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Ex Vivo Induction of Human Regulatory T Cells

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EX VIVO INDUCTION OF HUMAN REGULATORY T CELLS

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To GP: I hope you're proud of my accomplishments, but more importantly the woman I have become.

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LITERATURE REVIEW

I. Introduction to Immunological Tolerance

The concept of immunological tolerance is a complex one that has evolved for the past almost seventy years. This phenomenon was introduced by R. D. Owen in 1945 after his influential observation of cattle twins. Owen noted that dizygotic cattle twins can share a placenta, and the consequent anastamoses result in chimeras in which each twin maintains blood cells from its sibling into adult life. Each adult cow is therefore tolerant of the other twin's blood cells and sustains a "red cell mosaicism" whereby the immune system of one adult cow does not mount a response to the technically foreign cells of its twin (1). At the same time, another influential and historic immunologist, Medawar, documented that "foreign skin" could not be successfully or permanently used for skin graft in humans except in the case of monozygotic twins (2). These seminal observations exposed investigators to the concept of immunological tolerance and many speculated that one could develop tolerance towards antigens to which one is exposed prior to acquiring a fully developed immune system.

The previously mentioned observations postulated about immunological tolerance, but it was Medawar in 1953 who first reported actively acquired immune tolerance in experiments using fetal mice and chicken embryos (3). In these experiments, recipient mouse embryos were injected with a cell suspension from an adult mouse of a different donor strain. Eight weeks after birth, these mice were challenged with a skin

graft from the same donor strain of mouse. A similar experimental protocol was carried out with chicken embryos. The authors demonstrated that mice and chickens are not only tolerant to an original inoculum of foreign donor cells to which they are exposed in fetal life, but maintain this tolerance in adult life as demonstrated by tolerance of a skin graft of the same antigenic constitution. This actively acquired tolerance is notably antigen specific because mice and chickens retain the ability to reject a skin graft to which they have not been tolerized. These findings suggested that immune tolerance could be actively induced in an antigen specific manner after fetal exposure to a particular antigen and that this tolerance could be maintained in adult life. Medawar was awarded the Nobel Prize in Medicine in 1960 as a result of these findings. Medawar and his colleagues would go on to identify interleukin-2 (IL-2) and vitamin A acetate as critical to immunological tolerance long before a mechanism was revealed (4, 5) and his work would contribute significantly to the concept of immunological tolerance. The role of IL-2 and vitamin A in the immune system would be investigated in depth in years to come; however, at the time, the mediators and mechanisms of tolerance and rejection remained elusive.

The first observations of immune tolerance were not coincidentally made in the context of maternal-fetal tolerance. Indeed, the fetal and placental tissue is partially immunologically foreign to the mother, and therefore, the lack of an immune response against such tissue is an interesting immunological phenomenon of tolerance and can be considered a successful, though temporary, "transplant (6, 7)." There are numerous immunological mechanisms in place to maintain maternal-fetal tolerance such as expression of the non-classical HLA molecule HLA-G, and secretion of

immunomodulatory human chorionic gonadotropin (hCG), as well as many other mechanisms that directly promote a tolerogenic environment. For example, HLA-G expressing cells have been shown to selectively promote tolerance inducing regulatory T cell (Treg) differentiation (8-11) and hCG produced by placental tissue is chemoattractant to Tregs (12). It follows that breakdown of the immunological phenomena contributing to maternal-fetal tolerance have been shown to be responsible for conditions such as immune hemolytic disease of the newborn, preeclampsia and spontaneous abortion (13-16). Additionally, many mechanisms that promote maternal-fetal tolerance have also been shown to be beneficial in inducing tolerance in a transplant setting (6, 8, 11, 17). These critical clinical correlations highlight the importance of understanding the principles of immunological tolerance through research. Maternal-fetal tolerance is a fascinating phenomena and the tolerogenic nature of the placenta and umbilical cord blood provides the foundation out of which our research model developed.

II. The Adaptive Immune System: Focus on Thymocytes

The human immune system consists of two distinct branches: innate and adaptive. The innate immune system provides a first line of defense against an antigen responding very quickly and in a non-specific manner. Activation of the adaptive immune system occurs as a consequence of innate immune system mediators such as antigen presentation and cytokine production and release. The adaptive immune system, composed of highly specialized cells, then mounts a specific response against an antigen. An adaptive response takes longer to generate, but results in long lasting, specific and important memory against a particular immune stimulus. The main constituents of the adaptive immune system are T and B cells. B cells are critical in the immune system, functioning

as antigen presenting cells and producing antibody to facilitate the humoral immune response. Thymocytes, or T cells, mature in the thymus from bone marrow haematopoietic progenitors and undergo a complex process of positive and negative selection before emigrating from the thymus. Upon maturation, naïve T cells leave the thymus and home to secondary lymphoid organs, including the spleen, lymph nodes and mucosal associated lymphoid tissue. In the periphery, T cells constantly survey for foreign antigen and upon antigen recognition, function as critical mediators of cell mediated immunity. T cells can be further divided into two primary subsets: CD8+ cytotoxic T cells, responsible for the specific killing of damaged or infected cells, and helper CD4+ T cells, which will be the focus of this document.

Helper CD4+ T cells are essential players in orchestrating the adaptive immune response by directing other immune cells to execute their function and coordinating a complex and specific response to a pathogen. Activation of a CD4+ T cell occurs through engagement of the T cell receptor (TCR) and antigen presented in the context of an MHC class II molecule by an antigen presenting cell (APC). When a T cell encounters an APC and recognizes the antigen which it expresses, the TCR complex binds to the antigen/MHC complex. The CD4 molecule also participates in this interaction by binding a different portion of the MHC molecule. This interaction initiates T cell activation and is known as Signal 1. Signal 2 occurs via engagement on co-stimulatory molecule CD28 on the T cell surface and CD80 or CD86 on the APC cell surface. The nature of these interactions, including the type of APC, engagement of co-stimulatory molecules, and the cytokine milieu in which the cellular interaction occurs direct a naïve CD4+ T cell to differentiate into a specific CD4+ subset, each with distinct phenotypic markers and

effector function (18). The cytokine milieu is primarily determined by the APC, which is stimulated to secrete a variety of cytokines according to the antigen which it has encountered and against which it has mounted a response. The coordination of T cell activation is complex and results in mature T cells subsets functionally specialized to react to the original antigen.

Today, four primary CD4+ T cell populations are recognized. The first CD4+ T cell subset is Th1, which are functionally specialized to eliminate intracellular pathogens, and primarily secrete effector cytokines interferon-γ (IFN-γ) and interleukin-2 (IL-2) (18). One function of IFN-γ is to activate APCs such as macrophages, enhancing phagocytosis and antigen presentation and resulting in a positive feedback loop of APC activation and T cell stimulation (19). From a naïve CD4+ T cell, TCR stimulation and activation in the presence of IL-12 and IFN-γ promote differentiation to the Th1 subset. Transcription factors are also critical for T cell programming, and one principal transcription factor expressed by Th1 cells is T-bet (18, 20). Transcription factors such as T-bet are important in controlling the up- and down-regulation of subset specific gene expression.

Naïve CD4+ T cells are directed to differentiate into Th2 cells after TCR stimulation in the presence of IL-4 and IL-2 (18). Th2 cells respond to extracellular pathogens and play a critical role in the activation of B cells, production of antibody and promotion of humoral immunity. The Th2 subset also contributes to the pathogenesis of asthma through secretion of cytokines including, but not limited to, IL-4 and IL-5 (18, 19). The principal Th2 cell specific transcription factor is GATA-3 (18, 20).

The Th17 subset is controlled by the transcription factor ROR-γt and primarily secretes the effector cytokines IL-17A, IL-17F, IL-21 and IL-22 (18, 20) which promote Th17 mediated clearance of extracellular bacteria and fungi, as well as contribute to the development of autoimmune disease (18). IL-6, IL-21, IL-23 and transforming growth factor-β (TGF-β), primarily secreted by the interacting APC, direct the differentiation of Th17 CD4+ T cells.

Similar to Th17 differentiation, TGF-β also promotes the differentiation of the last primary subset of CD4+ T cells, regulatory T cells (Tregs). While plasticity has been reported between Th17 and inducible Treg subsets, their differentiation program and effector functions are antagonistic and mutually exclusive, in part through reciprocal negative regulation of the other subset by Th17 and Treg specific transcriptional regulators: ROR-γt and Foxp3, respectively (20, 21). Tregs themselves are quite diverse and are the focus of the work discussed herein. The differentiation and effector function of Tregs will be addressed in greater detail in the subsequent section.

Undoubtedly, CD4+ T cells are a functionally diverse class of adaptive immune mediators. Our knowledge of this class of cells is constantly evolving and the diversity even within Th1, Th2, Th17 and Treg subsets is becoming more apparent. It is important to note that the nature of the interaction between the APC and naïve CD4+ T cell, and the cytokine microenvironment in which that interaction occurs is critical in directing CD4+ T cell differentiation to each functionally specific subset.

III. The Last Subset: Suppressor to Regulator

The final subset of CD4+ T cells is known today as Tregs. As a cell population, Tregs are critical in the human immune system in the maintenance of self-tolerance and

immune homeostasis. This cell population is focus of this document and the research contained within. Though more detailed knowledge of Treg differentiation and effector function was forthcoming, their identification began with further examination of the aforementioned concept of immunological tolerance.

In an effort to uncover the mediators of immunological tolerance, Gershon executed an elegant experiment in mice consisting of thymectomy and lethal irradiation followed by bone marrow reconstitution with or without the simultaneous reconstitution of thymocytes. After evaluating response to antigenic challenge, the authors noted that a population of thymus derived lymphocytes induced tolerance in both other thymus derived lymphocytes as well as bone marrow derived lymphocytes (22). In future publications, Gershon *et al* observed that the suppressive thymocyte population was sufficient for suppression of the antigen response of other thymocytes (23), that the suppressive activity of these cells depends on the cell population which they are regulating (24) and that certain *in vitro* conditions favored the induction of suppressor T cells (25). Other groups also noted that thymocytes are capable of exerting a suppressive effect on immune responses, including Tada *et al.* who further promoted the field of suppressor T cells by specifically evaluating their influence on suppression of the B cell mediated antibody response, among other studies (26-31). Complicated experiments were conducted in which different populations of T and B cells were mixed together and the antibody response to a particular antigen measured, with a decrease in antibody response as evidence of suppressive activity. Numerous groups would go on to identify that depletion of a “suppressive” or autoimmune protective thymocyte subset promotes the development of autoimmune disease (32, 33). Likewise, investigators demonstrated

that adoptive transfer of a pathogenic CD4+ thymocyte subset induces autoimmune disease such as "wasting disease (34)." These findings suggested, and it was later shown, that not only does a pathogenic population of CD4+ T cells exist, but also that the activation of such a pathogenic CD4+ subset could be controlled or "suppressed" by an additional and distinct CD4+ T cell subset (35, 36). While the concept of suppressor T cells was controversial and heavily disputed among immunologists, research continued and identifying and differentiating these pathogenic and protective CD4+ T cell subsets became the goal of many investigators.

Despite widespread controversy, suppressor T cell studies had catalyzed research in immunological tolerance and the cell population responsible for maintenance of self-tolerance and immune homeostasis. After the discovery of actively acquired immune tolerance, investigators sought to distinguish between pathogenic and suppressive or autoimmune-preventive CD4+ T cells subsets. As previously mentioned, investigators had demonstrated a population of pathogenic CD4+ T cells, as well as the ability to control or suppress the activation of such a pathogenic CD4+ subset by an additional and distinct suppressive CD4+ T cell subset (35, 36). Additionally, depletion of suppressor T cells was shown to promote the development of autoimmune disease and anti-tumor immunity (37). The existence of a suppressive immune cell subset had been demonstrated in innumerable healthy and disease states in both mice and human research. Investigators unsuccessfully sought to understand the differentiation and function of such suppressor T cells; however, distinguishing features or markers of the cell subset remained elusive and the immunological field of suppressor T cells endured harsh criticism. "The concept of

antigen-specific suppression and suppressor factors simply became too complicated and was dismissed as artifact (38)."

It wasn't until 1995 when Sakaguchi revisited this concept and introduced regulatory T cells with the identification of CD25, the IL-2 receptor alpha chain, as a marker of the suppressive T cell subset (39). Sakaguchi removed CD4+ CD25+ cells from otherwise healthy mice and documented the development of extensive autoimmune disease. In confirmation, adoptive transfer of murine T cell suspensions depleted of the CD25+ suppressive T cells was sufficient to elicit autoimmune disease in athymic nude mice, some progressing to severe graft-vs-host-like wasting disease. Co-transfer of CD4+ CD25+ T cells with their CD25- counterparts prevented the development of autoimmunity in a dose dependent manner. These data suggested that CD4+ CD25+ T cells are responsible for maintaining self-tolerance and preventing autoimmune disease (39) and elegantly clarified previous research. Soon after Sakaguchi's seminal observations, investigators demonstrated that not only does depletion of Tregs (CD4+ CD25+) elicit autoimmunity, but also augments immune responses to non-self antigens (40). In addition, removal or reduction of Tregs also provokes potent tumor immunity in normal mice (37). It has also been shown that CD4+ CD25+ Tregs enriched from normal mice suppress allergy, promote graft tolerance, prevent graft-vs-host disease and maintain maternal-fetal tolerance (40-44). The dysregulation of Treg number and/or function is now known to play a critical role in the pathogenesis of cancer, allergy and various human autoimmune pathologies such as multiple sclerosis, psoriasis, rheumatoid arthritis and diabetes (41). This research established the critical role of Tregs in immune homeostasis and self-tolerance and additionally demonstrated their importance in

immune response to non-self antigens, autoimmune disease, cancer, allergy and maternal-fetal tolerance. These data superseded controversy surrounding suppressor T cells and incited forward progress in research in immunological tolerance.

IV. Differentiation and Effector Function of Regulatory T Cells

In addition to CD25, other markers have been suggested to distinguish Tregs from their potentially pathogenic counterparts such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (45), but the most consistent Treg marker is expression of the transcription factor Foxp3 (20, 42, 46). Foxp3 is said to be the master regulator of Treg development and function (47-49) and expression of Foxp3 is sufficient to confer suppressive activity to non-Treg CD4+ T cells (47, 48, 50). In these experiments, Foxp3 expression was both knocked-out of CD4+CD25+Foxp3+ Tregs and knocked-in to CD4+CD25-Foxp3- T cells, and subsequently, regulatory/suppressive function was lost or acquired in correlation with the loss or gain of Foxp3 expression. The importance of Tregs and Foxp3 in the human immune system was further illustrated by investigation of the severe autoimmune pathology of IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which develops as a direct result of mutation in the Treg specific transcription factor: Foxp3 (40, 42, 43, 51). This rare but devastating disease characterized by the dysfunction of Tregs is evident as autoimmune endocrinopathies such as diabetes, nail dystrophy, and skin pathology including alopecia, dermatitis and bullous pemphigoid. IPEX illustrates the role of Tregs in the human immune system in maintaining the normal homeostasis of diverse body systems and preventing the development of autoimmune disease.

It is important to note that human T cells transiently upregulate Foxp3 after T cell receptor (TCR) stimulation and only genuine Tregs maintain stable expression of the transcription factor (52). Investigators have shown that the difference between transient and stable Foxp3 expression may lie in the methylation status of the *Foxp3* locus, where stable Foxp3 expression is mediated by methylation of CpG-rich regions of the gene locus (53). The Foxp3 regulatory network is complex and wide reaching, including molecules such as transforming growth factor-β (TGF-β) and GARP (53), which will be discussed in a subsequent section. The factors which regulate Foxp3 expression and the transcriptional regulation which Foxp3 exerts itself remain to be elucidated and are the subject of much research. The complex Foxp3 regulatory network suggests the intricate regulation of Treg differentiation and effector function.

Since the identification of suppressor T cells, we have learned a lot about distinguishing Treg markers and their significance in maintaining self-tolerance and immune homeostasis, but there is still much to learn about Treg differentiation. Tregs can be produced in the thymus as a functionally mature subpopulation of T cells and can also be induced from naïve CD4+ T cells in the periphery (54, 55). Tregs that develop in the thymus are termed naturally occurring Tregs (nTregs) and mature through engagement of MHC/self-peptide ligands expressed by thymic medullary epithelial cells, as regulated by autoimmune regulator (AIRE) (55, 56). AIRE is a transcription factor which intricately regulates expression of self-antigens by thymic medullary epithelial cells. The affinity of the self-peptide/MHC/TCR interaction contributes to directing the differentiation of nTregs through positive selection. T cells which bind strongly to self-antigens and could potentially be self-reactive in the periphery are eliminated by negative selection. Upon

emigration from the thymus, like their naïve non-Treg CD4+ T cell counterparts, nTregs home to secondary lymphoid organs. Once established in the periphery, nTregs maintain immune homeostasis to self-antigens and are capable of suppressing self-reactive immune responses (55, 56). As previously mentioned, Tregs can also be induced from naïve CD4+ T cells in the periphery and are then called inducible Tregs or iTregs. Though nTregs and iTregs exert similar tolerogenic function in the human immune system, they are both essential cell subsets in maintaining immune homeostasis and self-tolerance.

The IL-2 receptor alpha chain, CD25 is one marker of functional Tregs and it follows that IL-2 is essential for iTreg differentiation and survival (18, 20, 55). TCR stimulation is also required for iTreg activation and differentiation including engagement of co-stimulatory molecule CD28 with the CD80 or CD86 molecule on an APC, similar to the activation process for other helper T cell subsets. Beyond TCR stimulation and co-stimulatory molecule engagement, exposure to molecules such as retinoic acid and cytokines such as TGF-β and IL-10 has been shown to promote iTreg differentiation in different models (55, 57-59). Furthermore, different APC subsets such as tolerogenic dendritic cells (DC) and myeloid derived suppressor cells (MDSC) have been shown to promote iTreg differentiation in mouse and rhesus macaque models (57, 58, 60-73). Literature about tolerogenic DCs and MDSCs cites the important role of IL-10 or TGF-β in promoting Treg differentiation, while other groups document the importance of expression of indoleamine 2,3-dioxygenase (IDO) (74) or thymic stromal lymphopoietin (TSLP) (75, 76). Regardless of the type of APC; TCR stimulation, co-stimulatory

molecule engagement and the cytokine milieu in which this interaction occurs are essential principles of human iTreg differentiation which are still under investigation.

While some groups have demonstrated the generation of human Tregs by methods such as stimulation with antibody coated plates (77), culture with a murine stromal cell line (78) or generation of Treg cell lines (79), it is clear that human iTreg differentiation is different from murine iTreg differentiation and that the principles learned in murine models cannot all be directly applied to a human system. One important example of this is that addition of soluble exogenous TGF-β to cultures of human naïve CD4+ T cells does not generate functional iTregs, where it does in murine cultures (80). Therefore, it is necessary to further examine human iTreg differentiation. Human umbilical cord blood (UCB) has been shown to be a source of iTregs and fetal CD4+ T cells have a strong tendency to differentiate into Tregs that actively promote self tolerance as well as maintain maternal-fetal tolerance (81-83). While human UCB is an inherently tolerogenic environment, the cellular interactions and molecular mechanisms of human Treg differentiation from both human adult peripheral blood and UCB remain elusive. Human UCB will be utilized in the work discussed herein and determining the differentiation program of human iTregs is the focus of the work described in this document.

After differentiation, Tregs maintain self-tolerance and immune homeostasis by exerting dominant suppression over self-reactive or hyper-proliferative immune responses via suppressing the activation, proliferation and/or effector function of a variety of immune system cells (42, 84). While the exact mechanisms of Treg mediated suppression are as yet unknown, it has been shown that Tregs can suppress other immune cells in an antigen-specific and/or –nonspecific manner. One suggested mechanism of

Treg-mediated suppression is cell contact-dependent inhibition of effector CD4+ T cells via direct engagement of cell surface molecules such as CTLA-4 or release of inhibitory cytokines such as TGF-β (40, 42, 84). Tregs have also been shown to directly inactivate APCs from further stimulating an immune response, and to kill effector T cells or APCs through the release of cytotoxic substances such as perforin and granzyme A (40, 42, 84) which results in termination of the self-reactive or hyper-proliferative immune response.

Indeed, Tregs are indispensible in the human immune system and there are many Treg-directed therapies already under investigation. Some groups are attempting to deplete or inhibit Tregs in an effort to enhance anti-tumor immunity via toxin conjugated IL-2 or anti-Treg specific monoclonal antibodies (42). Conversely, to abrogate autoimmune pathologies such as multiple sclerosis, psoriasis, inflammatory bowel disease or diabetes, other investigators are seeking to promote Treg proliferation by Treg-stimulating monoclonal antibodies (42). Additionally, scientists are striving to achieve *ex vivo* expansion of Tregs for patient transfusion to treat graft-vs-host disease or promote transplant tolerance (42). In mouse models, *ex vivo* expansion and re-infusion of Tregs has been shown to treat diabetes (85) and similar protocols are beginning to be tested in humans (86-88). While many avenues are being investigated to manipulate Tregs in the treatment of human disease, to date, scientists have been challenged by developing efficacious therapies. Increasing our knowledge of human Treg differentiation and development will be important in advancing our ability to treat numerous debilitating and sometimes fatal human diseases.

V. The Monocyte: A Specialized Antigen Presenting Cell

An antigen presenting cell (APC) is an immune cell that can process and present antigen in the context of a major histocompatibility complex (MHC) molecule in order to stimulate other cells of the immune system, typically T cells, to generate an adaptive immune response. Professional APCs are one class of APCs which are functionally specialized to take up, via phagocytosis or receptor mediated endocytosis, process and present antigen in the context of MHC. Professional APCs constitutively express MHC and co-stimulatory molecules and include cell subsets such as DCs, B cells and macrophages. Recently, monocytes have also been recognized as professional APCs and their role in human immunology has been further investigated.

Monocytes are circulating mononuclear cells, capable of phagocytosis and differentiation to macrophage or DC lineages after stimulation. It is largely accepted that after stimulation and extravasation from the bloodstream, a monocyte begins the differentiation program into macrophage or DC lineages and is no longer a monocyte, but a "monocyte-derived" cell (89, 90). Because monocytes are circulating cells, antigen encountered by a monocyte prior to extravasation can be retained, transported and presented in other organ systems (89, 91). Some groups have also suggested that circulating monocytes may transport tissue-restricted antigens to the thymus for display during T cell maturation (89, 92, 93). This tissue-restricted antigen expression in the thymus may promote differentiation of Tregs tolerant to such self-antigens which would not be expressed by traditional AIRE mediated thymic medullary epithelial cells.

While human monocytes are a heterogenous population of cells, the TLR-4 accessory molecule CD14 is expressed on most monocyte subsets. CD14 is a

glycosylphosphatidylinositol (GPI)-anchored protein expressed on the cell surface of monocytes and other APCs such as macrophages, DCs and neutrophils (94-96). CD14 enhances the responsiveness of APCs to lipopolysaccharide (LPS) by participating in LPS binding to TLR-4 (94-97). Indeed, investigators have suggested that the level of CD14 expression determines the ability of the cell to respond to LPS (98). Some reports have additionally shown that CD14 can respond to LPS without engagement of TLR-4 (95). CD14 can also interact with TLR-2 and can be shed as a soluble form: sCD14 (94, 97).

CD36 is another cell surface molecule expressed by monocytes and other cell subsets such as platelets, erythrocytes, adipocytes and some endothelial cells. CD36 is a member of the class B scavenger receptor family and also a TLR accessory molecule which can form oxidized lipid ligand induced heterodimers with TLR-2, TLR-4 and TLR-6 and promote sterile inflammation (94, 96, 97, 99-101). Reports show that CD36 is a signaling molecule itself (102) and has functions other than aiding in TLR recognition of oxidized lipid ligands. CD36 expression is tightly regulated during monocyte to macrophage differentiation and may play a role in further promoting foam cell formation (103); however, the mechanism of regulation is not known. Because of its ability to bind oxidized lipids, CD36 has a purported role in atherosclerosis and cardiovascular disease (100). It has additionally been shown to mediate internalization of erythrocytes parasitized by *Plasmodium falciparum*, the causative agent of malaria (104). The functions of CD36 are diverse, and CD36 can bind additional ligands including components of the extracellular matrix like collagen, and thrombospondin-1 (TSP-1) (103, 105).

As mentioned previously, human monocytes are a heterogenous population of cells. They can be divided into three main subsets based on expression of cell surface molecules CD14, CD16 (FcγRIII) and CD64 (FcγRI): classic, activated/mature and immunomodulatory/intermediate (91, 106-109). CD14+ CD16- monocytes are termed classic because they most closely resemble the phenotype of the originally described monocytes. Activated/mature monocytes are CD14+ CD16+ and more closely resemble tissue macrophages. Activated monocytes constitute a smaller percentage of total monocytes and are called inflammatory monocytes because they secrete more pro-inflammatory cytokines such as TNF-α than their CD16- counterparts (106, 110). CD16+ monocytes also express higher levels of CD32 (FcγRII) and MHC class II molecules and express a different chemokine-receptor profile as compared to CD16- monocytes (106). Both classic and activated monocytes differentiate into DC subsets after stimulation and similarly stimulate T cell activation and proliferation (106, 110). The third monocyte subset, immunomodulatory/intermediate, is distinguished by expression of CD14, CD16 and CD64 and has potent T cell stimulatory capability as this class expresses the highest levels of HLA-DR and co-stimulatory CD86 of any monocyte subset (111). CD14+ CD16+ CD64+ monocytes exhibit characteristics of both monocytes and DCs and have been suggested to be an intermediate phenotype between the two cell populations (106). While the *in vivo* significance of the different monocyte subsets is still under investigation, it is clear that there are distinct subsets according to expression of cell surface molecules and various functional parameters, which may influence their ability to induce CD4+ T cell subset differentiation.

