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LOYOLA UNIVERSITY CHICAGO

THE EFFECT OF VITAMIN D ON HUMAN T REGULATORY CELL DIFFERENTIATION

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN INFECITIOUS DISEASE AND IMMUNOLOGY

BY:

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CHICAGO, IL

MAY 2013

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To my parents for always believing in me

Life, if you live it right, keeps surprising you, and the thing that keeps surprising you the most…is yourself

Jane Austen

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Thank you all.

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LIST OF ABBREVIATIONS

Treg	Regulatory T cell
IBD	Inflammatory Bowel Disease
MS	Multiple Sclerosis
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome
DC	Dendritic cell
UCB	Umbilical cord blood
NRP-1	Neuropilin-1
nTreg	Naturally arising regulatory T cell
iTreg	Inducible regulatory T cell
RA	Retinoic Acid
LAP	Latency Associated Peptide
EAE	Experimental autoimmune encephalomyelitis
VDR	Vitamin D receptor
VDRE	Vitamin D responsive element
HSC	Hematopoietic stem cell
CMP	Common myeloid progenitor
MDP	Macrophage/DC progenitor
TLR	Toll like receptor

LPS	Lipopolysaccharide
TSP-1	Thrombospondin-1
VEGF	Vascular endothelial growth factor
PBMC	Peripheral blood mononuclear cells
MFI	Mean fluorescence intensity
PAMP	Pathogen associated molecular pattern
GVHD	Graft vs. host disease

ABSTRACT

Regulatory T cells (Tregs) are required for the induction and maintenance of immune homeostasis. Singh et al. demonstrated that depletion of Tregs in mice results in a loss of self-tolerance that manifests in the development of autoimmune diseases such as Inflammatory Bowel Disease (IBD) [1]. In humans, autoimmune diseases such as IBD, Multiple Sclerosis (MS), and Type 1 Diabetes are thought to occur due to a deficiency in the number or function of Tregs [2]. The importance of Tregs in modulating the human immune system is perhaps best exemplified by IPEX (immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome), a severe autoimmune disease characterized by a defect in the key Treg transcription factor Foxp3 [3].

Tregs unquestionably play an important role in regulating the immune system, but there is still more to learn about how Tregs are induced. In recent years, vitamin D has been identified as an important immunomodulatory molecule that may promote Treg differentiation [4]. *In vitro* studies have confirmed that vitamin D inhibits T cell proliferation and regulates dendritic cell (DC) maturation to promote a tolerogenic phenotype [5, 6]. Moreover, vitamin D deficiency is associated with an increased risk in the development of autoimmune diseases such as MS and Type 1 Diabetes [3, 7-10].

Our goal was to test the hypothesis that vitamin D enhances human Treg differentiation and to elucidate the mechanism by which vitamin D modulates immune responses. Here we report that there is a positive correlation between the serum concentration of vitamin D and the Treg frequency in adult peripheral blood. To further study the effect of vitamin D on human Treg differentiation, we used an *ex vivo* culture system to induce Tregs from human umbilical cord blood (UCB). We found that supplementation of vitamin D to human UCB enhanced Treg differentiation. Furthermore, addition of a vitamin D receptor inhibitor to human UCB decreased Treg differentiation. Taken together, these data indicate that vitamin D has a positive effect on human Treg differentiation. Additionally, our data suggest that vitamin D exerts its immunomodulatory effects by acting on monocytes. Vitamin D supplementation enhanced the expression of the monocyte cell surface molecule Neuropilin-1 (NRP-1), which functions to bind TGF-β at the cell surface [11]. TGF-β is an essential immunomodulatory cytokine to induce Tregs in this culture system.

We concluded that vitamin D enhances human Treg differentiation in part by altering the phenotype of monocytes to promote the expression of cell surface molecules important for Treg differentiation. The work encompassed here will contribute to our understanding of vitamin D as an immunomodulatory molecule and potentially aid scientific efforts to develop new therapeutics to treat autoimmune disease.

CHAPTER ONE

LITERATURE REVIEW

Gershon and Kondo's experiments in the early 70's suggested that thymus-derived lymphocytes were essential for the induction of immunological tolerance [12, 13]. Many investigators worked to delineate the cell population responsible for this phenomenon, and to this end, Tada identified suppressor T cells as a key thymus-derived cell subset responsible for the induction of immunological tolerance [14, 15]. Tada argued that suppressor T cells were a functionally distinct T cell subset that worked to suppress antigen induced CD4+ T helper cell responses as well as antibody responses [16, 17]. Moreover, Tada was one of the first to suggest that the inability of tumor bearing animals to mount an effective anti-tumor immune response was due to the active suppression of tumor immunity via suppressor T cells rather than a general state of immune unresponsiveness in these animals [18].Tada's work to further characterize suppressor T cells and to understand the mechanism of T cell mediated suppression was heavily challenged due to the inability to identify specific markers that could distinguish this population of T cells [19]. As a result, the hunt to understand the basis of immunological tolerance and the role of suppressor T cells was largely abandoned for some time.

Later work by Sakaguchi sparked a renewed interest in the study of immune regulation and tolerance. Sakaguchi discovered and characterized a new subset of

cells named regulatory T cells that were functionally similar to Tada's suppressor T cells. Sakaguchi et al. determined that T cells expressing the IL-2 receptor CD25 had regulatory activity by inoculating BALB/c athymic nude mice with CD4+ cell suspensions prepared from the lymph nodes and spleens of BALB/c nu/+ mice and depleted of CD25+ cells. All recipients of the CD25+ depleted cell suspensions developed autoimmune disease. Reconstitution of the CD4+CD25+ cell population within a certain amount of time prevented the development of autoimmune disease [20]. A decade after Tada's work arguing for the existence of suppressor T cells, Sakaguchi finally solidified the role of T cell mediated suppression in the induction of immunological tolerance. Moreover, Sakaguchi's work was fundamental in characterizing CD4+CD25+ regulatory T cells.

Our understanding of regulatory T cells was further advanced by the discovery that the transcription factor Foxp3 was the master regulator of Tregs. Hori et al. discovered that naïve T cells were converted to a regulatory phenotype after the induced expression of Foxp3 via retroviral gene transfer [21]. The importance of Foxp3+ Tregs in modulating the human immune system is perhaps best exemplified by IPEX, a severe autoimmune disease characterized by a defect in Foxp3 [3]. Loss of function of Foxp3 in mice also leads to the development of autoimmune disorders as seen in Scurfy mice [22]. In humans, other autoimmune diseases such as IBD, MS, and Type 1 Diabetes are thought to occur due to a deficiency in the number or function of Foxp3+ Tregs [2].

Naturally arising Tregs (nTregs) are generated in the thymus whereas inducible Tregs (iTregs) are generated in the periphery. Various factors influence the generation and function of iTregs including the vitamin A metabolite retinoic acid (RA) and TGF-β [23].The cytokine TGF-β is produced in a latent form that must be activated in order to exert its biological effects. Non covalent interactions between latent TGF-β and latency associated peptide (LAP) render the cytokine inactive [24]. TGF-β is activated upon proteolytic separation from LAP, and signals through SMADs to influence cellular transcription. Xiao et al. discovered that RA promotes the generation of Foxp3+ Tregs by increasing the expression and phosphorylation of SMAD3, a key component in the TGF-β signaling pathway [25]. Furthermore, Nakamura et al. showed that CD4+CD25+ cells expressed TGF-β at the cell surface and suppressed CD4+CD25- cells. Addition of anti-TGF-β abolished the suppressive function of the CD4+CD25+ population [26]. Taken together, these findings indicate that TGF-β is a critical factor for the generation and function of Tregs.

Recently, vitamin D has been shown to have an effect on Treg differentiation. Urry et al. discovered that vitamin D supplementation increased the frequency of Foxp3+ Tregs *in vitro* [27]. Indeed, vitamin D is considered to be an immunomodulatory molecule and vitamin D deficiency is associated with an increased risk in the development of autoimmune diseases such as MS [2[10]]. Moreover, vitamin D supplementation has been shown to inhibit the development of experimental autoimmune encephalomyelitis (EAE), a murine model of MS [3, 7, 8].

Additionally, vitamin D has been shown to slow the loss of renal-allograft function in kidney transplant patients, suggesting that the immunomodulatory properties of vitamin D may be harnessed in the future to prevent or slow graft rejection [9]. While vitamin D is considered to be an immunomodulatory molecule, the exact mechanisms underlying its immunosuppressive function are yet to be elucidated.

Humans acquire vitamin D through dietary intake or by exposure to sunlight. Vitamin D is metabolized to its biologically active form by hydroxylation in the liver and kidney. In the liver, vitamin D is metabolized by the enzyme 25-hydroxylase to form 25-hydroxyvitamin D-3, the predominant form of vitamin D in the circulation. Next, 25- hydroxyvitamin D-3 is metabolized in the kidney by the enzyme 1-α-hydroxylase to form 1,25-dihydroxyvitamin D-3, the biologically active form of the molecule. 1,25-dihydroxyvitamin D-3 crosses the plasma membrane of a cell and binds to the nuclear vitamin D receptor (VDR). The VDR forms a heterodimer with the retinoic acid-X-receptor and binds to the vitamin D responsive element (VDRE) in DNA, leading to the alteration of gene transcription [3, 4].

Various immune cells such as T and B lymphocytes, monocytes, macrophages, and DC's express a VDR and are therefore capable of responding to vitamin D [7]. Kang et al. demonstrated that 1,25-dihydroxyvitamin D3 promoted Foxp3 expression in CD4+ T cells. After vitamin D supplementation, the VDR bound directly to the VDRE within the Foxp3 gene to enhance promoter activity [28]. Additionally, Mayne et al. investigated the ability of vitamin D to inhibit EAE by acting on the VDR in CD4+ T cells. The incidence of EAE in mice with a functional

VDR in CD4+ T cells was decreased upon vitamin D supplementation. VDR gene inactivation in CD4+ T cells prevented this phenomenon, indicating that vitamin D can act on CD4+ T cells via the VDR to inhibit EAE [29].

Other studies have suggested that vitamin D can act on antigen presenting cells to influence Treg differentiation. Van der Aar et al. showed that immature dendritic cells stimulated with 1,25-dihydroxyvitamin D3 adopted a tolerogenic phenotype that promoted Treg differentiation in co-cultures with naïve CD4+ T cells [5]. Additionally, Ferreira et al. studied the effects of addition of 1,25-dihydroxyvitamin D3 on dendritic cell differentiation from the bone marrow cells of C57/Bl/6 mice. Dendritic cells differentiated in the presence of vitamin D displayed decreased levels of the DC activation markers MHCII, CD80, and CD86 as compared to control DC's differentiated in the absence of vitamin D. Moreover, the ability of the vitamin D treated DC's to stimulate T cell proliferation *in vitro* was reduced as compared to control DC's [30]. Taken together, these findings illustrate that vitamin D can act on antigen presenting cells to modulate the immune response.

Leukocytes such as monocytes, macrophages, and DC's are derived from hematopoietic stem cells (HSCs) in the bone marrow. HSCs give rise to lineage-restricted progenitors such as the common myeloid progenitor (CMP) and the macrophage/DC progenitor (MDP) that have the potential to further differentiate into monocytes, macrophages or DC's. Monocytes circulate through the blood and play an important role in innate immunity by producing cytokines and antimicrobial compounds that alert the immune system and combat the invading microorganism.

Monocytes can also leave the circulation and enter tissues where they function as a precursor to macrophages and DC's. Differentiation of monocytes to macrophages and DC's in tissue is understood to happen during steady-state conditions as well as in response to inflammation and bacterial infection. In mice, monocyte recruitment is required to combat infection by the intracellular bacterium *Listeria monocytogenes* as well as other bacterial, viral, and fungal pathogens. Additionally, in humans approximately 90% of monocytes express the cell surface molecule CD14, a Toll Like Receptor (TLR) that recognizes bacterial Lipopolysaccharide (LPS) and helps sound the alarm against bacterial invasion. Depending on the cytokine milieu, monocytes can either facilitate activation or inhibition of the immune response, and monocytes can also contribute to tissue healing [31-34].

In a recent manuscript currently under review, Jaeger et al. reported that monocytes expressing the cell surface molecule CD14 and high levels of CD36 were uniquely capable of inducing Foxp3+ Tregs from naïve CD4+ T cells in human UCB. The monocyte cell surface marker CD36 is a receptor for the ligand thrombospondin-1 (TSP-1). TSP-1 activates TGF-β by binding to the LAP/latent TGF-β complex and inducing a conformational change that somehow relieves the mature form of TGF-β from the latent complex [35]. In turn, TGF-β is a critical factor for the generation of Tregs. Additionally, Jaeger et al. used human UCB to study Treg induction because UCB has a greater affinity for Treg differentiation than adult peripheral blood. Indeed, for over fifty years investigators have understood that mammals exposed to foreign tissue in-utero have the ability to develop tolerance to

the foreign homologous tissue. For example, Medawar and colleagues described an experiment where each of the six fetuses of a pregnant female CBA mouse were injected intra-embryonically with cells from an adult male mouse of the A strain. Of the six fetuses, five healthy mice were born and all received skin grafts from an adult donor mouse of the A strain. The graft was accepted and incorporated into the skins of 3 of the five mice given the intra-embryonic injections. Additionally, the graft was still intact at day 11. In contrast, when adult CBA mice received a graft from an A-line donor the graft was rejected in approximately 12 days [36]. The work of Medawar and others was instrumental in understanding the phenomenon of neonatal tolerance. Additionally, Jaeger's work sheds new light on our understanding of how neonatal tolerance is induced.

