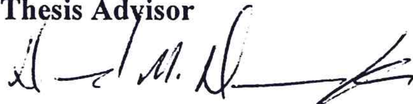
Human Monocyte-Derived Dendritic Cell Metabolic Phenotype

Thesis submitted to the Faculty of the
University of Michigan- Flint
in partial fulfillment of the
Master of Science in Biology degree
By

Brittany N. Douglas

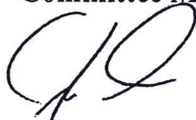
Approved by:

Dr. David Duriancik
Thesis Advisor



17 Aug 2018

Dr. Joe Sucic
Committee Member



17 Aug 2018

8/23/2018

Dr. Ron Stamper
Committee Member

Summer, 2018

Abstract

Lead (Pb) is an environmental contaminant associated with adverse health problems across the world that affects multiple body systems. Previous studies demonstrate chronic inflammation of intestinal, respiratory, and integumentary systems with even nominal levels of exposure. Monocyte-derived dendritic cells play an important role in innate and adaptive immunity because they have the ability to stimulate CD4+ and CD8+ T-cell responses and regulate B-cell immunoglobulin production. Successful development of a protocol for monocyte-derived dendritic cells from human peripheral blood mononuclear cells and isolation using anti-CD14 conjugated with magnetic particles. Flow cytometry was used to analyze surface cell markers of cultured cells and was consistent with phenotypical characteristics of dendritic cells. Increased expression of CD209 and CD1c on day 7 of cell culture was observed. In addition, reduced expression of CD14 and increased expression of costimulatory factors CD83/CD86 was also observed. It is our effort to determine the effects of lead acetate on the immune cell function by analyzing the ability of these monocyte-derived dendritic cells to metabolize vitamin A from the circulating form, retinol, to the steroid hormone transcription factor ligand, all-*trans* retinoic acid. We expect that in the presence of Pb, dendritic cells will produce more bioactive vitamin A resulting in more inflammatory immune response. In future efforts, our laboratory will determine the effects of Pb on STRA6 (stimulated by retinoic acid 6) expression, which is known as the receptor for retinol binding protein (RBP) and transports vitamin A to tissues, and use the Aldefluor assay as a marker for aldehyde dehydrogenase activity in monocyte-derived dendritic cells.

Acknowledgements

This work would not have been possible without the financial support provided by the University of Michigan – Flint Biology Department. I am grateful for receiving a graduate student research assistantship which has provided financial support to assist me in pursuing these academic goals.

To all those whom I have had the pleasure of learning from and working with on this and other projects, I am grateful. I would like to thank each of the members of my committee, Dr. Joe Sucic and Dr. Ron Stamper, as they have provided personal and professional guidance and have contributed greatly to my education.

To Dr. John Gerlach and Dr. Susan McQuiston at the Biomedical Laboratory Diagnostics program at Michigan State University, who patiently taught me techniques required for flow cytometry.

I am especially thankful to Dr. David Duriancik who has been supportive and actively guided me throughout the course of my graduate studies. As my teacher and mentor, he has continuously encouraged and provided training for this project. He has taught me laboratory skills that will follow me throughout my professional career. I am unable to show the appreciation I have for his endless support in words.

Lastly, to my wonderful family, your love and guidance motivates me in everything I pursue. I would not have made it this far without your unending inspiration.

Table of Contents

I. Literature Review
II. Methods
    a. Participants
    b. Samples
    c. Sample Processing
    d. Monocyte Isolation
    e. Monocyte Derived Dendritic Cell Culture
    f. Monocyte Derived Dendritic Cell Analysis
III. Results
    a. Cell Counts/Viability
    b. CD14 Magnetic Particle
        i. Concentration
        ii. Separation
    c. Flow Cytometry Observed Expression
        i. CD14
        ii. CD1c
        iii. CD209
        iv. CD83/CD86
    d. Figures
IV. Discussion
V. Appendices
    a. Explanation of Acronyms
    b. Informed Consent
    c. Recruitment Flyer
VI. References

Literature Review

The heavy metal, lead (Pb), is an environmental contaminant associated with adverse health problems across the world. Multiple body systems are affected by this toxic metal, including neurologic, renal, cardiovascular, hematologic, and gastrointestinal systems (WHO, 2017). Even relatively low levels of exposure can result in irreversible neurologic damage, with children being exceptionally vulnerable to its effects. Children are more susceptible to Pb toxicity because their gastrointestinal tract is capable of five to eight times the absorption of an adult with better retention in tissues (Mahaffey, 2009). Sources of the contaminant are commonly found in households but are particularly prevalent in developing countries. Cities within the United States are dealing with Pb, such as Flint, Michigan, whose river was treated improperly causing Pb from the aging pipes to leach into the water supply and the recent closure by Exide Technologies battery plant in Vernon, California where the improper recycling of Pb acid batteries resulted in pollution of neighborhoods in southeast Los Angeles county (Barboza, 2015). There are increasing efforts to reduce the use and minimize exposure, while effectively ensuring the safe recycling of Pb containing waste (WHO, 2017). However, the neurotoxin still poses a public health concern and the effects of exposure and long-term consequences require further investigation.

The physiological effects of Pb exposure on human health include damage caused by inflammation. Previous studies demonstrate that Pb may induce oxidative stress by increasing the production of reactive oxygen species (ROS), and antioxidants have an important role to reduce the effects of Pb toxicity (Gurer-Orhan, Sabir, and Ozgunes., 2004). Cell membrane damage caused by free radicals results in lipid peroxidation which, in turn, triggers the signaling cascades of the inflammatory process (Sirivarasai, et al., 2013). Blood Pb concentration has been

associated with increased white blood cell (WBC) count, increased levels of tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine (Kim, et al., 2007), and high-sensitivity C-reactive protein (hs-CRP), another marker of inflammation (Khan, et. al., 2008).