Various groups have reported that the functional immaturity, or antigen naïve status, of an APC contributes to its ability to promote Treg differentiation (112, 113). Others have documented modest differences between umbilical cord blood or newborn and adult monocyte populations which may contribute to Treg differentiation (98, 114). And the ability of circulating monocytes to express tissue-restricted antigen in the thymus may promote intra-thymic differentiation of nTregs (89, 92, 93). However, the role of monocytes in induction of human Treg differentiation is unknown. Of note, one clinical study reported an increased Treg frequency in patients with acute myelogenous leukemia (AML) where there are also increased numbers of circulating immature monocytes: monoblasts (115). The function of monocytes in human Treg differentiation warrants further investigation.

VI. Transforming Growth Factor β: An Immunomodulatory Cytokine

Cytokines are known to influence the differentiation of a naïve CD4+ cell to a specific effector subset and transforming growth factor-β (TGF-β) is one cytokine shown to preferentially promote Treg differentiation in murine and human models. The TGF-β superfamily consists of TGF-β1-3, bone morphogenetic proteins (BMPs), activins and other related proteins, all of which play a role in cell development. TGF-β itself is a pleiotropic cytokine with numerous roles in both modulating the immune response and physiologic processes such as angiogenesis.

TGF-β superfamily ligands signal through a family of transmembrane serine/threonine kinases divided into two subfamilies: type I and type II TGF-β receptors (116). Upon binding to its receptor, TGF-β signals through Smad-dependent and Smad-independent pathways including Rho-like GTPase and MAP kinase pathways (116-118).

Prior to binding receptor and initiating downstream signaling, TGF-β must be activated from its latent form. TGF-β is secreted and exists in the extracellular milieu bound to a propeptide, latency associated peptide (LAP) (116, 119). The TGF-β/LAP complex may be further bound by latent TGF-β binding proteins (LTBPs) and anchored in the extracellular matrix (119). Latent TGF-β has been shown, in some circumstances, to be present on the cell surface and mediate cell function (120, 121), but neither LAP nor TGF-β are transmembrane molecules.

Human GARP (LRRC32) is a transmembrane protein composed almost entirely of leucine rich repeat (LRR) domains (122). LRR proteins are a large family of molecules known to participate in protein-protein interactions and signal transduction (123). GARP has been shown to be essential for LAP/TGF-β cell surface expression, and to be expressed in humans only on activated Tregs (124-129). GARP undergoes signal peptide cleavage prior to cell surface localization, and upon surface expression, is capable of altering expression of other T cell activation markers (130). Furthermore, GARP has been shown to mediate some of the suppressive function of Tregs and ectopic expression of GARP induces expression of the Treg specific transcription factor: Foxp3 (121, 124-129). Therefore, GARP is an activated Treg marker that is important in mediating Treg specific cell surface expression of LAP/TGF-β and suppressive function.

As mentioned previously, the dissociation of LAP from latent TGF-β is a critical regulatory step required for downstream TGF-β signaling. Thrombospondin-1 (TSP-1) is a glycoprotein with a known role in angiogenesis (131, 132), that is also capable of activating latent TGF-β via inducing a conformational rearrangement which results in the release of LAP and biologically active TGF-β (131-135). CD36 serves as a cell surface

receptor for TSP-1 and "expresses" the enzyme on CD36+ cells (131, 134, 135). Indeed, CD36 has been shown to be required for TGF-β activation in a model utilizing rat alveolar macrophages (135, 136). Additionally, among human monocytes, TSP-1 is expressed highest in those that concomitantly express the highest levels of CD36 (109).

While TSP-1 and CD36 are important in TGF-β activation, TGF-β is likewise important in promoting human Treg induction (133, 137-140). With the understanding that dysregulation of Tregs is critical in autoimmune, and other, disease pathogenesis, TGF-β is already under investigation in the treatment of various human diseases including diverse autoimmune pathology (141-143) and fibrotic conditions like sclerosis (144). Moreover, GARP was found to be expressed in the mouse placenta, a known immunotolerant and Treg inundated environment (122), which relates this potential contributor of Treg biology back to the concept of maternal-fetal tolerance and the tolerogenic nature of the placenta and UCB. These findings all point to the significance of the TGF-β/GARP and TSP-1/CD36 axis in Treg biology and immune tolerance and homeostasis.

VII. The Role of Vitamin D in Human Regulatory T Cell Biology

The immune system is subject to influence by circulating endogenous factors as well as exogenous factors, such as environmental exposure to aryl hydrocarbons in cigarette smoke. One example of an endogenous factor that is shown to influence the cells of the immune system including Tregs is Vitamin D, which can be consumed in food products or synthesized upon exposure to sunlight.

Vitamin D deficiency is a highly prevalent, but relatively easily diagnosed and treatable condition. Reports have documented a rise in vitamin D deficiency recently to

as high as 50% of the white and up to 90% of the dark-skinned population in the United States being vitamin D insufficient or deficient, defined by a serum vitamin D level less than 30ng/mL (145). While vitamin D insufficiency is easily diagnosed and treated with simple oral supplementation, most patients remain both undiagnosed and untreated (145-149).

Nonambulatory, elderly, dark-skinned, institutionalized and chronically ill individuals are among the most at risk for development of vitamin D insufficiency, and would benefit the most from oral supplementation. The beneficial effects of vitamin D supplementation on bone homeostasis and skeletal health have long been known, but the favorable influence of vitamin D on the immune system is just recently being investigated. Vitamin D supplementation has been show to have broad anti-inflammatory effects, enhance cancer treatments, attenuate autoimmune reactions such as systemic lupus erythematosis, rheumatoid arthritis and multiple sclerosis, and even to decrease the severity of graft-vs-host disease (GVHD) (150-152), but the mechanism of these immunomodulatory effects is largely unknown. The immunomodulatory influence of vitamin D was also shown on peripheral blood human T cell constituents, where elevated serum vitamin D was correlated with increased peripheral CD4+ and CD8+ T cells, increased naïve T cells and increased levels of Foxp3 expression (153). It follows then, that in another study, supplementation of vitamin D in otherwise healthy individuals was associated with a significantly increased frequency of CD4+ Tregs (154) illuminating a clinical correlation between exogenous vitamin D and immune-regulation.

Vitamin D receptors are widely expressed by immune cells and responsiveness to vitamin D is cell type specific. The affect of vitamin D is mediated by binding and

activation of the vitamin D receptor (VDR), which promotes heterodimerization with the retinoid X receptor (RXR), nuclear translocation, binding of vitamin D response elements (VDRE) in gene regulatory regions and modulation of transcription of target genes (155). Vitamin D has been shown to promote monocyte differentiation into a macrophage phenotype (156) and also to promote the development of tolerogenic, Treg inducing DCs (146). Our focus will be on the influence of vitamin D on T cell differentiation, particularly on promoting Treg induction.

Vitamin D plays a role in controlling the Th17/Treg differentiation axis, favoring Treg induction as vitamin D directly inhibits transcription of IL-17A, a primary effector cytokine of the Th17 subset, and directly induces expression of Foxp3 (157, 158). These effects of vitamin D were found to ameliorate experimental autoimmune encephalomyelitis (EAE), a mouse model of autoimmune multiple sclerosis (158, 159). Vitamin D also stimulates expression of the Treg marker CTLA-4, directly promotes Treg differentiation (160-162) and enhances Treg suppressive function (163).

The mechanism of action of vitamin D in immunomodulation is relatively unknown, but Smad3, a downstream signaling molecule of TGF-β, is capable of interacting with and serving as a co-activator of the VDR (164-166). TGF-β influences CD4+ T cell differentiation and has a positive role in promoting Treg differentiation, so the interaction of Smad3 and the VDR may potentially be a mechanism of vitamin D mediated immunomodulation.

VIII. The Influence of Alcohol on the Human Immune System: Focus on Regulatory T Cells

Ethanol (EtOH) mediated impairments of immune competency have been demonstrated in laboratory studies and clinical observations, specifically related to EtOH exposure during fetal life. For example, animal models have shown dysregulated immune responses as a result of EtOH exposure (167-171). The immunoteratogenic effects of alcohol are apparent in that lymphocyte numbers and proliferative capacity in response to stimulation are impaired in adult rats exposed to EtOH only *in utero* (172). Furthermore, *in utero* EtOH exposure has long lasting effects, as adult mice exposed to alcohol only during gestation have increased influenza-associated morbidity and mortality and pulmonary viral titers, and decreased numbers of both B cells and influenza-specific CD8 T cells in the lungs following influenza infection (173). Secondary exposure to alcohol further exacerbated these effects, which supports literature citing the general immunosuppressive effects of alcohol exposure (173). Additionally, EtOH exposure has been shown to interfere with the kinetics of Foxp3 , RORγt and T-bet gene expression, the transcription factors required for human Treg and other CD4+ T helper subset differentiation (174). Together, these data demonstrate that not only does EtOH have significant immunosuppressive and immunoteratogenic effects, but also that alcohol exposure restricted to gestation has immediate and long lasting immunosuppressive effects through adult life in animal models.

Maternal alcohol consumption has been associated with a number of adverse effects on the developing human fetus as well. These adverse effects include spontaneous abortion, growth retardation, facial abnormalities, damage to the central nervous system,

behavioral disorders, cognitive disabilities and increased susceptibility to bacterial and viral infection (126, 175-179). Fetal alcohol syndrome (FAS) refers to the combination of distinguishing facial features, reduced birth weight and behavioral and cognitive impairment characteristic of children exposed to EtOH *in utero* (178). In addition to the neuropsychological and behavioral disorders inherent in FAS, significant impairment of immune function have been identified in these patients (168, 170, 171, 173, 175-177, 180). For these reasons, in 2005, the U.S. Surgeon General warned pregnant women and women who may become pregnant to completely abstain from alcohol consumption (178). Unfortunately, however, up to 30% of women report consuming alcohol at some time during their pregnancy (175, 181, 182) and the prevalence of FAS in the United States remains between 0.2-1.5 in every 1000 live births (126). Additionally, children diagnosed with FAS incur annual mean medical expenditures nine times as high as those of children without FAS (182).

Human FAS neonates have an increased incidence of bacterial infection, including pneumonia, sepsis and meningitis, and viral infection caused by agents such as influenza and cytomegalovirus (173, 175, 176). Increasing amounts of maternal alcohol consumption during the three months prior, or at any time during pregnancy, significantly increases the risk of neonatal infection by up to 6.83 fold (175). The increase in neonatal infection observed after maternal alcohol consumption was higher than increases in infection seen with mothers who smoke cigarettes or abuse cocaine or marijuana (175). When adjusted for race and smoking, any drinking 3 months prior to conception or in the first or second trimesters of pregnancy increased the risk of newborn infection by 2.5 fold

(175). These data suggest a profound influence of isolated *in utero* exposure to alcohol on the developing fetal/neonatal immune system.

While alcohol has been shown to influence the human immune system, very little is known about the effect of alcohol, *in utero* or otherwise, on Treg biology or TGF-β. Understanding these effects could be critical to comprehending certain human pathologies including FAS and will potentially reveal novel treatment strategies for addressing the increased risk of infection linked to FAS and the immunosuppression seen in adult alcoholics.

IX. The Placenta as a Source of Increasing Human UCB Cell Yields

The use of umbilical cord blood (UCB) as a source of hematopoietic stem cells is increasing (183, 184) in the treatment of hematologic malignancies and non-malignant disorders. UCB has presented a practical and beneficial alternative to bone marrow or peripheral blood stem cell transplantation as the time to transplant and HLA matching criteria for bone marrow and peripheral blood stem cell transplantation often present challenges (183-186). Among the advantages of UCB transplantation are ease of acquisition and availability of samples, leading to decreased time to transplant, less stringent HLA matching requirements (187, 188) and decreased incidence of graft-versus-host disease (GvHD) (185-187, 189, 190). The utility of UCB transplantation is reinforced in studies that demonstrate UCB as a source of CD34+ hematopoietic stem/progenitor cells (HSC) (190, 191); however, the limited HSC number obtained from a single UCB sample presents challenges to widespread therapeutic application. While UCB CD34+ HSC numbers are sufficient for transplantation of children, cell dose is a critical determinant of successful transplantation and UCB is inadequate in this regard for

the treatment of most adults (190, 192, 193). Investigators have pursued three primary avenues to combat this challenge: *ex vivo* expansion of UCB cell types (194), infusion of more than one UCB unit per recipient (195-197), and increasing cell recovery from UCB and placenta samples (198, 199). The importance of cell dose in successful transplant highlights the need for optimization of these techniques to enhance the efficacy of UCB transplantation.

In an effort to increase cell dose for UCB transplant, many groups have turned to the human term placenta and found an additional source of HSC (198-203). The success of UCB in clinical applications suggests that, if placenta derived cells are attainable and exhibit comparable phenotypic and functional characteristics as UCB derived cells, then placenta derived mononuclear cells would increase donor cell yield and further improve transplant success.

CHAPTER 2

RESULTS

A fundamental component of the tolerogenic nature of the fetal/neonatal immune system is the dominance of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (Tregs); however, the mechanism of induction and maintenance of such tolerance are largely undetermined. To understand the induction of fetal/neonatal tolerance, we examined T cell biology in human umbilical cord blood (UCB) samples. More specifically, I sought to determine the cellular interactions and molecular mechanisms which promote the induction of tolerogenic human Tregs.

I. CD14+ $CD36^{HI}$ Monocytes Are Sufficient to Induce Treg Differentiation from Human Naïve CD4+ T Cells

A. CD14+ cells promote human Treg induction from UCB mononuclear cells

To determine a mechanism by which the fetal immune system acquires tolerance, we evaluated the T cell response in human umbilical cord blood (UCB) upon antigen receptor stimulation. Freshly isolated UCB samples did not have a high starting frequency of CD4+ Tregs. Indeed, only ~0.17% of CD4+ cells from freshly isolated UCB were CD25+Foxp3+ Tregs (Figure 1). However, initial findings demonstrated that mononuclear cells isolated from UCB exhibited increased Treg differentiation as compared to mononuclear cells isolated from adult peripheral blood. Induced Tregs from UCB retained their Foxp3 expression and Treg phenotype for up to 62 days in culture,

demonstrating that these cells are stably induced Tregs.

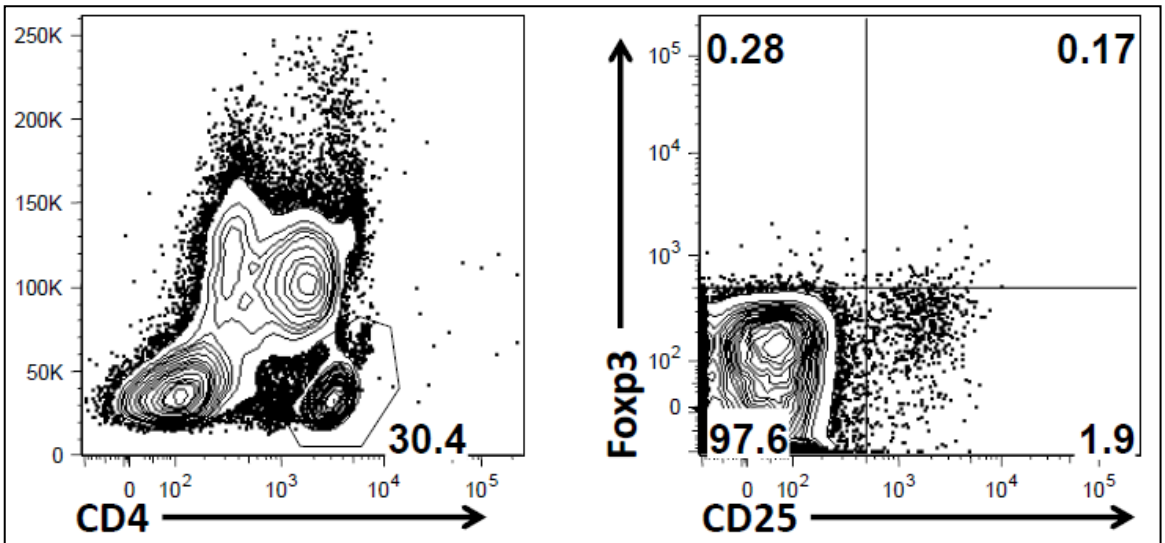


Figure 1. CD4+ Treg frequency in freshly isolated human UCB. Freshly isolated mononuclear cells were stained and analyzed by flow cytometry to determine the baseline, day 0, Treg frequency. The panel on the right shows the CD4+ population gated in the left panel among total live cells. Data are representative of 10 independent repetitions.

Previous studies in the laboratory demonstrated both an increased frequency of Treg differentiation from UCB mononuclear cells as compared to adult peripheral blood mononuclear cells, and that such Treg induction required a non-T cell population. Therefore, we sought to determine which non-T cell populations promote human Treg differentiation. After systematic depletion of various cell subsets, we found that only depletion of the CD14+ cell subset significantly reduced Treg induction from UCB mononuclear cells. In these experiments, whole UCB exhibited ~85% of CD4+ T cells differentiating to CD25+Foxp3+ Tregs, where the same frequency decreased to ~27% in the absence of CD14+ cells (Figure 2). Moreover, culture of enriched CD14+ cells and naïve CD4+ T cells resulted in a high frequency of CD4+ Treg differentiation (Figure 2).

We therefore concluded that the CD14+ cell subset is critical for promoting Treg differentiation from UCB mononuclear cells.

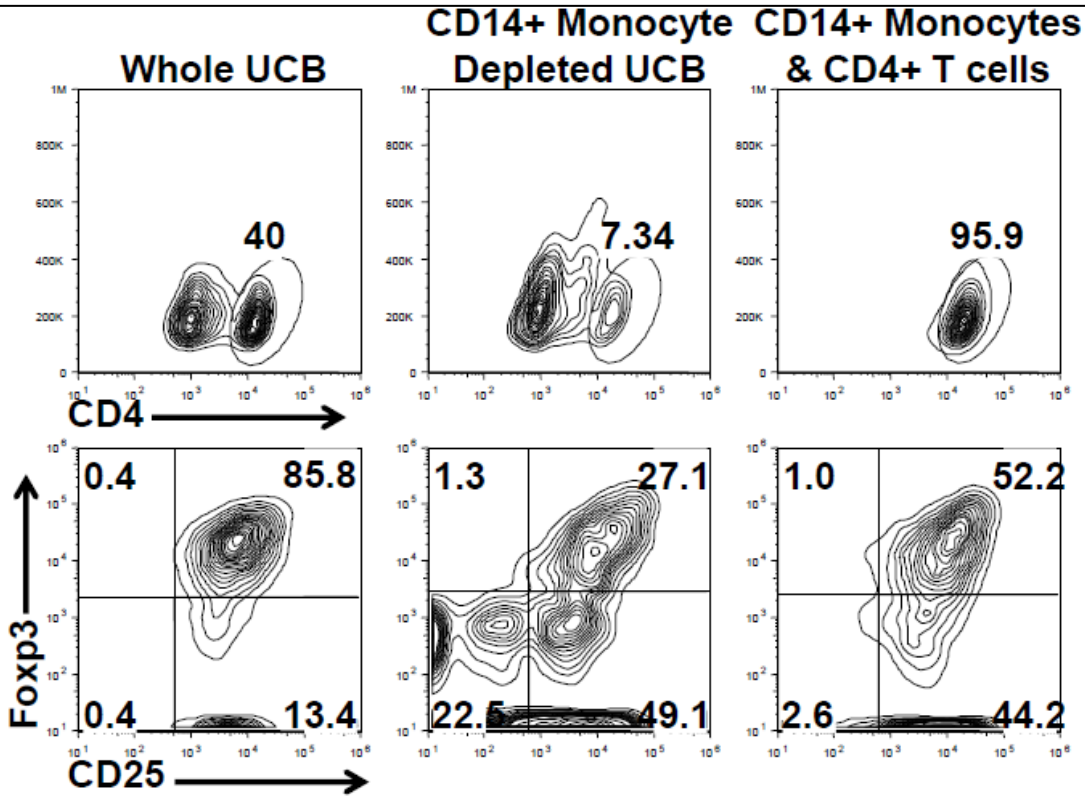


Figure 2. The effect of CD14+ cell depletion on Treg induction from whole UCB culture. The indicated human UCB cell populations were cultured and evaluated at day 14 for Treg differentiation by flow cytometry. The numbers in the top panels indicate the frequency of CD4+ cells of total live cells in each treatment group. The bottom panels depict CD25 and Foxp3 expression among total CD4+ cells. Data are representative of 3 independent experiments.

The increased frequency of Treg differentiation from UCB mononuclear cells could have also been a result of UCB CD4+ T cells being intrinsically programmed to become Foxp3+ Tregs after T cell receptor (TCR) stimulation. To test this hypothesis, we cultured UCB naïve CD4+ T cells with TCR stimulating beads. Human CD4+ non-Tregs (CD4+CD25-) failed to differentiate into Foxp3+ Tregs when cultured with TCR stimulating anti-CD3 and anti-CD28 coated polystyrene beads (Figure 3). These data

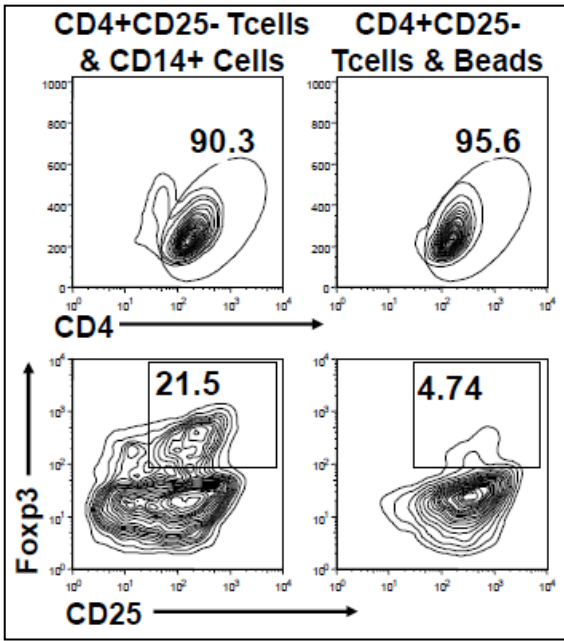


Figure 3. Stimulation of freshly isolated UCB CD4+ CD25- non-Tregs by CD14+ cells or anti-CD3 and anti-CD28 coated beads. Freshly isolated CD4+ CD25- non-Tregs were cultured for 14 days with either UCB CD14+ cells or anti-CD3 and anti-CD28 coated beads. Cultures were then analyzed by flow cytometry for differentiation of Tregs. The bottom panels show CD25 and Foxp3 expression among total CD4+ cells. Data are representative of 5 independent repetitions.

demonstrated that UCB CD4+ T cells are not intrinsically programmed to become Foxp3+ Tregs upon TCR stimulation, but require interaction with the CD14+ cell subset to induce such differentiation.