We used the human UCB model established by Jaeger et al. to study the effect of vitamin D on immune regulation and Treg differentiation. We hypothesized that addition of vitamin D to UCB would enhance Treg differentiation. Additionally, we worked to determine a mechanism of action of vitamin D in immune modulation and Treg differentiation. To this end, we determined that vitamin D has a positive effect on human Treg differentiation and we identified a monocyte cell surface molecule that we believe may be important in promoting Treg differentiation namely, NRP-1.

NRP-1 is a multi-functional transmembrane glycoprotein expressed on neuronal and endothelial cells as well as other cell types [37]. NRP-1 binds vascular endothelial growth factor (VEGF) as well as other growth factors, class 3 semaphorins, and TGF-β (both the active form and the LAP/ latent TGF-β complex).

Consequently, NRP-1 plays an important role in angiogenesis, axon guidance and growth, cancer, and immunity. In cancer biology, NRP-1 overexpression has been shown to promote tumor growth and metastasis and is indicative of a poor prognosis. NRP-1 has also been associated with immune inhibition and is expressed on murine CD4+ CD25+ Tregs as well as some activated human Tregs [11, 38, 39]. The effect of NRP-1 on cancer progression and immune inhibition is believed to correlate with the ability of NRP-1 to bind active and latent TGF-β. As previously mentioned, NRP-1 is able to bind the LAP/latent TGF-β complex and is suspected to activate TGF-β although the mechanism remains unclear and investigators are currently searching for an additional receptor that could mediate this effect. In turn, TGF-β is an important immunomodulatory cytokine that can promote tumor progression [11].

CHAPTER TWO

MATERIALS AND METHODS

Human UCB Collection

The nurses in the Birth Center at Gottlieb Memorial Hospital collected UCB samples in sterile BLOOD-PACK™ units (Fenwal, Inc., IL) that were anticoagulant treated. After collection, samples were transported to Loyola and processed as soon as possible.

Adult Blood Collection

Adult blood samples were collected in heparin coated tubes by Barb Sexton, RN, MS at Loyola University Medical Center following specific inclusion and exclusion criteria. Five vitamin D deficient and five vitamin D sufficient blood samples were collected for analysis where vitamin D deficiency was defined as less than 30ng/mL in accordance with clinically established parameters. Males and females age 18-80 with 25 OHD levels ≤20ng/dl and able to give informed consent and comply with study procedures were included in the study. Age-matched samples were obtained. Individuals on glucocorticoids or immunosuppressants were excluded from the study as were individuals with evidence of active malignancies in the past 5 years, estimated GFR< 30 mg/ml/min/1.73M2, alanine aminotransferase (ALT) >3x normal, congestive heart failure class 3 or 4, uncontrolled hyper/hypothyroidism, presence of an autoimmune disease, history of excessive alcohol consumption or drub abuse, or poor medical or psychiatric

risk for participation in clinical research in the opinion of the investigator. Women who were pregnant or nursing were also excluded from the study.

Mononuclear Cell Isolation from Human UCB and Adult Peripheral Blood

Blood was diluted with room temperature 1x PBS (1:1 ratio) and mononuclear cells were isolated by Ficoll Gradient Centrifugation using Lymphocyte Separation Medium (Cellgro). The buffy coat was carefully extracted by pipetting. For UCB, the centrifugation protocol was repeated twice to minimize red blood cell contamination. The mononuclear cells were then counted and prepared for downstream applications such as cell culture or flow cytometry.

Human UCB Cell Culture

Cells were cultured in RPMI 1640 Media (Thermo Scientific) supplemented with 10% FCS (Atlanta Biologicals), MEM essential and non-essential amino acids (Invitrogen), β-mercaptoethanol (50 uM), sodium pyruvate (1mM), L-glutamine (2mM), penicillin (100 I.U/mL) streptomycin (100 ug/ml), and HEPES (10 mM). Exogenous IL-2 (PeproTech) was added at 10ng/mL and soluble anti-CD3 (eBioscience) was added at 0.2ug/mL. Cells were cultured for up to 14 days and split approximately every two days. The concentration of IL-2 was maintained throughout. Where indicated, soluble 1,25-dihydroxyvitamin D3 was added at 1.6, 3.1, 6.2, 12.5, or 25nM. For overnight culture of UCB, 1,25-dihydroxyvitamin D3 was added at 25nM and incubated for up to 24 hours without IL-2 and anti-CD3.

Cell Enrichment and Co-culture

CD4+ T cells were enriched from whole UCB mononuclear cells via a total CD4+ BD IMag enrichment kit following the manufacturers protocol. For CD4+/THP1 cell co-culture experiments, THP1 cells were irradiated at 3000 rad and then cultured with CD4+ T cells in a 3:1 ratio.

Flow Cytometry

Prior to staining, cells were blocked with purified human IgG (R&D Systems) for 5 minutes in FACS buffer (1xPBS, 1% FCS, 0.1% sodium azide). Cells were then washed with FACS buffer and stained with the indicated mouse anti-human antibodies for 30 minutes. Foxp3 (BD Pharmingen clone 259D/C7), CD4 (Biolegend clone RPA-T4), CD8 (BD Pharmingen clone RPA-T8), CD25 (Biolegend clone BC96), CD36 (Biolegend clone 5-271), LAP (Biolegend clone TW4-2F8), CD14 (Biolegend clone HCD14), NRP-1 (Biolegend clone 12C2). The following isotype controls were used: LAP (Biolegend clone MOPC-21), NRP-1 (BD Pharmingen clone G155-178), CD25 and CD36 (Biolgengend clone MOPC-173), Foxp3 (Biolegend clone MOPC-21). For intracellular staining of the transcription factor Foxp3, a Foxp3 Fix/Perm buffer set (Biolegend) was used. Data were collected on FACS Canto II (Becton Dickinson) and ImageStreamX (Amnis Corp. Seattle, WA.) FlowJo software (Tree Star, Inc.) and Amnis Ideas 5.0 were used for data analysis.

CHAPTER THREE

EXPERIMENTAL RESULTS

We hypothesized that the immunomodulatory properties of vitamin D were a direct result of its ability to enhance human Treg differentiation. Our first goal was to determine if the serum concentration of vitamin D correlated with the Treg frequency in adult peripheral blood mononuclear cells (PBMC). Adult blood samples were collected by Barb Sexton, RN, MS at Loyola University Medical Center following specific inclusion and exclusion criteria. Males and females age 18-80 were included in the study as long as they were able to give informed consent and comply with study procedures. In contrast, individuals on glucocorticoids and immunosuppressants were excluded from the study, as were individuals with evidence of autoimmune disease, hypo/hyperthyroidism, and history of excessive drug or alcohol consumption. Additionally, equal numbers of vitamin D sufficient and deficient blood samples were collected for analysis where vitamin D deficiency was defined as less than 30ng/mL in accordance with clinically established parameters. Moreover, age-matched samples were obtained.

We expected that vitamin D sufficient individuals would have a higher peripheral blood Treg frequency as compared to vitamin D deficient individuals. PBMC's were first isolated by Ficoll gradient separation using lymphocyte separation medium (Cellgro). The freshly isolated mononuclear cells were then prepared for flow cytometric analysis by staining for the Treg cell surface molecules CD4 and CD25 and the transcription factor Foxp3. Tregs were identified as being $CD4^{+}CD25^{+}Foxp3^{+}$. Since blood was collected, processed, and stained on the same day, Treg frequency was reported at day 0.

Figure 1A depicts the Treg frequencies of vitamin D deficient (0-30 ng/mL) and sufficient (31-100 ng/mL) cohorts at day 0. In figure 1B the flow cytometry data were compiled to show the linear regression. In both plots the Treg frequency in adult PBMC was increased in vitamin D sufficient individuals as compared to deficient individuals, although the differences found by linear regression were not statistically significant. These results suggest that the serum concentration of vitamin D may be positively correlated with peripheral blood Treg frequency in adults.

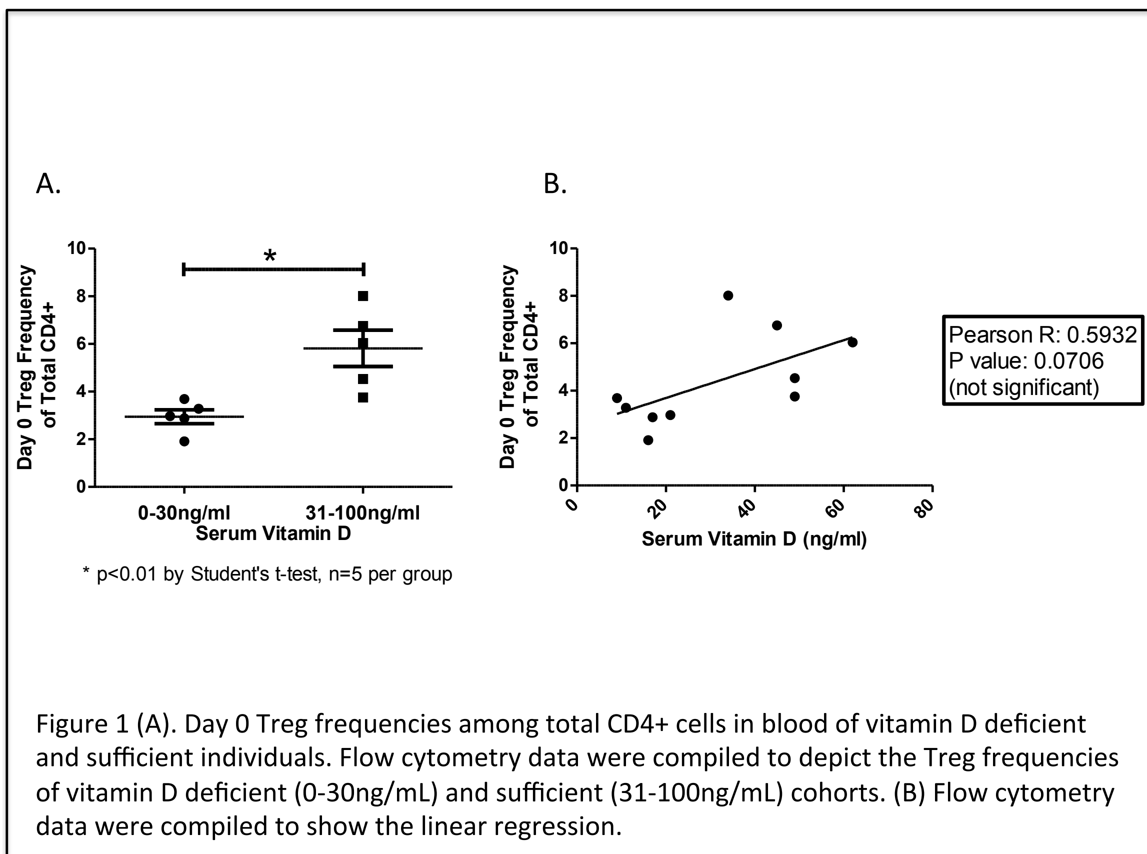

Figure 1 (A). Day 0 Treg frequencies among total CD4+ cells in blood of vitamin D deficient and sufficient individuals. Flow cytometry data were compiled to depict the Treg frequencies of vitamin D deficient (0-30ng/mL) and sufficient (31-100ng/mL) cohorts. (B) Flow cytometry data were compiled to show the linear regression.

Our results encouraged us to further investigate the effect of vitamin D on human Treg differentiation. Our hypothesis predicted that the addition of vitamin D to whole UCB would increase Treg differentiation. To test this hypothesis, we used an *ex vivo* human UCB culture system developed in our laboratory to determine the

effect of addition of vitamin D on Treg differentiation in whole UCB. Human UCB samples were obtained from Gottlieb Memorial Hospital and mononuclear cells were isolated by Ficoll gradient separation using lymphocyte separation medium (Cellgro). The freshly isolated mononuclear cells were then cultured for 14 days in medium supplemented with IL-2 and anti-CD3 with or without the addition of 1,25-dihydroxyvitamin D3. IL-2 is a T cell survival factor necessary for the growth, proliferation, and differentiation of Tregs and anti-CD3 simulates the T cell antigen receptor. Additionally, cells were cultured for 14 days because human T cells transiently express Foxp3 after activation for approximately 10 days (Seki et al., unpublished data). After 14 days of culture, cells were harvested and prepared for flow cytometric analysis by staining for the Treg cell surface molecules CD4 and CD25 and the transcription factor Foxp3. Tregs were identified as being $CD4^{+}CD25^{+}Foxp3^{+}$.

Figure 2 (A) depicts flow cytometry data of addition of 1,25-dihydroxyvitamin D3 to whole UCB. Each panel shows CD25 and Foxp3 expression among total CD4+ cells. Vitamin D supplementation to whole UCB resulted in a dose dependent increase in the Treg frequency. Moreover, the absolute number of Tregs was increased upon addition of vitamin D to whole UCB (Figure 2 B).

