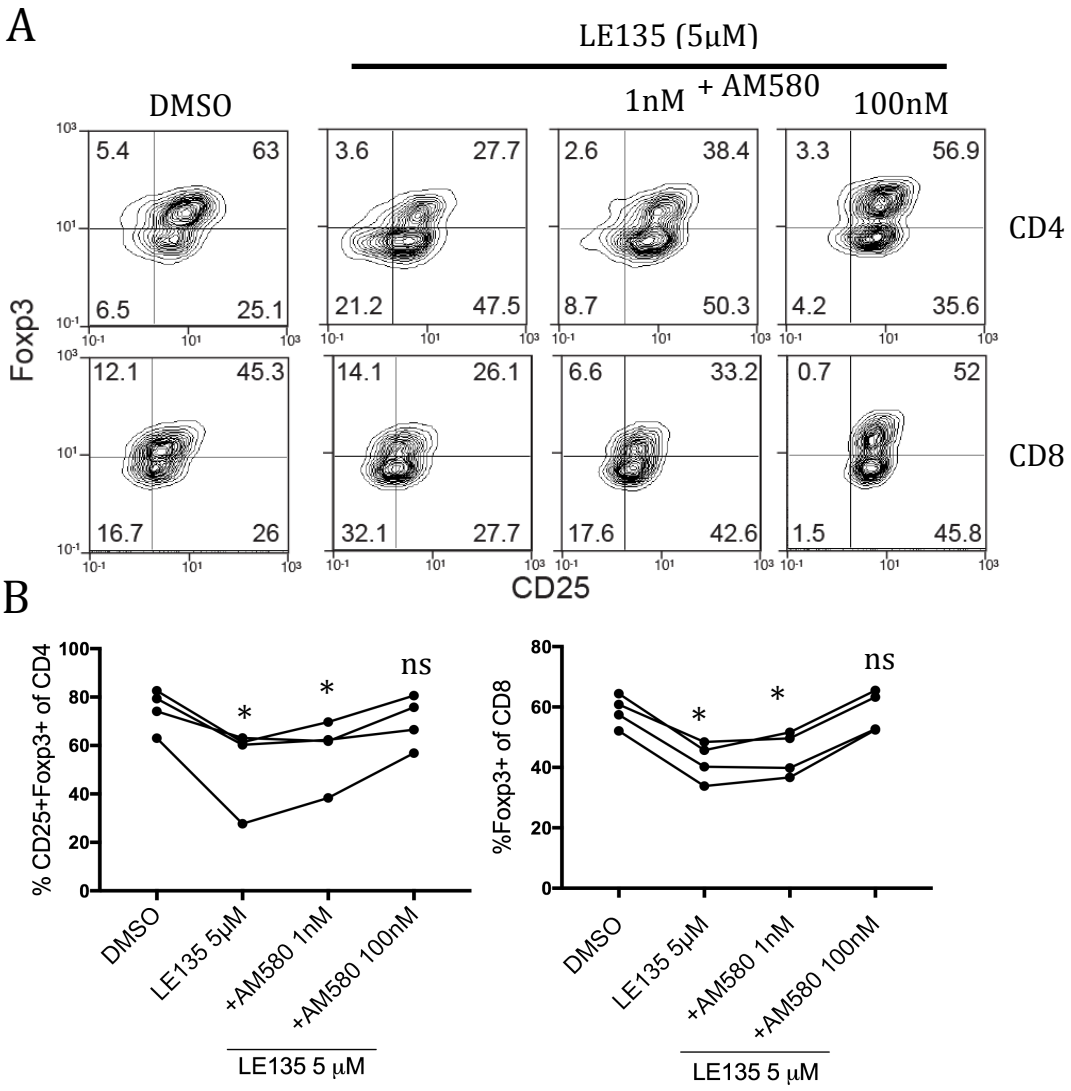


Figure 13. Modulation of UCB Treg generation by retinoic acid. (A-B) Inhibition of Treg differentiation by an RAR antagonist. Total UCB cells were stimulated with anti-CD3, IL-2 and an RAR antagonist, LE135. Where indicated, an RAR agonist, AM580, was added in addition to LE135. (A) Representative plot of Foxp3 expression analyzed on $CD4^+$ (upper panel) and $CD8^+$ (lower panel) cells. (B) Data summarized from 4 donors; * $p < 0.05$, ** $p < 0.01$, ns = not significant compared to DMSO control; one-way ANOVA with Dunnett's multiple comparison's test.

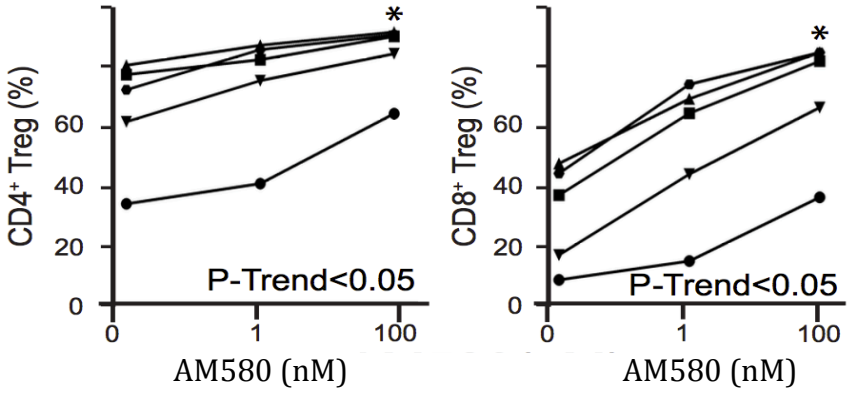


Figure 14. Enhancement of UCB Treg generation by an RAR agonist. UCB cells were cultured as in Fig. 13 with the addition of increasing concentrations of AM580. Each line represents the frequency of Foxp3^{+}CD25^{+} cells (Tregs) from one donor. An asterisk (*) indicates a significant increase of Tregs ($p<0.05$) with increasing AM580 concentration using Cuzick's Non-Parametric Test on the median of all samples.

Retinoic acid is oxidized from vitamin A by an alcohol dehydrogenase and aldehyde dehydrogenase (ALDH) (Fig. 15A). Since CD14^{+}CD36^{hi} monocytes induce Foxp3^{+} Tregs, we hypothesized they produce retinoic acid. We determined which UCB cells are capable of producing retinoic acid by determining which cells contain ALDH. ALDH activity can be measured using a diffusible fluorescent dye that is retained by cells when oxidized by ALDH (ALDEFLUOR assay). As a control, the ALDEFLUOR assay is also performed in the presence of an ALDH inhibitor. A large percentage of CD14^{+}CD36^{hi} and a small fraction of CD14^{+}CD36^{lo} monocytes retained the fluorescent dye above background levels, suggesting they express active ALDH (Fig. 15B-C). In contrast, a very minor fraction of granulocytic cells or CD4^{+}/CD8^{+} T cells had detectable ALDH activity. Together, our data suggest CD14^{+}CD36^{hi} monocytes are a primary producer of retinoic acid among UCB cells.

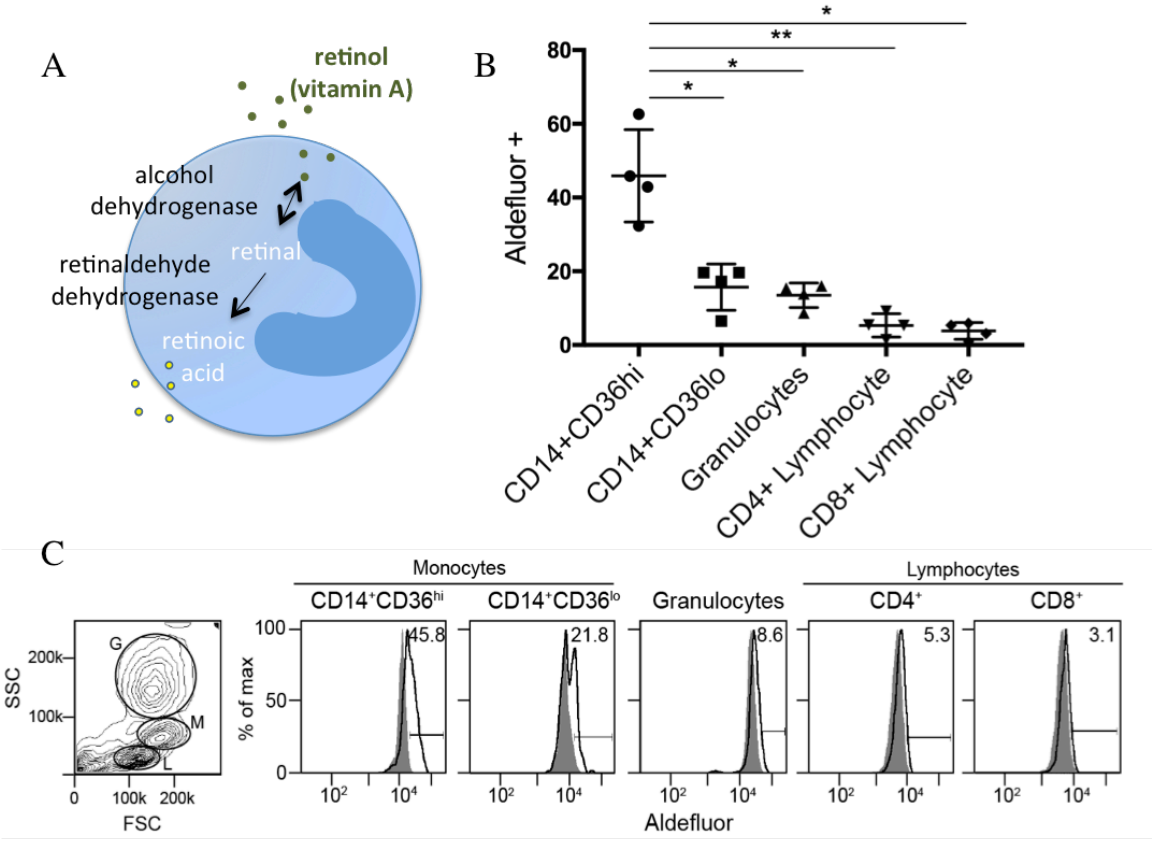


Figure 15. ALDH activity in CD14⁺CD36ʰⁱ monocytes. (A) Schematic of retinoic acid production. Retinol (vitamin A) diffuses into cells and is reversibly oxidized to retinal by an alcohol dehydrogenase. It is then irreversibly oxidized into retinoic acid by retinaldehyde dehydrogenase. **(B-C)** Aldehyde dehydrogenase activity was assessed in UCB monocytes (M), granulocytes (G), and lymphocytes (L) using the ALDEFLUOR™ assay, which labels cells based on their ability to oxidize an aldehyde-modified fluorescent molecule. (B) Summarized data from 4 donors and (C) a representative plot are shown. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA with Dunnett's multiple comparison test, n =4.

The Role of Notch Signaling in CD14⁺CD36ʰⁱ Monocyte-Induced Treg Generation

Notch signaling is a type of cell-to-cell communication that has been implicated in the differentiation of various T helper subsets, including Th1, Th2 and Treg cells (198, 199). Membrane-bound Notch ligands (in the Jagged or delta-like ligand families) induce the stepwise cleavage of a Notch receptor (Notch 1-4 in humans) by ADAM family metalloproteases and gamma secretase. Notch cleavage releases the intracellular domain

(ICD) of the Notch receptor and leads to its nuclear translocation and the transcriptional activation of target genes. Previous studies have demonstrated Notch crosstalk with the TGF-β signaling pathway. Specifically, the ICDs of Notch-1 and -4 can associate with Smad3, a target of the TGF-β receptor (200, 201). Furthermore, the Notch ICD can directly bind the *foxp3* locus along with Smads (47, 200, 201). Therefore, we asked whether Notch signaling contributes to monocyte-induced Treg differentiation.

To determine the role of Notch signaling in UCB Treg differentiation, we first asked whether UCB T cells express and activate Notch. We examined resting and stimulated T cells for the expression of Notch by flow cytometry and western blot. Consistent with previous reports in activated T cells (202–204), Notch-1 was highly upregulated 2 days after TCR stimulation in UCB $CD4^+$ and $CD8^+$ T cells (Fig. 16A-B). Furthermore, cleaved Notch-1 was detectable by western blot after T cell stimulation, demonstrating Notch-1 activation (Fig. 16C). Stimulated $CD4^+$ T cells also upregulated Notch-2 (Fig. 16D).

To determine whether Notch signaling contributes to monocyte-induced Treg differentiation, we cultured UCB in the presence of a gamma secretase inhibitor, DAPT, which prevents Notch cleavage and downstream signaling. DAPT efficiently blocked the generation of cleaved Notch-1 in activated UCB $CD4^+$ T cells (data not shown). Notch inhibition also led to a mild, but consistent, decrease in the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells (Fig. 17A-B), along with a reduction in the level of Foxp3 expressed (Fig. 17C-D). DAPT did not decrease the percentage of $Foxp3^+$ $CD8^+$ T cells (data not shown).

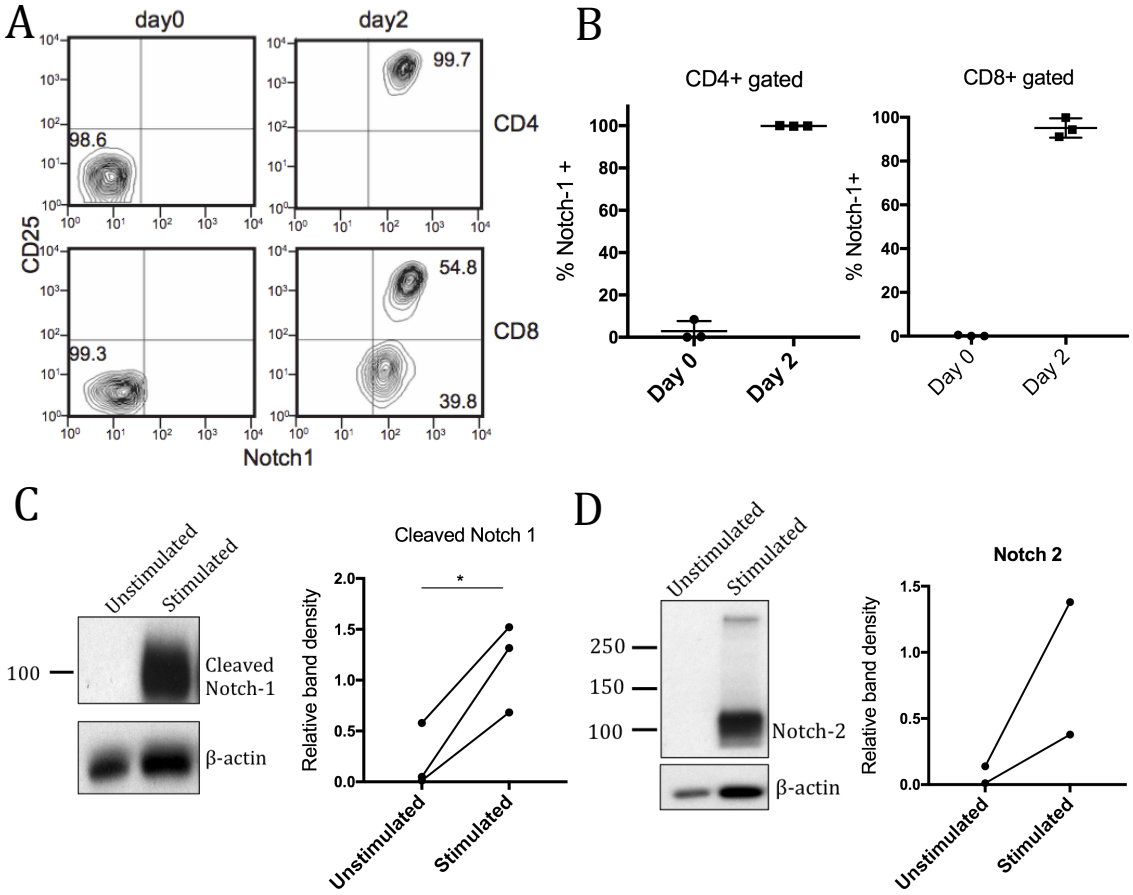


Figure 16. Notch expression and activation in stimulated UCB T cells. Total UCB mononuclear cells were analyzed for Notch-1 or Notch-2 expression either directly after isolation (unstimulated; day 0) or 1-3 days after stimulation with anti-CD3 and IL-2 (stimulated). Cells were **(A-B)** stained with anti-CD4, CD8, CD25 and Notch-1 antibodies and analyzed by flow cytometry. (A) Representative plots and (B) summarized data from 3 donors are shown. **(C-D)** $CD4^+$ T cells were enriched from cultures and analyzed by western blot for (C) cleaved Notch-1 and (D) Notch-2. The band intensities from 2-3 donors were measured and normalized to the beta actin control. Statistics were performed on the band density normalized to beta actin. * $p < 0.05$; paired Student *t* test.