There is abundant literature showing that Pb exerts adverse health effects on the human immune system and that it has the ability to increase susceptibility to bacterial and viral infections. The mechanism by which Pb exposure affects the immune response is not completely characterized; however, Pb exposure has been shown to result in reduced antibody titers, reduced antibody synthesis, and a decreased mitogen-induced activation of T-lymphocytes (Ewers, et al., 1982). A study consisting of 125 male subjects, 72 of them being workers in a battery plant exposed to Pb, investigated the relationship between the individual blood Pb level and the serum concentrations of complement C3, IgM, IgG, and IgA (Ewers, U., et al., 1982). On average the Pb workers had reduced IgM, IgG, and complement C3 levels, which is indicative of immune suppression. Another study demonstrates the toxic effects of Pb during early development by assessing the immune system of developing Fischer 344 rat embryos using *in vivo* and *ex vivo* experiments (Miller, 1998). The results indicate that exposure of mothers to moderate levels of Pb produces chronic immune modulation, including increased levels of IgE and decreased levels of interferon gamma (INF-γ) (Miller, 1998). The offspring total leukocyte counts were decreased significantly and this demonstrates that *in utero* exposure to Pb modulates the immune system. These offspring were later examined as adults and persistent effects of Pb on immune function were observed (Miller, 1998). Based on these results, early immune development can be compromised even with short-term exposure during pregnancy in rodents. We expect to similar results in other species, including humans. The specific immune alterations observed are that Pb alters the balance of T helper (Th) cell activity (Th1 & Th2) and that embryonic exposure

decreased Th1 function while elevating Th2 dependent activities (Miller, 1998). Th2 hyperresponsiveness increases the susceptibility of the host to infection and may predispose to allergic atopic manifestations and autoimmunity (Romagnani, 1995). Recent increases in the severity and prevalence of childhood asthma have impelled a search for environmental risk factors. There is a known role of other heavy metals facilitating autoimmune reactions. The Pb induced Th1-Th2 immunomodulation and the embryonic exposure results indicate a relationship between Pb exposure and increased the propensity of allergies and other chronic inflammatory disease (Miller, 1998).

Mice exposed to Pb had decreased Th1 responses and INF-γ levels (a main Th1 cytokine that can inhibit Th2 cell development). The effects of Pb on immunity were examined in INF-γ knockout (KO) mice, which displayed significantly enhanced serum IgG1 anti-keyhole limpet hemocyanin (KLH) (Gao, et al., 2005). Pb exposure significantly increased IgG1 and IgG2a anti-KLH production in the $INF\text{-}\gamma^{-/-}$. Pb enhanced the Th2 responses of the wildtype (WT) mice and altered CD4+ T-cell mediated immunity. These adverse effects on immune cell functions are how Pb compromises host resistance to bacterial infection (Gao, et al., 2005). This Th2-induced delayed type hypersensitivity (DTH) is suggested to be associated with asthma (Snyder, et. al., 2000). Production of INF-γ is used as an index for Th1 responses and Pb has been shown to inhibit INF-γ production. This further supports that Th1 and Th2 imbalance caused by Pb exposure is implicated in the development of allergies and autoimmune diseases (Gao, et al., 2005).

Studies suggest that vitamin A deficiency induces inflammation and worsens pre-existing inflammatory states, and that supplementation could ameliorate inflammation (Reifen, 2002). Supplementation of vitamin A was found beneficial in a number of selected cases of

inflammatory conditions such as acne vulgaris, broncho-pulmonary dysplasia, and some cancers (Reifen, 2002). Vitamin A plays a role in the function of both innate and adaptive immunity (Reifen, 2002). Vitamin A deficiency decreases monocyte numbers and compromises the ability of macrophages and neutrophils to migrate to sites of infection, phagocytize, and kill bacteria (Stephensen, 2001). Vitamin A deficiency in rats also decreases natural killer (NK) cell numbers. When treated with dietary retinoic acid, NK cell numbers returned to normal within a week (Adams et. al., 1995). Vitamin A is necessary for CD4+ Th cells lymphocyte development. Vitamin A deficiency enhances Th1 response and suppressed Th2 response. Pro-inflammatory responses that primarily kill intracellular parasites and initiate autoimmune responses are products of the Th1 cytokines TNFα, interleukin (IL) – 12, and IFNγ. This reaction may be uncontrolled, which can cause serious damage to tissues and functions; therefore, Th2 responses counteract this mechanism (Romagnani, 1992). The Th2 cytokines IL-4, IL-5, and IL-10 promote antibody class switching in B lymphocytes. There are other Th cell subsets that must remain in balance, including T regulatory (Treg) cells and Th17 cells. Vitamin A is important in the balance of Treg and Th17 cell development. As well as, IgE and also interleukin-10 (IL-10) which acts as an anti-inflammatory (Deo, et al, 2010). For optimal immune function, humans must possess a balance of Th1 and Th2 response. Immunobiologists regard allergies as a Th2 imbalance in the immune system resulting in inflammatory response (Romagnani, 1992). Antigen-presenting cells such as dendritic cells (DC) are important for directing the development of naïve Th cell into these various functional Th cell subsets.

Murine DC subset distribution and function is well characterized, but correlates to human DC remain difficult due to limited access to human tissue samples. In murine secondary lymphoid tissues, there are 3 main DC subsets; 2 conventional DC (cDC) subsets and 1

plasmacytoid DC subset. Plasmacytoid DC produce large amounts of type 1 interferon (IFNα/β) in response to intracellular toll-like receptor activation by ligands such as single stranded RNA and methylated cytosine and guanine DNA (Cantorna, et al., 1995). Plasmacytoid DC minimally function as antigen-presenting cells. Antigen-presentation function is mediated by the 2 cDC subsets (Shortman, 2010). CD11b+ cDC in mice primarily present extracellular antigens in the context of major histocompatibility complex II to naïve Th cells and develop Th2 responses. CD8a+ cDC in mice primarily present intracellular antigens in the context of major histocompatibility complex I to CD8+ T cells and develop Th1 responses (Shortman, 2010). However, CD8a+ cDC can cross-present intracellular antigens in MHC II to naïve Th cells. Interestingly, in mucosal tissues, CD103+ cDC are present in high numbers. These CD103+ cDC correspond to CD8a+ cDC in secondary lymphoid tissue (Shortman, 2010). CD103+ murine cDC express vitamin A metabolic activity (Cantorna, et al., 1995). Human myeloid DCs (mDCs), also known as a type of conventional DCs, contain a major subset easily identified by CD1c+ markers. The mDCs typically display antigens CD13, CD33, and CD11b. In humans, mDCs express CD11c and lack CD14 or CD16 and can be categorized as CD1c+ and CD141+ fractions (Collin, 2013). CD14+ DCs found in tissues are a subset of CD11c+ mDCs and more closely resemble monocytes than the CD1c+ and CD141+ types and may also originate from traditional monocytes. Understanding the various markers will allow us to determine which DC subsets are produced from the differentiation induced by GM-CSF and IL-4 (Teddler and Jansen, 2001). The lack of expression of CD83 and CD86 markers will allow us to confirm the presence of immature dendritic cells after differentiation as these co-stimulatory markers are increased upon DC maturation.