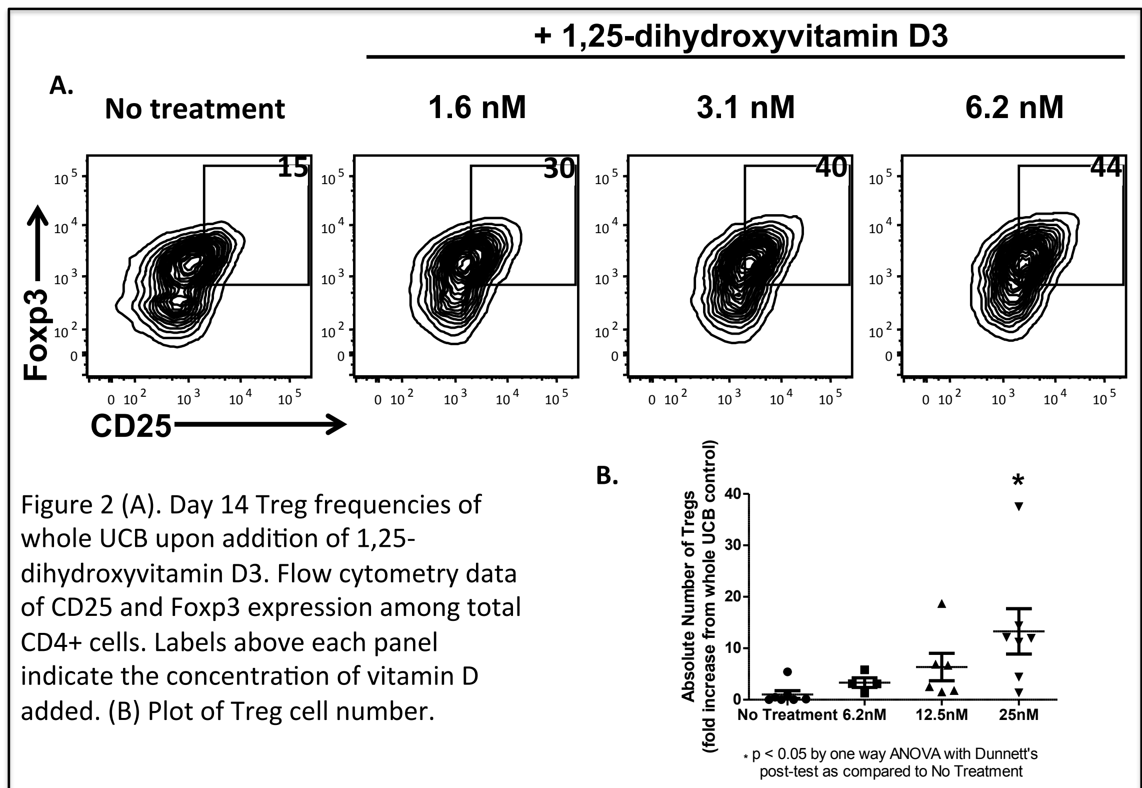

Figure 2 (A). Day 14 Treg frequencies of whole UCB upon addition of 1,25-dihydroxyvitamin D3. Flow cytometry data of CD25 and Foxp3 expression among total CD4+ cells. Labels above each panel indicate the concentration of vitamin D added. (B) Plot of Treg cell number.

We next tested the effect of addition of vitamin D on Treg differentiation in a more simplified co-culture system using UCB CD4+ T cells and THP1 cells. THP1 cells are a human monocytic leukemia cell line capable of inducing Tregs from UCB CD4+ T cells (Jaeger et al., under revision). We hypothesized that addition of vitamin D to CD4+/THP1 cell co-culture would result in an increase in Treg differentiation.

To test this hypothesis, human UCB samples were obtained from Gottlieb Memorial Hospital and mononuclear cells were isolated by Ficoll gradient separation. CD4+ T cells were then enriched from the freshly isolated mononuclear cells via a total CD4+ enrichment kit (BD Biosciences) that negatively selected for CD4+ T cells. THP1 cells were irradiated at 3000 rads to prevent their proliferation during the co-culture with CD4+ T cells. THP1 cells were cultured with CD4+ T cells in a 3:1 ratio in medium supplemented with IL-2 and anti-CD3 for 14 days with or without the addition of 1,25-dihydroxyvitamin D3. After 14 days of culture, cells

were harvested and prepared for flow cytometric analysis by staining for the Treg cell surface molecules CD4 and CD25 and the transcription factor Foxp3.

Figure 3 depicts flow cytometry data of CD4+/ THP1 cell co-culture stained at day 14 for Treg induction after addition of 1,25-dihydroxyvitamin D3. Tregs were identified as being $CD4^{+}CD25^{+}Foxp3^{+}$ and each panel depicts CD25 and Foxp3 expression among total CD4+ cells. As hypothesized, there was a dose dependent increase in the Treg frequency from approximately 24 to 53 upon addition of 1,25-dihydroxyvitamin D3. Moreover, we obtained similar results in four independent experiments.

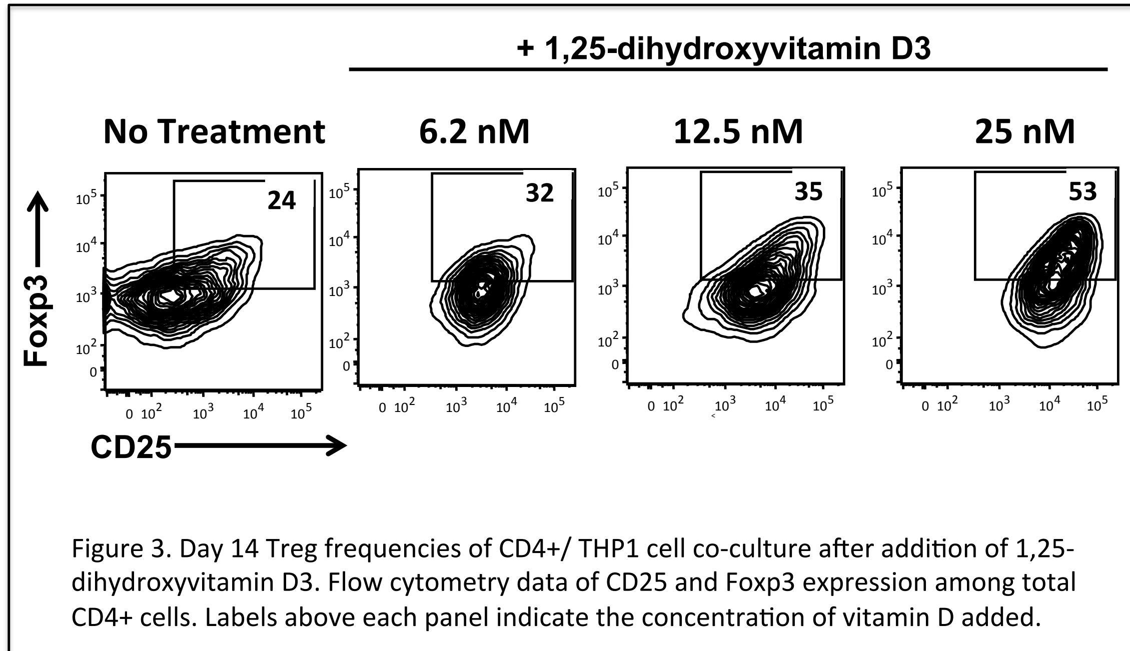

Figure 3. Day 14 Treg frequencies of CD4+/ THP1 cell co-culture after addition of 1,25-dihydroxyvitamin D3. Flow cytometry data of CD25 and Foxp3 expression among total CD4+ cells. Labels above each panel indicate the concentration of vitamin D added.

To verify the effect of vitamin D on human Treg differentiation, we added a vitamin D receptor inhibitor to CD4+/ THP1 cell co-culture. We hypothesized that addition of a vitamin D receptor inhibitor to CD4+/THP1 cell co-culture would result in a decrease in Treg differentiation. To test this hypothesis, human UCB CD4+ T cells were cultured with irradiated THP1 cells with or without the addition of the vitamin D receptor inhibitor ADMI3. After 14 days of culture, cells were

harvested and prepared for flow cytometric analysis by staining for the Treg cell surface molecules CD4 and CD25 and the transcription factor Foxp3.

Figure 4 depicts flow cytometry data of CD4+/THP1 cell co-culture after addition of the vitamin D receptor inhibitor ADMI3. Each panel depicts CD25 and Foxp3 expression among total CD4+ cells. When ADMI3 was added at a concentration of ½ IC_{50}, the Treg frequency was decreased from approximately 24 to 9. Since the ADMI3 stock solution was suspended in ethanol, an ethanol control was included in the experiment, and showed no change in the Treg frequency.

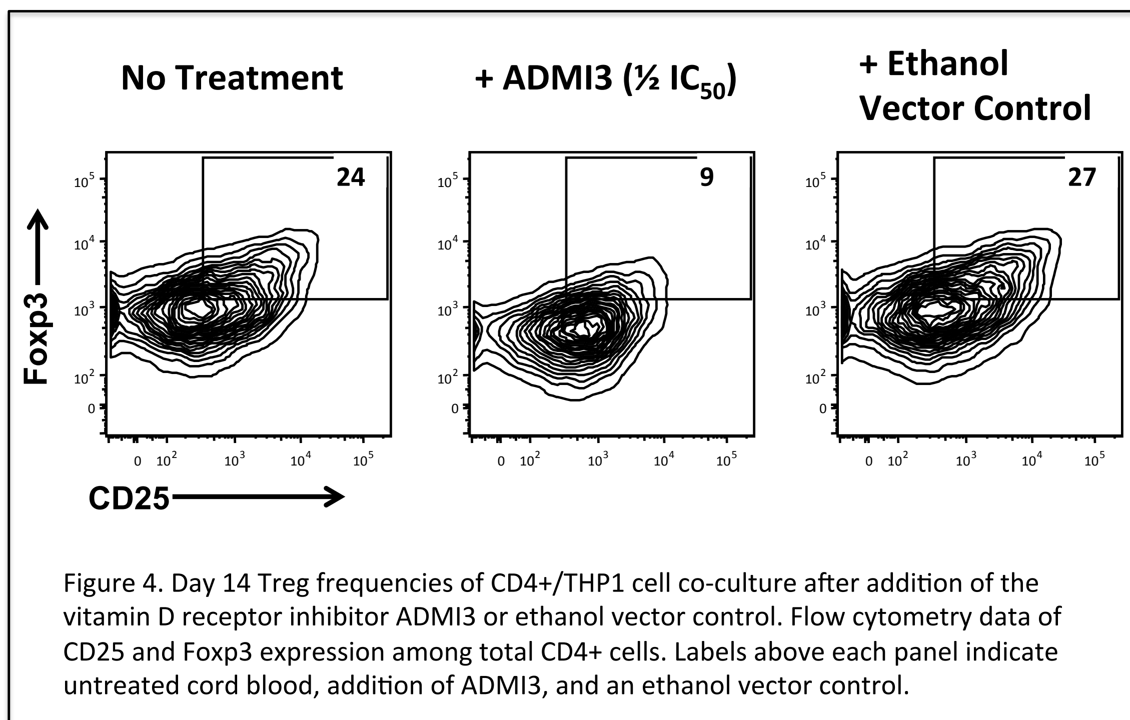

Figure 4. Day 14 Treg frequencies of CD4+/THP1 cell co-culture after addition of the vitamin D receptor inhibitor ADMI3 or ethanol vector control. Flow cytometry data of CD25 and Foxp3 expression among total CD4+ cells. Labels above each panel indicate untreated cord blood, addition of ADMI3, and an ethanol vector control.

To elucidate the mechanism by which vitamin D enhanced human Treg differentiation, we tested if vitamin D acted on monocytes and/or CD4+ T cells. We expected that if vitamin D acted on monocytes to enhance Treg differentiation, there would be an increase in the Treg frequency when vitamin D pre-treated THP1 cells were cultured with untreated CD4+ T cells. On the other hand, if vitamin D acted on CD4+ T cells to enhance Treg differentiation, then we would expect to see an

increase in the Treg frequency when vitamin D pre-treated CD4+ T cells were cultured with untreated THP1 cells.

To test this, UCB CD4+ T cells or THP1 cells were incubated overnight with 1,25-dihydroxyvitamin D3. The next day cells were washed twice in medium and THP1 cells were irradiated. Vitamin D pre-treated CD4+ T cells were then cultured with untreated THP1 cells. Additionally, vitamin D pre-treated THP1 cells were cultured with untreated CD4+ T cells. A control sample was also included in which untreated THP1 cells were cultured with untreated CD4+ T cells. Cells were cultured for 14 days in the presence of IL-2 and anti-CD3 and analyzed by flow cytometry for the expression of CD4, CD25, and the transcription factor Foxp3.

Figure 5 depicts flow cytometry data of CD4+/THP1 cell co-culture where either THP1 cells or CD4+ T cells were incubated overnight with vitamin D before co-culture. When vitamin D pre-treated THP1 cells were cultured with untreated CD4+ T cells, the Treg frequency was increased from approximately 34 to 49. In contrast, when vitamin D pre-treated CD4+ T cells were cultured with untreated THP1 cells, there was no change in the Treg frequency as compared to no treatment.