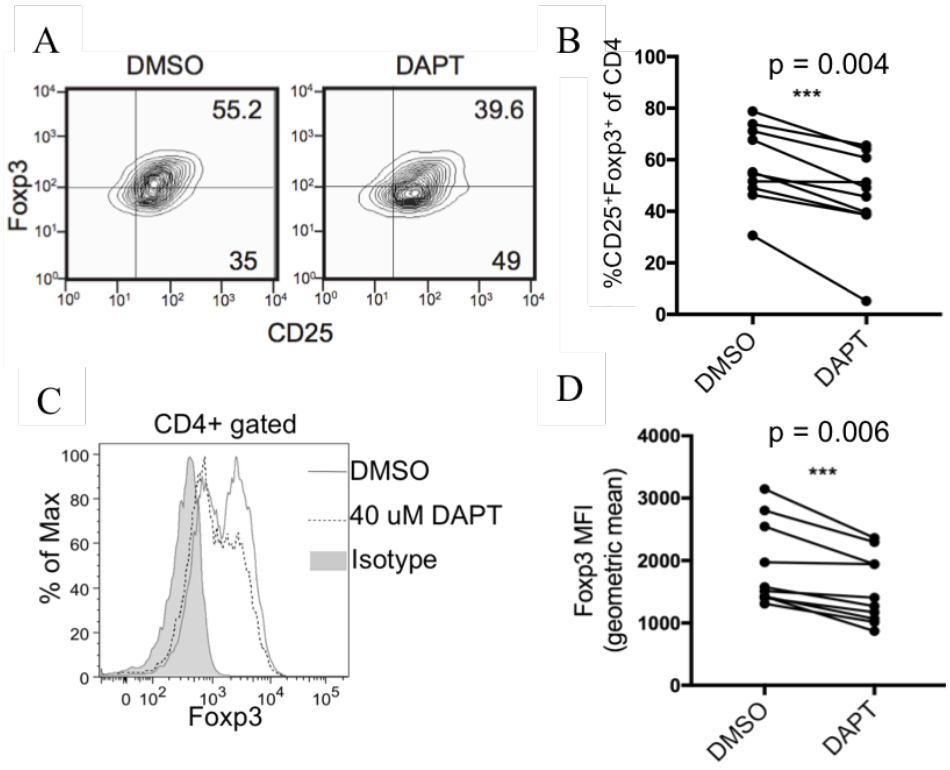


Figure 17. The role of Notch signaling in Treg differentiation. Total UCB mononuclear cells were cultured with anti-CD3, IL-2, and the gamma secretase inhibitor, DAPT, or a DMSO vehicle control. The cells were stained two weeks later for flow cytometry. **(A)** A representative plot of CD25 and Foxp3 expression on $CD4^+$ gated cells and **(B)** summary from 10 donors are depicted. **(C)** The intensity of Foxp3 expression on $CD4^+$ cells and **(D)** a summary of the geometric mean fluorescence intensity (MFI) are shown. Data were analyzed using the paired Student *t* test, *** $p < 0.001$, $n = 10$.

To determine whether $CD14^+CD36^{hi}$ monocytes provide Notch ligands to T cells, I first examined the expression of Notch ligands. Humans encode five Notch ligands: Jagged-1 and -2 and Delta-like ligand (DLL) 1, 3, and 4. Both $CD14^+CD36^{hi}$ and $CD14^+CD36^{lo}$ monocytes expressed DLL3 mRNA by RT-PCR analysis (Fig. 18A). Most $CD14^+CD36^{hi}$ monocytes expressed DLL3 protein on the cell surface (Fig. 18B-E). Fewer $CD14^+CD36^{lo}$ monocytes and $CD14^-$ cells expressed membrane DLL3. Furthermore, only $CD14^+CD36^{hi}$ monocytes co-expressed LAP with DLL3 on their cell surface (Fig. 18C). In some donors, $CD14^+CD36^{hi}$ monocytes also expressed Jagged-1,

but less consistently between donors and at a lower frequency than DLL3 (data not shown). These findings show $CD14^{+}CD36^{hi}$ monocytes co-express DLL3 with LAP at their cell surface. Previous data show monocytes provide TGF-β to T cells and that $CD14^{+}CD36^{hi}$ monocytes are the primary UCB cells expressing membrane-bound latent TGF-β. From these results, we propose a model in which $CD14^{+}CD36^{hi}$ monocytes induce Treg differentiation by presenting TGF-β concurrently with Notch ligands and retinoic acid to naïve T cells at the point of cell-to-cell contact.

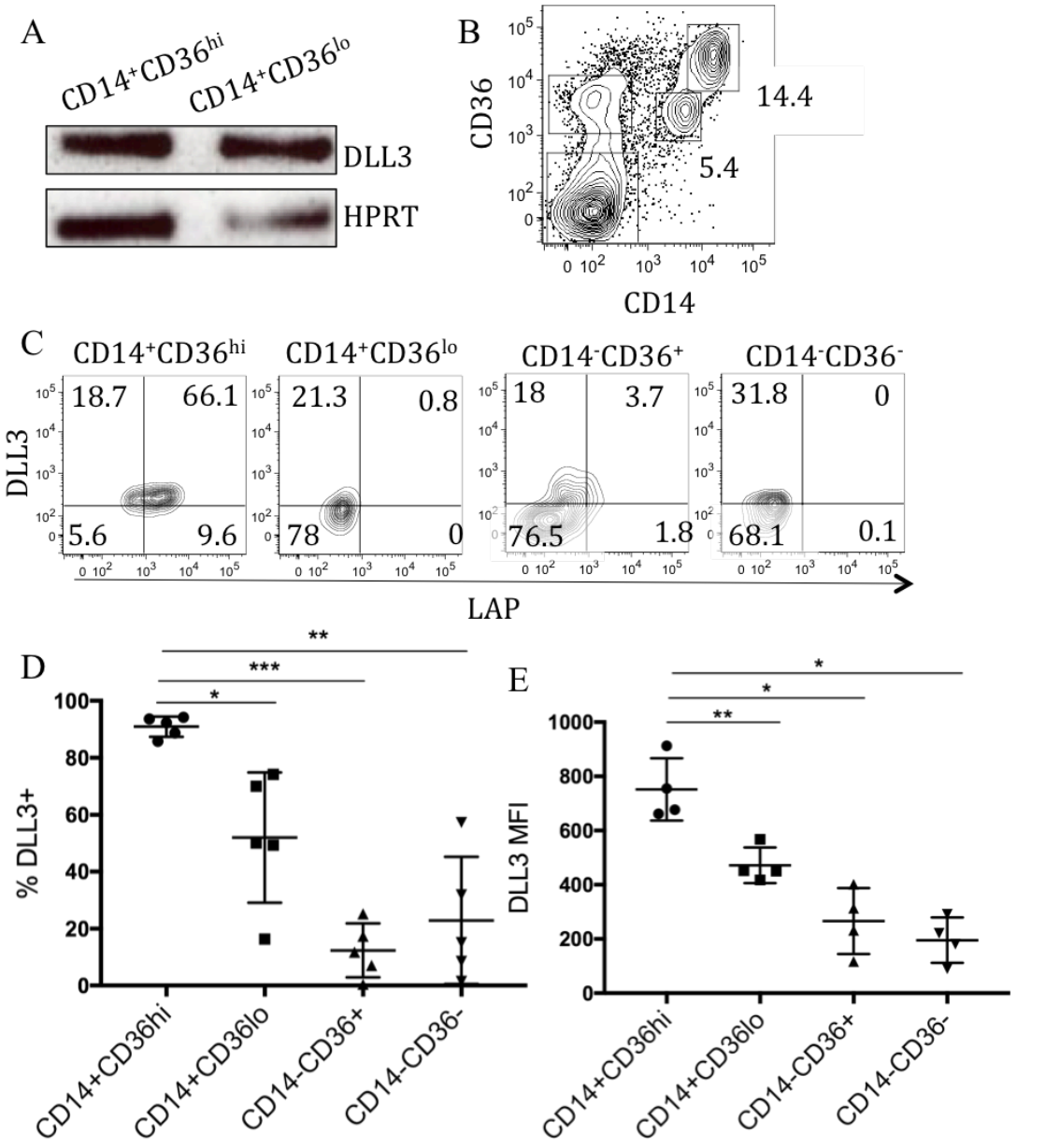


Figure 18. DLL3 and LAP expression on UCB mononuclear cells. (A) The mRNA expression of DLL3 was analyzed on sorted $CD14^{+}CD36^{hi}$ or $CD14^{+}CD36^{lo}$ monocytes by RT-PCR analysis and compared to the housekeeping gene, HPRT. Representative data from 2 donors. **(B)** Expression of CD14 and CD36 on freshly isolated mononuclear cells. **(C)** Expression of DLL3 and LAP on CD14/CD36 subsets shown in B. **(D)** Summarized frequency of DLL3 from 5 donors and **(E)** DLL3 MFI from 4 donors are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Dunnett's multiple comparisons test.

The Function of CD36 Ligands in $CD14^{+}CD36^{hi}$ Monocyte-Induced Treg Differentiation

Because monocytes expressing high levels of CD36 induce Tregs, but $CD36^{lo}$ monocytes do not, we asked whether CD36 plays a role in Treg differentiation. CD36 has many ligands that trigger a wide range of physiological responses when bound to CD36. We reasoned CD36 could have Treg-promoting or Treg-antagonizing roles, depending on its ligand and the environment. Thrombospondin-1 (TSP-1) is a CD36 ligand that is also known to activate latent TGF-β (144–147). We hypothesized that monocytes may activate membrane-bound TGF-β through TSP-1. To address this question, we first examined whether monocytes express TSP-1 on their cell surface. We found $CD14^{+}$ monocytes express TSP-1 mRNA (Fig. 19A), and a large percentage (62.2% ± 17.33) of $CD14^{+}CD36^{hi}$ monocytes express TSP-1 on their cell membrane. In contrast, very few $CD14^{+}CD36^{lo}$, $CD36^{+}CD14^{-}$ or $CD14^{-}CD36^{-}$ cells express membrane TSP-1 (5.55% ± 3.35, 3.02 ± 4.37, 0.18% ± 0.13, respectively) (Fig. 19B-C).

If monocytes utilize TSP-1 to activate membrane-bound TGF-β, then blocking TSP-1 should inhibit monocyte-induced Treg differentiation. To test this, I utilized several peptides which inhibit different aspects of TSP-1: LSKL, CVSTCG, and GGWSHW (Table 1) (161, 205–207). None of these peptides substantially altered Treg differentiation (Fig. 20A-D). Therefore, although $CD14^{+}CD36^{hi}$ monocytes express TSP-1 at the cell surface, we lack evidence for TGF-β activation by TSP-1.

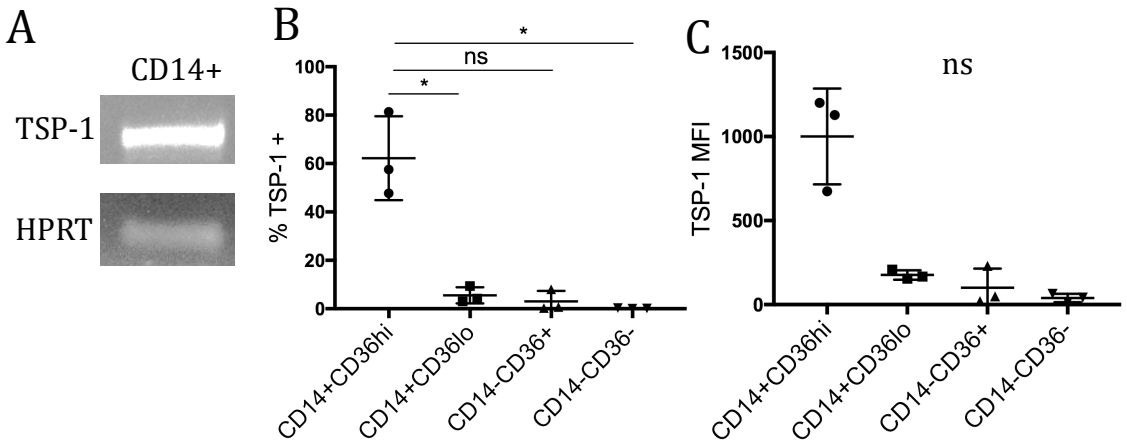


Figure 19. TSP-1 expression by UCB monocytes. (A) The mRNA expression of TSP-1 was analyzed on $CD14^{+}$ enriched UCB by RT-PCR analysis and compared to the housekeeping gene, HPRT; representative data from 3 donors. The **(B)** frequency and **(C)** MFI of TSP-1 by CD14/CD36 gated subsets are shown for 3 donors. * $p < 0.05$, ns = not significant, one-way ANOVA with Dunnett's multiple comparisons test.

Peptide	Description	Peptide Function	References
LSKL	Peptide sequence in LAP that binds both the RKPK sequence of TGF-β and the KRFK sequence of TSP-1. When TSP-1 binds the LSKL sequence of LAP, it presumably competes off TGF-β, activating the cytokine.	Competitively blocks TSP-1 mediated activation of TGF-β	(161, 205, 208)
GGWSHW	Peptide sequence found in the type I repeat of TSP-1 and thought to mediate initial binding to LAP/TGF-β prior to TSP-1 activation of TGF-β.	Inhibits TSP-1 binding to LAP/ TGF-β	(161)
CSVTCG	Peptide sequence found in the type I repeat of TSP-1 that mediates TSP-1 binding to CD36	Blocks TSP-1 binding to CD36	(209)

Table 1. TSP-1 blocking peptides. The identity and function of TSP-1 blocking peptides used in this study.