DCs express vitamin A metabolic activity. **Figure 1** demonstrates this pathway beginning with retinol, liberated from liver stores or absorbed from the diet, which circulates in the blood bound to retinol binding protein (RBP) and transthyretin. RBP binds to STRA6 (stimulated by retinoic acid 6) allowing retinol uptake into the cell. Cytosolic binding proteins, such as cellular retinol binding protein, bind to retinol or it is reversibly oxidized to retinaldehyde by non-specific alcohol dehydrogenase enzymes. Retinal binds to cellular retinal binding proteins or is irreversibly oxidized to retinoic acid. RALDH2 expression metabolizes retinol to all-*trans* retinoic acid (atRA). Retinoic acid binds to retinoic acid receptors (alpha, beta, or gamma), which will heterodimerize with nuclear transcription factors such as retinoid X receptor to regulate genes This is accomplished through binding to retinoic acid response elements in the promoter region of genes and will recruit co-activators or repressors.

STRA6 is known as the receptor for retinol binding protein (RBP) and transports vitamin A to tissues. STRA6 can be found in various tissues such as spleen, kidney, eye, placenta, and female reproductive tract where it functions as both a membrane transporter and a cell surface receptor. It is known primarily as a retinol binding protein and for transporting retinol to tissue in the eye (Berry et. al., 2013). Mutations in the STRA6 gene may result in several different abnormalities of the eye such as microphthalmia, anophthalmia, and coloboma (Casey et. al., 2011). The presence of STRA6 in multiple organ systems indicates its importance to tissues and functions outside of the eye. In fact, STRA6 mutations can be fatal in embryonic development (Ruiz et. al., 2012). It has been demonstrated that single nucleotide polymorphisms in STRA6 are associated with type 2 diabetes. This may be because STRA6 signaling activates the transcription factor, STAT5 (Kawaguchi, et al., 2015). Suppressor of cytokine signaling 3 (SOCS3) is a STAT5 target gene and it functions as a strong inhibitor of insulin signaling. As a

result, STRA6 signaling suppresses the response to insulin by inhibiting the phosphorylation of the insulin receptor by an influx of insulin (Noy, 2016). Patients with chronic inflammation, such as obese patients, are not able to produce as potent of an immunological response as a person with healthy body weight and this is partially due to the SOCS3. Increased RBP in an obese animal will increase STRA6 activity, resulting in insulin resistance. There is a positive correlation between the chronic inflammatory state of obesity and STRA6 expression. Our lab will be investigating the effect Pb exposure has on vitamin A production and metabolism of immune cells by analyzing the response of the STRA6 receptor. A study showed that polymorphonuclear leukocytes (PMN) harvested from the blood of workers exposed to Pb displayed reduced chemotactic activity (Valentino, et al., 1991). This locomotion is stimulated through a specific membrane receptor and was found to be impaired in the PMN of these workers. This reduction of chemotaxis is partially due to Pb related modification of plasma membrane lipids because PMN locomotion is influenced by fatty acids (Valentino, et al., 1991). This study demonstrates that even low Pb levels can damage the essential function of immune cells.

Culturing monocyte-derived dendritic cells (moDCs) with lead acetate supplemented media is expected to induce an inflammatory response, therefore increasing the expression of the surface membrane receptor, STRA6. The increase in receptors should allow for increased retinol metabolism resulting in enhanced cellular uptake of vitamin A. Determining the effect of lead on STRA6 expression in mo-DCs will allow for a potential therapeutic target to offset increased risk of allergies and asthma in lead exposed individuals.

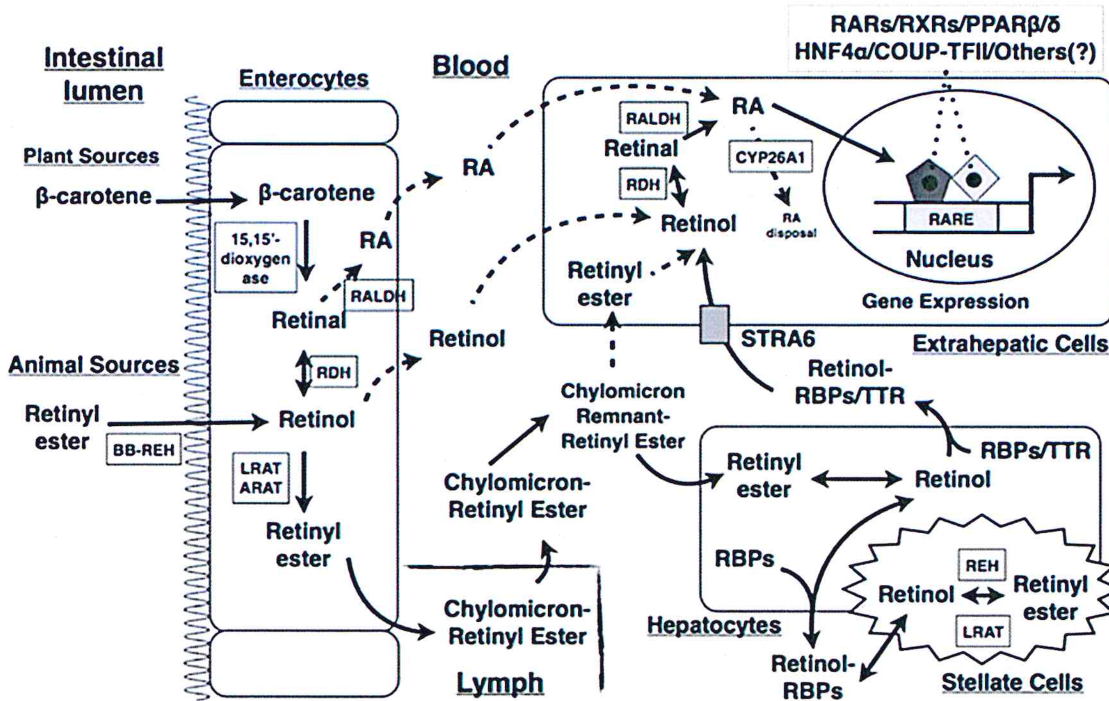


Figure 1: Pathway of vitamin A metabolism illustrating STRA6 as a membrane surface receptor and the steroid hormone transcription factor ligand function of all-*trans* retinoic acid (Chen and Chen, 2015).