B. CD14+ CD36^{HI} monocytes are sufficient to promote human Treg induction from naïve CD4+ T cells

CD14+ cells are critical for induction of Foxp3+ Tregs from UCB naïve CD4+ T cells; however, the CD14+ cell subset is a heterogenous population that includes many different cell types including monocytes, dendritic cells and macrophages (94, 95). We therefore sought to identify the specific subset of CD14+ cells sufficient to induce Treg differentiation. To further distinguish CD14+ cell subsets, we evaluated the expression of cell surface molecules, specifically CD36, on UCB CD14+ cells. CD36 is of interest because it is capable of binding a TGF-β activating enzyme, thrombospondin-1 (TSP-1) (131, 134, 135). Upon binding TSP-1, CD36 can subsequently tether this TGF-β

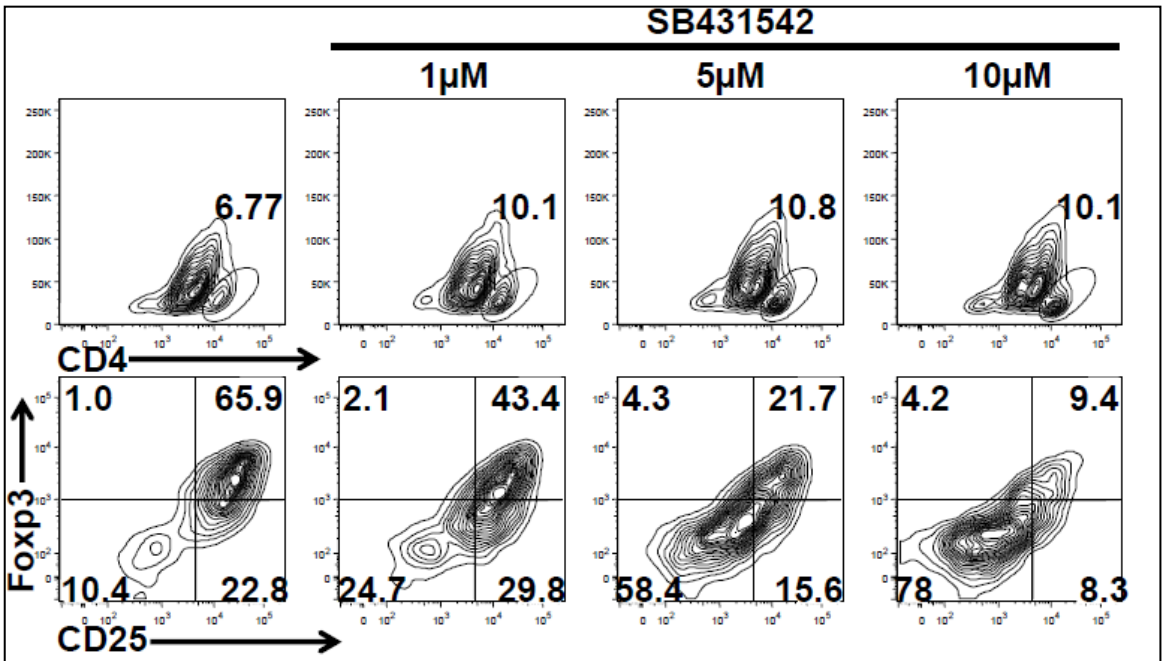


Figure 4. Inhibition of TGF-β signaling in whole human UCB culture. Whole UCB was cultured with a TGF-β type I receptor kinase specific inhibitor (SB431542) at the indicated concentrations and Treg differentiation was evaluated at day 14 of culture by flow cytometry. The numbers in the top panels indicate the frequency of CD4+ cells of total live cells in each treatment group. The bottom panels depict total CD4+ cells.

activating enzyme to the cell surface of CD36+ cells. This is important because TGF-β is produced in a latent form and requires proteolytic cleavage by enzymes such as TSP-1 to release biologically active TGF-β (119). Our group and others have demonstrated that active TGF-β is critical in promoting human Treg differentiation. In our model, inhibition of TGF-β signaling resulted in a significant and dose-dependent decrease in Treg induction from whole UCB cultures (Figure 4). In fact, inhibition of other molecules reported to promote Treg induction such as retinoic acid, aryl hydrocarbon receptor and IL-10 resulted in no change in Treg induction in UCB culture (Figure 5). Inhibition of TGF-β by an anti-TGF-β monoclonal inhibitory antibody resulted in similar abrogation of

Treg differentiation (data not shown), further supporting the critical role of TGF-β in human Treg induction.

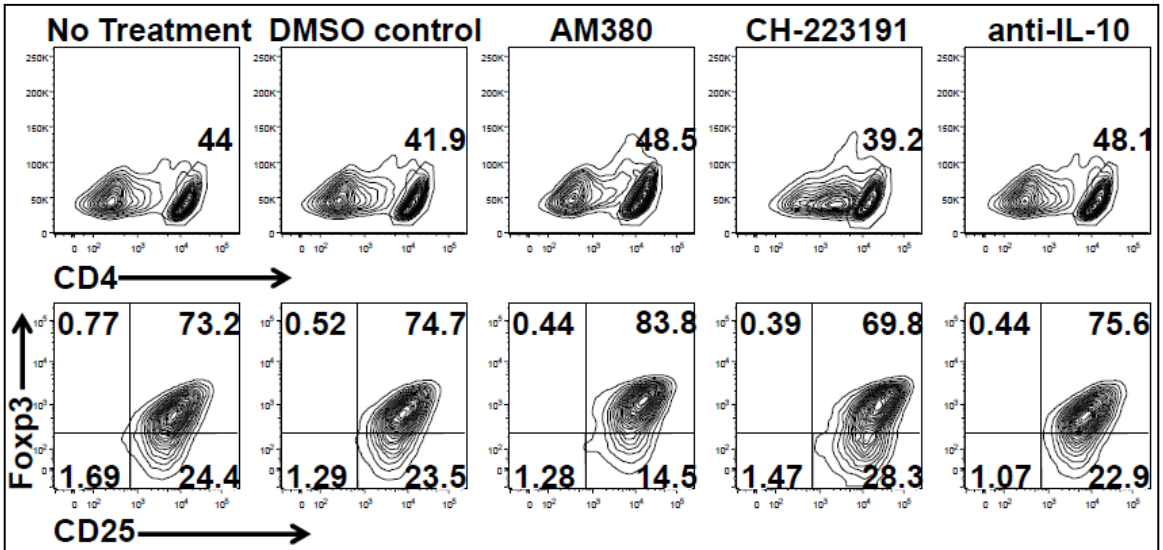


Figure 5. Inhibition of retinoic acid, aryl hydrocarbon receptor and IL-10 in whole human UCB culture. Whole UCB was cultured with either a DMSO carrier control, a retinoic acid inhibitor (AM380), an aryl hydrocarbon receptor antagonist (CH-223191) or anti-IL-10 antibodies. Treg differentiation was evaluated at day 14 of culture by flow cytometry. The numbers in the top panels indicate the frequency of CD4+ cells of total live cells in each treatment group. The bottom panels depict CD25 and Foxp3 expression among total CD4+ cells.

As mentioned, TSP-1 activates latent TGF-β via proteolytic cleavage, resulting in the release of LAP and biologically active TGF-β. CD36 serves as a cell surface receptor for TSP-1 and "expresses" the enzyme on CD36+ cells. In fact, CD36 has been shown to be required for TGF-β activation in a model utilizing rat alveolar macrophages (135, 136). To further investigate the role of TGF-β in our model of human UCB Treg induction, we evaluated expression of CD36 on UCB CD14+ cells. Flow cytometry showed that there were two distinct CD14+ cell subsets distinguished by their expression of CD36: $CD36^{HI}$ and $CD36^{LO}$ (Figure 6). Therefore, we hypothesized that the CD14+ $CD36^{HI}$ population was the CD14+ cell subset sufficient to induce human Treg

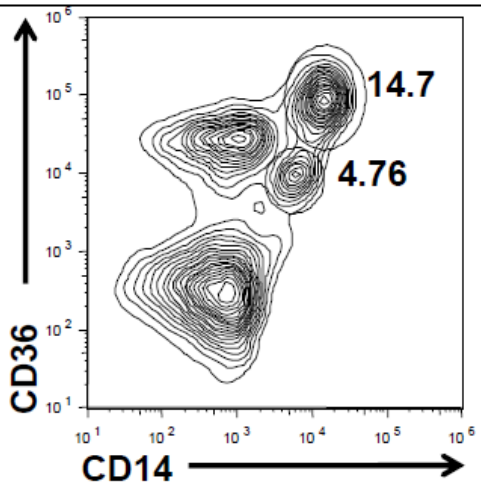


Figure 6. CD36 and CD14 expression on freshly isolated UCB mononuclear cells. Freshly isolated whole UCB was evaluated by flow cytometry for expression of CD14 and CD36. Numbers indicate the frequency of each population of total live cells. Data are representative of 15 independent experiments.

differentiation. To test the hypothesis that CD14+ CD36^{HI} monocytes are sufficient to promote human Treg differentiation from naïve CD4+ T cells, cell subsets were sorted from freshly isolated human UCB based on expression of CD14 and CD36 and cultured with enriched human UCB naïve CD4+ T cells at a ratio of 5:1. After 14 days, cultures were evaluated by flow cytometry for Treg differentiation. CD14+ CD36^{HI} cells were found to induce significantly increased human Foxp3+ Treg differentiation as compared to CD14+ CD36^{LO} and CD14- populations. Flow cytometry of these cultures at day 14 demonstrated that both whole UCB and culture with CD14+CD36^{HI} monocytes promotes a high frequency of Treg differentiation, 53 and 55.3% respectively, whereas culture with other cell subsets induces less than 20% Treg differentiation among total CD4+ T cells (Figure 7). When data from numerous cultures were compiled and normalized to the Treg frequency of total CD4+ cells induced by whole UCB, it became apparent that culture with CD14+CD36^{LO} and CD14- cells induced significantly less Treg differentiation than whole UCB or CD14+CD36^{HI} co-culture (Figure 8). At day 14 of culture, CD14+ CD36^{HI} monocytes also induced an increased absolute number of

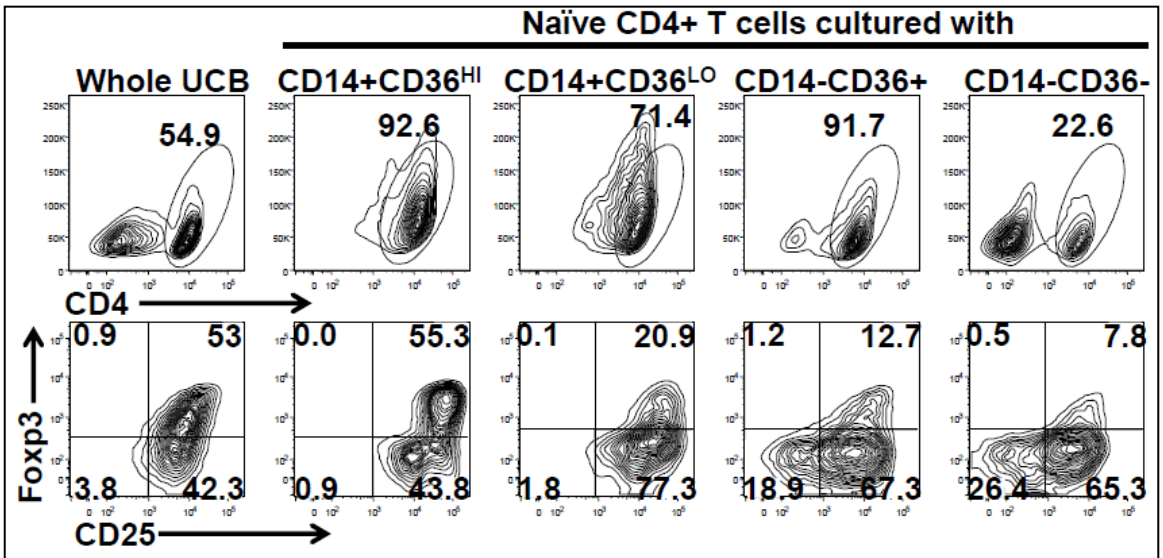


Figure 7. Culture of UCB naïve CD4+ T cells with UCB cell subsets distinguished by their expression of CD14 and CD36, including CD14+ CD36^{HI} monocytes. Naïve CD4+ T cells were isolated from whole UCB and cultured with the indicated population, sorted on expression of CD14 and CD36, at a ratio of 1:5, respectively. Cultures were evaluated at day 14 by flow cytometry. The numbers in the top panels indicate the frequency of CD4+ cells of total live cells in each treatment group. The bottom panels show total CD4+ cells. Data are representative of 6-12 independent experiments.

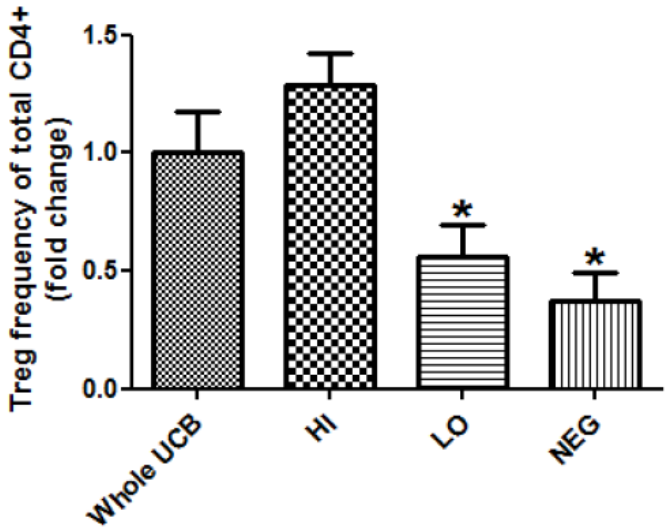


Figure 8. Analysis of Treg induction by UCB subsets distinguished by their expression of CD14 and CD36. Data from 6-12 independent experiments were pooled and evaluated based on frequency of Tregs (CD25+ Foxp3+) among total CD4+ cells. HI indicates culture with CD14+CD36^{HI} monocytes. LO and NEG indicate culture with CD14+CD36^{LO} and CD14- cells, respectively. Data are presented as fold change from whole UCB CD4+ Treg frequency.

Tregs as compared to CD14+ CD36^{LO} and CD14- subsets, correlating with the increased population frequency seen by flow cytometry. Additionally, Tregs induced by the CD14+ CD36^{HI} subset were functionally suppressive of syngenic responder CD4+ T cell proliferation as demonstrated by an *in vitro* suppression assay. Naïve UCB T cells proliferate in response to anti-CD3 antibody TCR stimulation; however, when either whole UCB or CD36^{HI} monocyte induced Tregs were added to culture, the proliferation of syngenic naïve T cells was reduced significantly in a dose dependent manner (Figure 9). Of note, CD14+ CD36^{HI} cells enriched from human adult peripheral blood were also sufficient to induce Treg differentiation from adult naïve CD4+ T cells (Figure 10), indicating that this phenomenon was not isolated to the human UCB environment.

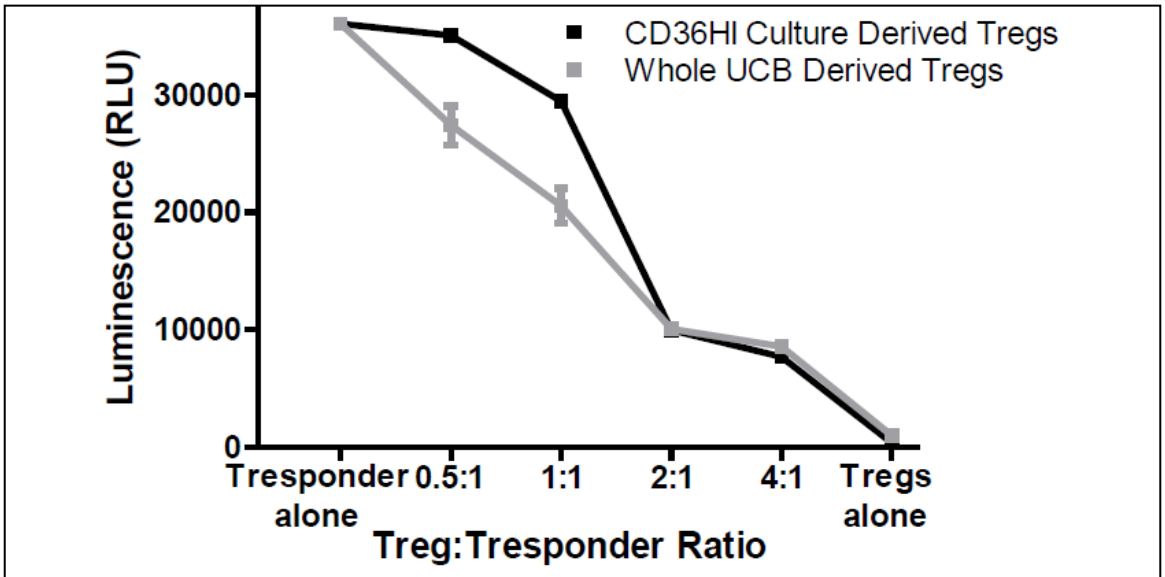


Figure 9. Functional suppression assay of CD14+ CD36^{HI} monocyte induced Tregs. Tregs were enriched from both whole UCB and CD14+ CD36^{HI} cultures at day 14, and cultured with syngenic total CD4+ T cells at the indicated ratios. Cell proliferation was measured after 7 days of culture by Cell Titer Glo Assay. Luminescence is a measure of cell proliferation.

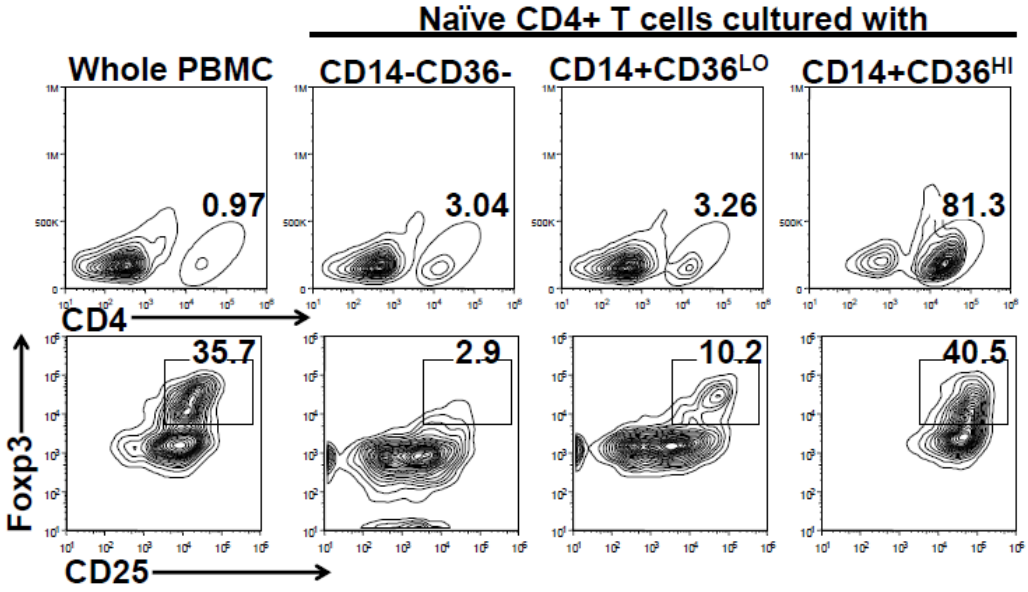


Figure 10. Culture of adult naïve CD4+ T cells with subsets sorted from adult peripheral blood by their expression of CD14 and CD36, including CD14+ CD36^{HI} monocytes. Naïve CD4+ T cells were isolated from adult PBMC and cultured with the indicated population, sorted on expression of CD14 and CD36, at a ratio of 1:5, respectively. Cultures were evaluated at day 14 by flow cytometry. The numbers in the top panels indicate the frequency of CD4+ cells of total live cells in each treatment group. The bottom panels have been gated on the CD4+ population. Data are representative of 3 independent experiments.

C. The role of TGF-β in CD14+ CD36^{HI} monocyte mediated Treg induction

Inhibition of TGF-β signaling significantly decreased Treg induction from human UCB mononuclear cells; therefore, we further pursued the potential role of TGF-β in CD14+ CD36^{HI} monocyte mediated Treg induction. Flow cytometry studies revealed an increased level of LAP, the TGF-β binding pro-peptide, on the cell surface of CD14+ CD36^{HI} monocytes as compared to their CD14+ CD36^{LO} counterparts (Figure 11A). Additionally, the majority (~57%) of LAP+ cells in freshly isolated human UCB were among the CD14+ CD36^{HI} subset (Figure 11B). Under physiological conditions, the TGF-β/LAP complex may be bound by latent TGF-β binding proteins (LTBPs) and

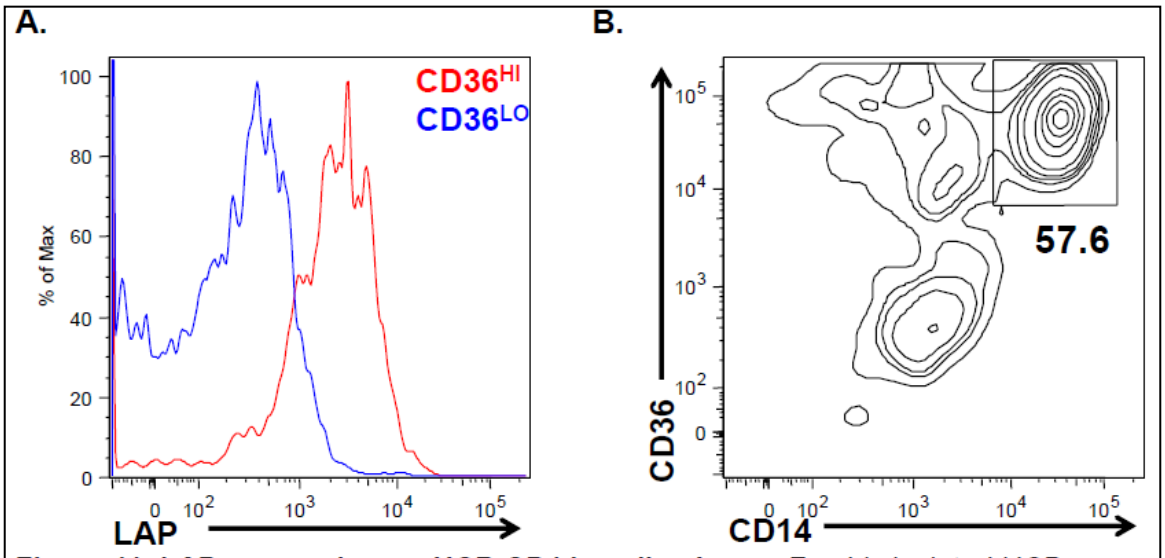


Figure 11. LAP expression on UCB CD14+ cell subsets. Freshly isolated UCB was stained by flow cytometry for cell surface expression of LAP. Data are representative of 8 independent experiments. (A) The mean fluorescence intensity (MFI) of anti-human LAP staining is depicted for the indicated CD14+ cell subsets. (B) This panel depicts CD14 and CD36 expression among LAP+ cells in whole UCB. The number indicates the frequency of CD14+ CD36^{HI} cells among the LAP+ subset.

anchored in the extracellular matrix or, in some circumstances, be present on the cell surface; however, neither LAP nor TGF-β are transmembrane molecules.

GARP, also known as LRRC32, is a transmembrane molecule required for cell surface expression of LAP-TGF-β by activated Tregs and is also reported to mediate some of the suppressive functions of human Tregs (124-129). Because of its ability to bind the LAP-TGF-β complex, GARP is, therefore, a means by which LAP-TGF-β could be tethered to the cell surface of CD14+ CD36^{HI} monocytes. To date, GARP is only known to be expressed in humans on the cell surface of activated Tregs (124-130). By flow cytometric analysis, GARP expression was identified on CD14+ cells. More specifically, studies revealed that CD14+ CD36^{HI} monocytes expressed higher levels of GARP than CD14+ CD36^{LO} cells (Figure 12A). Additionally, in freshly isolated UCB,

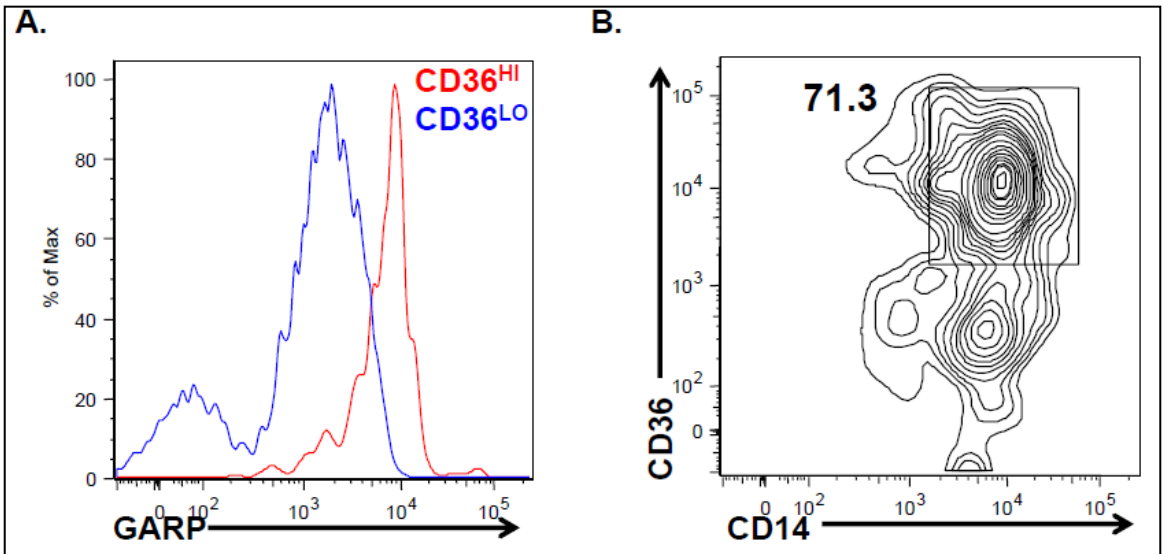


Figure 12. GARP expression on UCB CD14+ cell subsets. Freshly isolated UCB was stained by flow cytometry for cell surface expression of GARP. Data are representative of 6 independent experiments. (A) The mean fluorescence intensity (MFI) of anti-human GARP staining is depicted for the indicated CD14+ cell subsets. (B) This panel illustrates CD14 and CD36 expression among total GARP+ cells from whole UCB. The number indicates the frequency of CD14+ $CD36^{HI}$ cells among the GARP+ subset.

the majority (~71%) of GARP+ cells were CD14+ $CD36^{HI}$ (Figure 12B). GARP expression by CD14+ cells was confirmed by both Western blot and immunofluorescence (discussed in a later section). This is the first report of GARP expression on an immune cell subset other than activated Tregs and provides a mechanism by which CD14+ $CD36^{HI}$ cells could tether LAP-TGF-β to their cell surface.