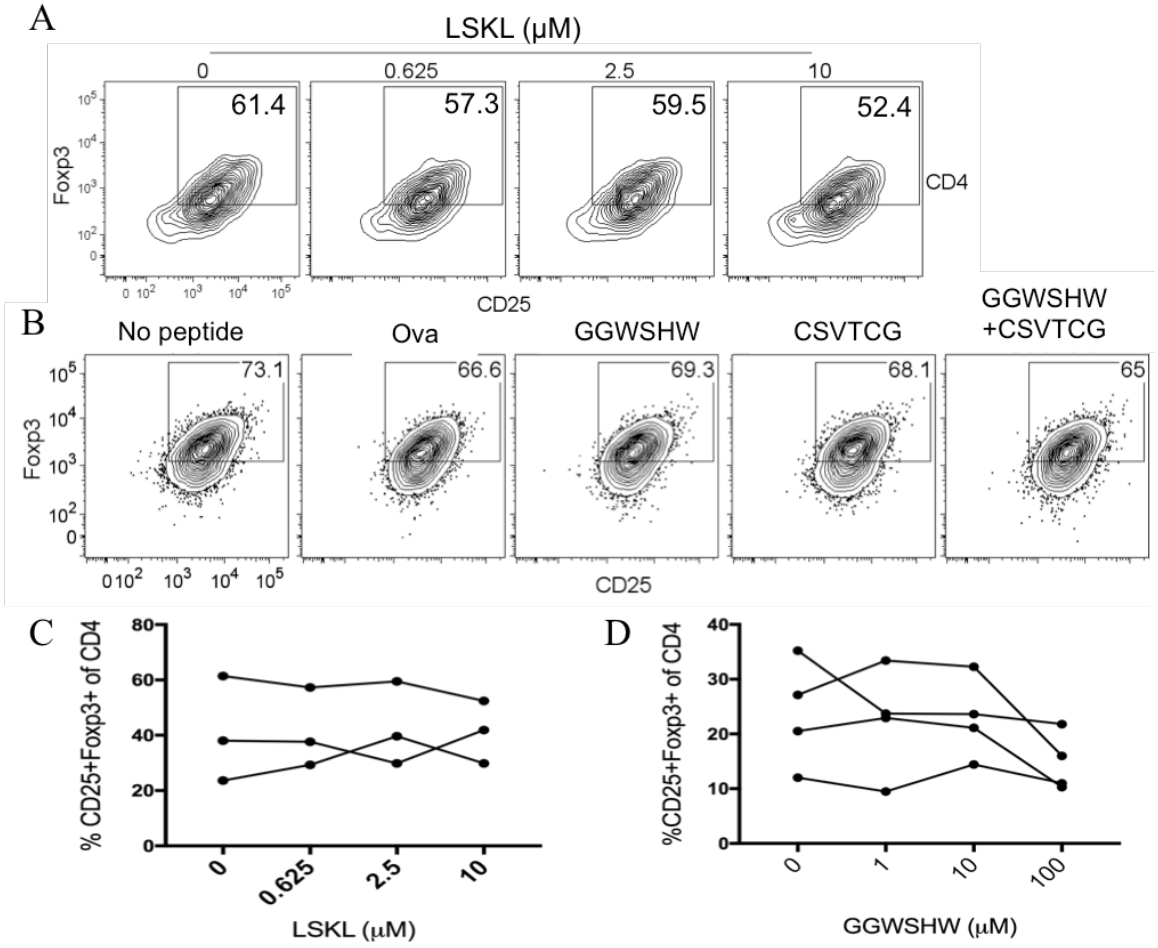


Figure 20. The role of TSP-1 in UCB Treg generation. (A-D) CD25 and Foxp3 expression on gated $CD4^+$ T cells cultured in the presence of TSP-1-blocking peptides. UCB cells were stimulated with anti-CD3 and IL-2 in the presence of (A) varying concentrations of LSKL or (B) 100μM ova, 100μM GGWSHW, 100μM CSVTCG, or 50 μM each of GGWSHW plus CSVTCG. (C-D) Summary data from 3 donors treated with varying concnetrations of (C) LSKL or (D) GGWSHW.

In addition to TSP-1, CD36 can bind ligands that mediate inflammatory processes. Low-density lipoproteins (LDLs) are carriers of cholesterol and other lipids in the blood. LDL can be modified to form more pathogenic species (210). Oxidized LDL (Ox-LDL) is a modified form of LDL that binds CD36 on macrophages and initiates

foam cell formation and atherosclerosis, a disease marked by systemic and local inflammation (174, 211). In contrast, Tregs protect against atherosclerosis (212).

To test whether ox-LDL alters the ability of $CD14^{+}CD36^{hi}$ monocytes to induce Tregs, I cultured UCB in the presence of LDL or ox-LDL (Fig. 21A). Ox-LDL mildly, but significantly, reduced $CD8^{+}Foxp3^{+}$ Treg generation compared to PBS control-treated cells. In contrast, unmodified LDL did not significantly reduce Treg generation. Ox-LDL decreased $CD4^{+}Foxp3^{+}$ Tregs in three of the four donors, but this decrease was not significantly different with this sample size. PMA and ionomycin restimulation of T cells from ox-LDL treated UCB demonstrated that ox-LDL increased IL-4 production from activated T cells, compared to PBS or unmodified LDL-treated UCB (Fig. 21B).

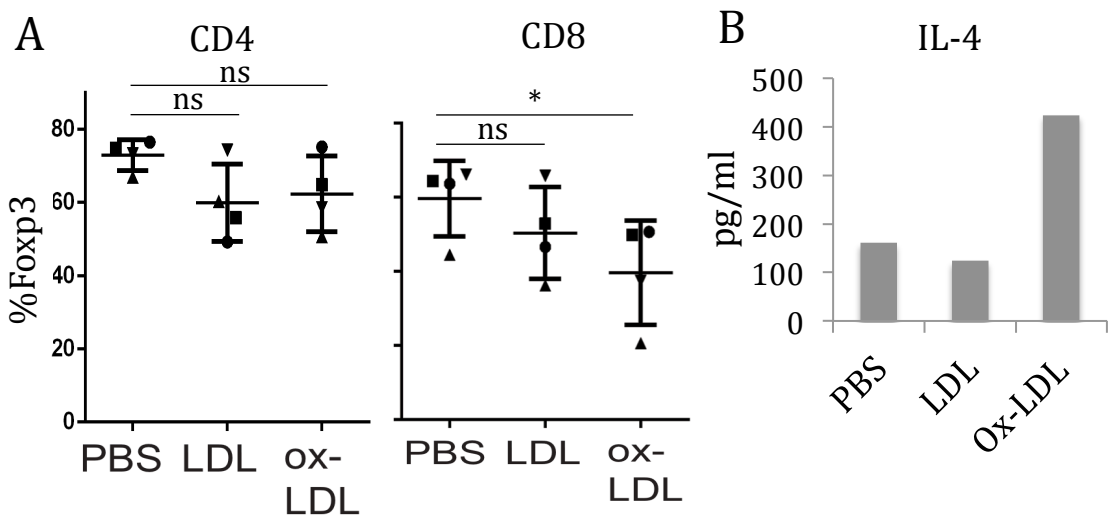


Figure 21. Ox-LDL modulation of monocyte-induced Treg differentiation and cytokine production. (A) CD4 (left) and CD8 (right) $Foxp3^{+}$ cell frequencies in UCB cells stimulated with anti-CD3 and IL-2 in the presence of LDL (50 µg/ml), ox-LDL (50 µg/ml), or a PBS carrier control as indicated. * $p < 0.05$, ns = not significant, one-way ANOVA with Tukey's post-test, n= 4. **(B)** IL-4 production from ox-LDL treated UCB T cells. UCB was stimulated as in (A). 2 weeks later, the cells were washed, restimulated with PMA and ionomycin, and the supernatants harvested for cytokine analysis, n=1.

Together, these data suggest an increase in serum ox-LDL may promote atherosclerosis not only through macrophage foam cell formation, but also by hindering the ability of $CD14^{+}CD36^{hi}$ monocytes to generate protective $CD8^{+}$ Tregs. Furthermore, ox-LDL increases T cell-derived IL-4. In the future, we will determine whether IL-4 neutralization restores Treg differentiation in ox-LDL treated UCB. Future studies will also be required to elucidate the mechanisms by which ox-LDL impairs Treg differentiation and modulates cytokine production.

Discussion

Membrane-Bound TGF-β.

Although the roles of TGF-β in peripheral Treg induction and immune regulation have been widely studied, very little is know about the context in which naïve T cells must encounter TGF-β or the mechanisms by which TGF-β is activated. This is partly because many studies that examined the role of TGF-β in peripheral Treg differentiation utilized high concentrations of exogenous active TGF-β, a situation that does not occur *in vivo*.

We differentiated Tregs *ex vivo* from naïve T cells without the need for exogenous TGF-β. Rather, monocytes were the major endogenous source of TGF-β for activated T cells. Although monocytes do not secrete high concentrations of active TGF-β, they present the latent form on their cell surface in a complex with LTBP-1.

Only a handful of the numerous publications on TGF-β describe a role for its membrane-bound form in establishing immune suppression and tolerance (185–191). A few reports describe $CD4^{+}$ Tregs utilizing membrane-bound TGF-β for contact-

dependent inhibition of inflammatory immune cells (185, 186, 213). Furthermore, $CD4^+$ cells that express membrane-bound TGF-β may have suppressive activity, even in the absence of CD25 or Foxp3(188, 189, 214). In the eye, membrane-bound TGF-β on retinal pigment epithelial cells induces Tregs(191). These studies suggest membrane-bound TGF-beta has the ability to stimulate TGF-β mediated processes.

In the context of monocytes, there are likely multiple advantages to expressing TGF-β on the cell surface. Monocytes can act as APCs. As such, they control the differentiation pathway of T cells through the cytokines they produce at the time of antigen presentation and costimulation. Membrane-bound TGF-β may be more potent than the soluble form due to its high local concentration at the site of cell-to-cell contact between monocytes and naïve T cells, ensuring TGF-β signaling is induced simultaneously with TCR activation. Furthermore, TGF-β is a pluripotent cytokine with biological effects on many cell types, affecting diverse processes, such as fibrosis, tumor metastasis, and inhibition of cell proliferation. The differentiation of multiple T helper subsets requires TGF-β, including the inflammatory Th17 and Th9 cells(215–220). Membrane localization of TGF-β may help limit the recipients of this cytokine and ensure that it is presented in the context of other Treg promoting factors, thereby limiting the differentiation of inflammatory T cell subsets. An unexplored area of TGF-β biology is whether the mode of TGF-β presentation (membrane-bound verses soluble) is one factor directing the pathway of T cell differentiation in response to this multifunctional cytokine.

In studies of membrane-bound TGF-β, it is unclear whether TGF-β exists in the active or latent form on the surface of cells. Because TGF-β and LAP are transcribed as one pro-protein and the two mature proteins associate to form latent TGF-β, current TGF-β antibodies on the market do not distinguish between the active and latent forms. Our studies used LAP expression on $CD14^{+}CD36^{hi}$ monocytes as a surrogate for latent TGF-β. We have also used phage display technology to generate fibronectin-based affinity reagents that specifically recognize active TGF-β (Volgina et al., manuscript in preparation). These reagents stain UCB monocytes positive for active TGF-β. Together, these results suggest monocytes present both active and latent TGF-β on the cell surface.

Although the functions of TGF-β have been widely studied, little is known about the mechanism(s) of TGF-β activation. Proposed models of activation involve proteolytic cleavage or conformational changes in LAP, which release the active cytokine (221). TSP-1 can activate TGF-β, presumably by competing for the TGF-β binding site on LAP (161, 205, 208). Integrins and LTBP-1 bind opposite poles of the latent TGF-β complex and are thought to pull LAP into a new conformation that releases active TGF-β (222–225). Furthermore, TGF-β can be activated in vitro by acidic conditions.

In our culture conditions, it is unclear where or how TGF-β is activated. We have shown LAP is expressed exclusively on monocytes in freshly isolated UCB cells. However, LAP is also upregulated on the surface of T cells after activation (data not shown). We know that monocytes express latent TGF-β and are required to initiate Smad2/3 phosphorylation in T cells. Therefore, monocytes may be required for TGF-β production, activation or both. Because monocytes exclusively express LAP in

unstimulated cells, and they also display active TGF-β on the cell surface, I hypothesize that TGF-β is activated at the monocyte plasma membrane and is presented in its active form to T cells. This could be tested experimentally using a TGF-β bioassay in which a TGF-β-deficient cell line containing a TGF-β response element reporter is co-cultured with monocytes in direct cell contact. TGF-β-deficient T cells may also be added to the assay to test whether TGF-β activation requires monocytes alone or interaction with activated T cells.

The Role of Notch in UCB Treg Differentiation.

Notch ligand binding triggers sequential cleavage of the Notch receptor, resulting in release of the Notch ICD (reviewed in (226)). The Notch ICD regulates target gene transcription through interaction with recombination-signal-binding protein J (RPB-J), also know as CBF1, Suppressor of Hairless, Lag-1 (CSL). In the absence of the Notch ICD, RPB-J directly binds target genes and inhibits transcription through recruitment of a co-repressor complex. Notch ICD binding to RBP-J displaces the co-repressor and allows for recruitment of a co-activator complex, resulting in gene transcription.

Multiple RBP-J binding sites have been identified in the Foxp3 promoter where the Notch ICD binds in conjunction with RBP-J to regulate Foxp3 expression (47, 227, 228). Some data suggests Notch ICD binding may be required for Smad3 recruitment to the Foxp3 promoter, as Smad binding is inhibited in the presence of a gamma secretase inhibitor (47). Notch may also regulate Treg differentiation through cross-talk with the TGF-β signaling pathway, as the ICD of Notch-1 and -4 can form a complex with Smad3 (200, 201).

Our data demonstrate that Notch signaling contributes to Treg differentiation from UCB. However, literature suggests Notch is also required for other T helper subsets. This may suggest Notch provides a general differentiation signal, not Treg specific (229–232). Alternatively, different Notch ligands may promote specific Th subsets. There is some evidence to support this. Multiple reports suggest that DLL4 promotes Th17 and Th1 differentiation, but inhibits Th2 and Tregs (229, 233–235). In contrast, Jagged-1 has been shown to induce Th2 and Treg differentiation and inhibit Th1 (45, 46, 52, 227, 232). Our data shows $CD14^{+}CD36^{hi}$ monocytes express DLL3. Interestingly, they also express Jagged-1, but at lower levels than DLL3 and less consistently between donors (data not shown). The mechanisms leading to different Th subset differentiation by different Notch ligands are still unknown.

One approach to studying the role of different Notch ligands and Notch receptors in T cell differentiation is to selectively express only one Notch receptor in T cells or provide only one Notch ligand. Gene expression profiling can then be used to determine the gene targets of specific Notch receptor/ligand pairs. It is possible that various Notch receptors and Notch ligands differentially regulate transcription factors involved in T helper cell differentiation.

Our data suggests Notch signaling contributes to UCB Treg differentiation. However, we did not determine the specific ligand involved, nor did we exclude other cellular sources of Notch ligands. We showed DLL3 was expressed by $CD14^{+}CD36^{hi}$ monocytes, but also at lower levels by $CD14^{+}CD36^{lo}$, $CD14^{-}CD36^{+}$, and $CD14^{-}CD36^{-}$ cells. Therefore, it is possible that other cells contribute to Notch activation in UCB T

cells. Monocytes, however, are sufficient to induce Tregs differentiation in the absence of other accessory cells and $CD14^{+}CD36^{hi}$ monocytes were the only cells observed to co-express Notch ligands with LAP.

The Role of Retinoic Acid in UCB Treg Differentiation.

Our data demonstrate that endogenous retinoic acid contributes to monocyte-induced Treg generation from UCB. Among UCB cells, $CD14^{+}CD36^{hi}$ monocytes are the primary cells that exhibit ALDH activity, the enzyme required for retinoic acid production. Previous studies showed retinoic acid enhances TGF-β-dependent Treg differentiation through several mechanisms. Retinoic acid enhances Smad-independent ERK phosphorylation in the presence of TGF-β and increases activating histone modifications at the Foxp3 promoter (54, 236). Retinoic acid also enhances peripheral Treg generation indirectly by decreasing the production of inhibitory effector cytokines by memory T cells (237).

The addition of an exogenous RAR agonist increases the efficiency of Foxp3 induction to over 80% of $CD4^{+}$ and $CD8^{+}$ T cells. Other studies demonstrated that retinoic acid improves nTreg and iTreg stability under inflammatory conditions (236, 238–240). This is especially important for *ex* vivo Treg generation for therapeutic applications because Tregs can lose $Foxp3^{+}$ expression and upregulate effector cytokines when they enter inflammatory environments (241–243). The use of retinoic acid in ex vivo Treg generation for therapeutic purposes may be useful for both generating a high frequency of Tregs and preventing Tregs from converting to inflammatory T helper subsets upon transfer.

Because retinoic acid is a vitamin A metabolite, it calls into question the effect of vitamin A deficiency on Treg development in the neonate. Vitamin A deficiency affects approximately one-third of infants and young children worldwide (244). Vitamin A deficiency causes blindness and deafness, and can increase the risk of serious diarrheal and respiratory infections. In children living in regions where vitamin A deficiency is prevalent, vitamin A supplementation has been estimated to decrease all cause mortality by 30%, and specifically improves survival during gastrointestinal and respiratory infections (245).