Methods

Participants

We recruited approximately 10 subjects for troubleshooting experiments. Human subjects were recruited from a convenience sample of University of Michigan-Flint faculty, students, and staff. Subjects were verbally screened for inclusion criteria. Participants were required to fast eight hours prior to blood donation. Inclusion criteria were: generally healthy (no known chronic or infectious disease and no known blood clotting issues), not currently pregnant, non-smoker, and between the ages of 18 to 70. All protocols were approved by the University of Michigan – Flint Institutional Review Board; approval HUM00130415.

Samples

Two volumes of 8-10 mL human blood were collected from each volunteer by standard venipuncture into heparinized vacutainer tubes. A certified phlebotomy technician (DMD) performed all blood draws.

Sample Processing

Heparinized whole blood was layered over 20mL Histopaque 1077 (Fisher Scientific) and density gradient centrifugation was performed by centrifuging at room temperature (25C) for 30 minutes at 400xg without braking. The buffy coat was removed and washed with sterile RPMI-1640 media and centrifuged at 320xg for 5 minutes. Cell pellets were resuspended in residual volume and washed three times with sterile RPMI-1640, first with 15 mL, then 10 mL, and finally 5 mL. Total cell numbers were obtained from hemocytometer cell counts with a 1:10 dilution using trypan blue. Cells were equally split into 2 separate tubes at this step.

Monocyte Isolation

Magnetic cell sorting was performed to isolate monocytes. Total mononuclear cells were incubated with 2.5uL of anti-human CD14 antibody per $1x10^6$ cells conjugated to magnetic particles for 30 minutes at room temperature. Cells were diluted with 1mL of sterile RPMI-1640. Cells were incubated on a magnet (BD Bioscience iMag) for 10 minutes. The negative fraction was discarded (or transferred to a different tube), and positive cells were resuspended in 1mL of sterile RPMI-1640. Cells were incubated on a magnet for 5 minutes. The negative fraction was discarded (or transferred to a different tube), and positive cells were resuspended in 1mL of sterile RPMI-1640. Cells were incubated on a magnet for 5 minutes. The negative fraction was discarded (or transferred to a different tube), and positive cells were resuspended in 3mL of sterile RPMI-1640. Previously, cells were equally split at this step. Cell pellets were formed by centrifugation at 320xg for 10 minutes at room temperature. Supernatant was removed, and cells were suspended in cell culture media described below.

Monocyte-Derived Dendritic Cell Culture

Cell were placed in 24 well plates and observed under microscopy. Positively selected monocytes were cultured at approximately $1x10^6$ cell/mL in complete RPMI-1640 (10% FBS, 1x Pen/Strep, 1x non-essential amino acids, 50uM beta mercaptoethanol) supplemented with 100ng/mL GM-CSF and 50ng/mL IL-4 and incubated at 37C, 5% CO_2 in air and humidity. On day 3 and day 5, one half of the culture media and any non-adherent cells were removed. Non-adherent cells were centrifuged for 10 minutes at 320xg and room temperature. Supernatant was removed, and non-adherent cells were resuspended in equal volume of complete RPMI-1640 supplemented with fresh cytokine and incubated at 37C, 5% CO_2 in air and humidity. Cells were harvested on day 7.

Monocyte-Derived Dendritic Cell Analysis

Cell culture plates were placed on ice. All media and cells were removed using ice cold fluorescence activated cell sorting (FACS) buffer (0.1% sodium azide, 5% Fetal Bovine Serum in Dulbecco's Phosphate Buffered Saline) and transferred to appropriately labeled FACS tubes. Culture wells were washed 3-5 times with cold FACS buffer, centrifuged at 320xg, 25C, for 5 minutes. Each well was visualized with microscopy to ensure all loosely adherent cells were removed from the well. Cells and compensation beads were stained with 2.5uL of each fluorochrome-conjugated antibody. The antibodies used were anti-Human CD209-FITC, anti-Human CD14-PE, anti-Human CD83-PE-Cy7, anti-Human CD86-PE-Cy7, and anti-Human CD1c-APC. The control contained 10µL of each sample to which no fluorochrome-conjugated antibodies were added. Each sample was incubated for 30 minutes on ice in the dark and vortexed 15 minutes into incubation. Each sample was washed three times with FACS buffer and centrifuged at 320xg, 4C, for 10 minutes. Cells were analyzed using a BD Bioscience FACS Calibur flow cytometer. Flow cytometry data were analyzed using FlowJo data analysis software.

Results

We obtained approximately $1x10^7$ PBMC from 16-20mL of whole blood. Approximately 10% of these cells were expected to be monocytes. Positive and negative fraction cells were analyzed by flow cytometry to determine the efficiency of magnetic selection. Cells were labeled with anti-CD14 conjugated to magnetic particles, underwent positive selection, and then flow cytometric analysis. Efficiency of monocyte positive selection (Figure 2) was determined by observing positive and negative fractions of cells after positive magnetic selection.

Optimal anti-CD14 concentration for efficient positive and negative selection was determined by analyzing the percent captures in both positive and negative fractions (Table 1).

Table 1. Anti-CD14 Concentrations

Anti-CD14 Concentration	Positive Fraction		Negative Fraction	
	Monocyte %	Lymphocyte %	Monocyte %	Lymphocyte %
1.25	81.4 ± 9.2	14.5 ± 7.8	16.4 ± 5.5	77.6 ± 4.6
2.5	81.1 ± 12.0	14.4 ± 11.0	14.9 ± 2.7	78.7 ± 1.8
5	80.1 ± 4.8	13.8 ± 5.6	14.9 ± 4.4	79.0 ± 4.2

Mean ± Std dev n=4. Percentages of monocytes and lymphocytes were collected from positive and negative fractions. Concentrations were 1.25, 2.5, and 5μL per million cells.

There were consistently more cells in the well from the positive fraction and fewer cells in the negative fraction that received half of the volume/cells after magnetic cell sorting. We obtained equal distribution of cells between the wells by splitting the cells equally prior to magnetic cell sorting.

Purified monocytes were used to generate monocyte-derived dendritic cells and on day 7 of culture were analyzed using flow cytometry. Gating was performed as depicted in Figure 2. Properties of the cultured cells were determined by analyzing the scatter properties (Figure 4). Monocyte-derived dendritic cells were confirmed as CD14 negative, CD209 positive, and CD1c positive (Figure 3). Also, the differentiated monocyte derived dendritic cells (mo-DCs) were found to be expressing high levels of co-stimulatory molecules CD83 and CD86 (Figure 3).