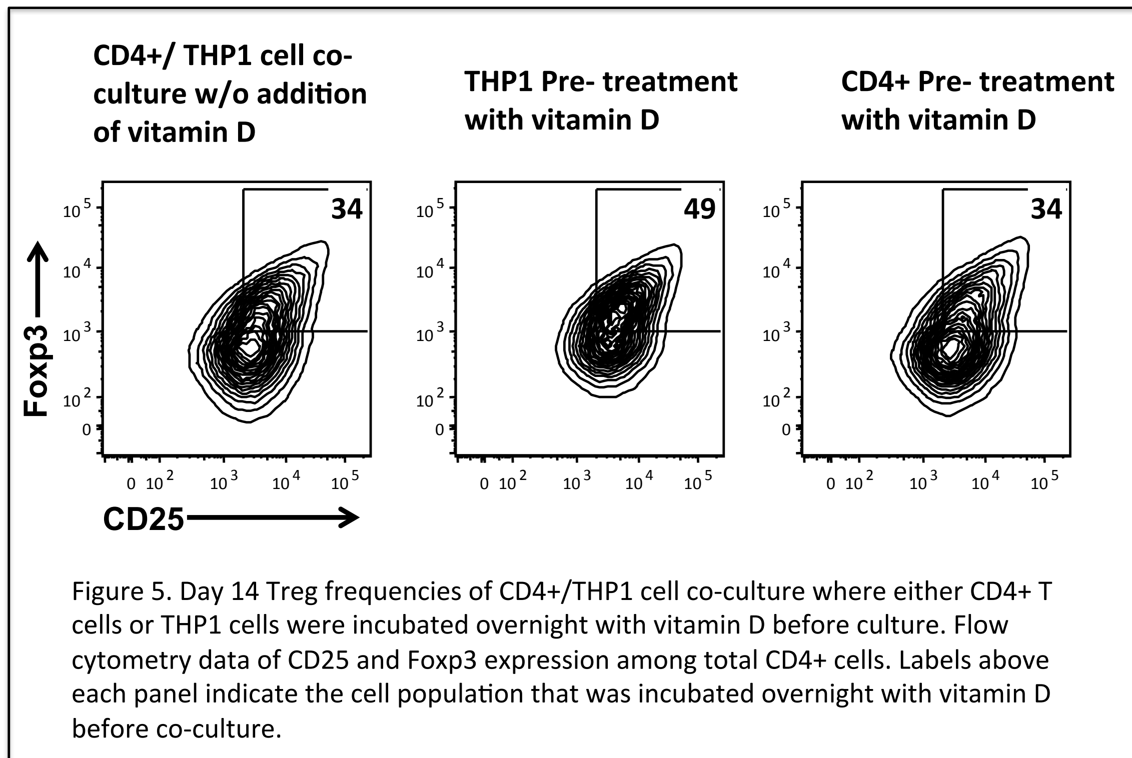

Figure 5. Day 14 Treg frequencies of CD4+/THP1 cell co-culture where either CD4+ T cells or THP1 cells were incubated overnight with vitamin D before culture. Flow cytometry data of CD25 and Foxp3 expression among total CD4+ cells. Labels above each panel indicate the cell population that was incubated overnight with vitamin D before co-culture.

These data suggest that vitamin D enhances human Treg differentiation by influencing monocytes. Recent work completed in our laboratory demonstrated that UCB $CD14^{+}CD36^{hi}$ monocytes were required for Treg induction from total UCB and this phenomenon was dependent upon TGF-β. Additionally, other investigators have reported that the cell surface molecule NRP-1 interacts with active TGF-β as well as the LAP/latent TGF-β complex to activate TGF-β and inhibit the immune response [11]. To understand the effect of vitamin D on monocytes and to elucidate a potential mechanism of action for Treg differentiation, we first tested if UCB $CD14^{+}CD36^{hi}$ monocytes expressed the cell surface molecules NRP-1 and LAP. Human UCB samples were stained for the monocyte cell surface molecules CD14 and CD36 as well as the cell surface molecules NRP-1 and LAP.

Figure 6A depicts flow cytometric analysis of expression of CD14 and CD36 in whole UCB. As previously observed in our lab, we detected the presence of two groups of monocytes differing by the expression of CD36. One group expressed high levels of CD36 whereas the other group expressed lower levels of CD36. When these

two subsets were compared for the expression of NRP-1, we found that the $CD14^{+}CD36^{hi}$ monocyte population expressed NRP-1 at a higher level than either the $CD14^{-}CD36^{lo}$ or the $CD14^{-}CD36^{-}$ population. The mean fluorescence intensity (MFI) of NRP-1 in the $CD14^{+}CD36^{hi}$ population was 840 as compared to 64 in the $CD14^{-}CD36^{lo}$ population and 47 in the $CD14^{-}CD36^{-}$ population, indicating more than a 10-fold increase in the expression of NRP-1. Similar results were repeated in five independent experiments. Similar to the expression of NRP-1, the $CD36^{hi}$ subset also expressed a higher level of LAP as compared to the $CD14^{-}CD36^{lo}$ population and the $CD14^{-}CD36^{-}$ population (Figure 6C). The MFI of LAP in the $CD14^{+}CD36^{hi}$ population was approximately 500 as compared to 145 in the $CD14^{-}CD36^{lo}$ population and 70 in the $CD14^{-}CD36^{-}$ population.

Taken together, these data demonstrate that the $CD14^{+}CD36^{hi}$ monocyte population expressed a higher level of NRP-1 and LAP as compared to the $CD14^{-}CD36^{lo}$ and the $CD14^{-}CD36^{-}$ populations, suggesting that the $CD36^{hi}$ population may utilize NRP-1 for the expression/activation of TGF-β.

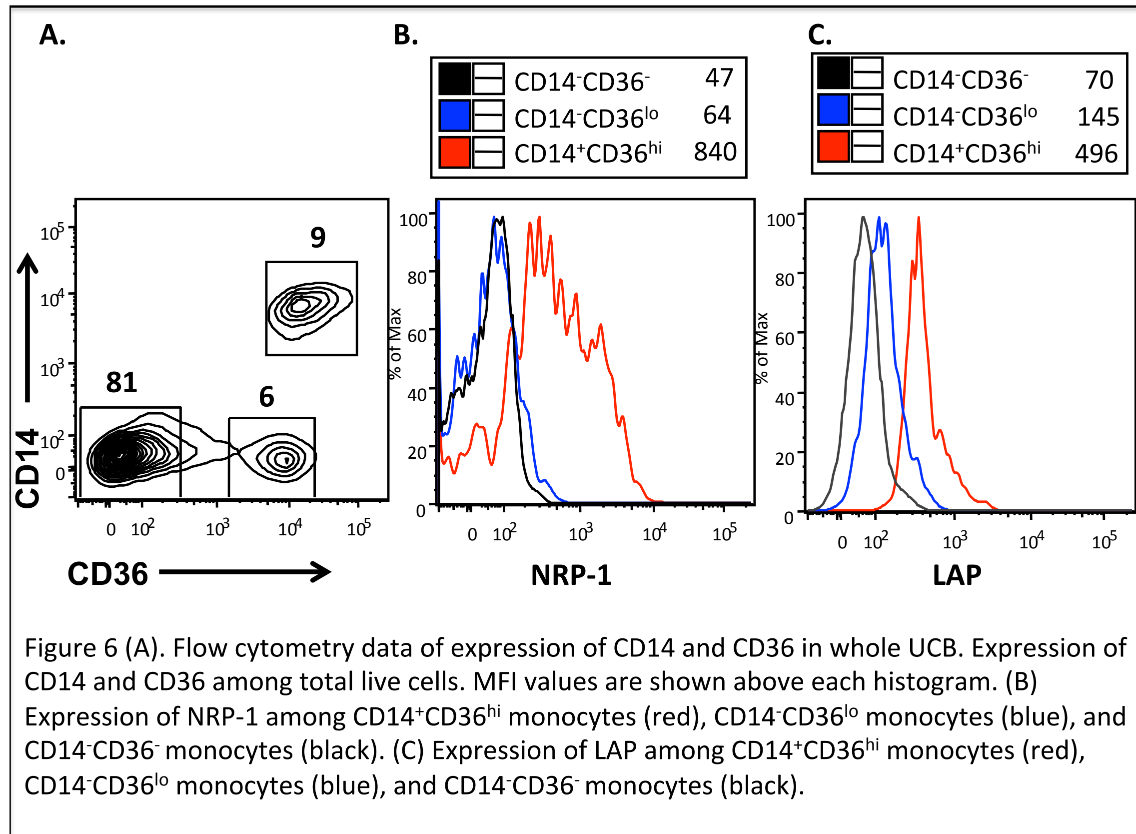

Figure 6 (A). Flow cytometry data of expression of CD14 and CD36 in whole UCB. Expression of CD14 and CD36 among total live cells. MFI values are shown above each histogram. (B) Expression of NRP-1 among $CD14^{+}CD36^{hi}$ monocytes (red), $CD14^{-}CD36^{lo}$ monocytes (blue), and $CD14^{-}CD36^{-}$ monocytes (black). (C) Expression of LAP among $CD14^{+}CD36^{hi}$ monocytes (red), $CD14^{-}CD36^{lo}$ monocytes (blue), and $CD14^{-}CD36^{-}$ monocytes (black).

Based on these data, we hypothesized that vitamin D enhanced human Treg differentiation by acting on monocytes to alter the expression of LAP and/or NRP-1. To test this hypothesis, we determined if addition of vitamin D altered the expression of NRP-1 and LAP. Freshly isolated UCB mononuclear cells were incubated overnight with or without the addition of 1,25-dihyrdoxyvitamin D3. The next day cells were harvested and prepared for flow cytometric analysis by staining for the monocyte cell surface molecules CD36, NRP-1, and LAP.

Figure 7 (A) depicts flow cytometry data of expression of CD36 and NRP-1 in whole UCB. Each panel shows CD36 and NRP-1 expression among total live cells. In both vitamin D treated and untreated UCB there was a $CD36^{hi}$ population. Under untreated conditions, 14% of the $CD36^{hi}$ population expressed NRP-1. When cells were treated with vitamin D, the frequency of the $CD36^{hi}$ NRP-1 positive population increased to 31%. Moreover, the $CD36^{hi}$ NRP-1 positive population became brighter as shown in panel B. After addition of vitamin D the expression of NRP-1 increased and the MFI increased from approximately 300 to approximately 1500.

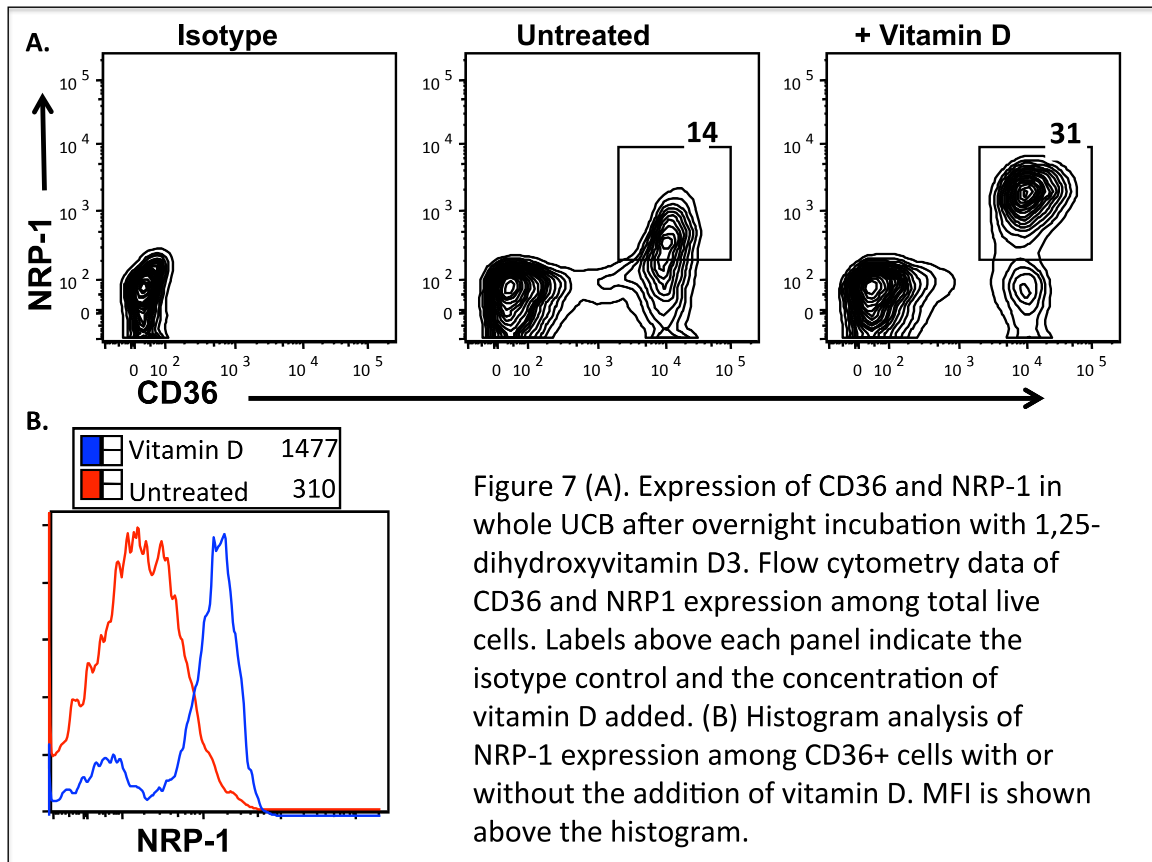

Figure 7 (A). Expression of CD36 and NRP-1 in whole UCB after overnight incubation with 1,25-dihydroxyvitamin D3. Flow cytometry data of CD36 and NRP1 expression among total live cells. Labels above each panel indicate the isotype control and the concentration of vitamin D added. (B) Histogram analysis of NRP-1 expression among CD36+ cells with or without the addition of vitamin D. MFI is shown above the histogram.