We also evaluated TGF-β production by CD14+ $CD36^{HI}$, CD14+ $CD36^{LO}$ and CD14- cell subsets. Cells were sorted based on expression of CD14 and CD36 as previously described and cultured for 24 hours without stimulation. At 24 hours, supernatants were subjected to TGF-β bioassay, which utilizes a secreated alkaline phosphate (SEAP) TGF-β reporter cell line to detect TGF-β present in culture supernatants. A TGF-β bioassay of supernatants from freshly isolated human UCB cell

populations revealed that CD14+ $CD36^{HI}$ monocytes produced significantly higher levels of TGF-β than either CD14+ $CD36^{LO}$ or CD14- populations (Figure 13). The average TGF-β concentration in CD14+ $CD36^{HI}$ 24 hour supernatants was 6.43 +/- 0.35 pg/ml (mean+/-SEM), while the average TGF-β concentrations in CD14+ $CD36^{LO}$ and CD14- 24 hour supernatants were 1.83+/-0.16 and 1.10+/-0.42 pg/ml (mean+/-SEM), respectively (Figure 13). Of note, TGF-β was only detected by this bioassay after acid treatment of supernatants, which activates latent TGF-β. The endogenously active form of TGF-β was below the detection level of this assay (~1pg/ml) for all cell subsets tested.

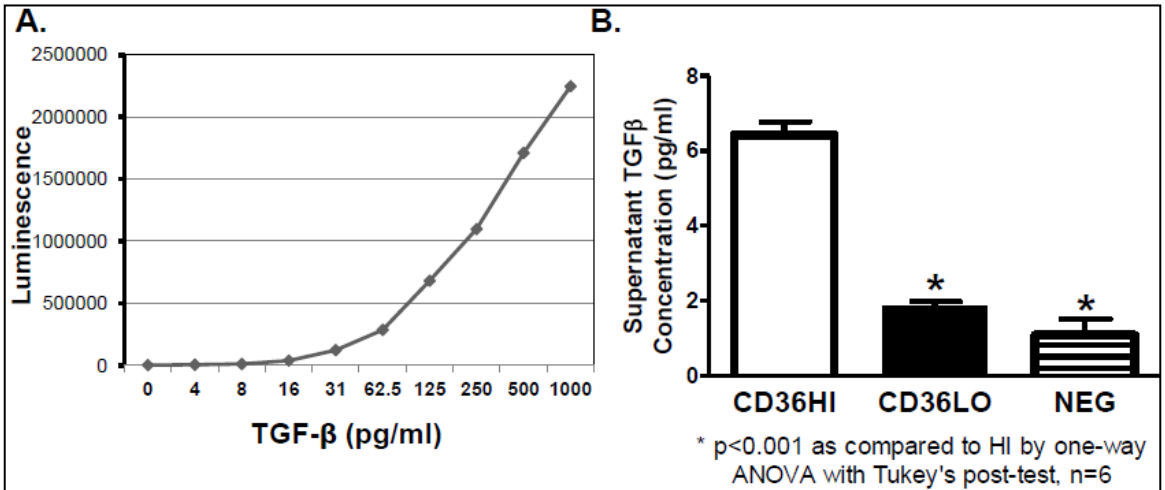


Figure 13. TGF-β bioassay of human UCB cell subsets. An engineered cell line, MFB-F11, was utilized in this bioassay and consists of a TGFβ1 KO MEF transfected with a SMAD-binding element promoter fused to a secreted alkaline phosphatase (SEAP) TGFβ reporter gene. Detection of SEAP was measured by a Chemiluminescent Reporter Gene Assay System whereby luminescence is a measure of SEAP, and therefore, TGF-β. (A) TGF-β control titration curve. (B) The indicated cell subsets were sorted from freshly isolated UCB and cultured for 24 hours without stimulation. MFB-F11 cells were exposed to the same volume of acid treated supernatant per cell subset and evaluated as described. TGF-β concentration was extrapolated from each independent experiment's titration curve.

Although we found that $CD14^{+}CD36^{HI}$ monocytes produce significantly higher levels of latent TGF-β than either $CD14^{+}CD36^{LO}$ or $CD14^{-}$ populations, the level of latent TGF-β production was not sufficient to explain the ability of $CD14^{+}$ $CD36^{HI}$ cells to

induce Foxp3^{+} Tregs. Addition of latent TGF-β to cultures of naïve CD4+ T cells and CD14+ CD36^{LO} cells did not restore Treg induction by this subset (Figure 14).

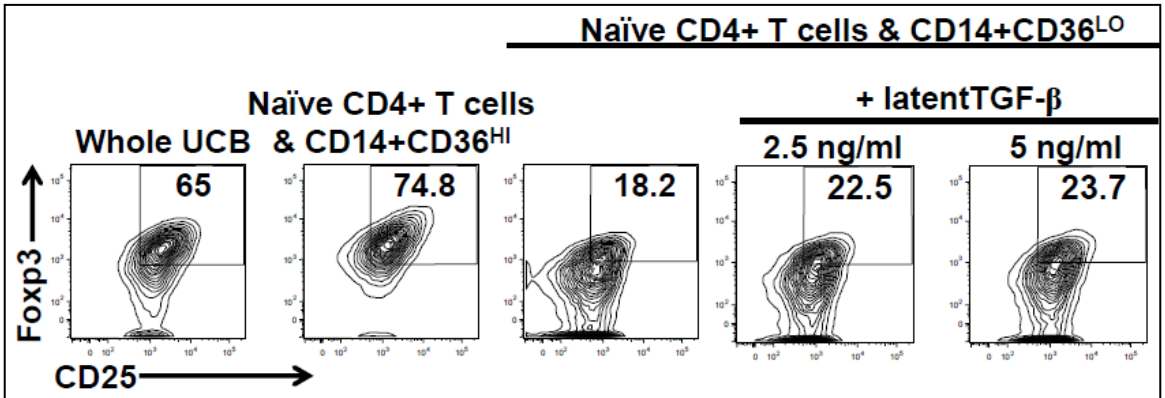


Figure 14. Addition of latent TGF-β to cultures of CD14+ CD36^{LO} cells and naïve CD4+ T cells. Naïve CD4+ T cells were isolated from whole UCB and cultured with the indicated population, sorted on expression of CD14 and CD36, at a ratio of 1:5, respectively. Cultures were evaluated at day 14 by flow cytometry. Panels have been gated on the CD4+ population. Numbers indicate the frequency of CD25+ Foxp3+ Tregs among CD4+ cells. Where indicated, latent recombinant human TGF-β has been added at 2.5 or 5 ng/ml.

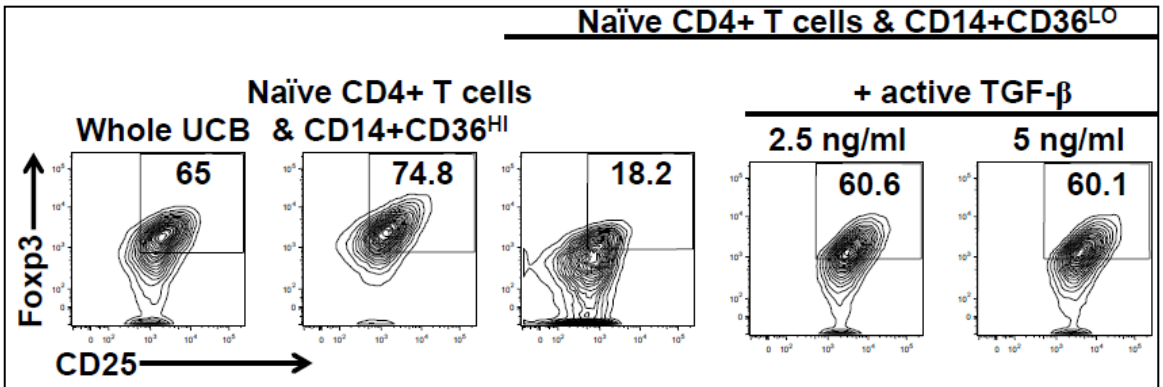


Figure 15. Supplementation of exogenous active TGF-β to cultures of CD14+ CD36^{LO} cells and naïve CD4+ T cells. Naïve CD4+ T cells were isolated from whole UCB and cultured with the indicated population, sorted on expression of CD14 and CD36, at a ratio of 1:5, respectively. Cultures were evaluated at day 14 by flow cytometry. Panels have been previously gated on CD4+ cells. Numbers indicate the frequency of CD25+ Foxp3+ Tregs among CD4+ cells. Where indicated, active recombinant human TGF-β has been added at 2.5 or 5 ng/ml.

Supplementation of up to 5.0 ng/ml of active TGF-β to cultures of naïve CD4+ T cells and CD14^{+} CD36^{LO} cells partially restored Treg induction to the level of CD14+CD36^{HI}

monocytes (Figures 15 and 16). The lowest concentration of active TGF-β added in these experiments (0.1ng/ml) was still approximately fifteen fold higher than that secreted by CD14+ $CD36^{HI}$ monocytes as measured by TGF-β bioassay (Figure 16). Because $CD14+CD36^{HI}$ monocytes produce high concentrations of latent TGF-β and supplementation of soluble latent TGF-β to cultures of $CD14+CD36^{LO}$ cells does not rescue Treg induction, we hypothesized that the cell surface bound latent form of TGF-β is required for $CD14+CD36^{HI}$ monocyte mediated Treg induction.

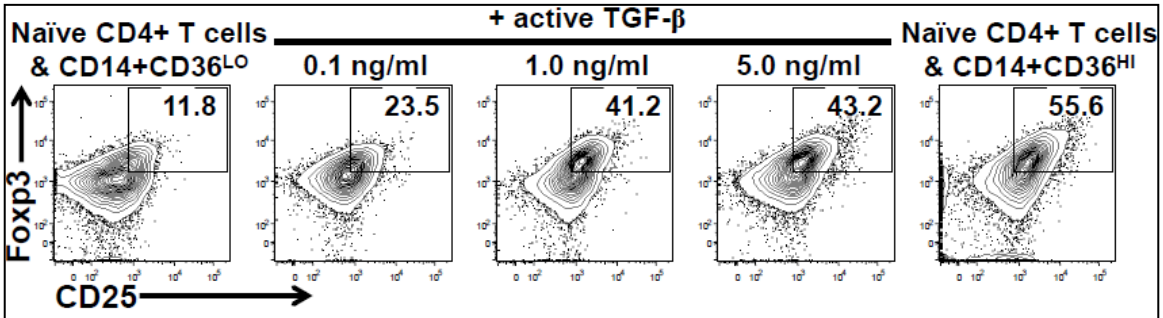


Figure 16. Addition of exogenous active TGF-β to cultures of CD14+ $CD36^{LO}$ cells and naïve CD4+ T cells. Naïve CD4+ T cells were isolated from whole UCB and cultured with the indicated population, sorted on expression of CD14 and CD36, at a ratio of 1:5, respectively. Cultures were evaluated at day 14 by flow cytometry. Panels have been previously gated on CD4+ cells. Numbers indicate the frequency of CD25+ Foxp3+ Tregs among CD4+ cells. Where indicated, active recombinant human TGF-β has been added.

D. The functional role of CD36 and GARP in human Treg induction

Data show that CD14+ $CD36^{HI}$ monocytes are sufficient to induce human Treg differentiation from naïve CD4+ T cells and that this subset of monocytes expresses higher levels of CD36, LAP and GARP and produces increased levels of latent TGF-β as compared to their $CD36^{LO}$ and CD14- counterparts. However, the functional role of CD36, GARP and cell surface bound TGF-β in Treg induction remained uncertain. To evaluate the role of these molecules in human Treg induction, we established a genetic

system using THP.1 cells: a human acute monocytic leukemia cell line. A fraction of THP.1 cells expressed CD36 and GARP (Figure 17A) and when cultured with UCB naïve CD4+ T cells, irradiated THP.1 cells induced Treg differentiation (Figure 17B).

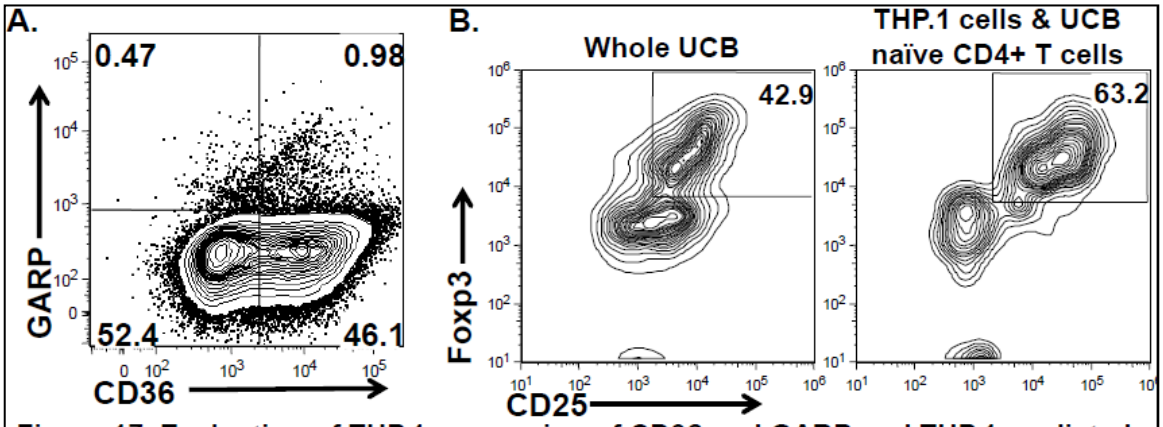


Figure 17. Evaluation of THP.1 expression of CD36 and GARP and THP.1 mediated Treg induction from naïve CD4+ T cells. (A) THP.1 cells were stained for expression of CD36 and GARP and evaluated by flow cytometry. (B) Whole UCB mononuclear cells were cultured in parallel to naïve CD4+ T cells isolated from the same UCB sample cultured with irradiated THP.1cells. Cultures were evaluated at day 14 by flow cytometry for differentiation of Tregs. Panels have been gated on CD4+ cells. Numbers indicate the frequency of CD25+ Foxp3+ Tregs among CD4+ cells.

Additionally, THP.1 induced Tregs were functionally suppressive as demonstrated by *in vitro* suppression assay (Figure 18).

To determine if CD36 and/or GARP play a functional role in human Treg induction, GIPZ lentiviral shRNA vectors were purchased from Thermo Scientific Open Biosystems targeting either CD36 or GARP, as well as a GFP expressing control vector. After spinoculation, THP.1 cells were cultured and evaluated for expression of CD36 and GARP at 72 hours post transduction. This transduction protocol was repeated 3-4 times each with fresh THP.1 isolates. Transduction with an anti-CD36 shRNA vector resulted in expression of GFP (Figure 19A) and a reduction in expression of CD36 as compared to GFP vector control transduced THP.1 cells (Figure 19B). Both GFP expression (Figure

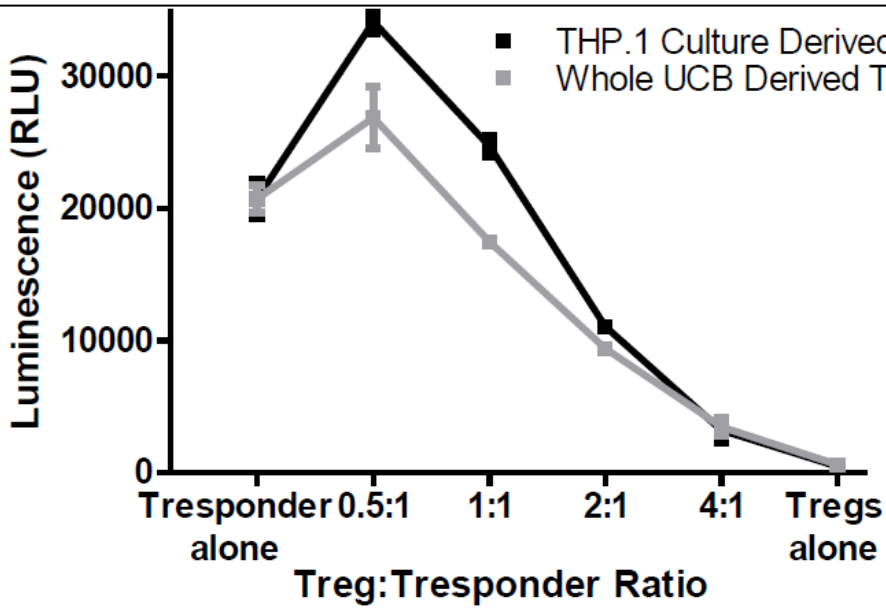


Figure 18. Functional suppression assay of THP.1 induced Tregs. Tregs were enriched from both whole UCB and THP.1 cultures at day 14, and cultured with syngenic total CD4+ T cells at the indicated ratios. Cell proliferation was measured after 7 days of culture by Cell Titer Glo Assay. Luminescence is a measure of cell proliferation.

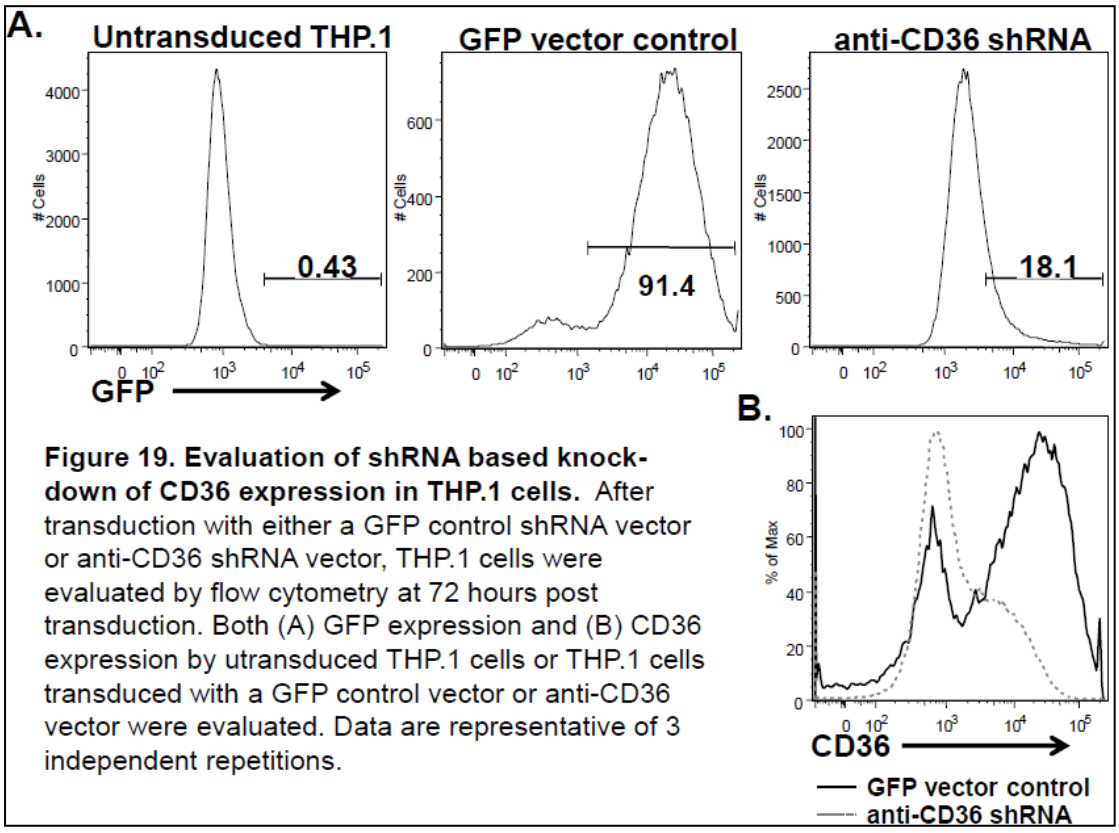


Figure 19. Evaluation of shRNA based knock-down of CD36 expression in THP.1 cells. After transduction with either a GFP control shRNA vector or anti-CD36 shRNA vector, THP.1 cells were evaluated by flow cytometry at 72 hours post transduction. Both (A) GFP expression and (B) CD36 expression by utransduced THP.1 cells or THP.1 cells transduced with a GFP control vector or anti-CD36 vector were evaluated. Data are representative of 3 independent repetitions.

20A) and a reduction in GARP expression as compared to GFP vector control transduced THP.1 cells (Figure 20B) were demonstrated for anti-human GARP shRNA vectors as well. GARP is rapidly recycled from the cell membrane, so cell surface and total GARP were evaluated, and both forms were reduced upon transduction with anti-human GARP shRNA vectors (Figure 20B). No vector achieved complete knock-down of either CD36 or GARP expression.

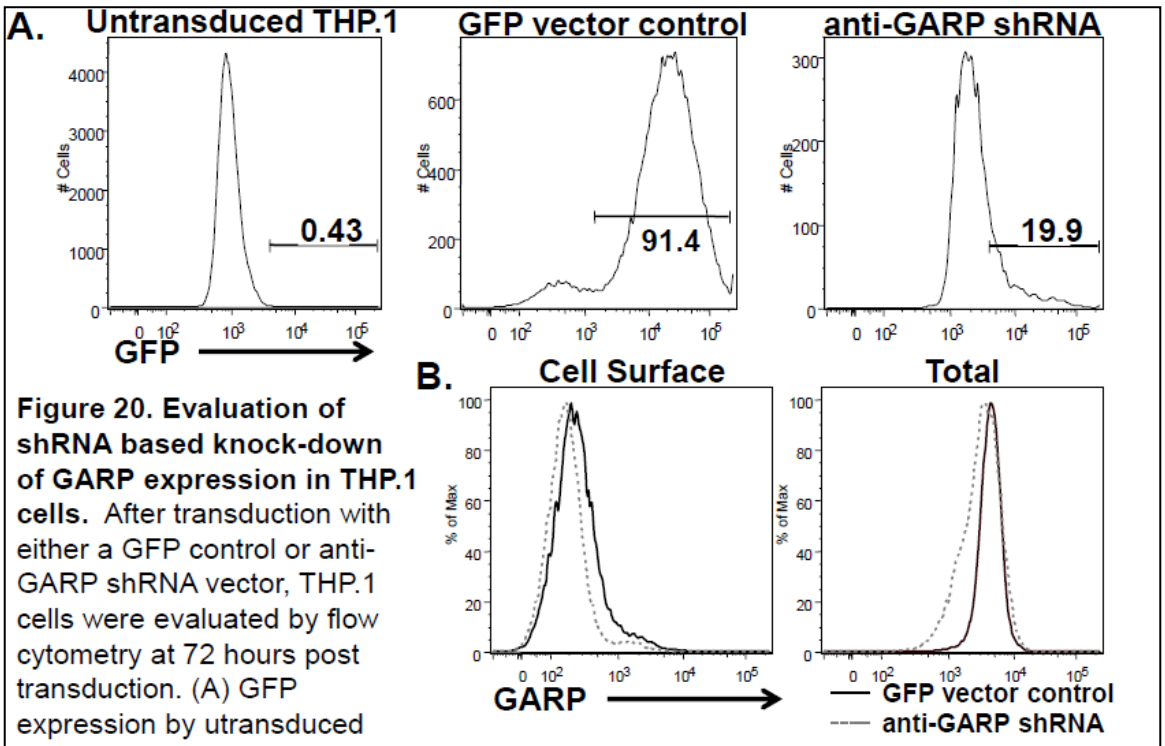


Figure 20. Evaluation of shRNA based knock-down of GARP expression in THP.1 cells. After transduction with either a GFP control or anti-GARP shRNA vector, THP.1 cells were evaluated by flow cytometry at 72 hours post transduction. (A) GFP expression by utransduced THP.1 cells or THP.1 cells transduced with a GFP control vector or anti-CD36 vector was evaluated. (B) Cell surface GARP expression or total GARP expression after fixation and permeabilization are compared between GFP control and anti-GARP shRNA vector transduced cells. Data are representative of 4 independent repetitions.

Transduced THP.1 cell populations were sorted based on GFP expression and maintained in culture with puromycin, an antibiotic resistance marker included in the GIPZ plasmid. If CD36 and/or GARP are required for human Treg induction from naïve CD4+ T cells as we hypothesize, then knock-down of expression of either of these

molecules will reduce Treg induction by that THP.1 population. Therefore, naïve CD4+ T cells were enriched from human UCB and cultured with untransduced THP.1 cells or THP.1 cells transduced with the GFP control vector or shRNA vectors targeting CD36 or GARP respectively. After 14 days of culture, untransduced and GFP vector control THP.1 cells induced a similar frequency of CD4+ Treg differentiation, while knock-down of CD36 or GARP expression resulted in a reduction of CD4+ Treg differentiation (Figure 21A). This experiment was performed with 5-10 independent human UCB samples and similar results were observed in all repetitions (Figure 21B). These data support that expression of both CD36 and GARP are required for THP.1 mediated human Treg induction.