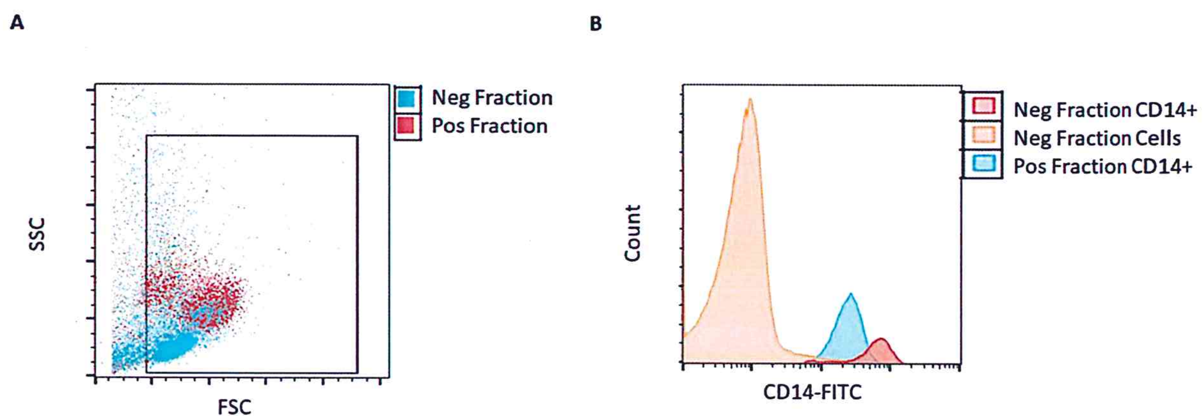


Figure 2. Efficiency of monocyte positive selection. A, forward (FSC) and side (SSC) scatter properties of positive (red) and negative (blue) fraction of cells after positive magnetic selection. Cells were labeled with anti-CD14 (MΦP9) conjugated to magnetic particles. B, Positive (blue) and negative (red and orange) fraction cells were labeled with anti-CD14 (M5E2) conjugated to FITC.

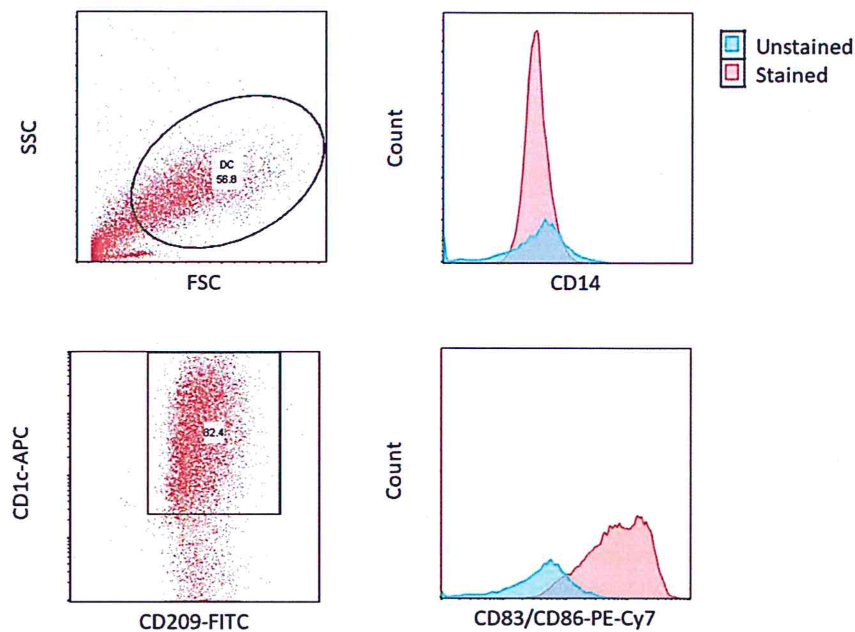


Figure 3

Figure 3. Gating of monocyte-derived dendritic cells. Forward scatter (FSC) and side scatter (SSC) display where gating was performed in relation to cell size and granularity. Monocytes were positively selected for CD14 expression using positive selection of anti-CD14 magnetic particles. Monocyte-derived dendritic cells were confirmed as CD14 negative, CD209 positive, CD1c positive. Monocyte-derived dendritic cells were expressing high levels of the co-stimulatory molecules CD83 and CD86.