The increase in frequency of $CD36^{hi}$NRP-1+ monocytes upon vitamin D supplementation also correlated with an increase in the cell number of $CD36^{hi}$NRP-1+ monocytes, indicating that the change in expression of NRP-1 was not caused by the loss of NRP-1 negative cells (Figure 8).

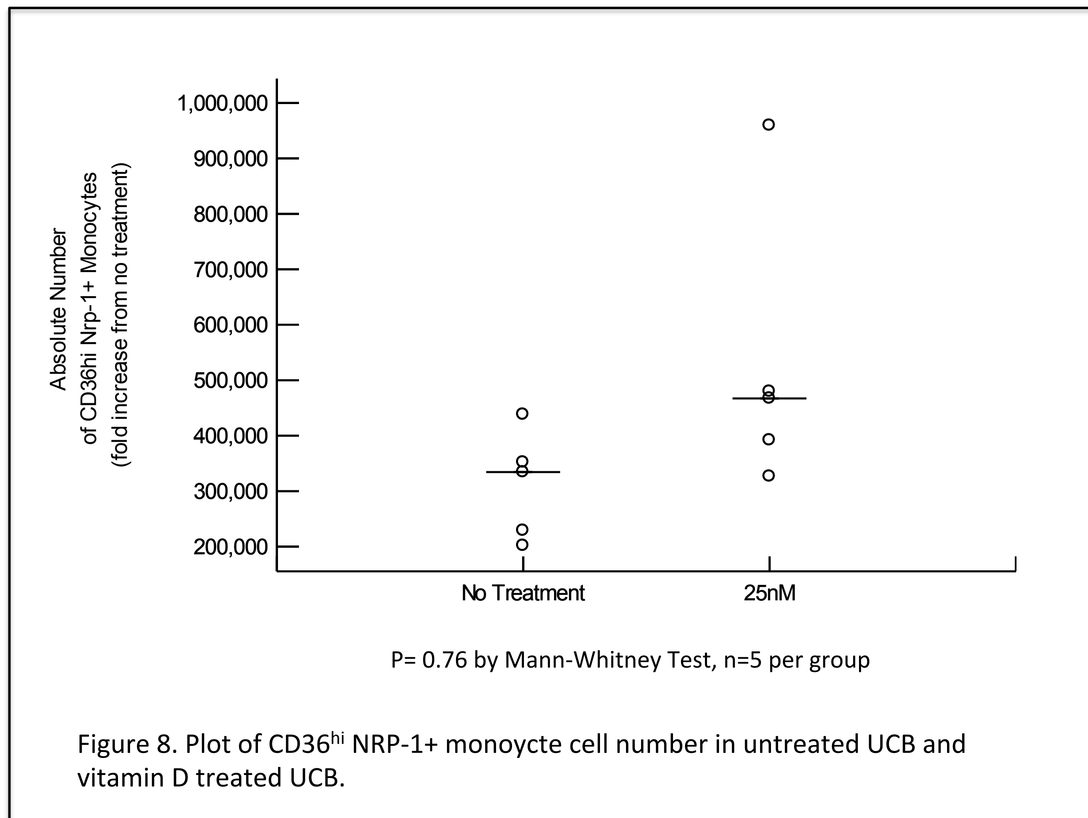

Figure 8. Plot of CD36^{hi} NRP-1+ monoycte cell number in untreated UCB and vitamin D treated UCB.

We next determined the effect of vitamin D on the expression of LAP by CD36^{hi} monocytes (Figure 9A). Each panel shows CD36 and LAP expression among total live cells. Under untreated conditions, 4% of the CD36^{hi} population expressed LAP. When cells were treated with vitamin D, the frequency of the CD36^{hi} LAP+ population increased to 11%. Moreover, the level of LAP expression became brighter as shown in Panel B. Vitamin D addition increased the MFI of LAP from approximately 270 to 340. Similar results were repeated in three independent experiments.

As observed with NRP-1+ cells, the increase in frequency of CD36^{hi}LAP+ monocytes upon vitamin D supplementation also correlated with an increase in the cell number of CD36^{hi}LAP+ monocytes, showing that the change in expression of LAP was not due to the loss of LAP- cells (Figure 10)

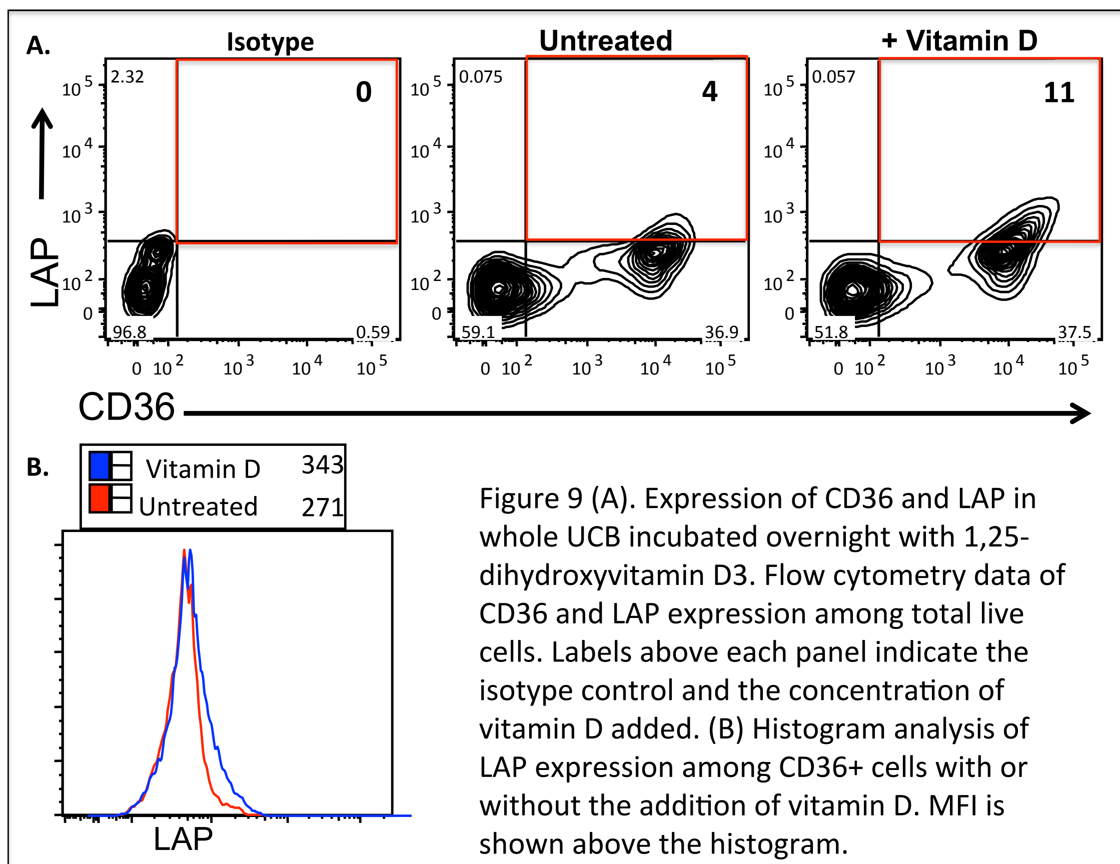

Figure 9 (A). Expression of CD36 and LAP in whole UCB incubated overnight with 1,25-dihydroxyvitamin D3. Flow cytometry data of CD36 and LAP expression among total live cells. Labels above each panel indicate the isotype control and the concentration of vitamin D added. (B) Histogram analysis of LAP expression among CD36+ cells with or without the addition of vitamin D. MFI is shown above the histogram.

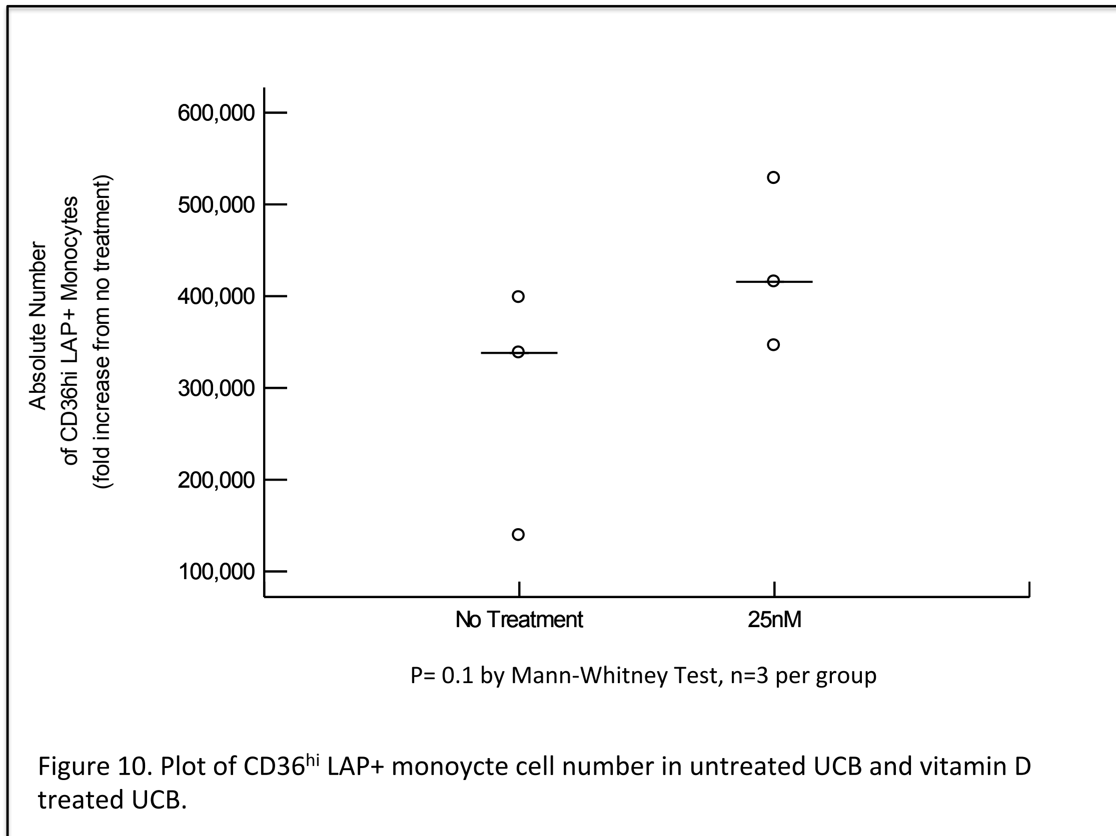

Figure 10. Plot of CD36^{hi} LAP+ monoycte cell number in untreated UCB and vitamin D treated UCB.

We next determined the effect of vitamin D on the distribution of CD36, LAP, NRP-1, and CD14 at the cell surface. Since NRP-1 is known to bind the LAP/latent TGF-β complex and CD36 is a receptor for TSP-1, which interacts with the LAP/latent TGF-β complex to activate TGF-β, our model predicts that these cell surface molecules localize in close proximity to activate TGF-β.

To test this hypothesis, we determined if vitamin D changed the localization of CD14, CD36, NRP-1, and LAP. Freshly isolated UCB mononuclear cells were incubated overnight with or without the addition of 1,25-dihydroxyvitamin D3. Distribution of these surface antigens was determined the next day using AMNIS, which detects the distribution of molecules at single cell level by combination of flow cytometry and microscopy. Additionally, the system allowed for quantitative analysis of co-localization between different molecules. Cells were stained for CD14, CD36, NRP-1, and LAP following the same protocol as preparation for flow cytometry.

Figure 11(A) depicts AMNIS image analysis of CD36 (green) a NRP-1 (red) with or without the addition of vitamin D. The merged image analysis shows co-localization between CD36 and NRP-1. Vitamin D treated UCB showed a significant level of co-localization between CD36 and NRP-1 as compared to untreated UCB. Panel B indicates histogram analysis of co-localization between CD36 and NRP-1 with or without the addition of vitamin D. Each histogram depicts co-localization among CD36+NRP-1+ cells. The R value denotes a percentage of cells where CD36 and NRP-1 were co-localized. In untreated UCB the R value was 7%. After vitamin D treatment the R value increased to 42%, indicating that CD36 and NRP-1 were co-localized on 42% of the cells. The median value shown below each histogram indicates the relative amount of co-localization per cell. The median values were very similar in vitamin D treated and untreated UCB, indicating that while vitamin D changed the percentage of cells that had co-localization, the level of co-localization between CD36 and NRP-1 was unchanged.