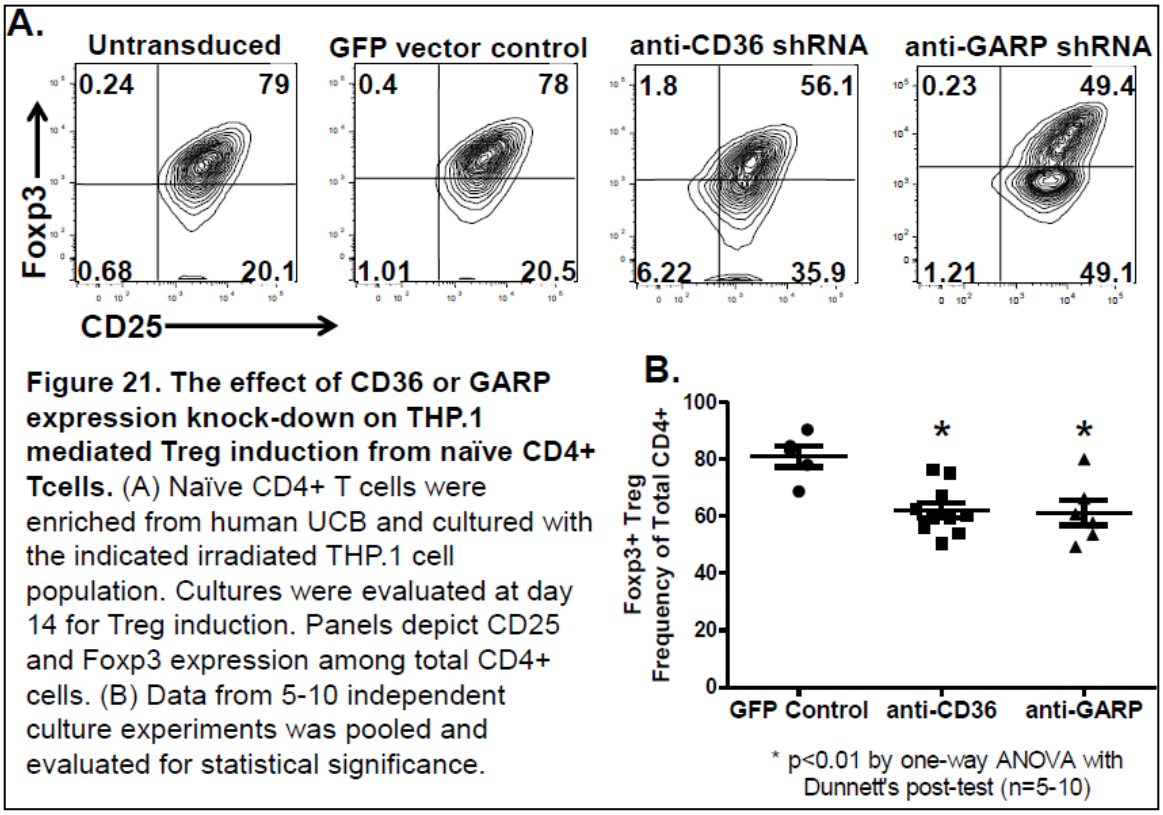


Figure 21. The effect of CD36 or GARP expression knock-down on THP.1 mediated Treg induction from naïve CD4+ Tcells. (A) Naïve CD4+ T cells were enriched from human UCB and cultured with the indicated irradiated THP.1 cell population. Cultures were evaluated at day 14 for Treg induction. Panels depict CD25 and Foxp3 expression among total CD4+ cells. (B) Data from 5-10 independent culture experiments was pooled and evaluated for statistical significance.

E. Confocal imaging studies of CD36 and GARP

Our data suggest that CD36 and GARP play critical roles in CD14+ CD36^{HI} monocyte mediated Treg induction. Potentially, co-localization of GARP and CD36 would place the inactive LAP-TGF-β complex bound to GARP in close proximity to the TGF-β activating enzyme, TSP-1, bound to CD36. To address whether or not CD36 and GARP are co-localized on the cell surface, we first enriched CD14+ cells from freshly isolated human UCB by magnetic bead isolation (Figure 22) and subjected the enriched cells to immunofluorescent labeling of CD36 and GARP. Confocal imaging studies and evaluation of the appropriate controls (Figure 23) revealed that GARP is expressed by CD14+ CD36+ cells as originally demonstrated by flow cytometry. Additionally, imaging demonstrated that CD36 and GARP are not co-localized on the cell surface of freshly isolated human CD14+ cells (Figure 24).

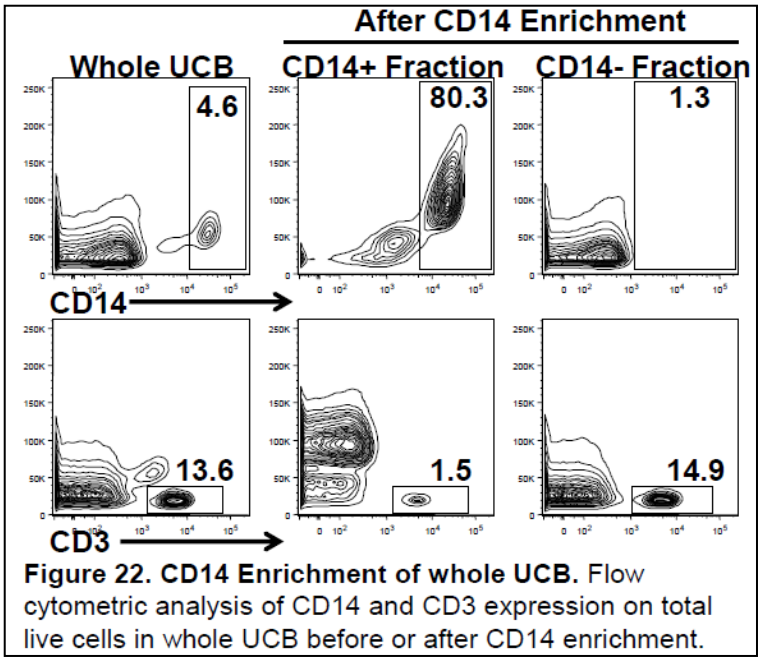


Figure 22. CD14 Enrichment of whole UCB. Flow cytometric analysis of CD14 and CD3 expression on total live cells in whole UCB before or after CD14 enrichment.

Also of note, by confocal microscopy, CD14+ CD36+ cells represented a homogenous population demonstrating monocytic nuclear morphology of a "U-shaped" or "horseshoe" nucleus (Figure 25). CD14+ CD36- cells did not stain positive for GARP

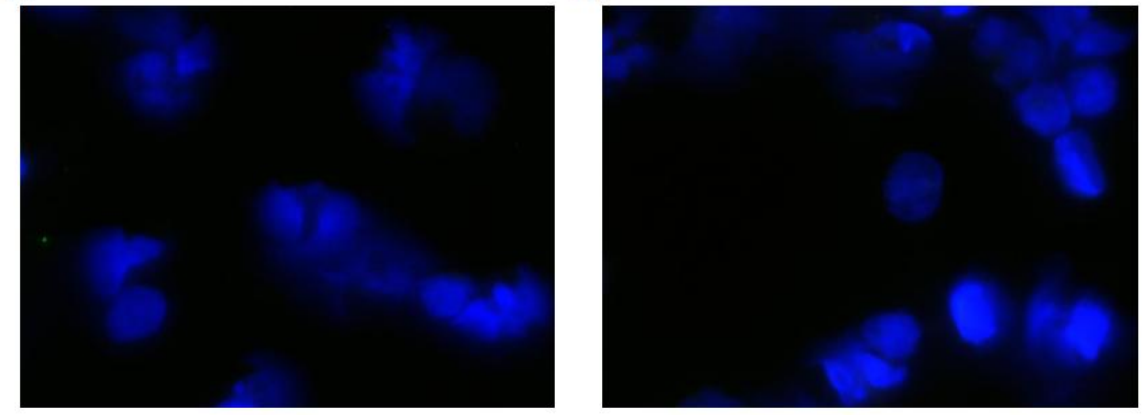


Figure 23. Immunofluorescent control staining of UCB CD14+ cells. After enrichment, CD14+ cells were fixed and subjected to immunofluorescent staining protocol. In both panels, green indicates anti-GARP staining, red indicates anti-CD36 staining and blue is Hoechst staining of nuclei. All channels are shown in these panels. (A) Anti-human GARP isotype control and corresponding secondary antibody. (B) Staining of CD14+ cells using only appropriate secondary antibodies to detect background, nonspecific staining.

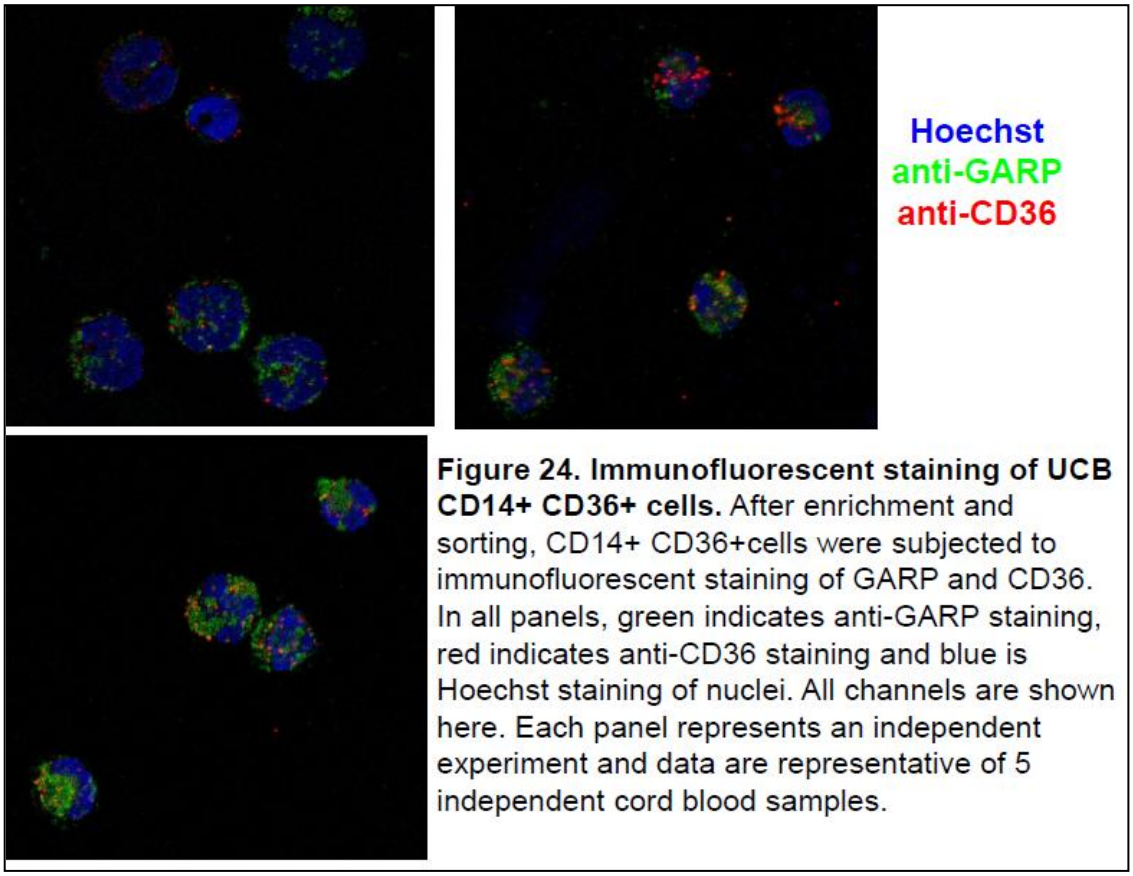


Figure 24. Immunofluorescent staining of UCB CD14+ CD36+ cells. After enrichment and sorting, CD14+ CD36+cells were subjected to immunofluorescent staining of GARP and CD36. In all panels, green indicates anti-GARP staining, red indicates anti-CD36 staining and blue is Hoechst staining of nuclei. All channels are shown here. Each panel represents an independent experiment and data are representative of 5 independent cord blood samples.

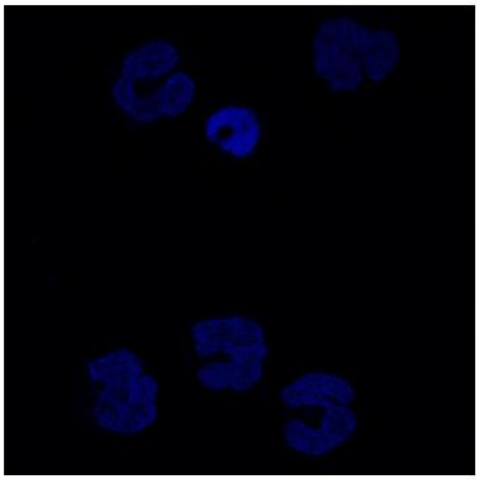


Figure 25. Comparison of Hoechst stained nuclei of CD14+ CD36+ cells and characteristic monocyte nucleus. (A) Hoechst stained nuclei of CD14+ CD36+ cells. (B) Monocyte stained by hematoxylin and eosin (H&E) depicting characteristic "U-shaped" or "horseshoe" nucleus (http://jeeves.mmg.uci.edu/immunology/Architecture/Monocyte.htm).

expression, as expected based on flow cytometry data (Figure 26). This experiment has been repeated with 5 independent human UCB samples.

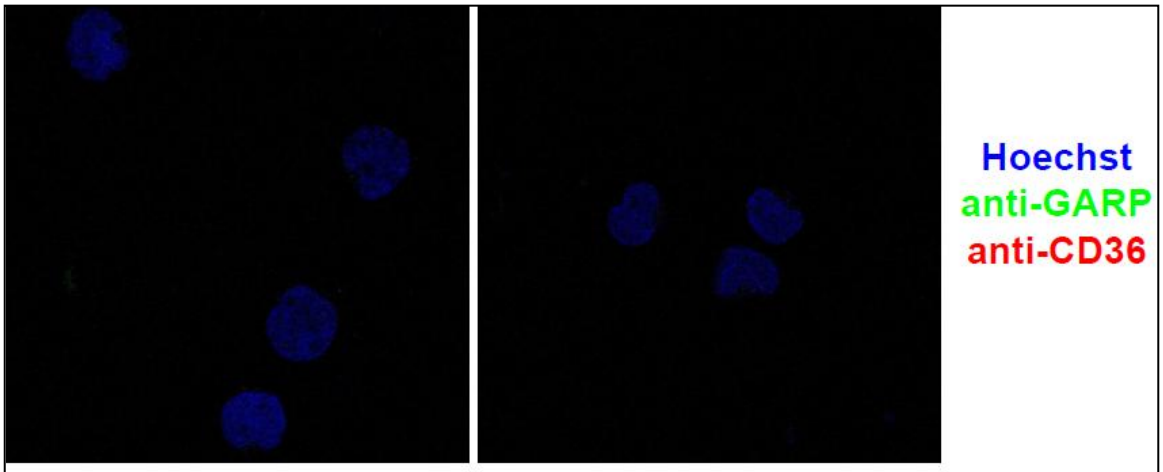


Figure 26. Immunofluorescent staining of UCB CD14+ CD36- cells. After enrichment and sorting, CD14+ CD36- cells were subjected to immunofluorescent staining of GARP and CD36. In all panels, green indicates anti-GARP staining, red indicates anti-CD36 staining and blue is Hoechst staining of nuclei. All channels are shown here. Each panel represents an independent experiment and data are representative of 5 independent cord blood samples.

F. CD14+ CD36^{HI} and CD36^{LO} cells represent distinct monocyte subsets

Evaluation of select cell surface markers also revealed differences between CD14+ CD36^{HI}, CD14+ CD36^{LO} and CD14- subsets. Human monocytes are a heterogenous class of immune cells and recent studies have identified subsets within the monocyte population based on expression of CD14, CD16 (FcγRIII), and CD64 (FcγRI). The CD14+CD16- cells are termed "classical" monocytes (91, 106, 108). CD14+CD16+ cells more closely resemble mature tissue macrophages and are considered "nonclassical." An additional class of monocytes, CD14+CD16+CD64+, has been identified more recently and is thought to be an intermediate phenotype between monocytes and dendritic cells. While there is still controversy in the field over the phenotypic characteristics and *in vivo* function of each monocyte subset, CD14+ CD36^{HI} and CD14+CD36^{LO} cells exhibited differential expression of CD16 and CD64. CD14+ CD36^{HI} monocytes were a relatively uniform population, exhibiting intermediate expression of CD16 and high expression of CD64 (Figure 27A and C). Conversely, the CD14+ CD36^{LO} population demonstrated heterogeneity, but the majority were CD16-CD64- (Figure 27B and C). While the *in vivo* significance of each monocyte subset is unclear, CD14+ CD36^{HI} and CD14+ CD36^{LO} populations represent different classes of monocytes based on expression of CD16 and CD64. Additionally, CD14+ CD36^{HI} monocytes express MHC class I and class II molecules. They do not express CD19, IgM or CD8α, demonstrating that they are neither B cells nor tolerogenic dendritic cells, respectively.

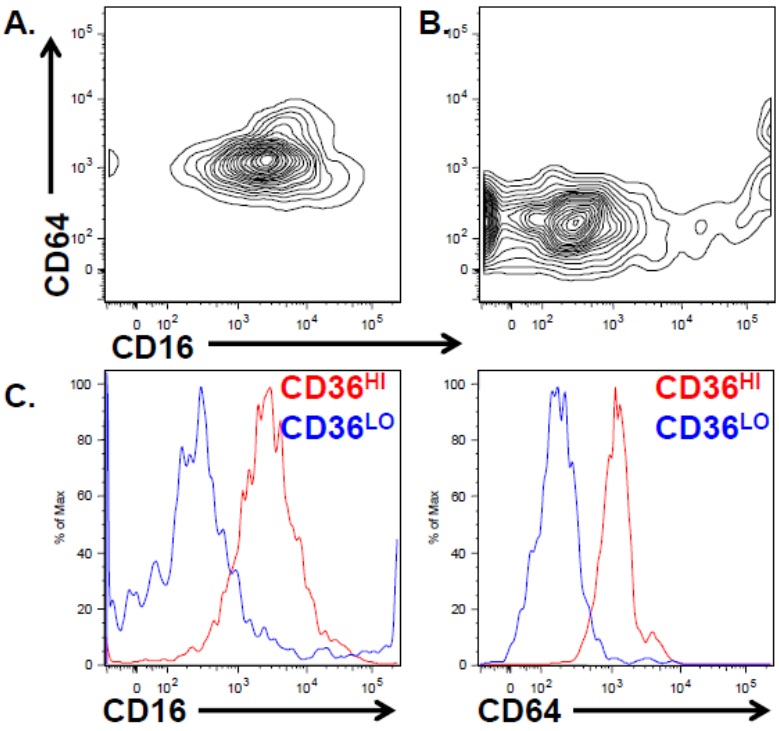


Figure 27. CD16 and CD64 expression on UCB CD14+ cell subsets. Freshly isolated UCB was stained by flow cytometry for expression of CD16 and CD64. Data are representative of 8 independent experiments. CD16 and CD64 expression are shown on (A) $CD36^{HI}$ and (B) $CD36^{LO}$ subsets. (C) The mean fluorescence intensity (MFI) of anti-human CD16 and CD64 staining is depicted for the indicated CD14+ cell subsets.

G. The effect of LPS stimulation on CD14+ $CD36^{HI}$ monocyte mediated Treg induction

Numerous groups have reported that the immature status of an antigen presenting cell contributes to its ability to promote Treg differentiation. The general immunologic immaturity of UCB suggests that the immaturity of antigen presenting cells isolated from UCB could contribute to their Treg inducing ability. Therefore, we cultured naïve CD4+ T cells with either freshly isolated CD14+ $CD36^{HI}$ monocytes or after LPS mediated activation of the same population with the hypothesis that LPS activation of this subset would decrease their ability to induce Treg differentiation. CD14+ $CD36^{HI}$ cells express

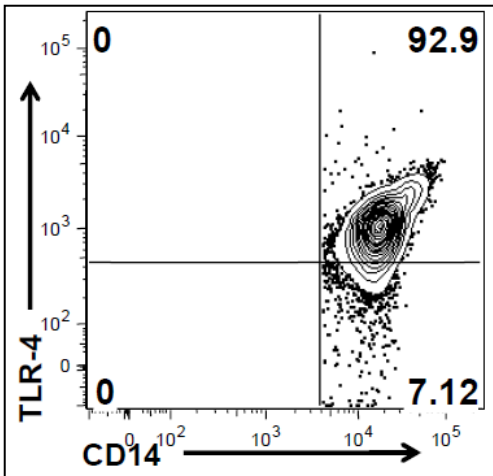


Figure 28. TLR4 expression on CD14+ UCB cells. Freshly isolated UCB was stained by flow cytometry for expression of CD14 and TLR4. This panel has been gated on CD14+ cells.

TLR4 and are therefore capable of responding to LPS (Figure 28). We found that not only did LPS treatment decrease the ability of CD14+ CD36^{HI} cells to induce Treg differentiation as hypothesized (Figure 29), but also decreased CD36 and GARP expression by this subset (Figure 30A). Data suggest that the LPS mediated decrease in expression of CD36 and GARP is specific, because expression of CD14 and CD47, another cell surface molecule capable of binding TSP-1, remained unchanged after LPS stimulation. LPS treatment also moderately decreased TGF-β production by CD14+ CD36^{HI} monocytes (Figure 30B).

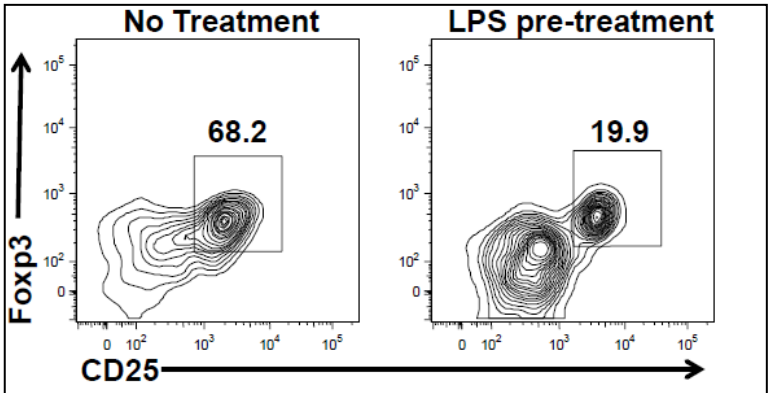


Figure 29. The effect of LPS pre-treatment of CD14+ CD36^{HI} monocytes before culture with naïve CD4+ T cells on their ability to induce Treg differentiation. Naïve CD4+ T cells were isolated from whole UCB and cultured with CD14+ CD36^{HI} monocytes at a ratio of 1:5, respectively. Cultures were evaluated at day 14 by flow cytometry. Panels have been gated on live, CD4+ cells. Where indicated, CD14+ CD36^{HI} monocytes were treated with 100ng/ml LPS for 24 hours and washed prior to culture with naïve CD4+ T cells. Data are representative of 3 independent experiments.

These data support that the relative immaturity, or antigenic naïvety, of CD14+ CD36^{HI} monocytes contributes to their ability to induce human Treg differentiation. While LPS activation may have additional unintended effects on CD14+ CD36^{HI}

monocytes, these data support previous studies demonstrating that expression of CD36 and GARP, as well as production of TGF-β, are important factors in CD14+ CD36^{HI} monocyte mediated Treg induction.

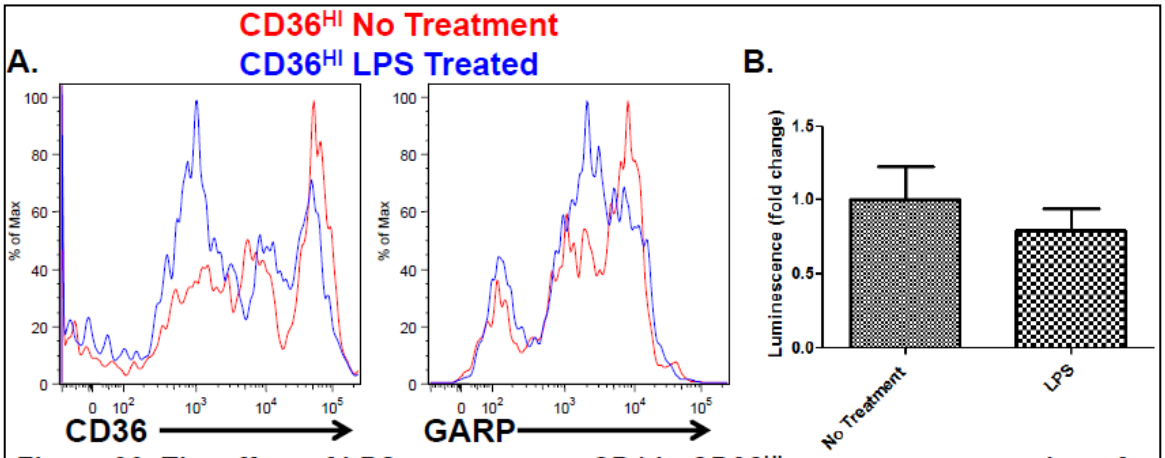


Figure 30. The effect of LPS treatment on CD14+ CD36^{HI} monocyte expression of CD36 and GARP and their production of TGF-β. (A) Sorted CD14+ CD36^{HI} monocytes were cultured for 24 hours +/- 100ng/ml LPS and evaluated for expression of CD36 and GARP by flow cytometry. The mean fluorescence intensity (MFI) of anti-human CD36 and GARP staining is depicted for the indicated treatment groups. Data are representative of 3 independent experiments. (B) A TGF-β bioassay was performed on acid treated supernatants from CD14+ CD36^{HI} monocytes cultured for 24 hours +/- 100ng/ml LPS. Luminescence is a measure of SEAP, and therefore, TGF-β production. Pooled data from 3 independent experiments is shown.