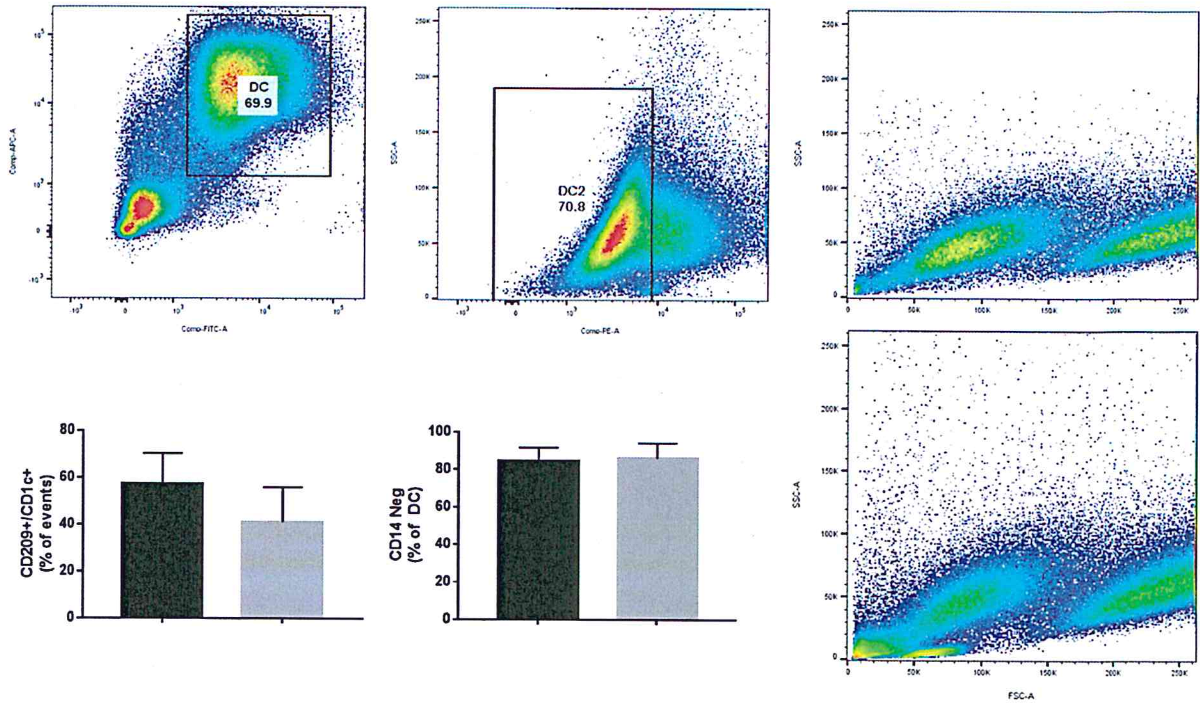


Figure 4. CD209+/CD1c+ percentages of cultured cells. Gates indicate selection of mo-DCs from culture. The flow plots on the right indicate where the gates capturing DCs and ungated cells are in relation to FSC and SSC. The percentage of CD209+/CD1c+ events for each FITC and PE and CD14 negative DCs are demonstrated.

Discussion

The potential to differentiate monocytes into dendritic cells is useful in providing quantities of cells functionally proportionate to allow for an understanding of their role in innate and adaptive immune response. Development of an efficient protocol to culture mo-DCs was the first step to determining the effect of lead acetate on immune cells. Anti-CD14 microbeads enabled isolation of viable CD14+ monocytes and these monocytes were differentiated and cultured effectively. Generation and phenotypic analysis of mo-DCs was successful using the 7-day protocol with supplementation of cytokines GM-CSF and IL-4.

Surface marker expression was analyzed using flow cytometry and confirmed characteristics consistent with conventional DCs. On day 7 of cell culture we observe increased expression of CD209 and CD1c. DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), also known as CD209, is a C-type lectin receptor which may be found on the surface of monocyte-derived DCs (Collin, et.al., 2013). CD1c+ myeloid dendritic cells (mDCs) are the major population of human DCs in blood, tissues and lymphoid organs (Collin, et.al., 2013). In humans both monocytes and mDCs express CD11c, but DCs lack CD14 or CD16 and may be split into CD1c+ and CD141+ fractions (Collin, et.al., 2013). We also observed reduced expression of CD14 that confirms successful differentiation from the CD14+ monocytes. Increased expression of costimulatory factors CD83/CD86, which are immunoglobulin proteins expressed on the surface of DCs, further confirms successful differentiation and culture.

In future efforts, the effects of lead acetate on these mo-DCs will be analyzed by the quantitation of the STRA6 receptor and Aldefluor assay in the metabolic pathway of vitamin A. Culturing the lead acetate supplemented media is expected to induce an inflammatory response,

therefore, increasing the surface membrane receptor for retinol binding protein. This will increase transport of vitamin A across cellular membranes resulting in enhanced uptake of vitamin A. We expect that in the presence of Pb, dendritic cells will produce more bioactive vitamin A resulting in more inflammatory immune response. Determining the effect of lead on STRA6 expression in monocyte-derived DCs will allow for a potential therapeutic target to offset increased risk of allergies and asthma in lead exposed individuals.

Appendices

Explanation of Acronyms:

Pb (lead)

DC (dendritic cell)

PBMC (peripheral blood mononuclear cell)

STRA6 (stimulated by retinoic acid 6)

RBP (retinol binding protein)

ROS (reactive oxygen species)

WBC (white blood cell)

TNF-α (tumor necrosis factor alpha)

hs-CRP (high-sensitivity C-reactive protein)

INF-γ (interferon gamma)

Th1 & Th2 (T helper cell types 1 and 2)

mo-DCs (monocyte-derived dendritic cells)

mDCs (myeloid dendritic cells)

KO (knockout)

DTH (delayed type hypersensitivity)

KLH (keyhole limpet hemocyanin)

IL (interleukin)

NK cell (natural killer cell)

GM-CSF (granulocyte macrophage-colony stimulating factor)

FSC (forward scatter)

SSC (side scatter)

at-RA (all-trans retinoic acid)

RALDH (retinaldehyde dehydrogenase)

SOCS3 (suppressor of cytokine signaling 3)

PMN (polymorphonuclear)

FACS (fluorescence activated cell sorting)

DC-SIGN or CD209 (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin)

Informed Consent:

Title of the research project

Human Monocyte Metabolic Phenotype & Function

Names of the researchers

Principle Investigator

David M. Duriancik, Assistant Professor of Biology, University of Michigan – Flint
Ph.D. Biochemical Human Nutrition, Michigan State University
B.S. Molecular Biology/Biotechnology, Clarion University of Pennsylvania

Description of the research

We are studying factors that affect immune cell function. Specifically, we are analyzing the ability of dendritic cells to metabolize vitamin A and vitamin D in the presence of lead (Pb). Dendritic cells are antigen presenting cells that stimulate immune response to pathogens. Bioactive forms of vitamin A and vitamin D produced by dendritic cells help to direct the immune response. We expect that in the presence of Pb, dendritic cells will produce less bioactive vitamin D and more bioactive vitamin A resulting in more inflammatory immune response.

Description of human subject involvement

Subjects will be screened via verbal questioning for inclusion/exclusion criteria including known infectious and chronic disease status, issues with blood clotting, and pregnancy. Determination of inclusion will be based on verbal response to the question "Is there any reason you should be excluded from the study (listing exclusion criteria)?" Upon determination of meeting inclusion criteria, subjects will be asked to return to 459 Murchie Science Building (MSB) fasted for 4-6 hours to have their blood drawn as well as completion of the demographic and anthropometric measures including sex, race/ethnicity, age, hours of exercise per week, fasting blood glucose, height, weight, and body composition using bioelectrical impedance analysis. The blood draw, demographic and anthropometric assessment should require no more than 1 hour for total subject participation of 5-7 hours.

Subjects will be asked to donate 2 tubes of 8 milliliters of blood one time. Subject blood will be processed for immune cells and plasma. Immune cells will be assessed for metabolic enzyme expression using real time quantitative polymerase chain reaction, biological activity of these enzymes, and phenotypic analysis of costimulatory molecules. Plasma will be used in the culture media to support immune cell development.