While it is clear that vitamin A deficiency impairs effective immune responses, little is known about the impact of vitamin A deficiency on Treg generation in humans. Because the vitamin A metabolite, retinoic acid, contributes to iTreg generation and stability, it is plausible to hypothesize that vitamin A deficiency would impair Treg generation or function. This could be particularly harmful during fetal development or shortly after birth, when there is enhanced Treg generation under normal conditions. Epidemiological studies are still needed to characterize Treg frequency and function in vitamin A deficient individuals, as well as to determine the impact of vitamin A supplementation in these individuals.

In mice, vitamin A deficient animals are more susceptible to a variety of infections and inflammatory conditions and this is associated with an increase in inflammatory cytokine production (246–249). While the data is limited on the effect of vitamin A deficiency on Treg generation, one study demonstrated that vitamin A deficient mice have decreased generation of oral tolerance and impaired $Foxp3^+$ Treg

induction by mesenteric DCs (250). However, a second study of viral upper respiratory tract infection suggested enhanced inflammatory cytokine production in vitamin A deficient mice is due to impaired viral clearance rather than decreased $Foxp3^+$ Tregs (246). Further studies are needed to understand the impact of vitamin A deficiency on human Treg development.

The Role of CD36 in UCB Treg Differentiation.

High expression of CD36 marks the monocytes capable of inducing Tregs, yet we lack evidence that CD36 itself is required for Treg differentiation. Multiple peptide inhibitors that block TSP-1 mediated activation of TGF-β or TSP-1 binding to CD36 did not inhibit Treg differentiation from UCB. In an effort to elucidate the role of CD36 expressing cells during Treg development *in vivo,* our lab sought to identify a murine counterpart to $CD36^{hi}$ cells capable of inducing Tregs, but without success. Our future goal is to knock down CD36 in human monocytes to test the role of CD36 in monocyte-induced Treg generation. However, gene deletion or knockdown in primary human monocytes is extremely challenging with our current technology. Monocytes are difficult to maintain in an undifferentiated state in substantial numbers. They easily die in culture without stimulation and do not proliferate sufficiently in the absence of differentiation. siRNA based knockdown in primary human monocytes is difficult prior to their differentiation into monocyte-derived macrophages or DCs.

Ox-LDL Modulation of UCB Treg Differentiation.

In our studies, the addition of ox-LDL to total UCB cultures blocked $CD8^+$ Treg differentiation. Ox-LDL can bind CD36, but also other surface receptors such as

scavenger receptor A (SR-A) (251). It is unclear in our studies whether Ox-LDL is modulating Treg differentiation through direct binding to CD36 on monocytes or through binding to other surface receptors. Ox-LDL can alter monocyte/macrophage phenotype, promoting foam cell formation (174). Other CD36 ligands, such as beta-amyloid, enhance inflammatory cytokine production and ROS in macrophages, and this may also be true of ox-LDL (145, 252, 253). Ox-LDL might convert the tolerogenic monocytes to a pro-inflammatory state and alter their cytokine production to favor the differentiation of other Th subsets rather than Tregs. T cells from ox-LDL-treated UCB upregulate IL-4, a Th2 cytokine. Future studies are needed to determine whether ox-LDL alters UCB cytokine production and whether these cytokines subsequently block Treg differentiation. To test this, ELISA or multiplex cytokine analysis can be used to analyze the cell supernatants of UCB stimulated in the presence or absence of ox-LDL. I hypothesize that ox-LDL increases the production of cytokines known to block UCB Treg generation, such as IL-4 and IL-12. To test whether increased production of these cytokines accounts for decreased Treg generation in ox-LDL treated UCB, neutralizing antibodies may be used. If ox-LDL impairs Treg generation through the upregulation of inhibitory cytokines, then neutralizing these cytokines should restore Treg differentiation.

CHAPTER FOUR

IL-4 REGULATION OF TGF-β SIGNALING AND UCB TREG GENERATION

Treg Generation after Birth and into Adulthood

Infection remains a leading cause of death among neonates and infants worldwide. In 2013, approximately 50% of deaths in children under 5 years old were from infection(254). Enhanced Treg differentiation by neonates could contribute to their increased risk for life-threatening infections. We have shown that the majority of UCB T cells acquire regulatory markers when stimulated with anti-CD3 and IL-2 *ex vivo* (Fig. 1). To determine if babies maintain the ability to efficiently generate Tregs after birth, PBMCs from 12 donors ages 7-180 days old were stimulated with anti-CD3 and IL-2. At these ages, babies maintained the ability to induce Treg differentiation at a higher level than adult PBMCs (Fig. 22, Fig. 1). Compared to UCB (day 0), the efficiency of $Foxp3^+$ Treg generation in babies diminished slowly over the first six months, with only a trend toward significance with this sample size.

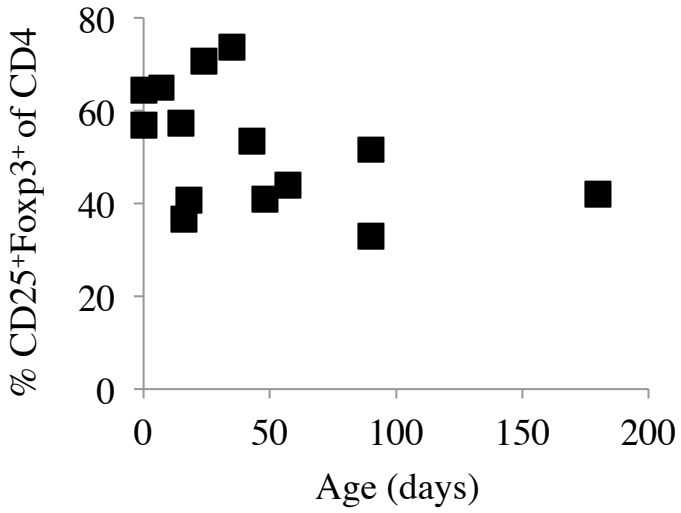


Figure 22. Induced Treg differentiation from neonates in the first six months of life. The percent of CD4+ cells expressing CD25 and Foxp3 was determined from UCB (0 days old) or neonatal PBMCs stimulated with anti-CD3 and IL-2 as in figure 1. Each dot represents a different donor.

To determine whether the efficiency of Treg generation correlates with the percent of $CD14^{+}CD36^{hi}$ monocytes, we compared the frequency of these cells between UCB, infant and adult PBMCs (Fig. 23). The frequency of $CD14^{+}CD36^{hi}$ monocytes decreased in infants (ages 5-24 months) compared to UCB (Fig. 23A). However, the percent of $CD14^{+}CD36^{hi}$ monocytes was not significantly different between adult blood and UCB (Fig. 23B). There was no correlation between the percent of $CD14^{+}CD36^{hi}$ monocytes and the frequency of induced Tregs (data not shown). Therefore, while it is possible that a loss of $CD14^{+}CD36^{hi}$ monocytes contributes to the gradual decrease of Treg generation in infants, there does not appear to be a direct relationship between the two. Furthermore, the loss of Treg differentiation in adult blood is not due to a loss of $CD14^{+}CD36^{hi}$ monocytes.

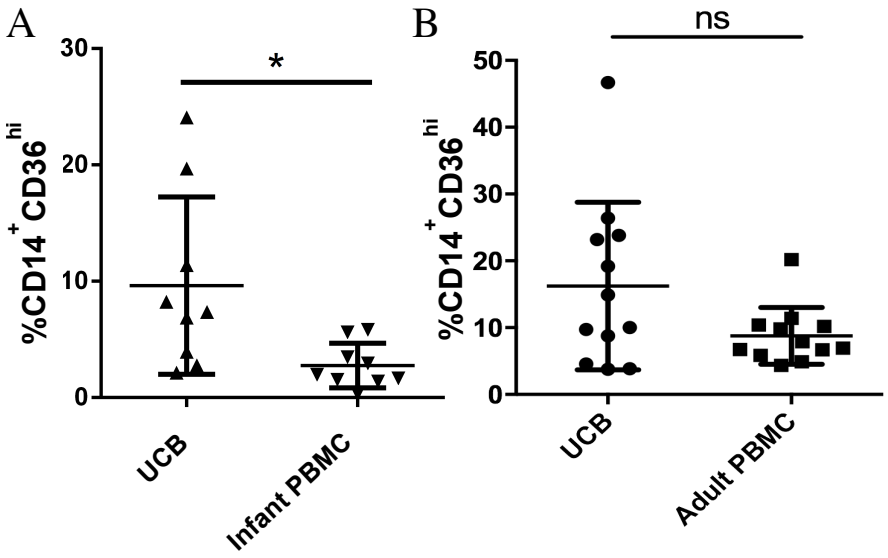


Figure 23. The frequency of $CD14^{+}CD36^{hi}$ monocytes in UCB, infant and adult PBMCs. The percent of $CD14^{+}CD36^{hi}$ monocytes was compared between **(A)** UCB and infant PBMCs (age 5-24 months), n=9; and (**B)** UCB and adult PBMCs, n = 12. * $p < 0.05$, ns = not significant, unpaired Student *t* test with Welch's correction.

We asked whether the loss of Treg generation in adult PBMCs is due to a lack of TGF-β, Notch or retinoic acid. A large percentage of adult $CD14^{+}CD36^{hi}$ monocytes express membrane-bound LAP and DLL3 (Fig. 24A-B). The vast majority of adult $CD4^{+}$ and $CD8^{+}$ T cells stimulated in the presence of other mononuclear cells upregulate pSmad2/3 and Notch-1 (Fig. 24C-D). Furthermore, the addition of an RAR agonist to adult PBMCs does not substantially increase $CD4^{+}$ Treg differentiation (Fig. 24E). The RAR agonist enhanced Foxp3 expression on $CD8^{+}$T cells, but the majority of cells remained $Foxp3^{-}$ (Fig. 24E). These data suggest that the lack of Treg generation from adult PBMCs is not primarily due to a lack of TGF-β, Notch or retinoic acid signaling.

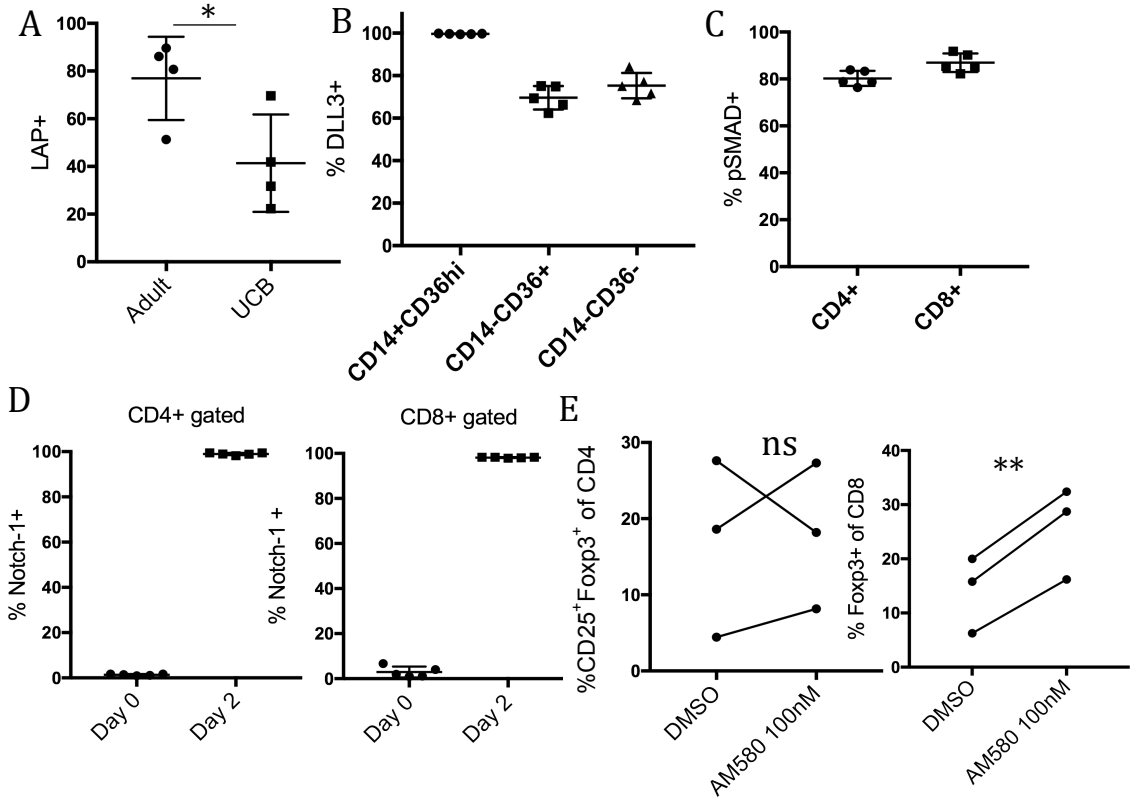


Figure 24. The presence of TGF-β, retinoic acid and Notch in adult PBMCs. **(A)** Comparison of LAP expression on UCB or adult PBMC $CD14^{+}CD36^{hi}$ monocytes. Student *t* test, * $p < 0.05$, n=4. **(B)** DLL3 expression on CD14/36 gated populations from adult PBMCs, n=5. **(C)** SMAD2/3 phosphorylation in $CD4^{+}$ and $CD8^{+}$ T cells from adult PBMCs stimulated for 2 days with anti-CD3 and IL-2, n=5. **(D)** Notch-1 upregulation on adult $CD4^{+}$ and $CD8^{+}$ T cells activated (Day 2) as in (C), compared to unstimulated T cells (Day 0); n=5. **(E)** Adult PBMC Treg generation in the presence of the RAR agonist, AM580. Adult PBMCs were stimulated with anti-CD3 and IL-2 in the presence of AM580 or the DMSO control and analyzed 2 weeks later for Foxp3 expression on $CD4^{+}$ and $CD8^{+}$ T cells. ** $p < 0.01$, ns = not significant, paired Student *t* test, n=3.

Because adult PBMCs do not lack monocytes or monocyte-derived signals to induce Tregs, we hypothesized that adult PBMCs produce a factor that actively inhibits Treg differentiation. To test this, we asked whether adult PBMCs inhibit UCB Treg differentiation. We co-cultured adult PBMCs with HLA-A2-mismatched UCB, to distinguish the two cell sources. As we have previously shown, anti-CD3 and IL-2

stimulation induces a lower percentage of $CD4^+$ and $CD8^+$ $Foxp3^+$ cells from adult PBMCs than from UCB (Fig. 25A). However, the percentage of $Foxp3^+$ cells between adult and UCB was the same when co-cultured. The percentage of $CD4^+CD25^+Foxp3^+$ cells from UCB also decreased when co-cultured with adult PBMCs in transwell compared to UCB cultured alone (Fig. 25B). These data suggest UCB is capable of producing factors to boost adult Treg generation. Likewise, adult PBMCs can produce a soluble factor(s) that dominantly suppresses UCB Treg generation.