H. Summary

Overall, data demonstrate that CD14+ CD36^{HI} monocytes are sufficient to induce Foxp3+ Treg differentiation from naïve CD4+ T cells. Our studies also suggest that CD14+ CD36^{HI} monocyte mediated Treg induction is intimately dependent on the TGF-β pathway. TGF-β is required for CD14+ CD36^{HI} monocyte mediated Treg induction as demonstrated by inhibition assays, and CD14+ CD36^{HI} monocytes produce significantly more latent TGF-β and express higher levels of CD36, LAP and GARP than CD14+ CD36^{LO} and CD14- cells. TGF-β supplementation experiments demonstrate that active

TGF-β supplementation partially increases UCB Treg induction by CD14+ $CD36^{LO}$ cells. However, CD14+$CD36^{HI}$ monocytes produce high concentrations of latent TGF-β and supplementation of soluble latent TGF-β to cultures of CD14+$CD36^{LO}$ cells does not rescue Treg induction. Therefore, we hypothesized that the cell surface bound form of latent TGF-β is required for CD14+$CD36^{HI}$ monocyte mediated Treg induction.

Through genetic manipulation of the THP.1 cell line, we have demonstrated that knock-down of expression of CD36 or GARP significantly decreases THP.1 mediated Treg induction. These studies suggest that CD36 and GARP expression are required for THP.1 mediated Treg induction and point to the importance of the cell surface bound latent form of TGF-β in CD14+ $CD36^{HI}$ monocyte mediated Treg induction. Additionally, while CD36 and GARP are not co-localized on freshly isolated CD14+ cells as demonstrated by confocal imaging, these studies have not ruled out a physical interaction between the two molecules.

In summary, we have shown that CD14+ $CD36^{HI}$ monocytes are sufficient to induce Foxp3+ Treg differentiation from naïve CD4+ T cells. CD14+ $CD36^{HI}$ monocytes produce significantly more latent TGF-β and express higher levels of CD36, LAP and GARP than CD14+ $CD36^{LO}$ and CD14- cells. We have also demonstrated that CD36 and GARP are required for THP.1 mediated Treg induction, illustrating the importance of cell surface bound form of latent TGF-β is required for CD14+$CD36^{HI}$ monocyte mediated Treg induction.

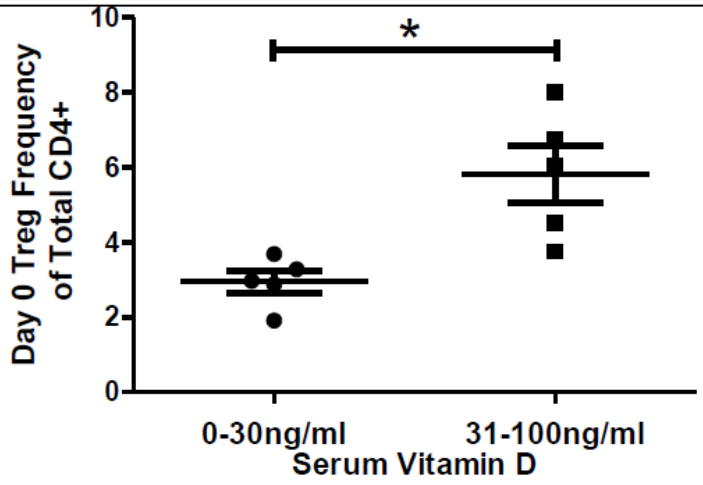
II. The Effect of Vitamin D on Human Treg Differentiation

Although vitamin D deficiency is a highly prevalent and easily diagnosed and treated condition, most deficient patients remain both undiagnosed and untreated (145-149). Treatment for vitamin D deficiency is oral supplementation of vitamin D and this treatment benefits bone homeostasis and skeletal health, as well as certain features of the immune system. The immune specific benefits of vitamin D supplementation, point to the importance of advancing our understanding of how vitamin D influences the immune response. Our focus is on the T cell constituents of the adaptive branch of the immune system and we sought to understand how vitamin D influences the CD4+ T cell subset: Tregs.

Previous studies demonstrated that human adult peripheral blood T cells are affected by serum vitamin D concentration, because increased serum vitamin D was correlated with increased peripheral CD4+ and CD8+ T cells, increased naïve T cells and increased levels of Foxp3 expression (153). Another clinical study demonstrated that supplementation of vitamin D in otherwise healthy adults was associated with a significantly increased peripheral frequency of CD4+ Tregs (154). With the collaboration of Pauline Camacho, MD and Barbara Sexton, RN, MS, at the Loyola University Medical Center, and working with Stephanie Chapman, we obtained peripheral blood samples from human volunteers between the ages of 18-80 years following specific inclusion and exclusion criteria in order to evaluate the relationship between vitamin D and human Treg biology. Exclusion criteria included, but were not limited to, presence of an active malignancy in the past 5 years, use of immunomodulatory medications, autoimmune

disease and pregnancy or lactation. Mononuclear cells were enriched from the peripheral blood samples within 24 hours of collection and evaluated by flow cytometry for CD4+ Treg frequency. Vitamin D deficient patients were described as having a serum vitamin D level of 0-30ng/mL and sufficient patients as having a serum vitamin D level of 31-100ng/mL, in agreement with clinically accepted parameters (145, 147-149, 204). In this pilot study, 5 vitamin D deficient patients and 5 age-matched vitamin D sufficient patients were evaluated. We found that vitamin D deficient patients had a significantly decreased CD4+ Treg frequency as compared to vitamin D sufficient controls ($p<0.01$) (Figure 31). Specifically, we found the Treg (CD25+ Foxp3+) frequency of total CD4+ cells in freshly isolated peripheral blood from vitamin D sufficient patients to be 5.82% +/- 0.76% and from vitamin D deficient patients to be 2.95% +/- 0.30% (mean +/- SEM) (Figure 31). These data are in agreement with previously published studies demonstrating a positive correlation between serum vitamin D concentration and Foxp3+ Treg frequency in adult peripheral blood.

After confirming a correlation between serum vitamin D and Treg frequency in adult peripheral blood, it was important to evaluate the influence of vitamin D in our model of human Treg induction from UCB. Additionally, one study reported that vitamin D levels do not correlate with Treg frequency in human UCB (205), which is in opposition to data published from adult peripheral blood studies. Through the addition of the biologically active form of vitamin D (1,25-dihydroxyvitamin D) to freshly isolated UCB cultures, we have demonstrated that supplementation of vitamin D to whole UCB cultures increases Treg frequency in a dose dependent manner as measured by flow



* p<0.01 by Student's t-test, n=5 per group

Figure 31. Treg frequency in freshly isolated peripheral blood from vitamin D deficient and sufficient human patients. Freshly isolated mononuclear cells from 5 vitamin D deficient and 5 age matched vitamin D sufficient patients were evaluated by flow cytometry for CD4+ Treg frequency. Frequency data are plotted here as Vitamin D deficient (0-30ng/ml) or sufficient (31-100ng/ml).

cytometry at day 14 of culture (Figure 32A). The absolute number of CD4+ Tregs also increased significantly with supplementation of 25nM vitamin D (Figure 32B).

The biological effect of vitamin D is cell type specific and mediated by binding and activation of the vitamin D receptor (VDR) (155). In support of vitamin D supplementation studies, VDR inhibition resulted in decreased Foxp3+ Treg differentiation from whole human UCB cultures (Figure 33). Supplementation and inhibition culture data are in agreement with previously published literature suggesting a positive role for vitamin D in promoting human CD4+ Treg induction, and disagree with the clinical study which reported no correlation between vitamin D levels and UCB Treg frequency (205). These studies are a novel demonstration of the role of vitamin D in promoting Treg induction in our model of human UCB culture.

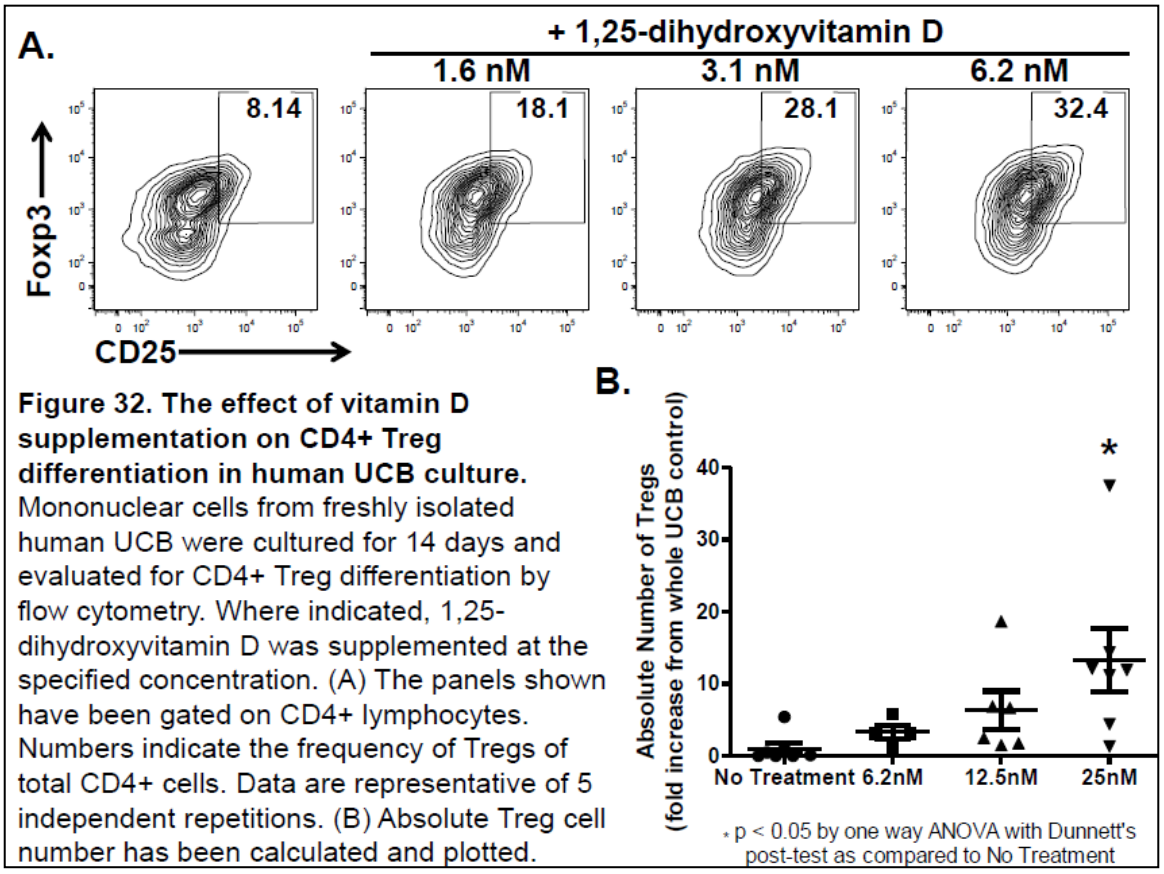


Figure 32. The effect of vitamin D supplementation on CD4+ Treg differentiation in human UCB culture. Mononuclear cells from freshly isolated human UCB were cultured for 14 days and evaluated for CD4+ Treg differentiation by flow cytometry. Where indicated, 1,25-dihydroxyvitamin D was supplemented at the specified concentration. (A) The panels shown have been gated on CD4+ lymphocytes. Numbers indicate the frequency of Tregs of total CD4+ cells. Data are representative of 5 independent repetitions. (B) Absolute Treg cell number has been calculated and plotted.

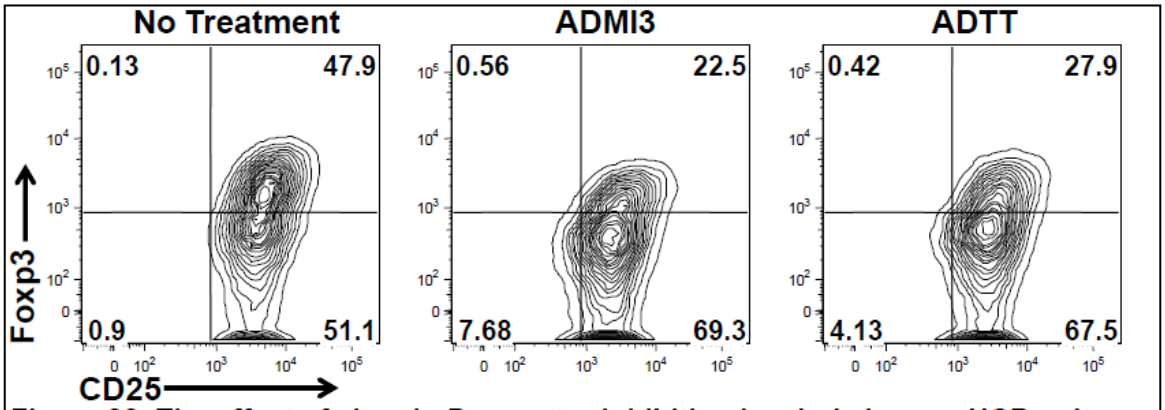


Figure 33. The effect of vitamin D receptor inhibition in whole human UCB culture. Mononuclear cells from freshly isolated human UCB were cultured for 14 days and evaluated for CD4+ Treg differentiation by flow cytometry. The panels show CD25 and Foxp3 expression among total CD4+ cells. ADMI3 and ADTT are independent inhibitors of the vitamin D receptor.

Our previous work has shown that CD14+ CD36^{HI} monocytes are sufficient to promote Treg induction from human naïve CD4+ T cells and recent data demonstrated that vitamin D promotes Treg induction in whole human UCB cultures. Therefore, we aimed to evaluate a possible relationship between vitamin D and CD14+ CD36^{HI} monocytes. We first evaluated expression of cell surface molecules CD36, GARP and LAP on CD14+ CD36^{HI} monocytes, and in data not shown, found no change in expression of these molecules after treatment with vitamin D. We also observed no difference in TGF-β production by whole UCB mononuclear cells with or without vitamin D treatment (data not shown). We next sought to evaluate CYP.27B1 (1 alpha-hydroxylase) expression, the enzyme that activates vitamin D to its biologically active form: 1,25-dihydroxyvitamin D3 or calcitriol (206). CYP.27B1 is known to be expressed in the kidney, skin, bone and some immune cells (206, 207). Flow cytometric studies demonstrated that CYP.27B1 was not only expressed by CD14+ cells from freshly isolated human UCB, but that it was expressed at a higher level in CD14+ CD36^{HI} monocytes as compared to their CD14+ CD36^{LO} and CD14- counterparts (Figure 34).

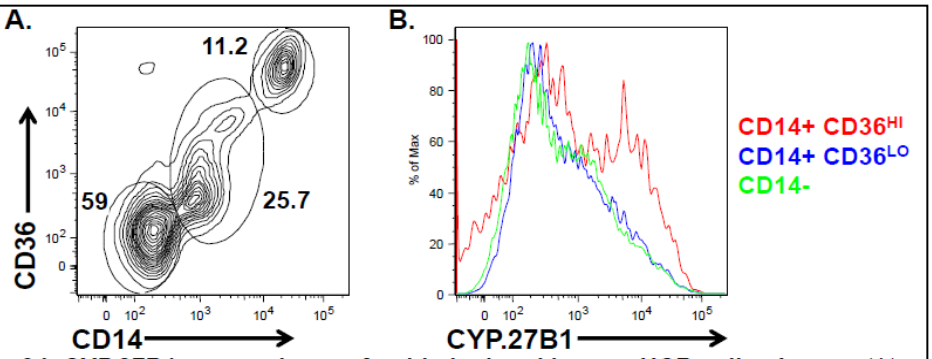


Figure 34. CYP.27B1 expression on freshly isolated human UCB cell subsets. (A) Freshly isolated mononuclear cells from human UCB were evaluated by flow cytometry for expression of CD14 and CD36. Numbers indicate the frequency of each population of total live mononuclear cells. (B) CYP.27B1 expression for the indicated populations is shown.

The observation of increased CYP.27B1 expression in CD14+ $CD36^{HI}$ monocytes suggested that vitamin D may play a role in CD14+ $CD36^{HI}$ monocyte mediated Treg induction. To evaluate this hypothesis, CD14+ $CD36^{HI}$, CD14+ $CD36^{LO}$ and CD14- CD36- subsets were sorted from human UCB as previously described and cultured with naïve CD4+ T cells enriched from freshly isolated human UCB. CD14+ $CD36^{HI}$ monocytes and naïve CD4+ T cells were cultured with or without an inhibitor of TGF-β signaling (SB431542) or a VDR inhibitor (ADMI3). SB431542 has been previously shown to significantly abrogate human Treg induction from whole UCB cultures and we have demonstrated that TGF-β plays a critical role in CD14+ $CD36^{HI}$ mediated Treg induction. ADMI3 has been shown to decrease human Treg induction from whole UCB cultures in our model (Figure 33). If vitamin D plays a role in CD14+ $CD36^{HI}$ monocyte mediated Treg induction, then VDR inhibition would decrease Treg induction in this culture similar to what is seen with TGF-β signaling inhibition. While VDR inhibition resulted in a reduction in Treg frequency, the decrease was not as substantial as with inhibition of TGF-β signaling (Figure 35A). Furthermore, TGF-β signaling inhibition decreased the mean fluorescence intensity (MFI) of Foxp3 expression among total CD4+ T cells as compared to the Foxp3 MFI of naïve CD4+ T cells cultured with CD14+ $CD36^{HI}$ monocytes alone. VDR inhibiton did not result in as considerable of a decrease in Foxp3 MFI among CD4+ T cells (Figure 35A). Additionally, supplementation of active vitamin D to cultures of CD14+ $CD36^{LO}$ or CD14- CD36- cells and naïve CD4+ T cells did not rescue Treg induction by these subsets (Figure 35B). These data suggest that while vitamin D promotes human Treg induction in a whole UCB model, it may not be a

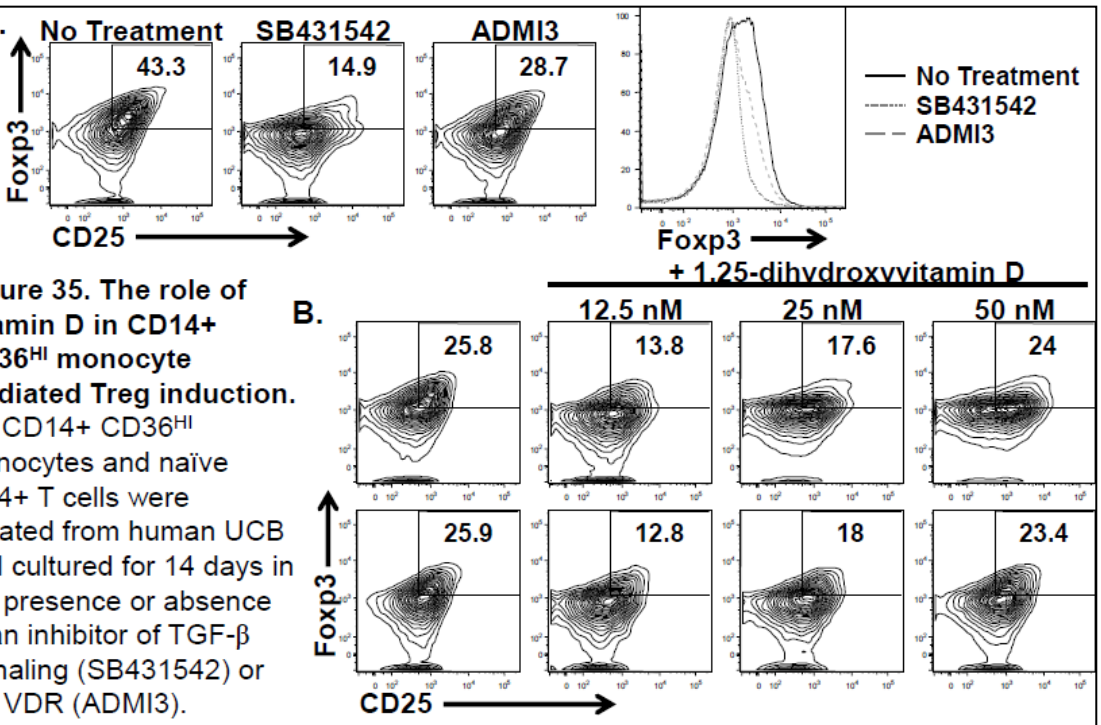


Figure 35. The role of vitamin D in CD14+ CD36^{HI} monocyte mediated Treg induction. (A) CD14+ CD36^{HI} monocytes and naïve CD4+ T cells were isolated from human UCB and cultured for 14 days in the presence or absence of an inhibitor of TGF-β signaling (SB431542) or the VDR (ADMI3). (B) CD14+ CD36^{LO} (top row) or CD14- CD36- (bottom row) cell subsets were cultured for 14 days with naïve CD4+ T cells. Where indicated, 1,25-dihydroxyvitaminD was added at the indicated concentrations. All data in (A) and (B) has been analyzed by flow cytometry for Treg differentiation. All panels have been gated on total CD4+ T cells.

critical component of CD14+ CD36^{HI} mediated Treg induction. However, vitamin D may play a role in maintaining the Treg inducing phenotype of the CD14+ CD36^{HI} monocyte.

III. The Influence of Ethanol Exposure on Human Treg Induction

FAS neonates have an increased incidence of bacterial and viral infection (126, 173, 175-177, 179, 182) and a potential explanation for these increased infection rates is dysregulation of Tregs, which are capable of suppressing immune responses to invading pathogens. To determine the effect of EtOH on human Treg biology, we utilized our previously described model of *ex vivo* human Treg induction from whole UCB. While this model does not exactly mimic the pathology of FAS, we believe this is a relevant

model to study the neonatal immune system and potentially better understand the increased risk of infection documented in association with FAS.

Increasing concentrations of EtOH were added to whole UCB culture wells from 25-50mM (167, 174, 180, 208-213). EtOH was added at the onset of culture only. At day 14 of culture, each treatment group was evaluated for Treg induction via flow cytometry. We observed significantly decreased Treg induction with an EtOH treatment of 50mM, measured as fold change from untreated whole human UCB control cultures (Figure 36). These data suggest that EtOH can dysregulate human Treg induction.

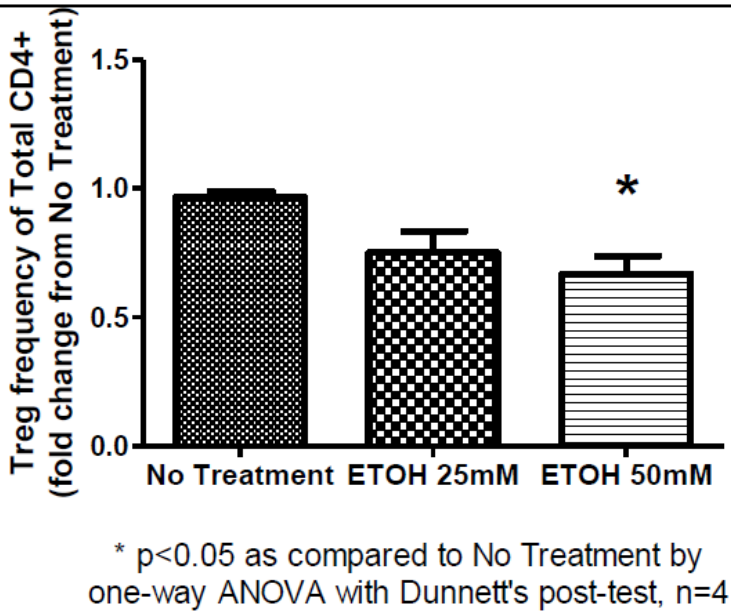


Figure 36. The effect of EtOH on CD4+ Treg differentiation in human UCB culture. Mononuclear cells from freshly isolated human UCB were cultured for 14 days and evaluated for CD4+ Treg differentiation by flow cytometry. Data are presented as fold change of CD4+ Treg frequency from no treatment control cultures. Where indicated, EtOH was added to culture at the indicated concentration.

Previous studies have demonstrated that TGF-β plays an essential role in human Treg induction, so we were interested in whether or not EtOH could alter TGF-β production by freshly isolated UCB mononuclear cells. To determine the influence of

EtOH on TGF-β production, we cultured freshly isolated mononuclear cells from human UCB in the presence or absence of EtOH and performed a TGF-β bioassay on the culture supernatants after 24 hours. This TGF-β bioassay experiment revealed that at the same EtOH concentration resulting in significantly decreased Treg induction, EtOH exposure resulted in significantly decreased TGF-β production by UCB mononuclear cells (Figure 37). These data suggest that EtOH may exert its influence on human Tregs via influencing the TGF-β pathway, which is known to play a critical role in human Treg differentiation.