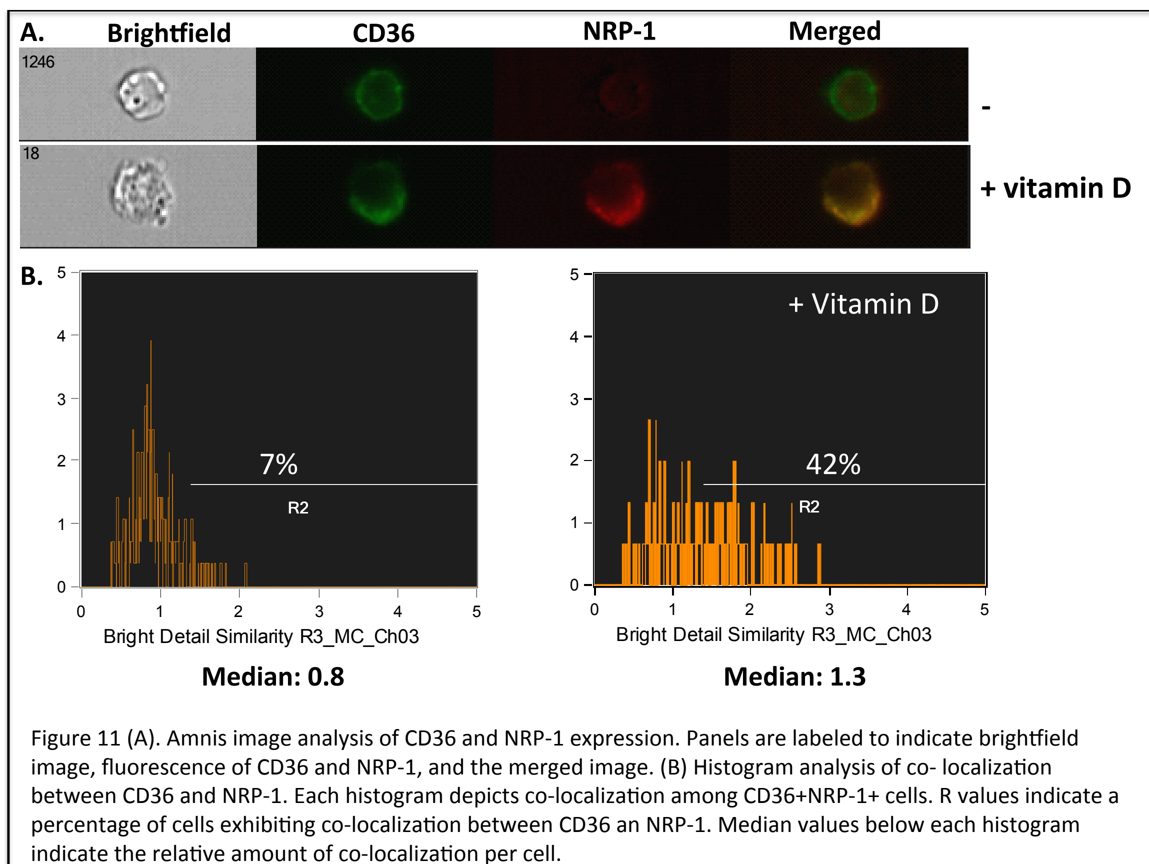

Figure 11 (A). Amnis image analysis of CD36 and NRP-1 expression. Panels are labeled to indicate brightfield image, fluorescence of CD36 and NRP-1, and the merged image. (B) Histogram analysis of co- localization between CD36 and NRP-1. Each histogram depicts co-localization among CD36+NRP-1+ cells. R values indicate a percentage of cells exhibiting co-localization between CD36 an NRP-1. Median values below each histogram indicate the relative amount of co-localization per cell.

We performed the same analysis for co-localization between CD14 and NRP-1 (Figure 12). The merged image analysis shows co-localization between CD14 and NRP-1 in vitamin D treated UCB. In contrast, there was no co-localization between CD14 and NRP-1 in untreated UCB. Panel B indicates histogram analysis of co-localization between CD14 and NRP-1 with or without the addition of vitamin D. Each histogram depicts co-localization among CD14+NRP-1+ cells. In untreated UCB CD14 was co-localized with NRP-1 on 3% of the cells. After vitamin D treatment CD14 and NRP-1 were co-localized on 75% of the cells. Additionally, the median values were very similar in vitamin D treated and untreated UCB, indicating that there was no change in the relative number of complexes of CD14 and NRP-1 per cell after addition of vitamin D.

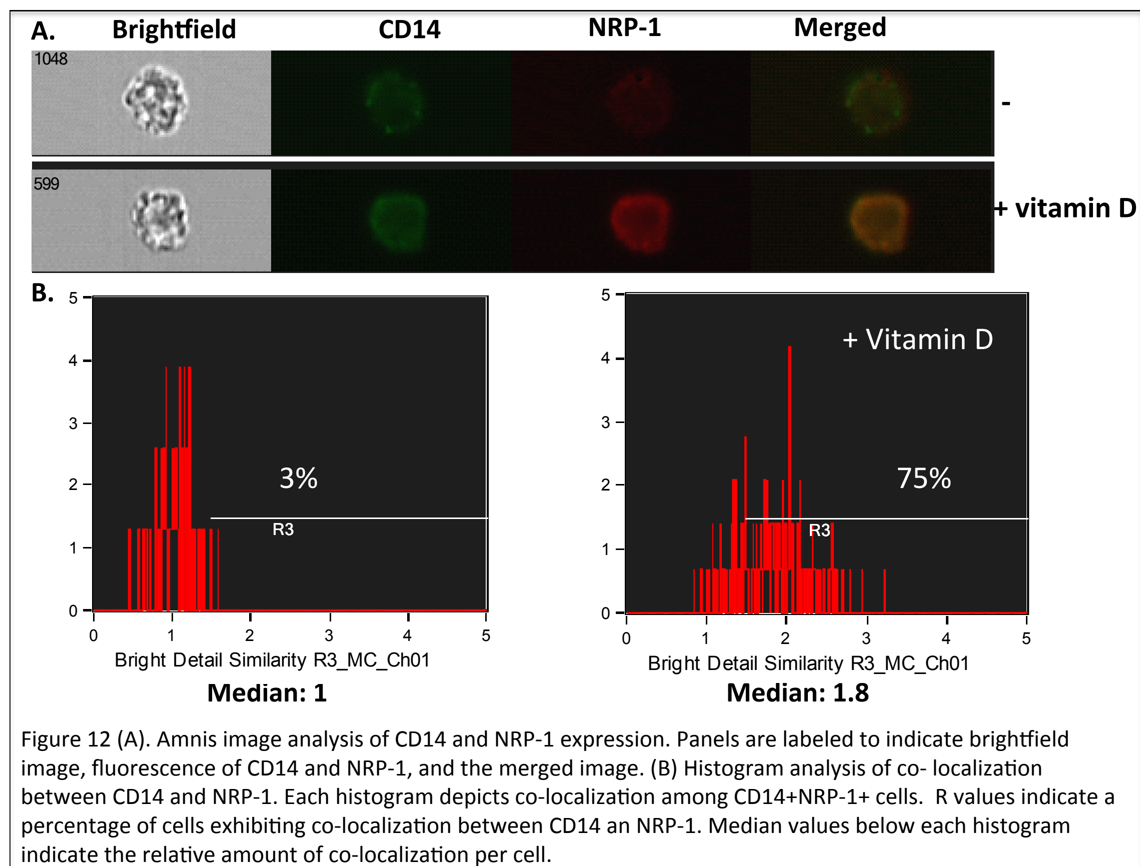

Figure 12 (A). Amnis image analysis of CD14 and NRP-1 expression. Panels are labeled to indicate brightfield image, fluorescence of CD14 and NRP-1, and the merged image. (B) Histogram analysis of co- localization between CD14 and NRP-1. Each histogram depicts co-localization among CD14+NRP-1+ cells. R values indicate a percentage of cells exhibiting co-localization between CD14 an NRP-1. Median values below each histogram indicate the relative amount of co-localization per cell.

These data indicate that CD36 and NRP-1 as well as CD14 and NRP-1 were co-localized after vitamin D treatment. We next determined the effect of vitamin D

on the co-localization between CD14 and CD36. CD14 and CD36 are both co-receptors for TLR-4 and TLR-2, and it's been well documented that these molecules form a complex at the cell surface [40, 41]. Indeed, we detected co-localization between CD14 and CD36 in both vitamin D treated and untreated UCB (Figure 13A). Panel B shows histogram analysis of co-localization between CD14 and CD36 with or without the addition of vitamin D. Each histogram depicts co-localization among CD14+CD36+ cells. The R values were very similar in vitamin D treated and untreated UCB, indicating that vitamin D had relatively no effect on the co-localization between CD14 and CD36. Additionally, the median values were very similar in both vitamin D treated and untreated UCB. Taken together, these data suggest that vitamin D promotes the recruitment of NRP-1 into a complex with CD14 and CD36 on the cell surface.

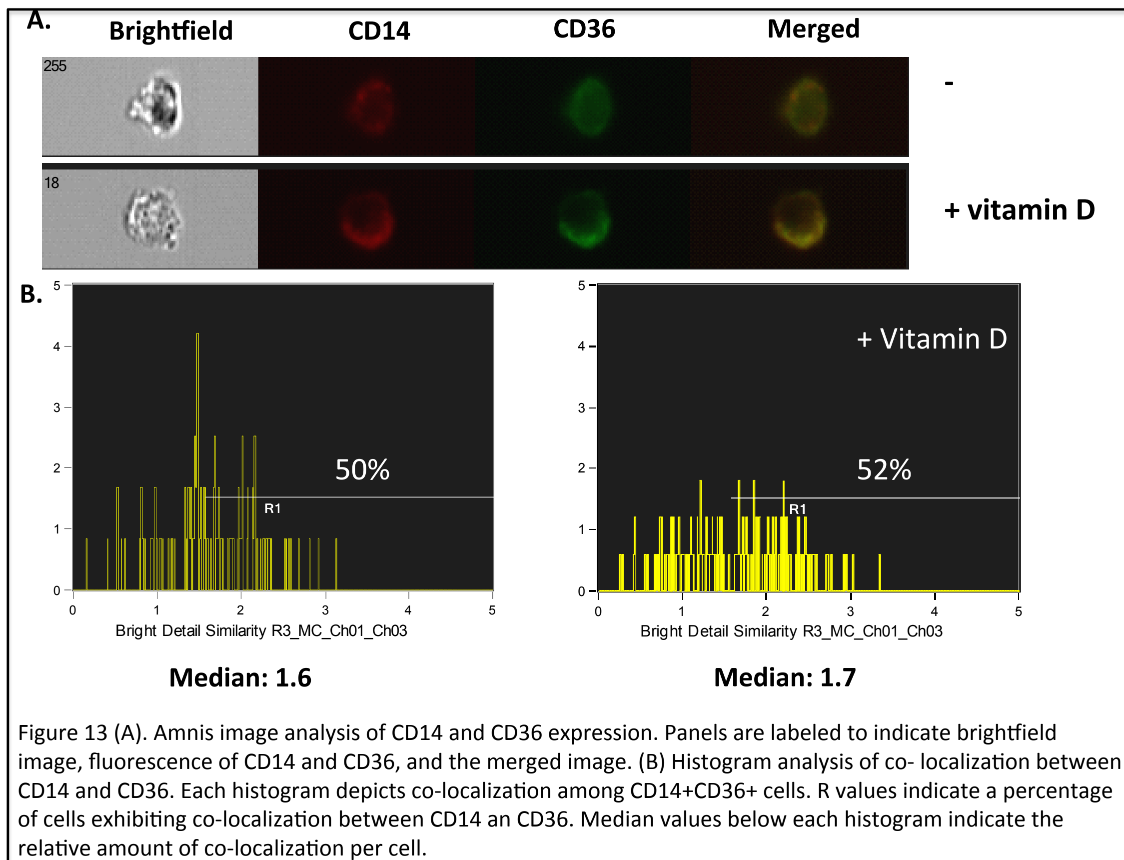

Figure 13 (A). Amnis image analysis of CD14 and CD36 expression. Panels are labeled to indicate brightfield image, fluorescence of CD14 and CD36, and the merged image. (B) Histogram analysis of co- localization between CD14 and CD36. Each histogram depicts co-localization among CD14+CD36+ cells. R values indicate a percentage of cells exhibiting co-localization between CD14 an CD36. Median values below each histogram indicate the relative amount of co-localization per cell.

Finally, we addressed the question if LAP was present in the complex with NRP-1 and/or CD14 and CD36 (Figure 14). In both vitamin D treated and untreated UCB there was no co-localization between LAP and NRP-1 detectable by the merged image analysis. Panel B shows histogram analysis of co-localization between LAP and NRP-1 with or without the addition of vitamin D. Each histogram depicts co-localization among LAP+NRP-1+ cells. In both vitamin D treated and untreated UCB there was no co-localization detectable between LAP and NRP-1. Additionally, the median values were very similar.