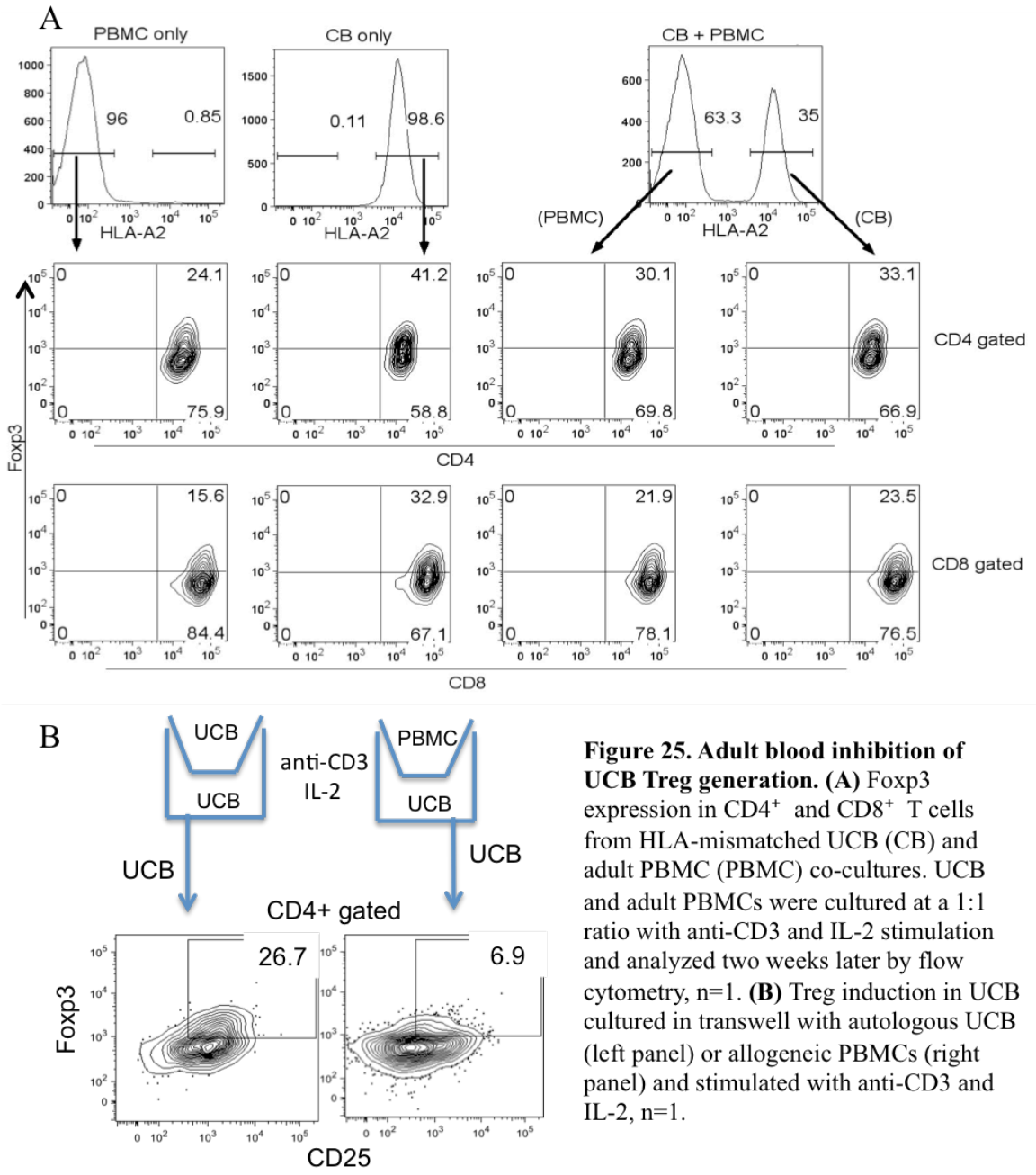


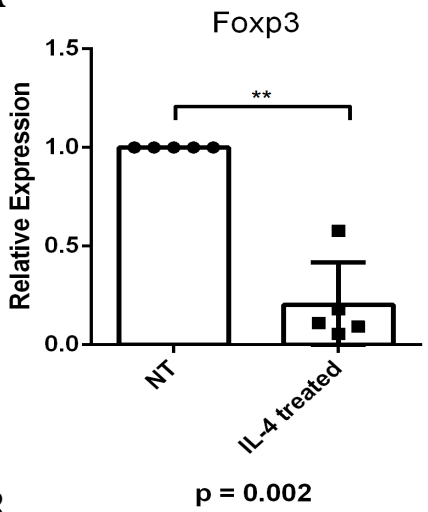
Figure 25. Adult blood inhibition of UCB Treg generation. (A) Foxp3 expression in $CD4^+$ and $CD8^+$ T cells from HLA-mismatched UCB (CB) and adult PBMC (PBMC) co-cultures. UCB and adult PBMCs were cultured at a 1:1 ratio with anti-CD3 and IL-2 stimulation and analyzed two weeks later by flow cytometry, n=1. **(B)** Treg induction in UCB cultured in transwell with autologous UCB (left panel) or allogeneic PBMCs (right panel) and stimulated with anti-CD3 and IL-2, n=1.

Inhibition of Treg Generation by IL-4

To identify the molecules expressed by adult PBMCs that may be inhibitory for Treg generation, we tested multiple cytokines known to abrogate Treg differentiation, stability or suppressive function in other experimental models (237, 255–262). These included the Th2 cytokines, IL-4 and IL-5; cytokines that induce Th1 differentiation or are produced by Th1 cells, IFN-γ, IL-12 and TNF-α; and the inflammatory cytokines, IL-6 and IL-1β. Addition of IL-4 at the time of T cell stimulation decreased Foxp3 mRNA expression 3 days after T cell stimulation (Fig. 26) and the percentage of Foxp3 expressing $CD4^+$ and $CD8^+$ T cells approximately 2 weeks after T cell stimulation (Fig 27A-D). IL-4 inhibition of Foxp3 expression occurred in a dose-dependent manner (Fig. 28). These data demonstrate the presence of IL-4 at the time of T cell stimulation potently inhibits UCB Treg differentiation.

We asked whether other cytokines inhibited UCB Treg differentiation similar to IL-4. A different Th2 cytokine, IL-5, did not inhibit Foxp3 expression to the same extent as IL-4 (Fig 27 A-D). IL-12, a Th1 inducing cytokine, substantially decreased the percentage of $CD25^+Foxp3^+$ expressing T cells (Fig. 27 A-D). IL-12 inhibition occurred primarily by decreasing CD25 expression, rather than Foxp3 (Fig. 27A-B). These data suggest IL-12 may decrease Treg differentiation by impairing T cell activation. This is in contrast to IL-4, which primarily decreased Foxp3 expression. IFN-γ, TNF-α, IL-1β, and IL-6 minimally impacted Treg differentiation.

A



B

	***Foxp3* Ct** (mean of 2-3 technical replicates)		***Hprt* Ct** (mean of 2-3 technical replicates)	
	NT	**+IL-4**	**NT**	**+IL-4**
Donor 1	21.95	24.55	22.52	21.945
Donor 2	21.89	25.48	22.57	23.68
Donor 3	23.545	26.64	24.38	24.04
Donor 4	22.7	23.51	23.28	23.3
Donor 5	22.61	26	23.39	22.62

Figure 26. IL-4 regulation of *Foxp3* mRNA expression. UCB T cells were stimulated with anti-CD3 and IL-2 in the presence (IL-4 treated) or absence (no treatment; NT) of 5 ng/ml IL-4. Three days later, $CD4^+$ T cells were enriched and analyzed for the expression of *Foxp3* by RT-qPCR. The **(A)** relative expression was calculated using the ΔΔCt method, normalized to the *Hprt* housekeeping gene. Statistics were calculated on the $2^{-\Delta Ct}$ values; ** $p < 0.01$, paired Student *t* test; n=5. **(B)** Ct values from each donor are shown.

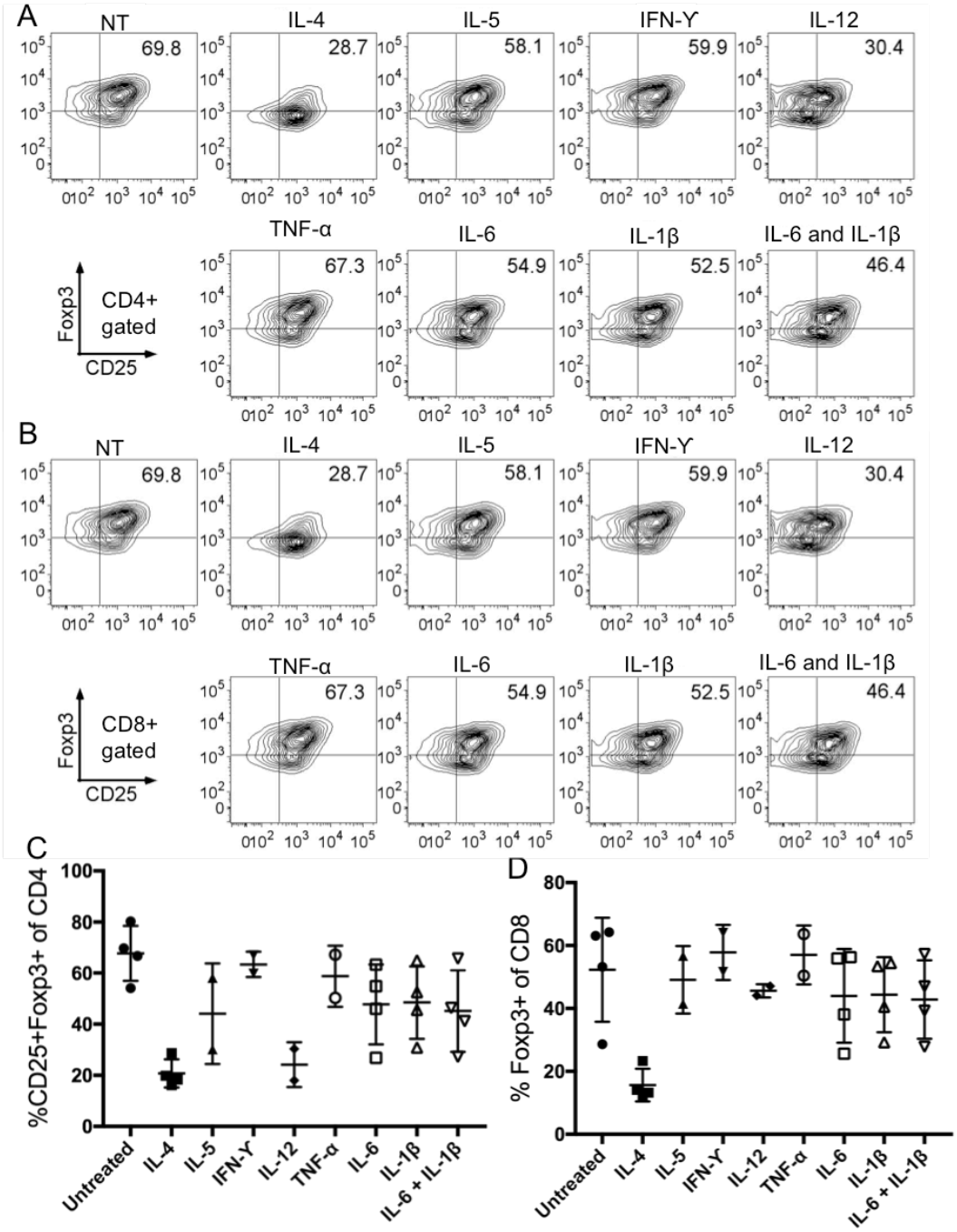


Figure 27. Cytokine modulation of UCB Treg differentiation. (A-D) UCB was stimulated with anti-CD3 and IL-2 in the presence of no additional cytokines (no treatment; NT) or with the indicated cytokines at 20 ng/ml. The frequency of $CD4^+$ (A, C) or $CD8^+$ (B, D) Tregs was analyzed approximately 2 weeks later. (C, D) Data are shown from 2-4 donors.

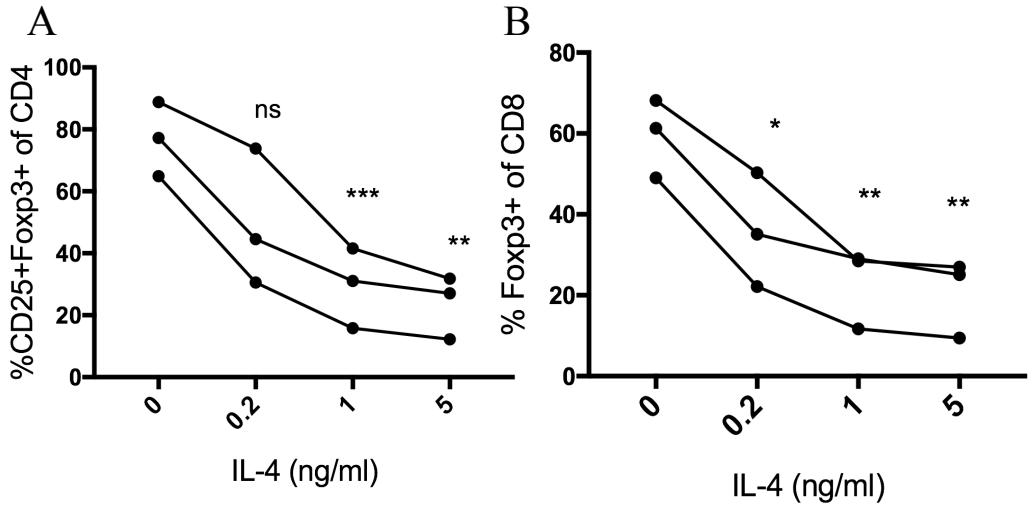


Figure 28. Dose-dependent inhibition of UCB Treg differentiation by IL-4. UCB was stimulated with anti-CD3 and IL-2 in the presence of varying concentrations of IL-4. The frequency of **(A)** CD4 or **(B)** CD8 Tregs was analyzed approximately 2 weeks later. Statistical differences were determined compared to 0 ng/ml of IL-4; * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, one-way ANOVA with Dunnett's multiple comparisons test; n=3.

IL-4 is a classic Th2 cytokine. To ask whether IL-4 blocks Tregs by diverting T cells toward Th2 differentiation, we stimulated UCB cells with anti-CD3 and IL-2 in the presence of IL-4. Two weeks later, we washed and restimulated the cells to examine their cytokine production. If IL-4 shifts T cell differentiation from Tregs toward Th2 cells, then stimulating T cells in the presence of IL-4 should increase the production of IL-4 upon restimulation. IL-4 was increased in the cell supernatant of IL-4-treated UCB (Fig. 29A). The percentage of cells expressing intracellular IL-4 also significantly increased in IL-4 treated cells (3.6% ± 2.3 no IL-4 treatment; 8.9% ± 6.1 with IL-4 treatment) (Fig. 29B-C). However, the majority of IL-4 treated cells did not produce detectable levels of IL-4. Some Th2 cells produce only IL-5 and IL-13, but the levels of these cytokines did not change in IL-4 stimulated UCB (data not shown). These data suggest only a small fraction of UCB cells differentiate into Th2 cells when stimulated in the presence of IL-4.

We also examined the production of other T helper cytokines to determine whether IL-4 is inducing a unique $CD4^+$ T cell subset. IL-10 is an immunosuppressive cytokine produced by Type 1 regulatory (Tr1) T cells. Tr1 cells can be induced in mice and man by IL-27 in combination with IL-21 (263). These cells have been shown to prevent allogeneic graft rejection and autoimmunity in multiple mouse models (263). IL-10 production was increased in the cell supernatants of IL-4 treated cells (Fig. 29A). IL-4 treatment also increased the percentage of cells expressing intracellular IL-10, however this increase was not significant with our sample size and the majority of cells lacked IL-10 (Fig. 29B-C). We also observed a small increase in IL-2 producing cells by intracellular staining, which was insignificant with our sample size. The majority of IL-4 treated cells remained negative for the Th1, Th17 and Th9 cytokines IFN-γ, IL-17a and IL-9, respectively. We conclude from these data that IL-4 directly inhibits Treg differentiation, rather than diverting naïve T cells toward a different T helper cell differentiation pathway.