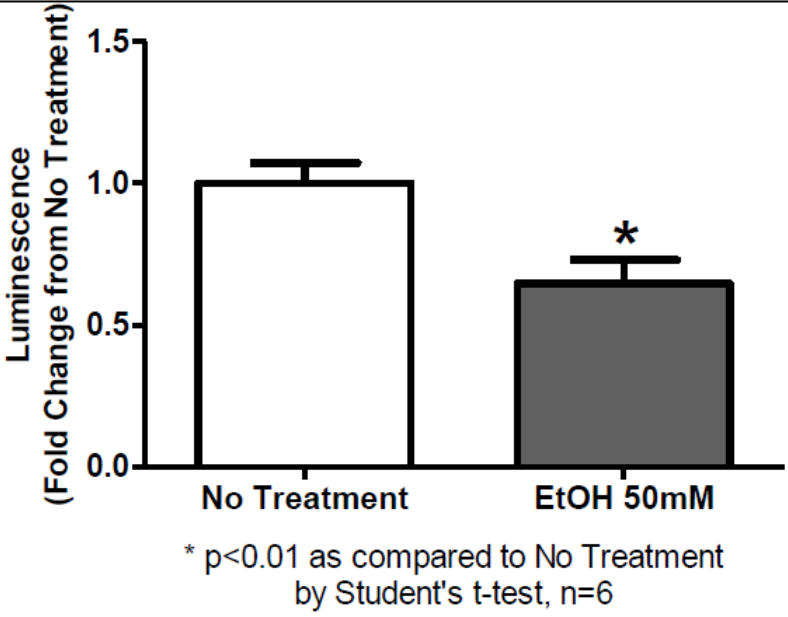


Figure 37. The effect of EtOH on freshly isolated UCB mononuclear cell TGF-β production. Mononuclear cells from freshly isolated human UCB were cultured for 24 hours in the presence or absence of 50mM EtOH. Supernatants were evaluated by TGF-β bioassay where luminescence is a measure of secreted TGF-β.

IV. A Method of Placental Perfusion to Increase Human UCB Donor Cell Yields and Evaluation of the Resulting Cellular Constituents

UCB is an established source of HSC for transplantation in the treatment of both hematologic malignancies and non-malignant disorders, and has many advantages in this application including a low incidence of GvHD (185-190). Transplant success is primarily dependent on HSC cell dose and the HSC yield derived from UCB is sufficient in the treatment of children, but largely inadequate for the treatment of adults (190, 192, 193). Therefore, investigators are evaluating the human placenta as an additional source of HSC (183, 188, 200, 202, 203) in an effort to increase total donor cell yields per transplant unit. To establish a method of increasing donor HSC per transplant unit, we evaluated whether a perfusion pump that mimics physiological blood flow would aid in obtaining cells from the placenta while maintaining the integrity and viability of the cellular constituents.

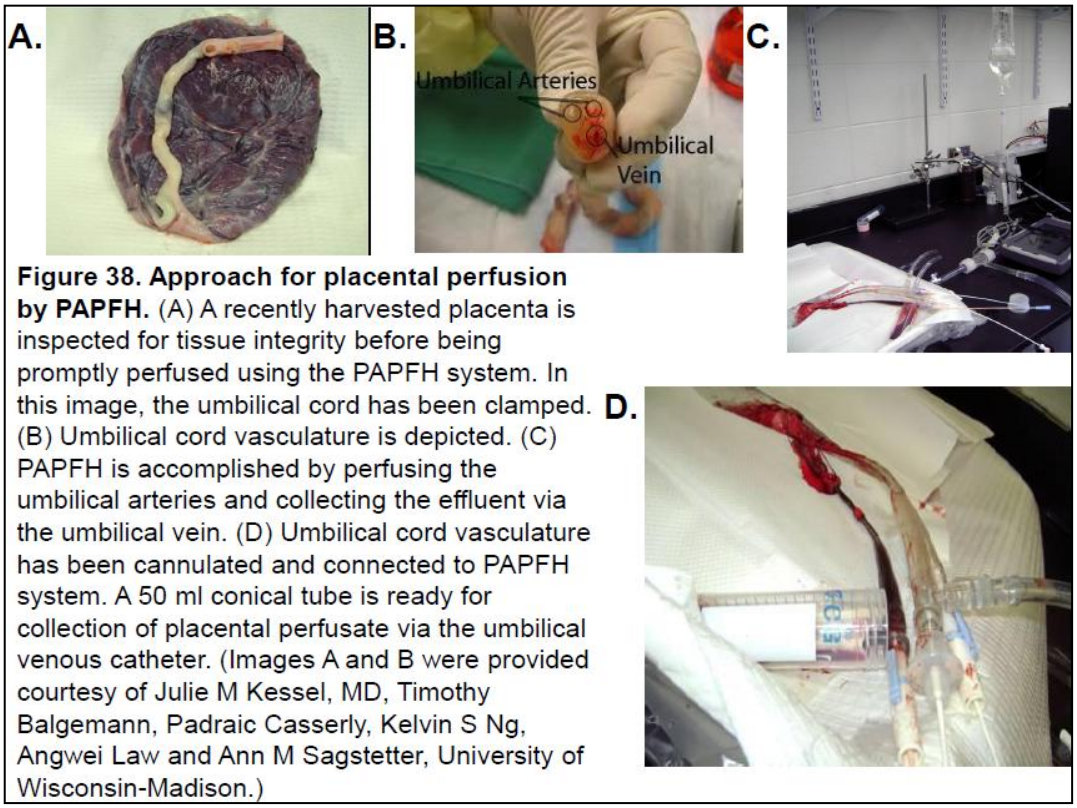


Figure 38. Approach for placental perfusion by PAPFH. (A) A recently harvested placenta is inspected for tissue integrity before being promptly perfused using the PAPFH system. In this image, the umbilical cord has been clamped. (B) Umbilical cord vasculature is depicted. (C) PAPFH is accomplished by perfusing the umbilical arteries and collecting the effluent via the umbilical vein. (D) Umbilical cord vasculature has been cannulated and connected to PAPFH system. A 50 ml conical tube is ready for collection of placental perfusate via the umbilical venous catheter. (Images A and B were provided courtesy of Julie M Kessel, MD, Timothy Balgemann, Padraic Casserly, Kelvin S Ng, Angwei Law and Ann M Sagstetter, University of Wisconsin-Madison.)

Each placenta was first placed in a sterile tray and inspected for tissue integrity of both the placenta and umbilical cord (Figure 38A). If the umbilical cord was not clamped prior to collection, a clamp was placed at the end of the cord. A slipknot was also tied with a silk suture around the umbilical cord close to its root from the placenta and tightened gently to occlude both umbilical arteries and the umbilical vein. The cord was cut proximal to the clamp and milked to remove all residual UCB into a sterile 50ml conical tube. The excess umbilical cord was then cut to leave only 6-8 inches adjacent to the slipknot. Sterile forceps, scissors, clamps and silk ties were used to make a two inch incision in the cord and dissect out the umbilical vessels.

After priming the perfusion circuit (Medical Engineering Company, LLC, Bishop, CA) with sterile saline, the single perfusion inflow tube was connected to the perfusate

reservoir bag: one liter of Perfadex (addmedica) mixed with 500 mcg of Prostin VR Pediatric at room temperature. Perfusate was pumped through the system and any air bubbles were removed with a syringe through the stop cock.

The umbilical vein was then cannulated with a 10 French (Fr) catheter and the arteries with 6 or 8 Fr catheters depending on the size of the vessels (Figure 38B). All catheters were secured with silk sutures. The placenta was then sterilely attached to the perfusion circuit by connecting the circuit outflow tubes to the umbilical arterial catheters and the slipknot was released.

Initial pumping parameters were upstroke volume 200; stroke volume 0.5; pulse rate 72. After initiating the perfusion pump, the perfusate flows through the arteries and the effluent flows out of the umbilical vein. The pumping parameters can be adjusted to achieve efficient perfusion. Samples were heparinized by the addition of 750 ul of 1,000 USP Units/ml heparin to each sterile 50 ml conical collection tube prior to collection. Perfusate was sterilely collected from the venous catheter into heparinized 50ml conical tubes (Figures 38C-D) until the effluent became clear. Samples were immediately placed on ice and processed further.

After PAPFH perfusion of the placenta, samples were subjected to density dependent centrifugation and isolation of mononuclear cells as described and further analyzed for their HSC content. As mentioned previously, HSC cell dose is paramount in successful UCB transplantation; therefore, determination of HSC frequency and absolute cell number is critical in establishing the placenta as a potential HSC source. HSC are self-renewing, multipotent cells that are additionally defined by their not having

committed to a specific differentiation program or cell lineage and expressing the cell surface marker CD34 (191, 194, 214). Therefore, each sample was stained with antibodies against CD34 and lineage markers (Lin: CD3, CD14, CD16, CD19, CD20 and CD56) and HSC were defined as being Lin- CD34+ (Figure 39A). The HSC frequency in UCB and placental perfusate samples was comparable, ranging from 0.6 to 1.6% of total live cells and averaging 1.1% and 0.9% in UCB and placenta samples respectively (Figure 39B). The average total cell number recovered from UCB samples was 2.2×10^8 and average HSC number was 2.3×10^6. The average total cell number recovered by placental perfusion was 3.0×10^7, a yield comparable to previously published methods (198), and average HSC number was 2.3×10^5.

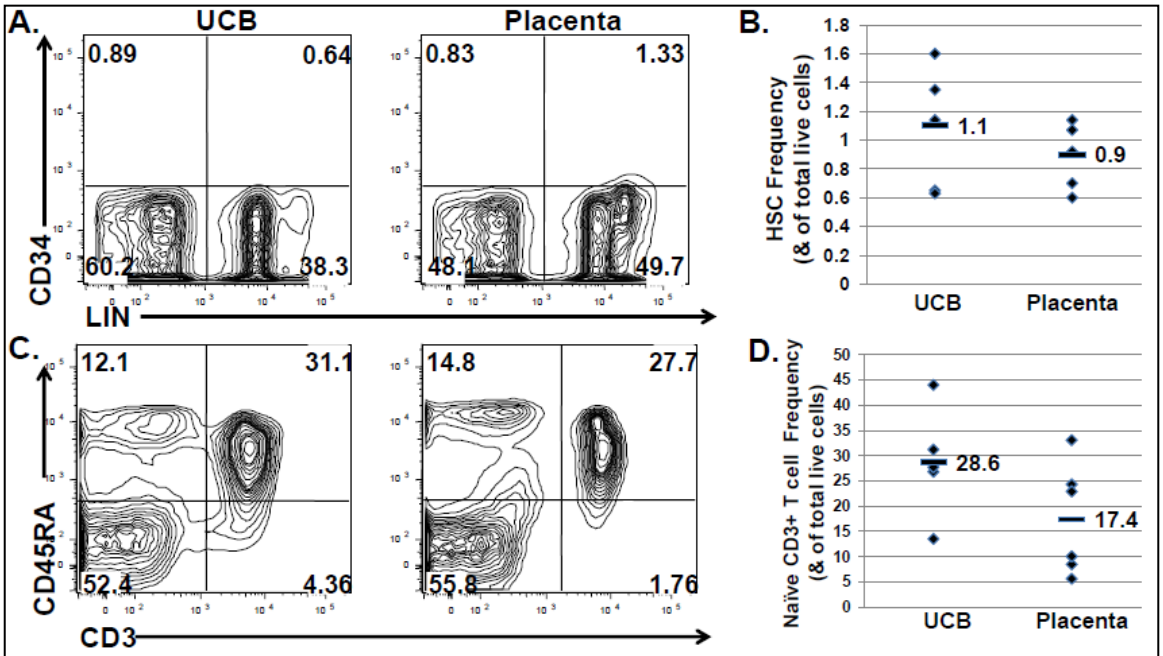


Figure 39. Staining of freshly isolated human UCB and placental perfusate. UCB and placental perfusate samples were processed as described and analyzed by flow cytometry. (A) Representative staining with HSC markers gated on total live cells. The lineage marker (LIN) identifies the following: CD3, CD14, CD16, CD19, CD20 and CD56. (B) HSC frequency of total live cells in both UCB and placenta samples. Numbers indicate average values. (C) Representative CD45RA and CD3 staining of UCB and placenta samples are shown gated on total live cells. (D) Naïve T lymphocyte frequencies of total live cells are shown. Numbers indicate average values.

One of the primary advantages of UCB transplantation, compared to bone marrow or peripheral blood, is a decreased incidence of GvHD (185-190). This is thought to be due, in part, to the immaturity of UCB derived lymphocytes. Therefore, we determined the frequency of naïve T lymphocytes in both UCB and placenta samples, which we defined as CD3+ CD45RA+. Similar to the HSC flow cytometry data, the frequency of naïve T lymphocytes in human UCB and placental perfusate samples was comparable (Figure 39C). Naïve T lymphocyte frequency ranged from 5.6 to 44% of total live cells in UCB and placental perfusate samples. The average naïve T cell frequency was 28.7% and 17.4% of total live cells in UCB and placenta samples respectively (Figure 39D).

T cell antigen reactivity could play a significant role in determining the level of GvHD as instigated by donor T lymphocytes. To compare the antigen receptor reactivity of T cells from both UCB and placental perfusate samples, mononuclear cells enriched from either UCB or placental perfusion were stimulated by the addition of an anti-CD3 antibody. After 7 days of stimulation, cultures were evaluated for T cell differentiation and proliferation. Expression of CD4 and CD8 was comparable between UCB and placenta derived live cells (Figure 40A).

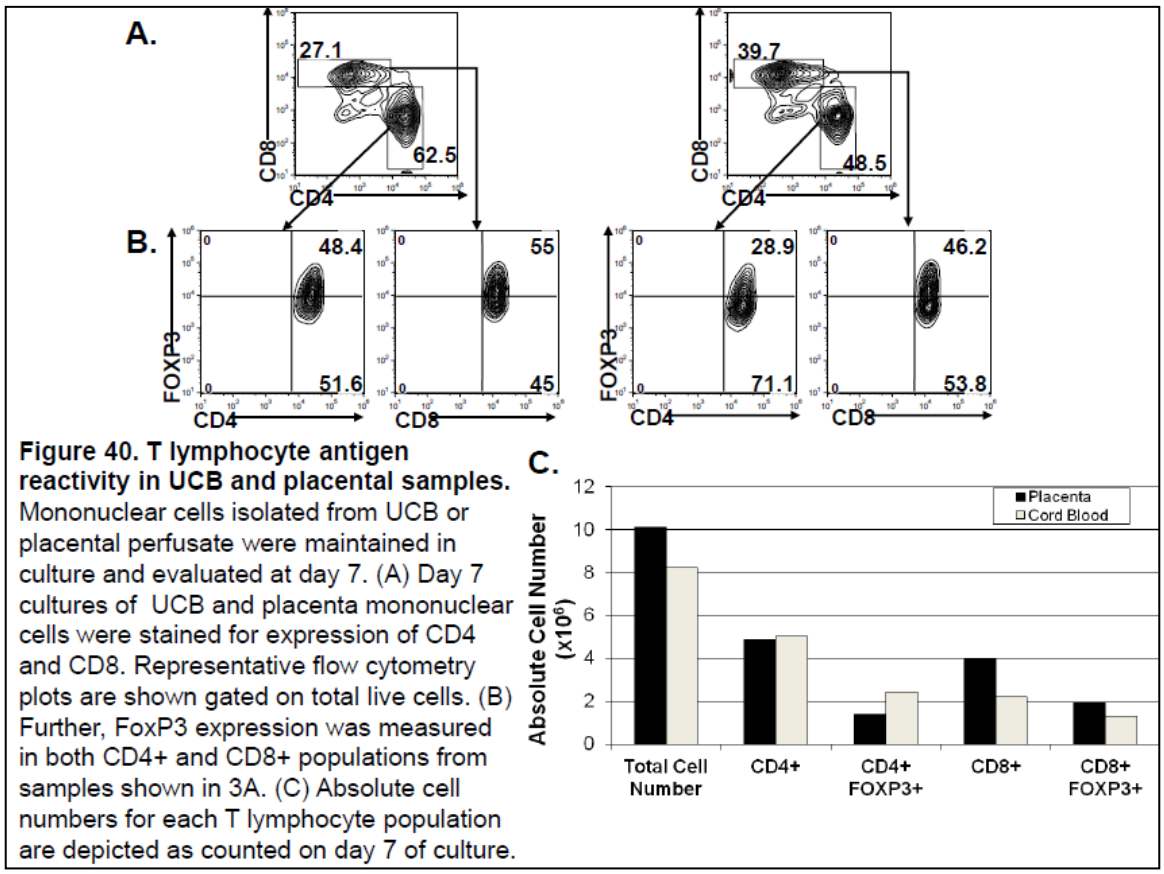


Figure 40. T lymphocyte antigen reactivity in UCB and placental samples. Mononuclear cells isolated from UCB or placental perfusate were maintained in culture and evaluated at day 7. (A) Day 7 cultures of UCB and placenta mononuclear cells were stained for expression of CD4 and CD8. Representative flow cytometry plots are shown gated on total live cells. (B) Further, FoxP3 expression was measured in both CD4+ and CD8+ populations from samples shown in 3A. (C) Absolute cell numbers for each T lymphocyte population are depicted as counted on day 7 of culture.

Expression of Foxp3 by activated T cells is required and sufficient for the establishment of Tregs, which suppress hyperproliferative and autoreactive immune responses (86, 215-217). Therefore, Foxp3 expression among both CD4+ and CD8+

populations was evaluated at day 7 of cell culture. After a week of stimulation, similar percentages of CD4+ and CD8+ T lymphocytes in both UCB and placental perfusate samples expressed the transcription factor Foxp3 (Figure 40B). The absolute cell numbers of each T lymphocyte population were calculated and found to be similar between cultures of human UCB and placental perfusate mononuclear cells (Figure 40C). Thus, T cell reactivity is similar in both human UCB and placental perfusate samples.

One challenge, as is found in all methods of UCB and placenta cell retrieval, is the prospect of maternal cell contamination. This issue is being addressed and techniques are being investigated to both detect and limit maternal cell contamination.

CHAPTER 3

DISCUSSION

I. CD14+ CD36^{HI} Monocyte Mediated Induction of Human Treg Differentiation from Naïve CD4+ T Cells

To date, the mechanism of induction and maintenance of human tolerogenic Tregs has remained elusive. In my dissertation work, I sought to determine the cellular interactions and molecular mechanisms which promote the induction of human Tregs, specifically as it relates to the fetal/neonatal immune system.

The data presented in this manuscript have shown that UCB CD14+ CD36^{HI} monocytes are sufficient to induce human Treg differentiation from UCB naïve CD4+ T cells, whereas CD14+ CD36^{LO} and CD14- populations are not capable of promoting the same induction. Through anti-TCR bead stimulation assays, we have additionally demonstrated that Treg differentiation from UCB naïve CD4+ T cells is not a result of intrinsic programming of UCB naïve CD4+ T cells, but that differentiation to Foxp3+ Tregs requires interaction with the CD14+CD36^{HI} cellular subset. Additionally, CD14+ CD36^{HI} monocytes enriched from adult peripheral blood are similarly capable of inducing Treg differentiation from adult peripheral naïve CD4+ T cells. Therefore, CD14+ CD36^{HI} monocytes are uniquely sufficient to induce human Treg differentiation from naïve CD4+ T cells: a phenomena which applies to both human UCB and adult peripheral blood. Because monocytes are important circulating antigen presenting cells in

the human immune system, capable of surveying the peripheral environment and transporting antigen back to distant organ systems (89, 91, 106-108), I hypothesize that CD14+ $CD36^{HI}$ monocyte mediated Treg induction is a critical mechanism by which the human immune system promotes peripheral tolerance.

TGF-β has long been known to play a positive role in human Treg induction (57, 59, 120, 138, 142) and inhibition studies demonstrated that TGF-β; not IL-10, retinoic acid, or AhR, is intimately involved in our model of human Treg induction as well. TGF-β is secreted and exists in the extracellular milieu in its latent form, bound by LAP (116, 119). The TGF-β/LAP complex may be further bound by latent TGF-β binding proteins (LTBPs) and anchored in the extracellular matrix (119). Latent TGF-β has also been shown, in some circumstances, to be present on the cell surface (120, 121). GARP is a transmembrane molecule known to bind the latent TGF-β-LAP complex and tether it to the cell surface of activated Tregs (124-130). Previously, GARP was only known to be expressed in humans on activated Foxp3+ Tregs, and even shown to mediate some of their suppressive function (126, 127, 129). Flow cytometric labeling of CD14+ $CD36^{HI}$ monocytes revealed that they express increased levels of CD36, LAP and GARP as compared to their CD14+ $CD36^{LO}$ and CD14- counterparts. Based on an exhaustive PubMed search, this is the first demonstration of GARP expression on a human cellular subset other than activated Tregs. Furthermore, GARP mediated cell surface expression of LAP-TGF-β on activated Tregs plays a functional role in their suppressive function. Therefore, so too could the cell surface "presentation" of LAP-TGF-β by GARP on CD14+ $CD36^{HI}$ monocytes mediate their ability to induce Treg differentiation.

In keeping with the importance of TGF-β in our model, CD14+ $CD36^{HI}$ monocytes produce significantly increased levels of latent TGF-β as compared to CD14+ $CD36^{LO}$ and CD14- cellular subsets. TGF-β was only detected in this bioassay after acid treatment of supernatants, which signifies that the TGF-β that was secreted was in its latent form. The active form of TGF-β was below the detection level of this assay as measured for all cellular subsets. Therefore, CD14+ $CD36^{HI}$ monocytes produce a significant amount of latent TGF-β which could be secreted into the supernatant or tethered to the cell surface by GARP. Taken together, these data indicate that latent TGF-β, potentially in its cell surface bound form, is critical in human Treg induction.

Through supplementation of active and latent TGF-β to cultures of CD14+ $CD36^{LO}$ cells, we have demonstrated that high concentrations of active TGF-β partially restore Treg differentiation from cultures of CD14+ $CD36^{LO}$ cells and naïve CD4+ T cells. Supplementation of soluble latent TGF-β did not increase Treg differentiation in the same cultures. It is possible that CD14+ $CD36^{LO}$ cells cannot activate latent TGF-β to promote Treg differentiation. The inability of CD14+ $CD36^{LO}$ cells to activate latent TGF-β and promote Treg induction could potentially be explained by their decreased ability to bind and present latent TGF-β by GARP, and their decreased expression of CD36, which binds the TGF-β activating enzyme TSP-1. It is also possible that the cell surface bound form of latent TGF-β is the form required for induction of Treg differentiation. We hypothesize that while soluble TGF-β may contribute to human Treg induction, cell surface-bound latent TGF-β, presumably tethered to the cell surface by GARP, is required for CD14+ $CD36^{HI}$ monocyte mediated Treg induction from naïve

CD4+ T cells. I hypothesize that the importance of cell surface bound TGF-β in our *ex vivo* model of human Treg induction points to its potential significance under physiological conditions as well. This is one possible avenue by which research and clinical therapeutics directed at soluble TGF-β and human Treg induction could potentially be improved. Perhaps instead of therapeutics which simply supplement soluble TGF-β, delivery methods which provide TGF-β presented by GARP would be more effective.

Further supporting the importance of cell surface bound TGF-β in human Treg induction, genetic manipulation of CD36 and GARP expression in THP.1 cells has shown that decreased expression of either molecule by THP.1 cells significantly decreases the ability of that cell population to induce Treg differentiation. These data suggest that both CD36 and GARP are required for THP.1 mediated human Treg induction from naïve CD4+ T cells. These studies represent not only a novel demonstration of GARP expression on a human cell subset other than activated Tregs, but also a novel functional role of GARP expression in human Treg induction.

Confocal microscopy imaging studies demonstrated that CD14+ cells express GARP and CD14- cells do not, as expected, but also that GARP and CD36 are not co-localized in freshly isolated CD14+ UCB cells. We have seen in other flow cytometry experiments, that the majority of GARP exists intracellularly and that only a small portion is expressed on the cell surface. These data suggest a tight regulation of GARP localization in the cell and that, perhaps, very specific stimuli promote cell surface expression of GARP. My hypothesis is that after interaction with a naïve CD4+ T cell,

the CD14+ CD36^{HI} monocyte receives a signal which promotes the cell surface co-localization of CD36 and GARP and subsequent activation of TGF-β, resulting in induction of Treg differentiation. It is also possible that CD36 and GARP do not need to physically interact to promote human Treg induction. Imaging studies of CD14+ CD36+ monocytes after 24 hours of culture with naïve CD4+ T cells are ongoing to determine whether or not interaction with naïve CD4+ T cells promotes the co-localization of CD36 and GARP.

LPS treatment of CD14+ CD36^{HI} monocytes decreases their ability to promote Treg induction, their CD36 and GARP expression, and decreases their TGF-β production. This LPS model represents a physiologic activation of CD14+ CD36^{HI} monocytes by antigenic stimulation and suggests that as this monocyte subset is naturally exposed to antigen, it decreases Treg induction, potentially in favor of the desired effector CD4+ T cell response, but this hypothesis has not been directly tested. While LPS activation may have additional unknown effects on CD14+ CD36^{HI} monocytes, these data support the hypothesis that expression of CD36 and GARP, as well as production of TGF-β, are critical factors in CD14+ CD36^{HI} monocyte mediated Treg induction. These data also suggest that the relative immaturity, or antigenic naïvety, of CD14+ CD36^{HI} monocytes contributes to their ability to induce human Treg differentiation.

Taken together, we have demonstrated that CD14+ CD36^{HI} monocytes are functionally specialized and sufficient to induce human Treg differentiation from naïve CD4+ T cells, dependent on expression of CD36 and GARP. These findings suggest that the cellular interaction between CD14+ CD36^{HI} monocytes and naïve CD4+ T cells plays

an important role in the generation of peripheral tolerance and perhaps plays a role in establishment and/or maintenance of maternal-fetal tolerance as both CD36 and GARP are expressed in the placenta (122, 218, 219). The ability to induce human Treg differentiation makes CD14+ $CD36^{HI}$ monocytes and cell surface bound TGF-β unique therapeutic targets for the treatment of autoimmune conditions, transplant rejection, allergy and conditions of disrupted maternal-fetal tolerance. Additionally, inhibition of this cell subset and/or the mechanisms involved in CD14+ $CD36^{HI}$ mediated Treg induction could prove beneficial in impeding cancer pathogenesis.