If fasting blood glucose is elevated, subjects will be excluded from the study and notified that their blood glucose was elevated and should be check by a physician. Fasting blood glucose will be determine using standard techniques and glucometer. Briefly, the ring finger of non-dominant hand will be sterilized with an alcohol pad, lanced with single use disposable lancets, and no more than 50 microliter of blood will be collected into a capillary tube coated with heparin and sodium fluoride. The lanced finger will be bandaged. An aliquot of the capillary blood will be injected into an Analox system that measures blood glucose. Fasting blood glucose will be recorded in the datasheet and reported to the subject.

Length of human subject participation

The estimated duration for each subject will be 5-7 hours total. Only 1 session of participation is required, so total time across the project will also be 5-7 hours and only 60 minutes for screening, anthropometric measures, and blood draw. The majority of subject participation (4-6 hours) will be spent outside of the lab in the fasted stated. We will schedule morning blood draws to limit disruption to normal daily activities.

Risks & discomforts of participation

This study poses no more than minimal risk to the subject. A trained phlebotomist will draw the blood, but bruising of the collection site may occur. The volume of blood is minimal and not associated with negative consequences. Subjects may experience temporary light headedness to syncope during the blood collection.

Measures to be taken to minimize risks and discomforts

Subject will be seated in a chair in case of light headedness. A trained phlebotomist will be employed to draw the blood, but no more than 3 needle sticks will be allowed without drawing blood to minimize potential for bruising. The phlebotomist will sterilized the collection site, use sterile technique for blood collection, and bandage the collection site to minimize the potential for infection.

Expected benefits to subjects or to others

Although you may not receive direct benefit from your participation, others may ultimately benefit from the knowledge obtained in this study.

Costs to subject resulting from participation in the study

There is no cost to the subject associated with participation in this study.

Incentives to subject for participation in the study

There is no incentive for participating in this study.

Confidentiality of records/data

You will not be identified in any reports on this study. No identifying data will be used. Subjects will be given a subject ID number. The coding (subject name and associated subject ID number) will be kept by the PI in a locked filing cabinet for the duration of the study. After the study, all codes will be destroyed by shredding. Records will be kept confidential to the extent provided by federal, state, and local law. However, the Institutional Review Board, the sponsor of the study (i.e. NIH, FDA, etc.), or university and government officials responsible for monitoring this study may inspect these records. No genetic analysis will be performed and no samples will be saved for later use.

Management of Physical Injury

In the unlikely event of physical injury resulting from research procedures, the PI will provide first aid medical care to control bleeding, prevent further harm, and contact emergency medical services. By signing this form, you do not give up your right to seek additional compensation if you are harmed as a result of participation in this study. In the

event of lightheadedness or syncope, UM-Flint public safety will be contact. The subject will remain under the supervision of the PI until public safety arrives and dismisses the subject. The PI has basic knowledge of first aid procedures.

Contact Information

David M. Duriancik, Ph.D.
Assistant Professor
810-766-6684
durianci@umflint.edu

Required IRB Contact Information

Should you have questions regarding your rights as a research participant, or wish to obtain information, ask questions, or discuss with someone other than the researcher(s), please contact Mary Mandeville in the Institutional Review Board, 4204 William S White Bldg., Flint, MI., 48502, 810-762-3383
(For International Studies: US Country Code: 001)
email: *irb-flint@umflint.edu*.

Voluntary nature of participation

Your participation in this project is voluntary. Even after you sign the informed consent document, you may decide to leave the study at any time without penalty or loss of benefits to which you may otherwise be entitled.

Documentation of the consent

One copy of this document will be kept together with the research records of this study. Also, you will be given a copy to keep.

Consent of the subject:

I have read [or been informed] of the information given above. Dr. David Duriancik has offered to answer any questions I may have concerning the study. I hereby consent to participate in the study.

____

Printed Name Consenting signature date

Recruitment Flyer:

Volunteers Needed
Participate in a research study

Purpose
We are studying factors that affect immune cell function. Specifically, we are analyzing the ability of dendritic cells to metabolize vitamin A and vitamin D in the presence of lead. Dendritic cells are antigen presenting cells that stimulate immune response to pathogens. Bioactive forms of vitamin A and vitamin D produced by dendritic cells help to direct the immune response. We expect that in the presence of lead, dendritic cells will produce more bioactive vitamin A and less bioactive vitamin D resulting is more inflammatory immune response.

Summary of Inclusion/Exclusion Criteria
You are eligible to participate if you meet the following criteria;

1. Generally healthy such as;
   a. No known chronic or infectious diseases
   b. No known blood clotting issues
2. Not currently pregnant
3. Fasted state (No food, caffeine, or alcohol for 4-6 hours prior to blood donation)
4. Between the ages of 18 and 70 years old

Procedures
You will be asked to give 2 volumes of 8 milliliters of blood via blood draw, complete a demographics questionnaire regarding your sex, age, race/ethnicity, hours of exercise per week, and be measured for fasting blood glucose, height, weight, and body composition (1 hour of participation). We ask that the day of blood donation you come to 459 Murchie Science Building (MSB) fasted for at least 4-6 hours to avoid complications of processing the blood sample (fasting blood glucose will be measured for confirmation of fasted state). We will subsequently analyze the response of your blood cells to lead. Blood cells will be cultured for 5-8 days and analyzed for messenger RNA expression of metabolic enzymes, protein expression of these metabolic enzymes, and cell survival, proliferation, and phenotype. Upon collection of the data, your samples will be destroyed. No genetic analysis will be performed and no samples will be saved for later use.

Participation in the experiment will take no more than 5-7 hour total, the majority of which is spent fasting (4-6 hours) outside of the lab. You will be asked a series of verbal questions to determine inclusion and demographics, read and sign an informed consent document, be measured for height, weight, and body composition using bioelectrical impedance, and donate blood (1 hour of participation). Therefore, the total subject participation time is 5-7 hours (4-6 hours fasting plus 1 hour of data collection/blood donation). Your information and participation will remain confidential.

Compensation
Participation is completely voluntary and there is no compensation for participation.

Study Location & Contact Information

Dr. David Duriancik
durianci@umflint.edu
810-766-6684
459 Murchie Science Building - Biology Department
University of Michigan – Flint
303 E. Kearsley St.
Flint, MI 48507

References

Sirivarasai, Jintana, Winai Wananukul, Sming Kaojarern, Suwannee Chanprasertyothin, Nisakron Thongmung, Wipa Ratanachaiwong, Thanyachai Sura, and Piyamit Sritara "Association between inflammatory marker, environmental lead exposure and glutathione S-Transferase gene." Toxicology Letters, vol. 221, 2013

Gurer-Orhan H, Sabir HU, Ozgunes H. "Correlation between clinical indicators of lead poisoning and oxidative stress parameters in controls and lead-exposed workers." Toxicology. 2004;195(2-3):147–154.