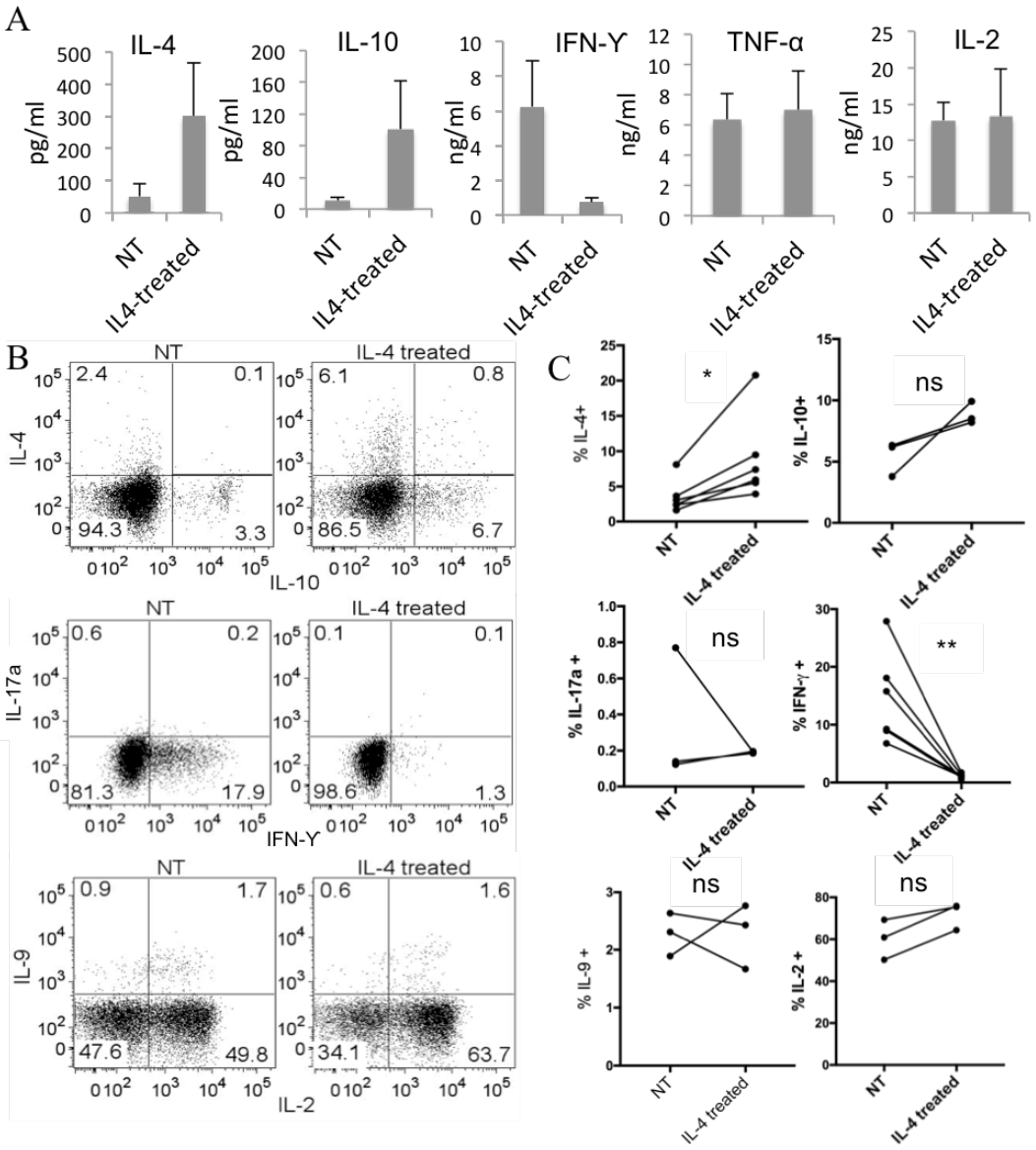


Figure 29. Cytokine production from IL-4-treated UCB cells. UCB was stimulated with anti-CD3 and IL-2 in the presence of no cytokines (no treatment; NT) or with the 5ng/ml of IL-4. Approximate two weeks later, the cultures were washed and restimulated with **(A)** PMA and ionomycin and the cytokine production was analyzed from the supernatants. Data are summarized from 3 donors. **(B-C)** Alternatively, the cells were restimulated with PMA, ionomycin and monensin and cytokine production was analyzed by intracellular staining in $CD4^+$ gated cells. (B) Representative plots and (C) cumulative data from 3-6 donors are shown. * $p< 0.05$, ** $p< 0.01$, ns = not significant, paired Student *t* test.

IL-4 binding to its receptor leads to the activation and nuclear translocation of STAT6 and the subsequent regulation of target genes. STAT6 upregulates GATA3, the master transcription factor of Th2 cells. IL-4 has been reported to block Foxp3 expression through several mechanisms, including direct binding of both STAT6 and GATA3 to the Foxp3 promoter (259, 264). However, it is unknown whether IL-4 regulates TGF-β signaling upstream of Foxp3.

To test whether IL-4 regulates TGF-β signaling, we examined Smad2 and Smad3 in UCB $CD4^+$ T cells cultured for 3 days in the presence or absence of IL-4. We examined this early time point, since TGF-β signaling is required during the first few days of T cell stimulation for Treg generation (Fig. 9). IL-4 increased the expression of Smad2, but decreased Smad3 (Fig. 30A-B).

The IL-4 receptor is expressed by T cells, but also by other mononuclear cells such as B cells and monocytes(265–267). IL-4 in combination with GM-CSF triggers monocyte differentiation into DCs (268). Therefore, we asked whether IL-4 acts directly or indirectly on T cells to regulate Smad2 and Smad3 by stimulating UCB $CD4^+$ cells with plate-bound anti-CD3 and anti-CD28. Consistent with IL-4 treatment of total UCB cells, IL-4 upregulated Smad2 and decreased Smad3 in isolated $CD4^+$ cells (Fig. 30C-D). The changes in Smad2 and Smad3 were clear by day 2 post-stimulation. Together, these data demonstrate that IL-4 differentially regulates Smad2 and Smad3 expression. Foxp3 is a known target of Smad2 and Smad3. Therefore, IL-4 regulation of Smad2 and Smad3 may alter Foxp3 transcription and Treg generation.

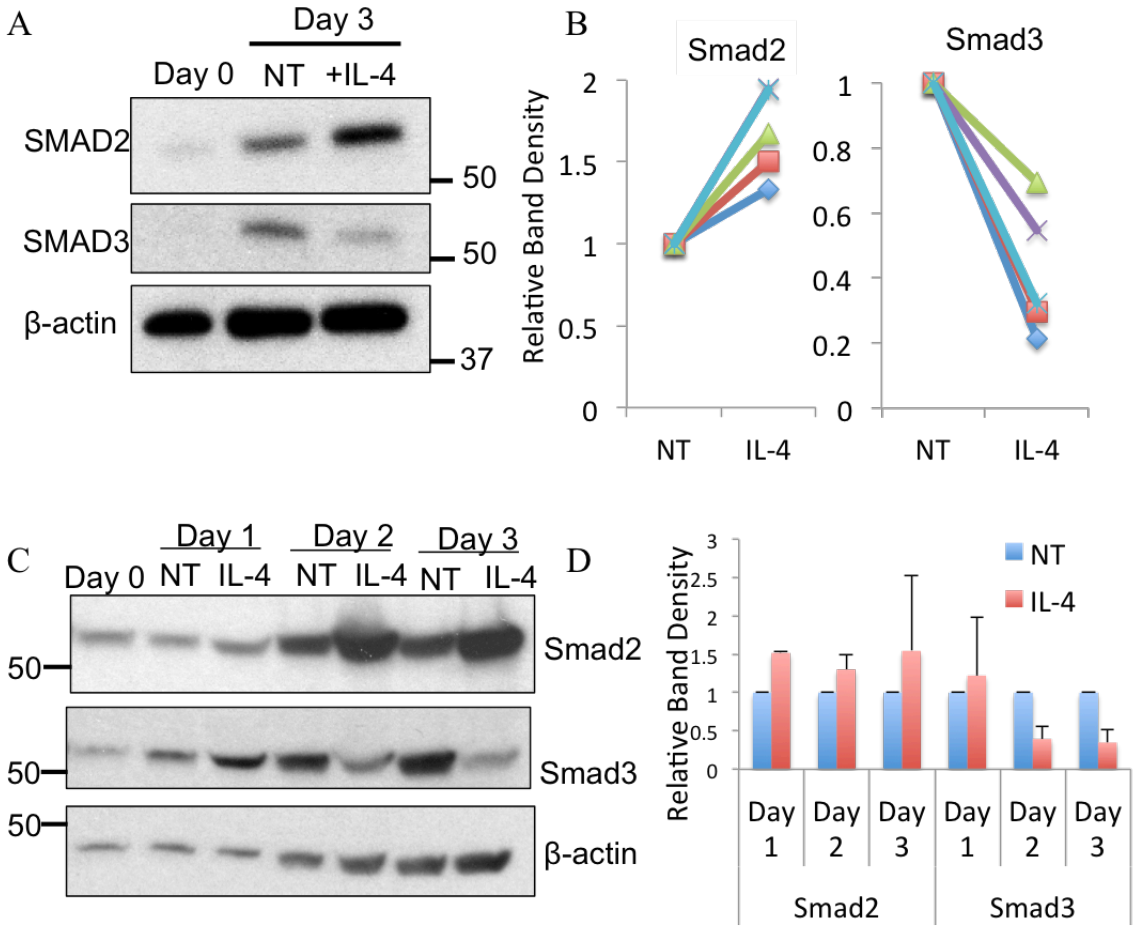


Figure 30. Differential regulation of Smad2 and Smad3 protein by IL-4. (A-B) $CD4^+$ T cells were enriched from unstimulated UCB (Day 0) or from UCB stimulated with anti-CD3 and IL-2 in the presence of no cytokines (no treatment; NT) or 5ng/ml of IL-4 for 3 days. Whole cell lysates were analyzed for Smad2 and Smad3 expression by western blot. Band densities from five independent donors were quantified by ImageJ software and graphed as the relative density compared to NT, normalized to beta actin. Each line represents one donor. **(C-D)** Expression of Smad2 and Smad3 by UCB unstimulated $CD4^+$ T cells (day 0) or $CD4^+$ T cells stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of no cytokines (no treatment; NT) or 5ng/ml IL-4. (C) Representative blot and (D) data summarized from 2 donors are shown.

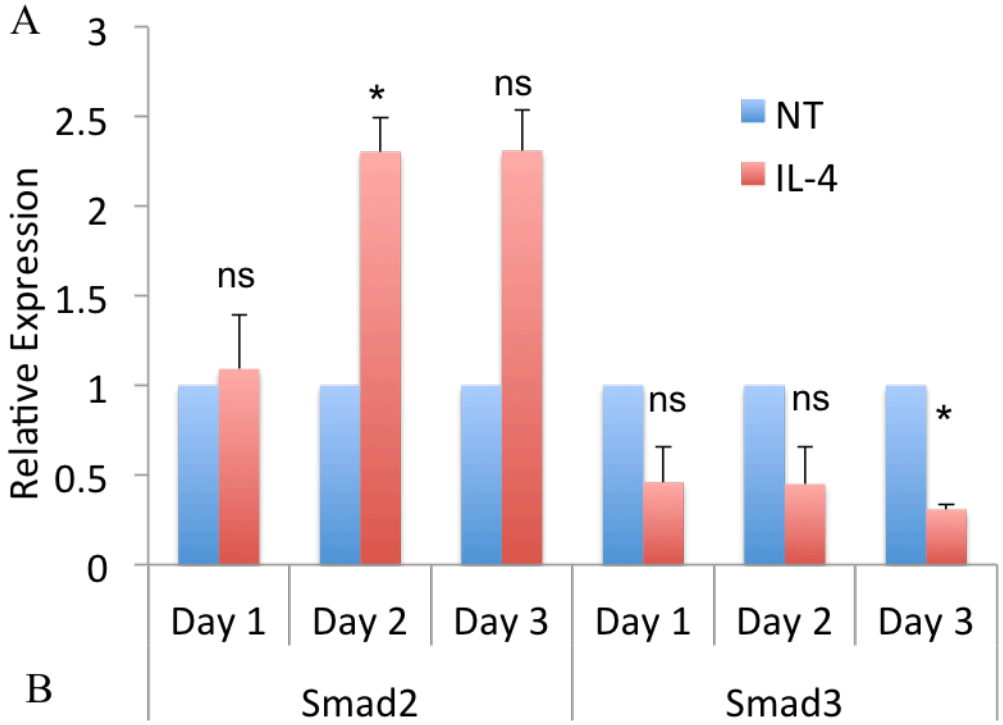
To determine how IL-4 differentially regulates Smad2 and Smad3, I determined the levels of *Smad2* and *Smad3* transcripts at various times after $CD4^+$ T cell stimulation with plate-bound αCD3 and αCD28 in the presence or absence of IL-4, with the

expectation that if IL-4 differentially regulates *Smad2* and *Smad3* at the transcript level, we should observe an increase in *Smad2* and decrease in *Smad3* transcripts in IL-4 treated cells. In parallel to the protein levels, IL-4 significantly increased *Smad2* and decreased *Smad3* transcripts (Fig. 31). These data demonstrated that IL-4 differentially regulates Smad2 and Smad3 at the transcript level.

Knockout studies in mice suggest that single deletion of either Smad2 or Smad3 is sufficient to decrease, but not completely abolish, Treg induction from naïve T cells *in vitro* (269–272). These data suggest Smad2 and Smad3 have both redundant and distinct roles in inducing Foxp3 expression in the periphery. For nTreg generation, single Smad2 or Smad3 deletion does not decrease $Foxp3^+$ cells in the thymus, spleen or mesenteric lymph node, while double deletion substantially decreases the percent of Tregs in these tissues(272). In humans, the relative roles of Smad2 and Smad3 in Treg differentiation are unknown.

My data demonstrates that IL-4 downregulation of Smad3 correlates with impaired Foxp3 expression and Treg differentiation. To test whether a loss of Smad3 is sufficient to block Treg generation, I used Smad3 siRNA and induced Tregs using THP-1 monocytic leukemia cells. This bypassed the need to freeze primary monocytes during siRNA knockdown in T cells. Freshly isolated UCB $CD4^+$ T cells were transfected with Smad3 siRNA and stimulated with irradiated THP-1 cells in the presence of IL-2 and anti-CD3.

A



B

		Smad2 Ct	Smad3 Ct	Hprt Ct
Day 1	NT	28.73	29.6	27.38
	IL4	27.27	30.78	26.42
Day 2	NT	24.19	25.13	22.79
	IL4	23.26	27.2	23.17
Day 3	NT	24.15	24.64	22.3
	IL4	22.57	26.02	22.17

Figure 31. Differential regulation of *Smad2* and *Smad3* transcripts by IL-4. RT-qPCR analysis of *Smad2* and *Smad3* in UCB $CD4^+$ T cells stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of no cytokines (no treatment; NT) or 5ng/ml IL-4. **(A)** Statistical analysis of data from 3 independent experiments was conducted as in Fig. 26 and graphed as the relative expression of the target gene compared to untreated cells, normalized to *Hprt.* * $p < 0.05$, ns = not significant, one-way ANOVA with Sidak's multiple comparisons test, n=3. **(B)** Representative Ct values from one donor.

Introduction of Smad3 siRNA resulted in mild reduction of Smad3 protein, as assessed by western blot (Fig. 32A). Despite mild knockdown, Smad3 siRNA moderately decreased the percentage of Foxp3 expressing $CD4^+$ T cells when co-cultured with THP-1 cells (Fig. 32B). These data suggest Smad3 may play a non-redundant role in monocyte-induced Treg differentiation. Furthermore, downregulation of Smad3 by IL-4 may be sufficient to decrease Treg generation.

Smad2 and Smad3 have opposing roles in some biological contexts (273). For example, Smad3 mediates TGF-β autocrine signaling to enhance TGF-β production by DCs, while Smad2 opposes it(274). Therefore, it is possible that Smad2 and Smad3 have opposing roles in monocyte-induced Treg differentiation. Our data suggests Smad3 promotes Treg differentiation. To examine the role of Smad2 in UCB Treg differentiation, we used siRNA-mediated knockdown of Smad2. Three of the four siRNAs tested decreased Smad2 protein in UCB $CD4^+$ T cells (Fig. 32A). After stimulation with THP-1 cells, Foxp3 expression was decreased in the $CD4^+$ T cells with effective Smad2 knockdown (Fig. 32C). These data suggest that both Smad2 and Smad3 contribute to Treg differentiation in UCB T cells in a non-redundant manner. Furthermore, IL-4 may inhibit Treg differentiation in part by reducing Smad3 expression. The role of increased Smad2 by IL-2 in UCB Treg generation remains to be determined.