II. The Role of Vitamin D in Human Treg Induction

Our data suggest that CD14+ $CD36^{HI}$ monocyte mediated Treg induction is an important contributor to peripheral induced tolerance. This process, however, is certainly not the only mechanism promoting peripheral tolerance and is also presumably subject to influence by other immune cells and plasma factors.

Because supplementation of vitamin D increases, and VDR inhibition decreases human Treg differentiation in whole UCB culture, vitamin D may be one such plasma factor that promotes human Treg induction. Although CD14+ $CD36^{HI}$ monocytes express high levels of the vitamin D activating enzyme, our data suggest that activation of vitamin D is neither sufficient nor required for CD14+ $CD36^{HI}$ monocyte mediated Treg induction. Inhibition of the VDR does not significantly abrogate Treg induction in this model and supplementation of vitamin D to cultures of naïve CD4+ T cells and either CD14+ $CD36^{LO}$ or CD14- APCs does not rescue Treg induction by these subsets. Vitamin D treatment also does not alter TGF-β production by whole UCB mononuclear

cells, another important aspect of CD14+ CD36^{HI} monocyte biology and Treg induction. While vitamin D may not directly mediate CD14+ CD36^{HI} monocyte Treg induction, it may play a role in maintaining the Treg inducing phenotype of the monocyte itself or have an additional role in human Treg differentiation not yet identified.

Perhaps, CD14+ CD36^{HI} monocytes express high levels of CYP27B1 and are capable of activating vitamin D to its biologically active form, but this vitamin D acts via other cell subsets and mechanisms to induce Treg differentiation. It is possible that vitamin D produced by circulating CD14+ CD36^{HI} monocytes acts on other APC subsets or naïve CD4+ T cells directly, but that CD14+ CD36^{HI} monocytes themselves are not affected. It is also possible that CD14+ CD36^{HI} monocytes are affected by vitamin D in a manner that we have not yet tested. The TGF-β downstream signaling molecule, Smad3, is capable of interacting with and serving as a co-activator of the VDR (164-166), which provides a point of communication between vitamin D and TGF-β signaling. Our data demonstrate that TGF-β is critical to the biology of CD14+ CD36^{HI} monocytes, so the interaction of Smad3 and the VDR could have unique consequences for this cellular subset. The effect of vitamin D is very cell specific and it has already been shown to promote monocyte differentiation (156) and the development of tolerogenic, Treg inducing DCs (146). Therefore, vitamin D may uniquely maintain the Treg inducing phenotype of CD14+ CD36^{HI} monocyte by an as yet unknown mechanism. Future directions of this research include determining the influence of vitamin D on CD14+ CD36^{HI} monocytes and the mechanism by which vitamin D promotes human Treg induction.

Though many questions remain, we have demonstrated that vitamin D positively regulates Treg differentiation in human whole UCB cultures. This is an important concept to acknowledge because vitamin D deficiency is a highly prevalent condition, most common in nonambulatory, elderly, dark-skinned, institutionalized and chronically ill individuals. Additionally, vitamin D deficiency may have much further reaching consequences beyond bone homeostasis and skeletal health. In fact, vitamin D is known to be highly immunomodulatory. Vitamin D supplementation has been show to have broad anti-inflammatory effects, enhance cancer treatments, attenuate autoimmune reactions such as systemic lupus erythematosis, rheumatoid arthritis and multiple sclerosis, and even to decrease the severity of graft-vs-host disease (GVHD) (150-152). Therefore, our data suggest that management of vitamin D deficient patients and monitoring of vitamin D oral supplementation should consider the patient's immune status and an effort should be made to evaluate and maintain immune homeostasis. Further research could lead to the development of new or improvement of current therapeutics with vitamin D for conditions such as autoimmune disease where the increase of Treg populations is a desired clinical outcome.

III. The Effect of EtOH on Human Treg Induction

Vitamin D is an essential fat-soluble vitamin that can be ingested or synthesized in our bodies after sun exposure, and therefore represents and endogenous means of influence on human Treg induction. On the contrary, alcohol exogenously influences the human immune system. Ethanol (EtOH) exposure dysregulates the immune response and is immunosuppressive, moreover EtOH exposure only *in utero* significantly impairs

immune competency and is immunoteratogenic (167-173). Human maternal alcohol consumption is detrimental to the developing fetus, resulting in spontaneous abortion, growth retardation, facial abnormalities, damage to the central nervous system, behavioral disorders, cognitive disabilities, FAS and increased susceptibility to bacterial and viral infection (126, 175-179). FAS refers to the combination of distinguishing facial features, reduced birth weight and behavioral and cognitive impairment characteristic of a child exposed to EtOH *in utero* (178).

FAS neonates have significant impairments in immune function (168, 170, 171, 173, 175-177, 180) and the risk of neonatal infection correlates with maternal alcohol consumption; however, little is known about the direct influence of EtOH on the immune system. Even less is known about the influence of EtOH exposure on human Treg biology. While our model of *ex* vivo human Treg induction does not exactly mimic the conditions of FAS, we believe that it provides a unique avenue to investigate this question.

To that effect, we have demonstrated that at a concentration of 50mM, EtOH exposure significantly decreases Treg induction from whole human UCB mononuclear cell cultures. These data suggest that EtOH can significantly dysregulate the human immune response by specifically blocking Treg differentiation. Human UCB is a close physiological model to an *in utero* or neonatal environment and we believe that our data supports that EtOH exposure via maternal consumption can have a significant effect on the immune system of a developing fetus. Because the maternal fetal environment is uniquely tolerogenic, dependent in part on Tregs, these data suggest that *in utero* EtOH

exposure could potentially disrupt maternal fetal tolerance. Indeed, maternal alcohol consumption has been associated with an increased rate of spontaneous abortion (178, 181).

TGF-β plays an essential role in human Treg induction and could be a means by which EtOH alters human Treg differentiation. We cultured freshly isolated mononuclear cells from human UCB in the presence or absence of EtOH and performed a TGF-β bioassay on the culture supernatants after 24 hours. We found that at the same EtOH concentration resulting in significantly decreased Treg induction, EtOH exposure results in significantly decreased TGF-β production by UCB mononuclear cells. Therefore, EtOH may act via the TGF-β pathway to decrease human Treg induction. TGF-β is not only known to promote human Treg induction, but also to play a critical role in the maintenance of maternal fetal tolerance. Therefore, these data further support the detrimental influence that EtOH can exert on a developing fetus.

The significant decrease in both human Treg induction and TGF-β production from human UCB mononuclear cells after EtOH exposure is thought-provoking and incites a number of future directions. While we know that Treg frequency is decreased after EtOH exposure, Treg function may be similarly impaired. It is also important to determine the influence of EtOH on Treg function by exposing Tregs to EtOH and directly evaluating suppressive activity. If EtOH exposure impairs Treg effector function as we expect, then we will observe an increase in responder cell proliferation when cultured with EtOH treated Tregs as compared to non-EtOH treated Tregs.

We have demonstrated that CD14+ $CD36^{HI}$ monocytes are sufficient to induce Foxp3+ Treg differentiation from naïve human CD4+ T cells. Therefore, I would like to evaluate the effect of EtOH on CD14+ $CD36^{HI}$ monocyte expression of CD36, GARP and LAP: molecules critical for human Treg induction. I would also like to directly incubate either CD14+ $CD36^{HI}$ monocytes or naïve CD4+ T cells with EtOH and wash prior to setting up a co-culture. EtOH treatment of only one cell subset prior to cell culture would test whether EtOH acts on the CD14+ $CD36^{HI}$ monocyte and/or naïve CD4+ T cell to decrease Treg induction. Additionally, CD14+ $CD36^{HI}$ monocyte mediated Treg induction is abrogated by pretreatment of the $CD36^{HI}$ monocytes with LPS, suggesting that exposure to immune activating signals alters the function of this monocyte subset. It has been shown that EtOH blocks human monocyte activation in response to LPS stimulation (168, 180, 208, 209, 211-213, 220). I hypothesize that EtOH can similarly block the LPS mediated activation of the CD14+ $CD36^{HI}$ subset, which could influence not only Treg induction, but also numerous other aspects of immune activation.

While we have demonstrated that EtOH significantly reduces human Treg induction and TGF-β production from human UCB mononuclear cells, many questions remain. Understanding the effect of EtOH on the human immune system, particularly Treg biology, is critical to comprehending the intricate maintenance of maternal fetal tolerance and pathogenesis of FAS. It is necessary to continue this line of research because, unfortunately, up to 30% of women report consuming alcohol at some time during their pregnancy (175, 181, 182) and the prevalence of FAS in the United States

remains between 0.2-1.5 in every 1000 live births (126). Advancing this research could potentially reveal novel treatment strategies for preventing spontaneous abortion and attenuating the increased risk of infection and immunopathology associated with FAS.

IV. Placental Perfusion as a Means to Increase Human UCB Donor Cell Yields

Vitamin D and EtOH may influence human Treg biology and have therapeutic applications relevant to numerous human disease pathologies, but human UCB is itself a therapeutic, providing a source of hematopoietic stem cells in the treatment of hematologic malignancies and non-malignant disorders (183, 184). The advantages of transplanting UCB compared to bone marrow or peripheral blood include ease and availability of sample acquisition leading to decreased time to transplant, less stringent HLA matching requirements (187, 188) and decreased incidence of graft-versus-host disease (GvHD) (185-187, 189, 190).

As mentioned, UCB is a viable source of CD34+ hematopoietic stem/progenitor cells (HSC) (190, 191); however, the limited cell number obtained from a single UCB sample presents challenges to widespread therapeutic application. Investigators have pursued three primary avenues to combat this challenge: *ex vivo* expansion of UCB cell types (194), infusion of more than one UCB unit per recipient (195-197), and increasing cell recovery from UCB and placenta samples (198, 199). The human term placenta has been identified as an additional source of HSC (198-203) and we sought to evaluate whether a perfusion pump that mimics physiological blood flow would aid in obtaining cells from the placenta while maintaining the integrity and viability of the cellular constituents.

Since supplementing UCB transplantation with HSC obtained from placental perfusion is the clinical goal, determining HSC frequency and absolute number is critical in establishing the placenta as a potential cell source of HSC. Via PAPFH (Medical Engineering Company, LLC, Bishop, CA), we determined that UCB and placental perfusate contain a comparable HSC (Lin-CD34+) frequency averaging 1.1% and 0.9% in UCB and placenta samples, respectively. The average HSC number recovered from UCB samples was 2.3×10^6. The average total cell number recovered by placental perfusion was 3.0×10^7, a yield comparable to previously published methods (198), and average HSC number was 2.3×10^5. These data indicate that the placenta, as perfused by PAPFH, represents a feasible source of HSC to increase total donor HSC number by 10% per transplant unit. While we do not know the clinical significance of this increase, the critical importance of cell dose in successful transplant points to the value of any increase in HSC cell number.

After determining that placental perfusate is a viable and potentially important source of HSC to increase total donor cell yields from UCB alone, we sought to evaluate other parameters of successful transplantation as they applied to placental perfusate. Decreased incidence of GvHD is one of the primary advantages of UCB as compared to bone marrow or peripheral blood transplantation (185-190). This is thought to be due, in part, to the immaturity of UCB derived lymphocytes. Therefore, we evaluated the frequency of naïve T lymphocytes in both UCB and placenta samples and found that the frequency of naïve T lymphocytes in human UCB and placental perfusate samples was comparable. While naïve CD4+ T cell frequencies were similar in UCB and placental

perfusate, these data did not necessarily imply similar reactivity among naïve CD4+ T cell populations. In order to compare the antigen receptor reactivity of T cells from both UCB and placental perfusate samples, mononuclear cells enriched from either UCB or placental perfusion were stimulated by the addition of an anti-CD3 antibody. Cultures were evaluated for T cell differentiation and proliferation and we found that CD4 and CD8 expression was comparable between UCB and placenta derived cultures. Additionally, similar percentages and absolute cell numbers of CD4+ and CD8+ T lymphocytes in both UCB and placental perfusate samples expressed the transcription factor Foxp3. Therefore, not only do UCB and placental perfusate have similar naïve T cell composition, but also T cell reactivity is similar in both samples, including differentiation of immunoregulatory Foxp3+ T cells. These data suggest that the risk of GvHD as mediated by donor T lymphocytes would be comparable between UCB and placental samples: an important advantage of UCB transplantation. The exact explanation of why UCB has a decreased incidence of GvHD is unknown, but our data suggest that UCB is a uniquely tolerogenic environment, dominated by CD14+ $CD36^{HI}$ monocyte mediated TGF-β production and Treg induction. Therefore, in future studies, I would like to evaluate CD14+ $CD36^{HI}$ monocyte composition and function in placental perfusate. Interestingly, GARP, a molecule that we have demonstrated to be required for human Treg induction by CD14+ $CD36^{HI}$ monocytes, is expressed in the endothelial cells of the mouse placenta (122). This finding suggests to me that some of the same principles observed in UCB could be applied to the tolerogenic, Treg rich environment of the placenta and the maintenance of maternal fetal tolerance. I would be very curious to

explore the role of GARP in the human placenta and determine if it functions in a similar Treg inducing capacity as we have observed on CD14+ $CD36^{HI}$ monocytes.

Taken together, our data demonstrate that PAPFH is an efficient and feasible method of placenta perfusion that increases HSC donor cell yields as compared to UCB samples alone. The importance of cell number in successful UCB transplantation highlights the need for optimization of these techniques to enhance HSC retrieval and the efficacy UCB transplantation. Additionally, these data suggest that placenta derived lymphocyte populations by this method are phenotypically and functionally comparable to UCB cells. These findings propose that the therapeutic advantages of UCB, including decreased incidence of GvHD, may also be applicable to placenta derived cells. PAPFH is a practical method of obtaining additional HSC per transplant unit, and we believe this study represents an important advancement in the field of UCB transplantation.

V. Conclusion

While we believe that our research is a significant contribution to understanding the human immune system and specifically, fetal tolerance, there are certainly many questions remaining, some of which are already under investigation in our laboratory. The importance of CD14+ $CD36^{HI}$ monocyte induced Treg differentiation and the requirement of CD36 and GARP in this model have far reaching influence and potential application in numerous clinical scenarios. For example, it is conceivable that treatment of bone marrow or solid organ transplant tissue with a therapeutic to promote GARP expression would, in turn, enhance transplant tolerance. Perhaps GARP and CD36 expression can be induced in the human placenta and prevent spontaneous abortion, not

limited to that seen as a consequence of maternal alcohol exposure. What if tolerance to an antigen could be induced by systematic exposure to said antigen in the context of a CD36 and GARP expressing vector? And what if this became a customized means to prevent or treat allergy? I would be thrilled and truly blessed to watch the evolution of these concepts and hopefully be involved in some of the research and clinical application.

CHAPTER 4

MATERIALS & METHODS

UCB Collection

UCB samples were collected by nurses in the Birth Center at Gottlieb Memorial Hospital, a Loyola University Health System affiliate. UCB was collected into sterile, anticoagulant treated BLOOD-PACK™ units (Fenwal, Inc., IL) and processed as soon as possible after collection.

Mononuclear Cell Isolation from UCB and Placental Perfusate

Heparinized UCB samples were diluted 1:1 with room temperature 1 x PBS. Mononuclear cells were then enriched by density dependent centrifugation using Lymphocyte Separation Medium (Cellgro). The buffy layer containing mononuclear cells was then carefully extracted by pipetting. The centrifugation protocol was repeated 2-3 times until the buffy layer contained minimal red blood cell contamination. Mononuclear cells were then collected, counted and further subjected to flow cytometry cell sorting or cell culture protocol.

Flow Cytometry

Cells were washed in 1 x PBS with 1.0% FCS, 0.1% sodium azide, and stained with the indicated mouse anti-human antibodies (all Biolegend) for 30 minutes. A Foxp3 Fix/Perm Buffer Set (Biolegend) was utilized for intracellular staining of the transcription factor Foxp3. For anti-GARP staining, cells were washed as described, incubated with a

purified anti-human GARP antibody (Biolegend) for 30 minutes at 37˚C, washed and subsequently incubated with a goat anti-mouse IgG antibody (Biolegend) for 30 minutes. Data was collected using FACS Canto II (Becton Dickinson) and data analysis was completed on FlowJo software (Tree Star, Inc.).

Cell Sorting and Enrichment

Freshly isolated mononuclear cells from blood samples were washed in 1 x PBS with 2.0% FCS and incubated with mouse anti-human CD14-FITC and CD36-PE antibodies (Biolegend) for a minimum of 30 minutes in the dark at room temperature. Cells were washed and resuspended in 1 x PBS with 2.0% FCS and sorted using FACS Aria (Becton Dickinson).

Total CD4+ T cells, naïve CD4+ T cells and CD14+ monocytes were enriched from whole UCB mononuclear cells via BD IMag Enrichment Sets according to BD protocol.

Cell Culture

Enriched cells were cultured in 48 well or 96 well U-bottom plates in RPMI-1640 Medium (Thermo Scientific) with 10% FCS (Atlanta Biologicals), β-mercaptoethanol (50 uM), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 I.U./ml), streptomycin (100 ug/ml), HEPES (10 mM), and MEM essential and non-essential amino acids (Invitrogen). Cultures were supplemented with exogenous IL-2 (PeproTech) at 10 ng/ml and soluble α-CD3 (eBioscience) at 0.2 ug/ml. Cultures were continued for up to 21 days. Media was changed every 2-3 days and IL-2 concentrations were maintained throughout.

Polystyrene beads were incubated with 10ug anti-human CD3 and 10ug anti-human CD28 per 100ul bead suspension overnight at room temperature, washed in 1 x PBS and blocked in 1 x PBS with 10% BSA for 2 hours. After blocking, beads were washed again and resuspended in cell culture medium to 2 x 10^6 beads per 50ul media. For stimulation, 1 x 10^6 cells were incubated with 50ul of prepared bead suspension for 1 hour at 37˚C before transferring to cell culture plates. When polystyrene beads were used in culture, no additional soluble anti-human CD3 was added.

Where indicated, an inhibitor of TGF-β signaling, SB431542 (Sigma-Aldrich) was added to cell culture at 1-10μM concentrations. Latent or activated recombinant human TGF-β (R&D Systems) was added to cell cultures at the indicated concentrations.

For LPS pretreatment, sorted CD14+ $CD36^{HI}$ cells were cultured overnight in cell culture media with 100ng/ml LPS. After 24 hours, cells were washed in media three times and used in cell culture or flow cytometric applications.

TGF-β Bioassay

Cells were cultured in bioassay medium, DMEM with penicillin (100 I.U./ml), streptomycin (100 ug/ml) and 1 x Nutridoma (Roshe), and supernatants harvested for analysis. MFB-F11 cells (TGFβ1 KO MEFs transfected with a SMAD-binding element promoter fused to a secreted alkaline phosphatase (SEAP) TGFβ reporter gene) were maintained in culture with MFB-F11 culture media, DMEM with penicillin (100 I.U./ml), streptomycin (100 ug/ml) and 10% FCS. One day prior to harvesting supernatants for analysis, MFB-F11 cells were counted and seeded at 4 x 10^4 cells per well of a 96 well flat bottom tissue culture plate and incubated overnight at 37˚C. The next day, seeded

wells were washed twice with 100ul room temperature 1 x PBS and 100ul bioassay medium was added. MFB-F11 cells were allowed to rest for 2 hours in bioassay medium before the addition of culture supernatants. TGF-β titration controls were established up to 1000pg/ml using human recombinant activated TGF-β (R&D Systems). To prepare culture supernatants, 5ul 1N HCl was added per 100ul supernatant and incubated at room temperature for 15 minutes before neutralization with 5ul 1N NaOH per 100ul supernatant. After acid treatment and neutralization, 100ul culture supernatant or TGFβ control was added to each MFB-F11 seeded well and the plate was incubated at 37˚C overnight.

Detection of SEAP was measured by a Chemiluminescent Reporter Gene Assay System (Applied Biosystems) according to protocol. Luminescence data were converted to corresponding supernatant TGF-β concentrations in pg/ml according to the TGF-β titration curve for each individual experiment.

Proliferation Assay

To perform a proliferation assay, CD4+ CD25+ Tregs were enriched from indicated day 14 cultures via a Human Regulatory T Lymphocyte Separation Set – DM (BD IMag). Additionally, total CD4+ T cells were enriched as previously described from syngenic samples that had been frozen since the day of collection. Cells were cultured in 96 well U-bottom plates at increasing Treg : Responder Tcell ratios in cell culture media with soluble α-CD3 (eBioscience) at 0.2 ug/ml. The same number of responder CD4+ T cells were cultured in each well with an increasing ratio of Tregs added to each subsequent well. Wells of either responder CD4+ T cells or Tregs alone were cultured as controls for

proliferation. Culture was maintained for 7-10 days at 37˚C until measure of cell proliferation by Cell Titer Glo® Luminescent Cell Viability Assay (Promega) according to protocol.

shRNA Based Knock Down of CD36 or GARP Expression

shRNA based knock-down of either CD36 or GARP in the human monocyte cell line, THP.1, was carried out using GIPZ lentiviral shRNA vectors (Thermo Scientific Open Biosystems) targeting either CD36 or GARP and an empty vector control. These vectors arrived as individual bacterial clones. Each clone was cultured, plasmids were prepared using a Midi Prep Kit (Qiagen), and restriction digests were performed to confirm plasmid integrity. Using Lipofectamine 2000, 293T cells were transfected with each shRNA plasmid and the resulting viral supernatants were used in spinoculation of THP.1 cells. After spinoculation, THP.1 cells were cultured and evaluated for expression of CD36 and GARP by flow cytometry at 72 hours post transduction. Transduced THP.1 populations were sorted based on GFP expression and maintained in culture with puromycin, an antibiotic resistance marker included in the GIPZ plasmid. Prior to culture with naïve CD4+ T cells, THP.1 cells were irradiated at 3,000 rad.

Confocal Staining and Imaging

The desired cell population for imaging was washed in 1 x PBS and resuspended to 50,000 cells per 100ul sterile 1 x PBS. 100ul of the cell suspension was cytospun for 10 minutes onto Superfrosted PLUS Slides (VWR). Cell suspension was encircled with a PAP pen and allowed to dry. Cells were fixed with 37˚C 4% PFA (filtered) for 15 minutes and washed 3 times with sterile 1 x PBS for 5 minutes each wash. To block, a

solution of 10% BSA and 3% normal goat serum in sterile 1 x PBS was added to slides and allowed to incubate at room temperature for a minimum of 30 minutes. Blocking solution was gently poured off, and without washing, primary antibodies were diluted in sterile 1 x PBS and added to slides: mouse anti-human GARP at 1:100 and rabbit anti-human CD36 at 1:800. Slides were incubated overnight in the dark at 4˚C.

After overnight incubation, slides were washed 3 times with sterile 1 x PBS for 5 minutes each wash. Secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG, respectively) were diluted at 1:500 in sterile 1 x PBS, added to slides and incubated in the dark at room temperature for 1 hour. Slides were washed 3 times with sterile 1 x PBS for 5 minutes. Hoescht nuclear stain was diluted at 1:20,000 and incubated on slides for 2 minutes before slides were washed again 3 times in sterile 1 x PBS. Slides were allowed to dry for a few minutes before adding mounting media and carefully applying coverslips. Slides were stored at 4˚C until imaging.

Placenta Collection and Perfusion

Full term placentas were collected in sterile containers in the Labor and Delivery Department at Loyola University Medical Center and processed immediately. The processing protocol is described in the main text.

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VITA

Kathleen Elizabeth Jaeger was born on November 28th, 1984 in St. Louis, Missouri to David and Tracy Mishler. She received her secondary education at Visitation Academy in St. Louis, Missouri. In 2003, Kathleen began her undergraduate education at Miami University, Ohio and graduated in 2007 with a BA in Microbiology and a Minor in Molecular Biology. While at Miami University, Kathleen was awarded the Harrison Scholarship throughout her attendance, Cum Laude Latin Honors and University Honors. She also received an Undergraduate Research Award for her work on a project entitled "Increased mRNA Stability in Dexamethasone Enhanced Indoleamine 2,3-Dioxygenase Activity." Kathleen also worked on a project aimed at categorizing SNP mutations associated with the development of breast cancer at The Genome Institute at Washington University, St. Louis, MO.

In August of 2007, Kathleen matriculated into the MD/PhD program at Loyola University Chicago Stritch School of Medicine, and in the summer of 2009, joined Dr. Makio Iwashima's laboratory. Under Dr. Iwashima's mentorship, Kathleen pioneered a project examining the CD14+ $CD36^{HI}$ monocyte mediated induction of human T regulatory cells from naïve CD4+ T cells. Kathleen was awarded a pre-doctoral fellowship position on an Alcohol Research Program training grant administered by Dr. Elizabeth J. Kovacs. In 2012, Kathleen was awarded a pre-doctoral fellowship Ruth L. Kirschstein National Research Service Award through the National Institutes of Health.

She also served on the Dean's Medical Council as the MD/PhD Program representative in 2012. Upon completion of her graduate studies, Kathleen will return to her third and fourth year medical school clerkships, after which she will continue on to residency training.