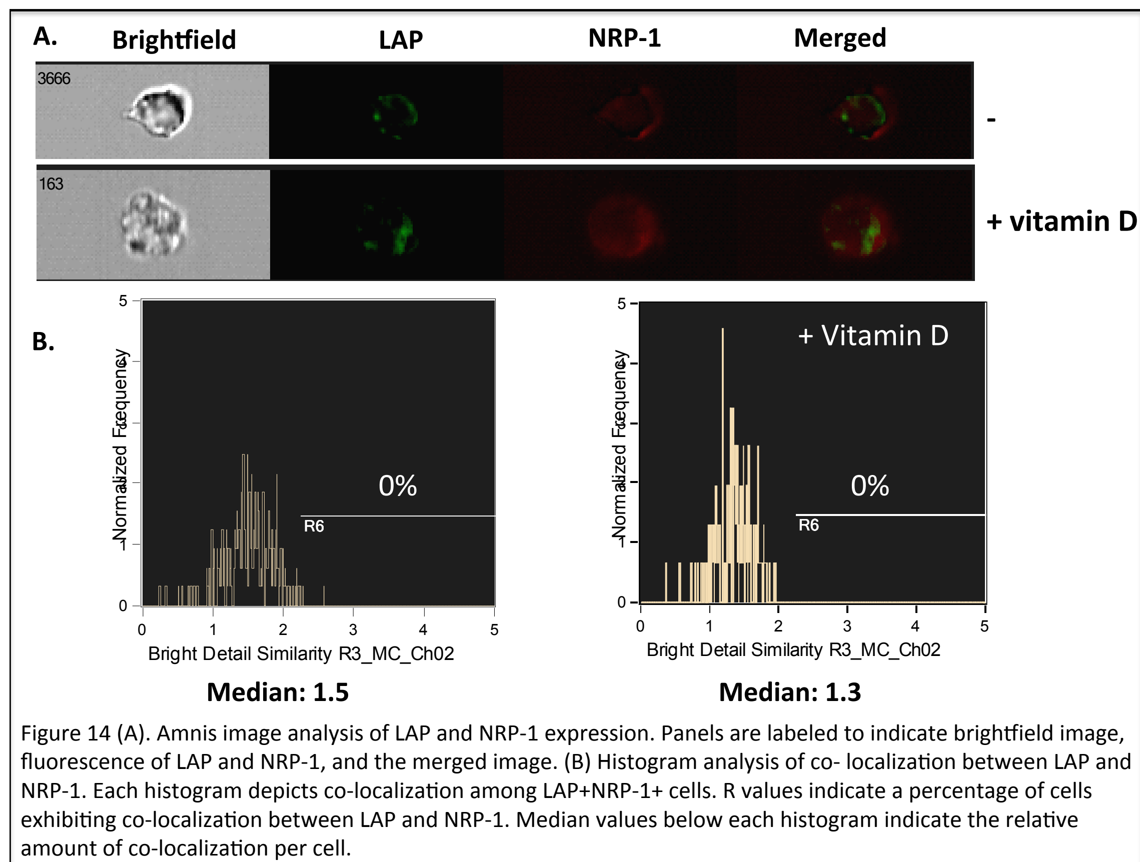

Figure 14 (A). Amnis image analysis of LAP and NRP-1 expression. Panels are labeled to indicate brightfield image, fluorescence of LAP and NRP-1, and the merged image. (B) Histogram analysis of co- localization between LAP and NRP-1. Each histogram depicts co-localization among LAP+NRP-1+ cells. R values indicate a percentage of cells exhibiting co-localization between LAP and NRP-1. Median values below each histogram indicate the relative amount of co-localization per cell.

Similarly, we could not detect co-localization between LAP and CD36 (Figure 15) or LAP and CD14 (Figure 16) by AMNIS analysis.

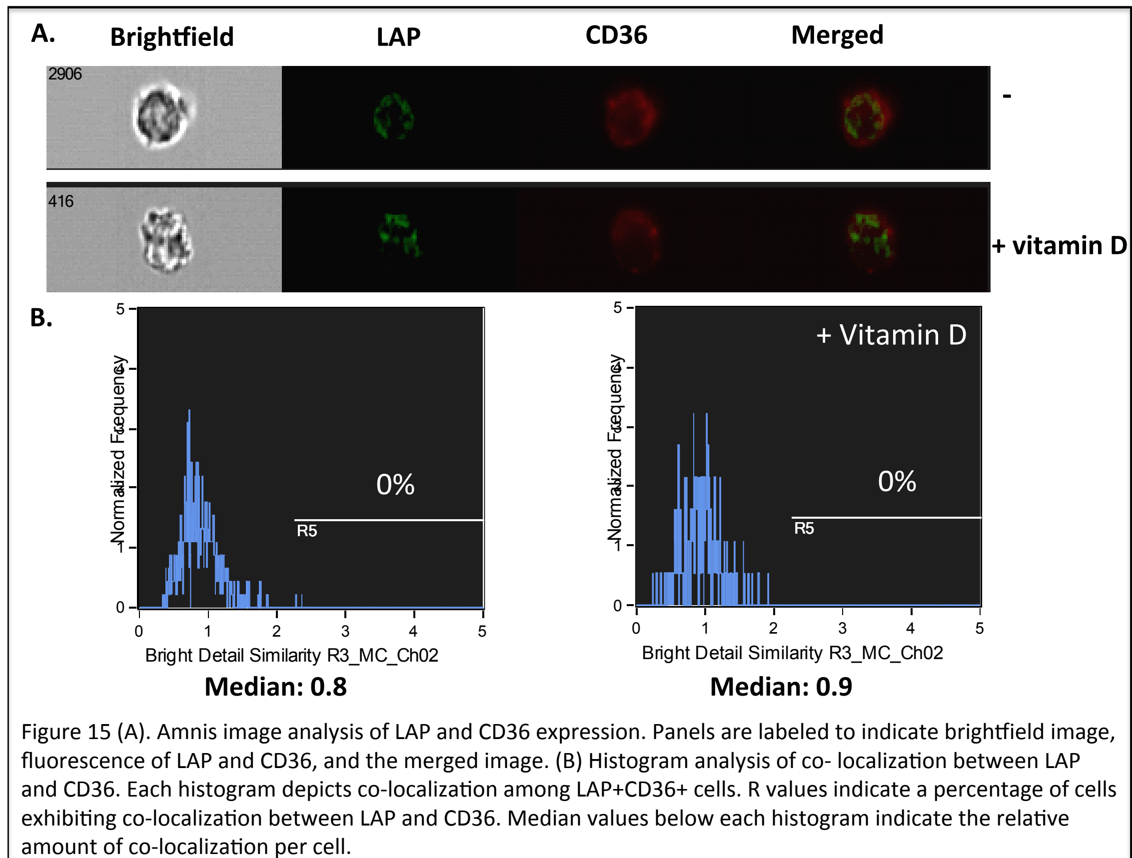

Figure 15 (A). Amnis image analysis of LAP and CD36 expression. Panels are labeled to indicate brightfield image, fluorescence of LAP and CD36, and the merged image. (B) Histogram analysis of co- localization between LAP and CD36. Each histogram depicts co-localization among LAP+CD36+ cells. R values indicate a percentage of cells exhibiting co-localization between LAP and CD36. Median values below each histogram indicate the relative amount of co-localization per cell.

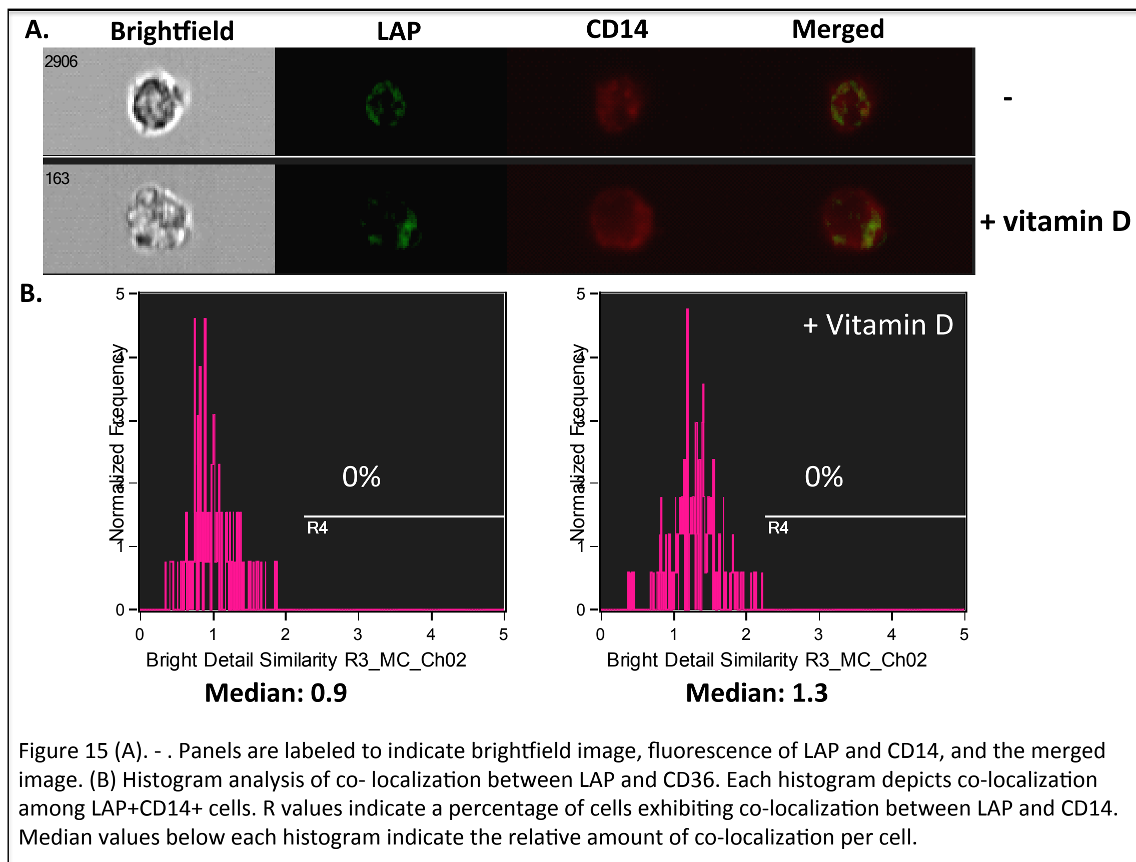

Figure 15 (A). - . Panels are labeled to indicate brightfield image, fluorescence of LAP and CD14, and the merged image. (B) Histogram analysis of co- localization between LAP and CD36. Each histogram depicts co-localization among LAP+CD14+ cells. R values indicate a percentage of cells exhibiting co-localization between LAP and CD14. Median values below each histogram indicate the relative amount of co-localization per cell.

Taken together, these data indicate that vitamin D promotes co-localization between NRP-1 and the pre-existing CD14/CD36 complex. This complex does not include LAP, suggesting that either LAP is excluded from this complex or LAP is immediately internalized and/or degraded after binding to the cell surface in proximity to this complex.

CHAPTER FOUR
DISCUSSION

Vitamin D is considered to be an immunomodulatory molecule with important implications in autoimmunity and transplantation [2, 10]. The exact mechanisms underlying the anti-inflammatory and immunosuppressive properties of vitamin D are not fully understood, however. Many clinicians and researchers have questioned whether or not vitamin D supplementation may be an effective form of treatment for individuals with autoimmune disease and graft vs. host disease (GVHD). Indeed, numerous clinical research studies have focused on vitamin D supplementation as a way to suppress the immune response and prevent graft rejection in transplant patients. For example, vitamin D supplementation has been shown to slow the loss of renal-allograft function in kidney transplant patients [9]. Additionally, various clinical trials have investigated the merits of vitamin D supplementation in M.S patients as a way to decrease inflammation, slow disease progression, and improve the overall quality of life in these patients [42]. To this end, it's been reported that vitamin D supplementation at certain concentrations decreased the incidence of disease relapse in M.S patients. In contrast, some studies showed that vitamin D had no effect on the disease progression of M.S [42]. There are more clinical trials currently underway to help evaluate the potential of vitamin D supplementation as a therapeutic for autoimmune diseases such as M.S [43]. In order to explain either the success or failure of such clinical trials, it will be beneficial to have a greater understanding of the mechanisms by which vitamin D exerts its immunomodulatory effects.

Our data predict that vitamin D regulates immune responses by enhancing human Treg differentiation. First, we found a potential positive correlation between

the serum concentration of vitamin D and the Treg frequency in adult peripheral blood. Additionally, we found that vitamin D supplementation to whole UCB as well as CD4+/THP1 cell co-culture boosted Treg differentiation. Taken together, these data suggest that the reason vitamin D may be an effective form of treatment for autoimmune disease and GVHD is because vitamin D enhances human Treg differentiation, thereby promoting an anti-inflammatory and regulatory environment. In the future, we plan on supplementing adult blood cultures with vitamin D to test if we can also boost the Treg frequency in adults. We are currently collecting equal numbers of vitamin D sufficient and deficient blood samples. Our data demonstrate that vitamin D deficient individuals have a lower peripheral blood Treg frequency as compared to vitamin D sufficient individuals. We hypothesize that addition of vitamin D to deficient adult blood cultures will increase the Treg frequency.

As previously mentioned, there appears to be a positive correlation between the serum concentration of vitamin D and the Treg frequency in adult peripheral blood (Figure 1). Noticeably, there was a discrepancy in the p-value when the data were presented by grouping analysis (Figure 1A) or as a linear regression plot (Figure 1B). The p-value was 0.01 when the data were presented by grouping analysis as compared to 0.07 in the linear regression plot. It's possible that the small sample size of our study contributed to this discrepancy since we only had 10 patients enrolled in the study. We are currently repeating this experiment to confirm our results and double our sample size. Importantly, even though the p-value was slightly different, we still saw the same trend in the data where vitamin D sufficient individuals had a higher Treg frequency as compared to vitamin D deficient individuals.