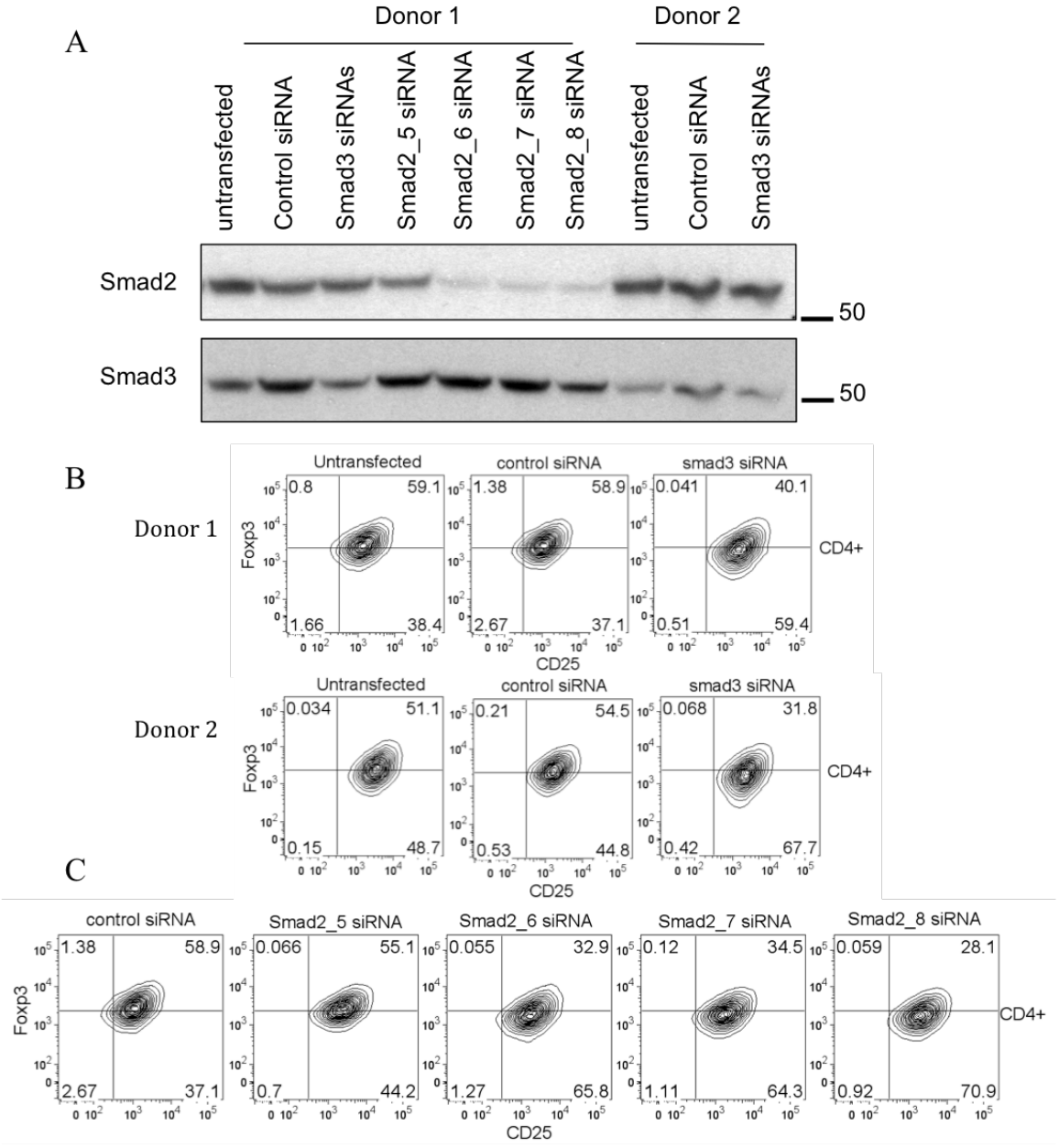


Figure 32. The effect of Smad3 and Smad2 siRNA on UCB Treg differentiation. $CD4^+$ T cells were enriched from UCB and transfected with control, Smad3 or one of four Smad2 (Smad2_5, _6, _7, or _8) targeting siRNAs. **(A)** Transfected T cells were stimulated with plate-bound anti-CD3 and anti-CD28 and the knockdown efficiency was tested by western blot. **(B-C)** CD25 and Foxp3 expression from $CD4^+$ T cells transfected with (B) Smad3 siRNA (n=2) or (C) Smad2 siRNAs (n=1) and co-cultured with irradiated THP-1 cells in the presence of anti-CD3 and IL-2.

Discussion

Treg Inhibition by IL-4.

Inflammatory cytokines can inhibit Treg differentiation or function in various mouse and human models (237, 255–262). However, UCB Treg differentiation is apparently resistant to many of these cytokines. IL-5, IFN-γ, TNF-α, IL-6, IL-1β, and IL-6 plus IL-1β had little ability to block $CD4^+$ or $CD8^+$ $Foxp3^+$ cell generation. This suggests UCB has mechanisms to promote Treg differentiation even in the presence high levels of inflammatory cytokines. In contrast, both IL-12 and IL-4 substantially decreased the percentage of $CD25^+Foxp3^+$ T cells, suggesting UCB Treg generation is not resistant to inhibition under Th1 and Th2 inducing conditions.

IL-12 lowered the percentage of Tregs primarily by decreasing CD25 expression, which is a T cell activation marker. Therefore, IL-12 may impair Treg differentiation through decreased T cell activation. This could be tested in the future by examining the expression of other activation markers at various times after UCB stimulation in the presence or absence of IL-12.

IL-4 strongly suppressed UCB Foxp3 expression. Neonatal mice have Th2-skewed immune responses, characterized by the production of Th2 cytokines upon immune stimulation (275, 276) and an intrinsic lack of Th1 cytokine production (277). It is less clear whether human neonates are also Th2-prone (278). However, several studies suggest human neonatal T cells may epigenetically favor Th2 cytokine production over Th1 (279, 280). Furthermore, Th1 responses are dampened in human neonates during stimulation by allogeneic T cells (281), which may lead to a relative increase in IL-4.

Babies encounter numerous microorganisms during their first few months of life, some of which are pathogenic. Therefore, they may require this relative increase in IL-4 to mount protective immune responses by shutting down the strong fetal tendency to induce Tregs.

To test whether IL-4 plays a major role in inhibiting Treg generation after birth, the effect of an anti-IL-4 neutralizing antibody on Treg generation from infant PBMCs could be tested. Neutralizing anti-IL-4 antibodies could also be used in neonatal mice, to test whether IL-4 inhibits Treg generation *in vivo*. If IL-4 plays a role in inhibiting Treg generation in infants, then we would expect that anti-IL-4 treatment would lead to increased Treg generation and enhanced immune tolerance in young mice.

Differential Regulation of Smad2 and Smad3 by IL-4.

Our data show IL-4 differentially regulates Smad2 and Smad3 at the transcript level. IL-4 can block Treg differentiation via direct binding of Stat6 or GATA3 to the Foxp3 locus (255, 258, 259, 264). PU.1, a transcription factor transiently expressed during Th2 differentiation, also inhibits Foxp3 expression and Treg differentiation (255). Therefore, IL-4 uses multiple mechanisms to inhibit Foxp3 expression. My data suggest that IL-4 has an additional layer of regulation on the Foxp3 locus through upstream modulation of the TGF-β signaling components Smad2 and Smad3 (Fig. 33). IL-4 increased Smad2 and decreased Smad3 and this correlated with decreased Treg generation.

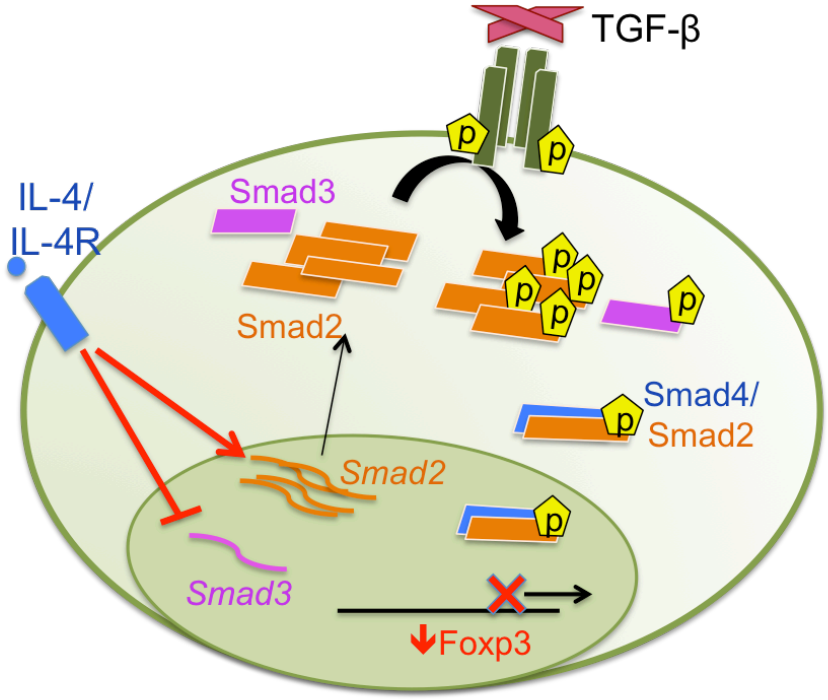


Figure 33. IL-4 regulation of the TGF-β signaling pathway. TGF-β binding to its receptor leads to the phosphorylation of Smad2 and Smad3. Phosphorylated Smad2 and Smad3 then dimerize with the co-Smad, Smad4, and translocate to the nucleus to regulate target genes, including Foxp3. IL-4 leads to an increase in Smad2, but a decrease in Smad3 transcripts and protein. This relative increase in Smad2 compared to Smad3 correlates with decreased Foxp3 expression and impaired Treg differentiation.

We are continuing to investigate whether the differential regulation of Smad2 and Smad3 by IL-4 contributes to the loss of Tregs. siRNA knockdown of Smad3 resulted in mild inhibition of Tregs, suggesting that IL-4 downregulation of Smad3 may contribute to IL-4 inhibition of Foxp3 expression and Treg differentiation. However, IL-4 also increased Smad2, a second mediator of TGF-β signaling. Like Smad3, our data suggests Smad2 is required for optimal Treg generation. Furthermore, previous reports suggest Smad2 and Smad3 are partially, but not completely, redundant. Therefore, it is possible that the increase in Smad2 by IL-4 compensates for the loss of Smad3. Alternatively, increased Smad2 may contribute to the loss of Tregs because dimerization with the co-

Smad, Smad4, is required for nuclear localization of both Smad2 and Smad3. Increased levels of Smad2 in IL-4 treated cells could outcompete Smad3 for Smad4 binding, effectively inhibiting Smad3 nuclear localization. These possibilities can be tested in the future using Smad2 overexpression in primary T cells or a T cell line.

The question remains of how IL-4 differentially regulates *Smad2* and *Smad3* transcripts. IL-4 signaling leads to the activation of Stat6, which has multiple target genes in T cells including other transcription factors such as Gata3 and Batf (282). Stat6 itself may directly bind to the Smad2 and Smad3 locus to differentially regulate their transcription. However, Stat6 is generally thought to be a transcriptional activator and would be less likely to repress Smad3. Furthermore, Smad2 upregulation peaks two days after stimulation in the presence of IL-4, whereas Smad3 is maximally downregulated three days post-stimulation. These results are consistent with different mechanisms regulating Smad2 and Smad3 downstream of IL-4. Stat6 target genes may contribute to Smad2 and/or Smad3 regulation. For example, Gata3 has known activity as a transcriptional repressor (259, 283), and may be responsible for downregulation of Smad3.

Apart from transcriptional regulation, *Smad3* transcripts may also be decreased by IL-4 through increased production of Smad3-targeting micro-RNAs. Smad2 and Smad3 have highly similar protein sequences and share regulation by some micro-RNAs, such as miR-136 (284). However, not all micro-RNAs equally regulate the two targets. For example, miR-133 decreases Smad3, but not Smad2 (285). IL-4 may decrease Smad3 transcripts by upregulating Smad3-targeting micro-RNAs. One micro-RNA of interest is

miR-145. Smad3 is a known target of miR-145, and this micro-RNA is upregulated by IL-4 in microglia (286–289). Future studies are needed to determine whether IL-4 exerts transcriptional or post-transcriptional regulation on Smad2 and Smad3 and which transcription factors or micro-RNAs are involved.

The purpose of differential Smad2 and Smad3 regulation by IL-4 remains a question. TGF-β contributes to the differentiation of several Th subsets, including Th17, Th9, Tr1, and potentially T follicular helper (Tfh) cells (215–220). Several studies demonstrate Th17 induction in mice requires Smad2 (270, 271), whereas the role of Smad3 remains controversial (269, 290). Both IL-4 and TGF-β contribute to Th9 cell generation in mice and humans (219, 291, 292). Considering the differential regulation of Smad2 and Smad3 by IL-4, I predict that human Th9 differentiation relies on Smad2, but not Smad3. Overall, IL-4 may block Treg differentiation, while still allowing the induction of other TGF-β dependent subsets.

TGF-β has many functions in addition to inducing Tregs. It instructs embryologic axis formation, inhibits cell proliferation, stimulates fibrosis, and induces epithelial to mesenchymal transition (EMT). During tumor development, tumors can become insensitive to TGF-β-mediated growth arrest (293). As the tumor progresses, it can produce TGF-β, promoting tumor metastasis through EMT and immunosuppression (293). Studies suggest TGF-β-mediated growth arrest predominantly requires signaling through Smad3 compared to Smad2 (294–296). Smad3 is also required for EMT from murine hepatocytes, while Smad2 knockout spontaneously triggers EMT (294). The IL4 receptor is expressed by a variety of hematologic and solid tumors(297–300). Therefore,

IL-4 upregulation of Smad2 and downregulation of Smad3 may modulate tumor responses to TGF-β.

IL-4 protects tumors from apoptosis, enhances tumor metastasis and promotes cancer stem cell survival (301, 302). Tumors genetically modified to overexpress IL-4 undergo more rejection compared to tumors not expressing IL-4 (301, 303, 304). However, in mouse models of lymphoma and lung carcinoma, tumor growth was impaired in IL-4 knockout mice compared to wild-type, suggesting IL-4 enhances tumor development (305, 306). Consistent with the these studies, IL-4 stimulated the growth of various colon and pancreatic cancer cell lines *in vitro* and a deficiency of the IL-4 receptor protected mice from two models of colon cancer *in vivo* (307, 308). Furthermore, the IL-4 receptor is commonly overexpressed on tumor cells and cancer patient PBMCs have increased Th2 compared to Th1 cytokine production (301, 302, 309, 310). An intriguing question is whether IL-4 acts as a tumor growth factor by rendering cells insensitive to TGF-β. My data suggests this is a possibility, since IL-4 decreases Smad3, which reportedly plays a dominant role in TGF-β mediated growth arrest.

Loss of Treg Generation in Adult Blood.

We show that neonates maintain much of the tendency to induce Treg generation during the first three to six months of life. However, anti-CD3 and IL-2 stimulation of adult blood is not effective at generating Tregs. We reasoned that this deficiency in adults could be due to a lack of monocytes or of the monocyte-derived signals needed to promote Treg differentiation. Indeed, others have suggested UCB APCs are better at inducing Treg differentiation than adult APCs (90). However, my results showed adult

monocytes resemble UCB in their frequency and their ability to produce TGF-β, retinoic acid and Notch ligands. Furthermore, previous studies in the lab showed adult monocytes are capable of inducing Tregs from purified naïve T cells (data not shown). Therefore, we have no evidence that the lack of Treg generation in adults is due to intrinsic differences in their naïve T cells or monocytes.

Adult blood has a higher frequency of memory T cells than UCB. Memory T cells potently proliferate in response to antigen stimulation. In adult blood, these cells may overtake the cultures in the conditions we use. Moreover, memory T cells could produce factors that are inhibitory to Treg differentiation or expansion. Indeed, our data suggest adult blood produces inhibitory factors that reduce the efficacy of Treg differentiation from UCB. The identity of these factors and their cellular source are under ongoing investigation in our lab. Cells of interest include IL-4 producing eosinophils, basophils, innate lymphoid cells, and memory T cells. To test the role these populations in inhibiting Treg differentiation, we will examine whether their depletion restores adult blood Treg generation. Conversely, we will test whether the same cells inhibit Treg generation from UCB.