"Lead." World Health Organization, (WHO), www.who.int/ipcs/assessment/public_health/lead/en/. 2017

Barboza, Tony. "How a battery recycler contaminated L.A.-Area homes for decades." Los Angeles Times, 21 Dec. 2015.

Khan DA, Qayyum S, Saleem S, Khan FA. "Lead-induced oxidative stress adversely affects health of the occupational workers." Toxicology and Industrial Health. 2008;24(9):611–618

Reifen, Ram. "Vitamin A as an anti-Inflammatory agent." Proceedings of the Nutrition Society, vol. 61, no. 03, 2002, pp. 397–400

William R. Adams, John Edgar Smith, Michael H. Green1 "Effects of N-(4-Hydroxyphenyl) Retinamide on Vitamin A Metabolism in Rats." Experimental Biology and Medicine, vol. 208, no. 2, Jan. 1995, pp. 178–185

Duriancik, D. M., D. E. Lackey, and K. A. Hoag. "Vitamin A as a Regulator of Antigen Presenting Cells." Journal of Nutrition 140.8 (2010): 1395-399.

Romagnani, S. “Human TH1 and TH2 Subsets: Regulation of Differentiation and Role in Protection and Immunopathology.” International Archives of Allergy and Immunology, vol. 98, no. 4, 1992, pp. 279–285

Deo SS1, Mistry KJ, Kakade AM, Niphadkar PV. “Role played by Th2 type cytokines in IgE mediated allergy and asthma.” Lung India, vol. 27, no. 2, 2010, p. 66

Casey J1, Kawaguchi R, Morrissey M, Sun H, McGettigan P, Nielsen JE, Conroy J, Regan R, Kenny E, Cormican P, Morris DW, Tormey P, Chróinín MN, Kennedy BN, Lynch S, Green A, Ennis S. “First implication of STRA6 mutations in isolated anophthalmia, microphthalmia, and coloboma: A new dimension to the STRA6 phenotype.” Human Mutation, vol. 32, no. 12, 2011, pp. 1417–1426.

Berry DC, Jacobs H, Marwarha G, Gely-Pernot A, O'Byrne SM, DeSantis D, Klopfenstein M, Feret B, Dennefeld C, Blaner WS, Croniger CM, Mark M, Noy N, Ghyselinck NB. “The STRA6 Receptor Is Essential for Retinol-Binding Protein-Induced Insulin Resistance but Not for Maintaining Vitamin A Homeostasis in Tissues Other Than the Eye.” Journal of Biological Chemistry, vol. 288, no. 34, Sept. 2013, pp. 24528–24539

Kawaguchi R, Zhong M, Kassai M, Ter-Stepanian M, Sun H. “Vitamin A transport mechanism of the multitransmembrane cell-surface receptor STRA6.” Membranes (Basel). 2015;5(3):425-453.

Ruiz A, Mark M, Jacobs H, Klopfenstein M, Hu J, Lloyd M, Habib S, Tosha C, Radu RA, Ghyselinck NB, Nusinowitz S, Bok D. “Retinoid Content, Visual Responses, and Ocular Morphology Are Compromised in the Retinas of Mice Lacking the Retinol-Binding Protein Receptor, STRA6.” Investigative Opthalmology & Visual Science, vol. 53, no. 6, 2012, p. 3027.

Noy, Noa. "Vitamin A Transport and Cell Signaling by the Retinol-Binding Protein Receptor STRA6." Subcellular Biochemistry, The Biochemistry of Retinoid Signaling II, 2016, pp. 77–93.

Blaner, William S. "STRA6, a Cell-Surface Receptor for Retinol-Binding Protein: The Plot Thickens." Cell Metabolism, vol. 5, no. 3, 2007, pp. 164–166.

Tedder, T F, and P J Jansen. "Isolation and generation of human dendritic cells. Current protocols in immunology.", U.S. National Library of Medicine, May 2001

Collin, Matthew, Naomi Mcgovern, and Muzlifah Haniffa. "Human Dendritic Cell Subsets." Immunology 140.1 (2013): 22-30.

Beijer MR, Kraal G, den Haan JM. "Vitamin A and dendritic cell differentiation." Immunology., U.S. National Library of Medicine, May 2014

Ewers U, Stiller-Winkler R, Idel H. "Serum immunoglobulin, complement C3, and salivary IgA levels in lead workers." Environmental Research, vol. 29, no. 2, 1982, pp. 351–357

"Metabolism of Vitamin D in Lead Poisoning." Nutrition Reviews, vol. 39, no. 10, 2009, pp. 372–373

Mahaffey, Kathryn R. "Nutritional Factors in Lead Poisoning." Nutrition Reviews, vol. 39, no. 10, 2009, pp. 353–362

Miller, T. "Developmental Exposure to Lead Causes Persistent Immunotoxicity in Fischer 344 Rats." Toxicological Sciences, vol. 42, no. 2, 1998, pp. 129–135

Valentino M, Governa M, Marchiseppe I, Visonă I. "Effects of lead on polymorphonuclear leukocyte (PMN) functions in occupationally exposed workers." Archives of Toxicology, vol. 65, no. 8, 1991, pp. 685–688

Gao D, Kasten-Jolly J, Lawrence DA. "The Paradoxical Effects of Lead in Interferon-Gamma Knockout BALB/c Mice." Toxicological Sciences, vol. 89, no. 2, Sept. 2005, pp. 444–453

Snyder, J. E. "The Efficiency of Maternal Transfer of Lead and Its Influence on Plasma IgE and Splenic Cellularity of Mice." Toxicological Sciences, vol. 57, no. 1, Jan. 2000, pp. 87–94

Stephensen CB. Vitamin A, infection, and immune function. Annu Rev Nutr. 2001;21:167–92.

Cantorna MT, Nashold FE, Hayes CE. "Vitamin A deficiency results in a priming environment conducive for Th1 cell development." Eur J Immunol. 1995;25:1673–9

Shortman K, Heath WR. "The CD8+ dendritic cell subset." Immunol Rev. 2010;234:18–31