In the human body, the main circulating form of vitamin D is 25-hydroxyvitamin D3 [3, 4]. We added the active form of vitamin D, 1,25-dihyrodxyvitamin D3 to cultures of whole UCB as well as CD4+/THP1 cell co-culture to enhance Treg differentiation. Importantly, the vitamin D activating enzyme, 1-α hydroxylase (CYP27B1) is expressed by a variety of extrarenal tissues including immune cells such as monocytes, DC's, and T cells [44, 45]. Vitamin D activation in

the kidney is important to maintain bone health and regulate calcium metabolism, but researchers suspect that vitamin D activation in extrarenal tissues such as immune cells may have other important biological effects [46]. Indeed, vitamin D activation in DC's has been shown to modulate T cell responses, thereby regulating the inflammatory response [45]. In preliminary data not shown here, we determined that human UCB monocytes expressed the enzyme CYP27B1 and are therefore capable of activating 25-hydroxyvitamin D3 to 1,25-dihydroxyvitamin D3. Moreover, there are low levels of 25-hydroxyvitmain D3 in our RPMI culture media that could mediate vitamin D activation in our culture system. We found that addition of exogenous, active vitamin D enhanced Treg differentiation in our culture system.

Additionally, our data suggest that vitamin D enhances Treg differentiation by acting on monocytes to increase the expression of the monocyte cell surface molecules NRP-1 and LAP, and by promoting co-localization between CD36 and NRP-1 as well as between NRP-1 and CD14. The effect of vitamin D on the co-localization between these molecules may be functionally important for the activation of TGF-β and therefore the differentiation of Tregs in our culture system.

First, co-localization between CD36 and NRP-1 may promote TGF-β activation and enhance Treg differentiation. As previously mentioned, NRP-1 is a multifunctional protein capable of binding TGF-β at the cell surface. Moreover, researchers suspect that NRP-1 may interact with an unidentified receptor to activate latent TGF-β and inhibit the immune response [11]. CD36 is an integral membrane protein and scavenger receptor that is involved in several important physiological processes such as angiogenesis. Moreover, CD36 binds several different classes of ligands including TSP-1 [47]. In turn, TSP-1 is a matricellular glycoprotein that interacts with the LAP/latent TGF-β complex to activate TGF-β, thereby modulating immune responses [48]. We propose that NRP-1 interacts with the CD36/TSP-1 complex to facilitate the activation of TGF-β and enhance Treg differentiation in response to vitamin D.

We also demonstrated that vitamin D promoted co-localization between CD14 and NRP-1. CD14 is a cell surface molecule that functions as a co-receptor with

TLR-4 for LPS. LPS is a pathogen associated molecular pattern (PAMP) found on the outer membrane of gram negative bacteria that stimulates inflammation and activation of the innate immune response. Moreover, preliminary studies in our lab have shown that LPS addition to human UCB results in a decrease in Treg differentiation. We hypothesize that NRP-1 and CD14 form a complex at the cell surface that is regulated by LPS such that in the presence of LPS, NRP-1 is down regulated and Treg induction is prohibited. In contrast, when LPS is absent NRP-1 would be up regulated and Treg induction enhanced. We are currently working to determine the effect of LPS addition on NRP-1 expression. We plan to add soluble LPS to UCB for up to 24 hours and assess the level of NRP-1 expression by flow cytometry. We would expect to see decreased levels of NRP-1 upon LPS addition.

We were surprised to find that there was no co-localization detectable between LAP and NRP-1 because it's been documented that NRP-1 can bind to the LAP/latent TGF-β complex [11]. It's possible we were unable to detect co-localization between NRP-1 and LAP because our flow antibodies created steric hindrance that occluded this interaction. Additionally, we found that LAP is generally hard to detect by flow cytometry. As previously shown, the frequency of CD36^{hi} LAP+ cells in untreated UCB was approximately 4%. One possibility for why LAP was hard to detect at the cell surface is that LAP was rapidly internalized or removed by TSP-1 after delivering TGF-β to the NRP-1 complex. This could also explain why we were unable to detect co-localization between CD36 and LAP or CD14 and LAP. Interestingly, Glinka and colleagues determined that NRP-1 and LAP co-localized on the membrane of MDA-MB-231 cells (a human breast adenocarcinoma cell line) and they showed that the complex was internalized within minutes [49]. Additionally, it's been suggested that NRP-1 may not be the primary receptor for LAP. Researchers have found that LAP has an RGD integrin binding site and LAP can bind other receptors at the cell surface including the molecule GARP [11]. Moreover, it's previously been shown in our lab that UCB CD14^{+}CD36^{hi} monocytes express GARP (Jaeger, under revision). It's therefore possible that LAP binds to some other molecule such as GARP at the cell surface to deliver TGF-β to the NRP-1 complex in our culture system.

Cancer cells frequently express NRP-1 and RGD binding integrins [49]. NRP-1 expression by cancer cells has also been linked to tumor metastasis and is indicative of a poor prognosis [49, 50]. Parikh et al. studied the relationship between colon cancer and NRP-1 expression using human colon adenocarcinoma tissue specimens. NRP-1 was expressed in all adenocarcinoma tissue specimens collected. In contrast, NRP-1 was not expressed in colon samples taken from the adjacent nonmalignant tissue. Furthermore, Parikh and colleagues found that over expression of NRP-1 in KM12SM/LM2 cells (human colon cancer cells) promoted an increase in tumor growth [51]. NRP-1 expression has also been implicated in a number of other cancers including breast, prostate, lung, and pancreatic cancer [52]. In many cases the functional role of NRP-1 in promoting tumor survival and growth remains unclear. Researchers have demonstrated that NRP-1 expression by breast cancer cells facilitated tumor survival by inhibiting hypoxia-induced apoptosis [52].

Researchers suspect another way NRP-1 may function to aid tumor progression is by activating TGF-β at the cancer cell membrane [49]. Glinka and colleagues studied the ability of NRP-1 to activate TGF-β at the cell surface using a breast cancer cell line. They determined that NRP-1 could activate LAP/ TGF-β after the latent complex had attached to an αvβ3 integrin or GARP at the cell surface. They also demonstrated that NRP-1 bound TGF-β receptors at the cell surface, including the TβRI and TβRII receptors. TβRI and TβRII normally form a complex with TGF-β at the cell surface, signaling through SMAD's to activate cellular transcription. NRP-1 was found to be co-localized with TβRI at the cell membrane of breast cancer cells. Additionally, TβRI, TβRII, and NRP-1 were co-internalized upon addition of TGF-β. Knockdown of NRP-1 in breast cancer cells has also been shown to greatly reduce TGF-β signaling. These data suggest that NRP-1 can bind and activate TGF-β as well as augment TGF-β signaling in cancer cells [49].

In addition to cancer, NRP-1 may also play a role in autoimmunity. MS is a progressive inflammatory disease affecting the central nervous system (CNS). The underlying cause of MS in humans is still relatively unclear. It's been demonstrated that antigen specific CD4+ T cells migrate to the CNS and destroy the myelin sheath

of nerves in EAE, a mouse model of MS. The inflammation and destruction of myelin caused by CD4+ T cells results in a decline of motor function culminating in paralysis [53]. As previously mentioned, NRP-1 is expressed on mouse CD4+CD25+ Treg cells and by some activated human Tregs [11, 38, 39]. Solomon et al. demonstrated that overexpression of NRP-1 in mouse CD4+ T cells correlated with a decrease in the disease progression of EAE. Additionally, NRP-1 knockout mice developed more severe disease [53]. NRP-1 expression has also been studied in the context of the autoimmune disease IBD. Murine CD4+CD25+ Treg cells expressing Foxp3 and NRP-1 have been identified as a critical T cell subset in the regulation of the aberrant gut inflammatory response characteristic of IBD [54]. These data indicate that NRP-1 may play an important role in regulating the immune response and protecting against the development of various autoimmune diseases.

The link between vitamin D and cancer risk is still poorly understood. One hypothesis is that the anti-proliferative effects of vitamin D are protective against tumor development and progression [55]. In fact, epidemiological studies indicate that vitamin D levels inversely correlate with the development and/or fatality of breast cancer [56]. Additionally, there are reports indicating that certain VDR polymorphisms are associated with an increased risk in the development of breast cancer [55, 57]. The *FokI* single nucleotide polymorphism in the VDR correlates with decreased transcriptional activation of the VDR. McKay et al. found that the *FokI* polymorphism was associated with an increased risk in the development of breast cancer [55]. In contrast, Bretherton-Watt and colleagues determined that there was no correlation between the *FokI* polymorphism and breast cancer risk in Caucasian women from the United Kingdom [58]. Admittedly, there are many discrepancies in the literature regarding the link between particular VDR polymorphisms and breast cancer risk. As a caveat to these findings, it's understood that allelic frequencies of VDR polymorphisms vary by ethnicity. It's possible that the link between vitamin D levels and cancer may differ depending on sex, ethnicity, or other factors.

Researchers have also investigated a potential link between cancer and the expression of the vitamin D activating and catabolizing enzymes, CYP27B1 and CYP24A1 respectively. As previously mentioned, the enzyme CYP27B1 converts 25-

hydroxyvitamin D3 to 1,25-dihydroxyvitamin D3, the biologically active form of vitamin D. Vitamin D activation is regulated by the enzyme CYP24A1, which functions to inactivate 1,25-dihydroxyvitamin D3. CYP27B1 and CYP24A1 are both expressed in the kidney as well as by a variety of other cell types including immune cells and cancer cells [59]. Overexpression of CYP24A1 has been associated with colorectal cancer [60]. Expression of CYP27B1 and CYP24A1 has also been studied in the context of breast cancer. Townsend et al. compared the RNA levels of CYP27B1, the VDR, and CYP24A1 in normal breast tissue and cancerous breast tissue among Caucasian females. Expression of CYP27B1, the VDR, and CYP24A1 were all up-regulated in breast cancer tissue as compared to normal breast tissue. Furthermore, both CYP27B1 and CYP24A1 were shown to be functionally active in the normal and cancerous tissues [59]. Admittedly, the up-regulation of both the vitamin D activating and catabolizing enzymes in breast cancer cells as reported by Townsend et al. makes it difficult to conclude that vitamin D has a protective effect against breast cancer. Others have reported that there are multiple VDR binding sites within the promoter of the CYP24A1 gene, and the number of VDR binding sites was increased in malignant mammary cells as compared to normal mammary cells. These results suggest that the catabolism of vitamin D may be increased selectively in cancerous cells to promote disease progression [61].

Interestingly, our data suggest that high levels of vitamin D may increase the risk of cancer. We demonstrated that vitamin D increased the expression of NRP-1 in human UCB. Moreover, NRP-1 expression by cancer cells is associated with increased tumor growth and metastasis [49]. If vitamin D increased the expression of NRP-1 in cancer cells, then high levels of vitamin D could contribute to increased tumor growth and metastasis. It's possible that the effect of vitamin D on the expression of NRP-1 in adult cancer cells is different than in human UCB, however. In fact, we found that while vitamin D increased the expression of NRP-1 in human UCB, the effect of vitamin D on NRP-1 expression in THP1 cells was not as strong Furthermore, the transcriptional and/or translational regulation of NRP-1 in cancer cells may be different than in healthy UCB mononuclear cells.

Lastly, it's still unknown whether vitamin D effects the expression of NRP-1 at the transcriptional level or the protein level in our culture system. Regulation of transcription of the NRP-1 gene is controlled, in part, by the CCAAT box upstream of the transcription start site [62, 63]. There are many CCAAT binding proteins that activate transcription. In mice, the transcription factor nuclear factor Y (NF-Y) is an important CCAAT binding protein that activates transcription of various genes. The VDR is also known to promote activation of certain genes with a CCAAT box in the promoter region. NF-Y and the VDR were shown to work synergistically to activate transcription of the osteoclast differentiation factor (ODF) gene in mice. Kabe et al. determined that transcriptional activation of the ODF gene by vitamin D required the presence of the CCAAT box. Moreover, NF-Y was required to recruit RNA Pol II to the promoter region [64].

In HeLa cells (cervical cancer cell line), the transcription factors SP1 and AP-1 as well as the CCAAT box have been shown to be important for the transcriptional activation of the NRP-1 gene [63]. There is little work characterizing the transcriptional regulation of NRP-1 in human immune cells, however, and there is no literature on the regulation of NRP-1 expression in UCB. To understand the molecular mechanism by which vitamin D increases the expression of NRP-1 in UCB, we performed an NCBI Gene search, finding multiple VDRE's within the NRP-1 gene. We hypothesize that upon supplementation of vitamin D, the VDR may bind a VDRE within the NRP-1 gene to activate gene transcription. Efforts to determine if vitamin D effects NRP-1 at the transcriptional level are underway. We incubated whole UCB for up to 24 hours with or without the addition of vitamin D. NRP-1 mRNA levels will be assessed by RT-PCR. If vitamin D acts at the transcription level to increase the expression of NRP-1, then we should see an increase in NRP-1 mRNA by RT- PCR in vitamin D treated UCB. If vitamin D has no effect on NRP-1 at the transcription level, we will evaluate the effect of vitamin D on NRP-1 at the protein level. We can incubate whole UCB overnight with or without the addition of vitamin D and then run a western blot probing for NRP-1. If vitamin D acts at the protein level to increase the expression of NRP-1, then we should see an increase in the amount of NRP-1 protein by western blot in vitamin D treated UCB.

In conclusion, by using an *ex vivo* human UCB culture system we determined that vitamin D has a positive effect on human Treg differentiation. Additionally, our data suggest that vitamin D enhances Treg differentiation by increasing the expression of the monocyte cell surface molecules NRP-1 and LAP and by promoting co-localization between NRP-1 and CD14 as well as NRP-1 and CD36. Our data predict that the co-localization between these molecules is important for the activation of TGF-β and therefore the differentiation of Tregs in our culture system.

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