Our studies found IL-12 and IL-4 effectively inhibit CD25 and Foxp3 expression respectively, but it is unclear whether these are the factors produced by adult cells that inhibit UCB Treg generation. Multiple approaches can be taken to address this question. First, if adult IL-4 and/or IL-12 contribute to UCB Treg inhibition, then we would expect the levels of these cytokines to be higher in the media from stimulated adult PBMCs than from UCB. This could be tested by ELISA or by multiplex cytokine analysis. Second, if

IL-4 and IL-12 contribute to adult inhibition of UCB Tregs, then neutralizing IL-4 and IL-12 should enhance UCB Treg generation when co-cultured with adult PBMCs. Finally, anti-IL-4 and anti-IL-12 antibodies could be administered to adult mice to determine whether peripheral Treg generation is enhanced by blocking these cytokines.

CHAPTER FIVE

FINAL DISCUSSION

Monocyte-Induced Treg Generation

Together, my data leads us to a model in which $CD14^{+}CD36^{hi}$ monocytes induce Tregs by producing three factors: TGF-β, retinoic acid and Notch ligands (Fig. 34). No other UCB cell observed could simultaneously provide all three signals to T cells. The roles of each signal were discussed in detail above. To summarize, Smad2/3, the Notch ICD, and RAR all promote Foxp3 transcription through binding to the Foxp3 locus. Some cross talk between these pathways may also occur. For example, Smad3 was previously shown to forma a complex with the Notch ICD and Notch signaling was required for Smad binding to the Foxp3 promoter (47, 200, 201).

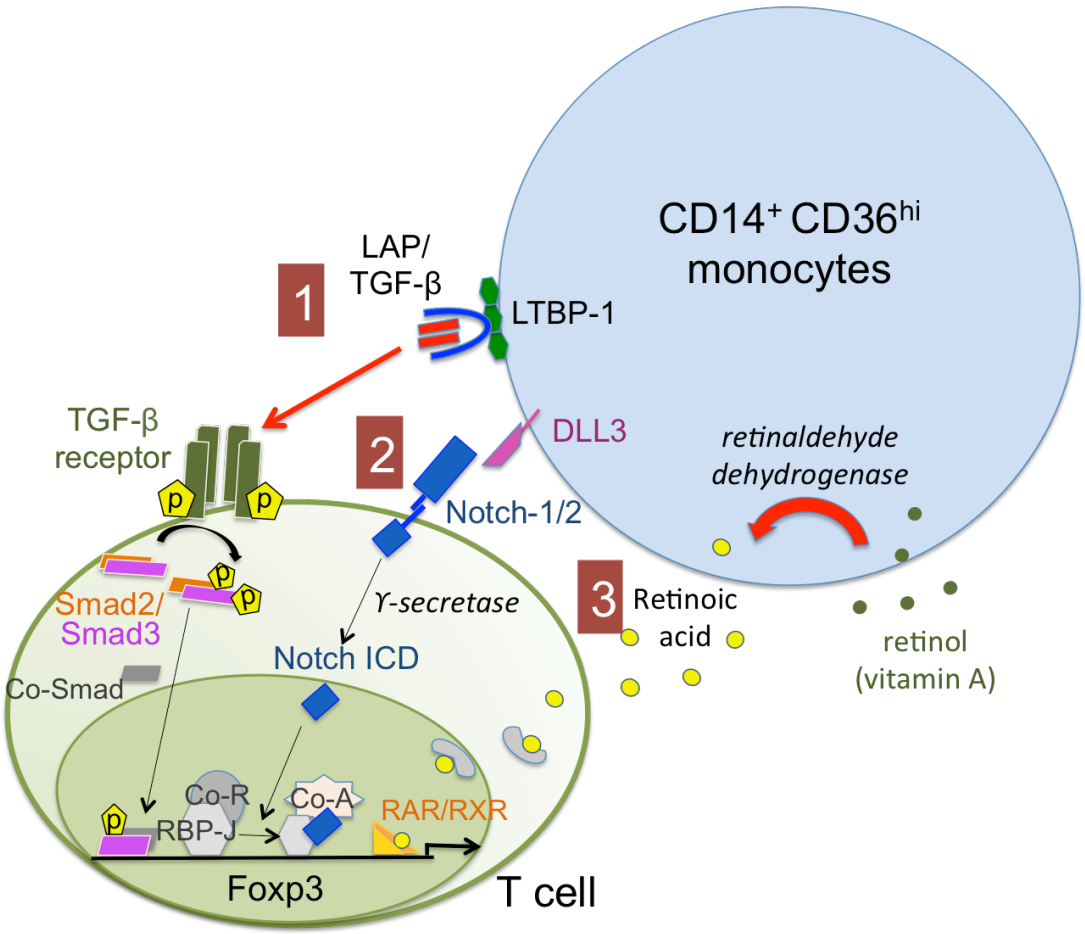


Figure 34. Model of monocyte-induced Treg differentiation. $CD14^{+}$ $CD36^{hi}$ monocytes provide at least 3 signals to T cells to promote Treg differentiation. **1)** $CD14^{+}$ $CD36^{hi}$ monocytes express surface latent TGF-β in a complex with LTBP-1 and induce TGF-β signaling in T cells, leading to Smad2 and Smad3 phosphorylation, nuclear translocation and regulation of Foxp3 transcription. **2)** $CD14^{+}$ $CD36^{hi}$ cells express the Notch ligand, DLL3. Activation of the Notch receptor leads to its cleavage by Υ-secretase and release of the Notch ICD. After nuclear translocation, the Notch ICD binds RBP-J at the Foxp3 promoter, displacing the co-repressor complex (Co-R) and allowing for recruitment of a co-activator complex (Co-A). **3)** $CD14^{+}$ $CD36^{hi}$ monocytes convert retinol into retinoic acid through retinaldehyde dehydrogenase. Retinoic acid binds the RAR/retinoid x receptor (RXR) heterodimer in the nucleus, resulting in regulation of target genes, including Foxp3.

Treg Generation During Early Development

Our studies and others have shown that the propensity for Treg generation changes throughout the lifespan. A healthy immune system relies on the balance between immune tolerance and the generation of protective immune responses against pathogens. The relative need for suppressive verses inflammatory immune responses likely changes throughout development, depending on the most abundant new antigens encountered (Fig. 35).

In the womb, the developing fetal immune system primarily encounters self-antigens and maternal antigens. The pre-term fetus has an increased frequency of Tregs and although the percentage drops in the full-term fetus (70, 71), the tendency of naïve T cells to differentiate into Tregs upon antigen stimulation remains high. This immunoregulatory program is likely important for generating life-long self-tolerance and for preventing inflammation against maternal antigens during the fetal period.

The strong tendency of the fetus to generate immune tolerance poses little risk to the baby, since few pathogens cross the placenta and fetal infection is infrequent. However, when fetal infection does occur, the results can be detrimental since the fetus has little ability to mount a protective immune response. This is clearly seen in the recent Zika virus outbreaks in South America, where maternal infection can result in transfer to the fetus and viral invasion of the fetal central nervous system (311–313).

At birth, the baby's immune system likely encounters the largest onslaught of new antigens that the individual will ever experience in his or her lifetime. This flood of antigens comes from many sources, most of which are harmless: food, pollen, pet dander

and commensal bacteria to name a few. The tendency to generate Tregs in the newborn is likely important in preventing overwhelming inflammation in the context of massive immune stimulation. However, it also leaves the infant in a precarious situation. The baby is no longer in the protective environment of the womb, and now encounters a variety of potentially harmful pathogens. Indeed, infections are a leading cause of death in infants worldwide (254).

After babies have already generated tolerance to the most common harmless antigens in their environment, it becomes advantageous to increase their ability to mount protective immune responses against pathogens. To do this, infants require a mechanism to decrease the immunoregulatory tendency of the fetus and newborn. It is generally accepted that infants have Th2-skewed immune responses, compared to Th1. In mice, stimulating neonatal naïve T cells results in robust Th2 cytokine production, including IL-4 (276). Our data demonstrate that UCB Treg differentiation is resistant to inhibition by a variety of inflammatory cytokines. However, UCB Foxp3 expression is substantially decreased by IL-4. Therefore, we propose that one purpose for Th2 dominance in infants may be to inhibit the Treg dominant program of the fetus and newborn.

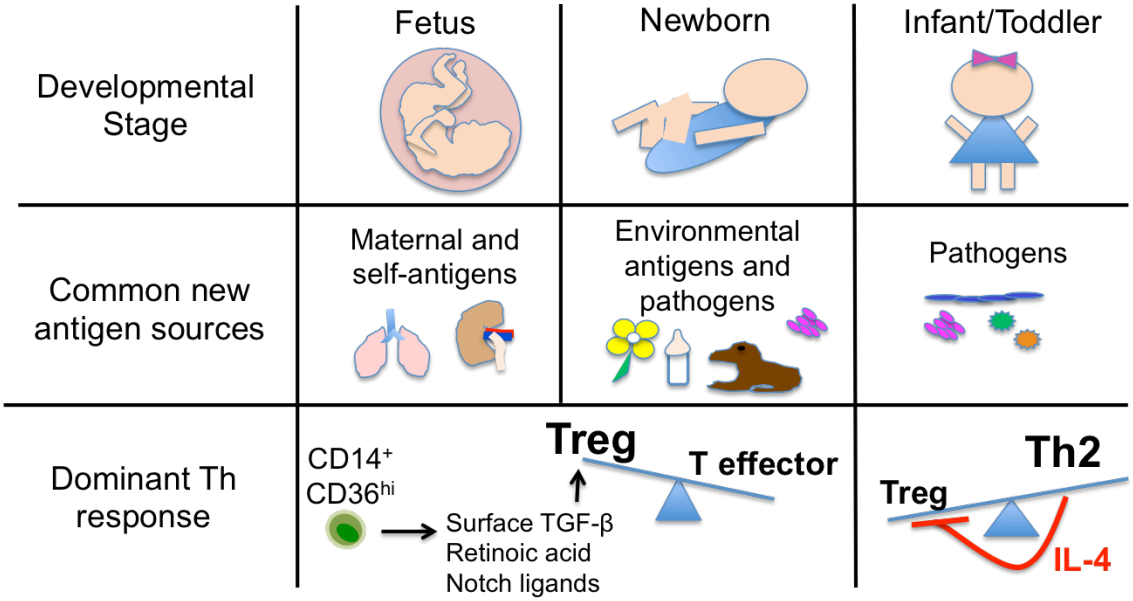


Figure 35. Regulation of T cell responses across early development. In the womb, fetuses primarily encounter maternal and self-antigens**.** At birth, newborns encounter many new antigens including those from food, pollen, pet dander and microorganisms (both commensal and pathogenic). Upon antigen exposure, fetal and newborn T cells predominantly differentiate into Tregs in a manner dependent on signals from $CD14^{+}CD36^{hi}$ monocytes. As a baby grows, its immune system has already encountered the most prevalent self and environmental antigens, but the baby still encounters new pathogens. Strong Th2 responses at this age lead to a relative increase in IL-4, which potently inhibits Foxp3 expression and Treg differentiation.

Monocytes and Vascular Immune Regulation

Since the discovery of monocytes in the early 1900s, they have been recognized as sentinels of the blood, playing an important role in protecting against extracellular pathogens and initiating inflammatory immune responses. More recently, monocytes have been shown to maintain vessel homeostasis by patrolling the blood vessel walls, clearing dead cells and debris and recruiting inflammatory immune cells during infection or injury (117, 120). While the roles of monocytes have been extensively studied during inflammation, infection, and tissue repair, their immunoregulatory properties have been largely unrecognized.

Although adults are not as likely as fetuses or neonates to generate tolerance upon antigenic stimulation, they maintain this capacity in certain microenvironments. The gut is a well-recognized tolerogenic niche that prevents allergy and inflammation to food antigens and commensal bacteria (314). A major contributor to oral tolerance is $CD103^+$ DCs in the small intestine lamina propria and mesenteric lymph nodes, which produce retinoic acid and TGF-β to induce Tregs (43, 44). Inflammatory bowel disease patients have decreased $CD103^+$ DCs and $ALDH^+$ cells in inflamed tissues compared to unaffected areas, highlighting the role of these cells in maintaining intestinal homeostasis (315). An intriguing question is whether other tissues have analogous populations of APCs that prevent aberrant inflammation.

Our data show that circulating $CD14^+CD36^{hi}$ monocytes from UCB and adults produce TGF-β and retinoic acid and promote Treg differentiation, similar to $CD103^+$ DCs in the gut. These data suggest monocytes may be an important immunoregulatory cell in the vasculature. Although blood vessels are often viewed as a transportation system for immune cells and nutrients, immune responses within the vasculature itself must also be regulated.

Atherosclerosis is increasingly recognized as an inflammatory and autoimmune state, rather than just a metabolic disease (211). LDL deposits in vessel walls activate endothelial cells at sites of turbulent blood flow, leading to increased adhesion molecule expression and chemokine production (316). Platelets recruited to these sites enhance the migration of other blood cells into the vessel wall, including macrophages and T cells. Mice deficient in the ApoE gene develop spontaneous atherosclerosis (316, 317).

However, knockout of the IFN-γ receptor along with ApoE significantly protected mice from disease (318). This was recapitulated in male IFN-$\gamma^{-/-}$ApoE$^{-/-}$ mice, in a gender-specific manner (319). Low-density lipoprotein receptor (Ldlr) deficient mice with a high-fat diet are commonly used as a second mouse model for atherosclerosis. In these mice, a loss of the Th1 master regulator, T-bet, also decreased disease severity (320). These studies demonstrate Th1 cells are pathogenic during atherosclerosis.

In contrast, accumulating evidence suggests Tregs mitigate atherosclerosis (212, 321–324). Oral feeding of anti-CD3 in ApoE$^{-/-}$ mice leads to CD4^{+}CD25^{+}Foxp3^{+} Treg induction, decreased Th1 and Th2 responses, and decreased atherosclerotic lesion size in the aortic sinus (324). In two other studies, Ldlr$^{-/-}$ mice were irradiated and reconstituted with ICOS$^{-/-}$, CD28 $^{-/-}$, or CD80$^{-/-}$CD86$^{-/-}$ bone marrow(321, 323). These mice have impaired Treg development and function and have greater atherosclerotic lesions. Co-transfer of CD4^{+}CD25^{+} Tregs with CD28$^{-/-}$ bone marrow reduced the lesion size, while depleting CD25 cells in ApoE$^{-/-}$ mice enhanced it(321). These data clearly demonstrate a protective role of CD4^{+} Tregs during atherosclerosis. Therefore, monocytes may protect the vasculature against atherosclerosis or other inflammatory diseases by inducing Treg differentiation.

Cholesterol and other lipids are transported in the blood as various lipoproteins that may be biochemically modified to more pathogenic forms(211). Monocytes and macrophages phagocytose ox-LDL via CD36 or SR-A, and this process leads to fatty streak formation in vessel walls. Our data show that ox-LDL also inhibits monocyte-induced CD8^{+} Treg differentiation. Therefore, pathogenic LDL species like ox-LDL may

promote atherosclerosis not only through foam-cell formation, but also by blocking the ability of $CD14^{+}CD36^{hi}$ monocytes to induce Tregs, exacerbating inflammation.

As a scavenger receptor, CD36 initiates monocyte/macrophage phagocytosis of multiple entities, including beta-amyloid, pathogens, and apoptotic cells. Uptake of these ligands can trigger an inflammatory program in monocytes. Beta-amyloid fibrils are deposited during Alzheimer's disease and stimulate inflammation in macrophages and microglia, contributing to disease(145, 253). Our lab is currently examining whether beta-amyloid also impairs monocyte-induced Treg differentiation. Overall, various CD36 ligands may have a dual role during inflammation and autoimmunity by activating an inflammatory program in phagocytes, and by impairing the ability of monocytes to induce Tregs.

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VITA

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In 2010, Jessica matriculated into medical school at Loyola University Chicago, Stritch School of Medicine. Shortly after, she joined the M.D./Ph.D. program and began her research studying neonatal regulatory T cell differentiation under Dr. Makio Iwashima. She received an Arthur J. Schmitt Dissertation Fellowship in 2014 and has presented her research at several conferences. She has published as a co-author prior to graduate school and her dissertation research is currently in review.