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The *in vivo* characterization of spleen dendritic cell populations of vitamin A-deficient C57BL/6J mice

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THE *IN VIVO* CHARACTERIZATION OF SPLEEN DENDRITIC CELL POPULATIONS OF VITAMIN A-DEFICIENT C57BL/6J MICE

By

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ABSTRACT

The *in vivo* characterization of spleen dendritic cell populations of vitamin A-deficient C57BL/6J mice

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Vitamin A deficiency affects 125 million preschool children each year. Vitamin A-deficient populations have decreased T-dependent antibody responses and unaffected or increased cell-mediated immune responses. The impaired T-dependent antibody response is due to decreased stimulation by T helper (Th) 2 cells as well as cross-regulation by increased Th1 cells. In the mouse, $CD11b^+$ myeloid dendritic cells (DCs) stimulate Th2 responses while $CD8\alpha^+$ lymphoid DCs stimulate Th1 responses. In addition, vitamin A is important for myelopoiesis and differentiation of myeloid progenitors into dendritic cells (DCs) and neutrophils. Therefore, we hypothesized that vitamin A-deficient mice would have decreased numbers of myeloid DCs and increased numbers of neutrophils compared to vitamin A-sufficient mice.

We developed a multicolor flow cytometry protocol for identifying DC populations from individual C57BL/6J mouse spleen. The protocol is novel, lacking any enrichment procedures or DC proliferating conditions. Previously, researchers have used various enrichment procedures, pooling of tissues from multiple animals, or stimulating DC proliferation to obtain sufficient numbers of cells to analyze. These procedures potentially skew analysis of DCs to select DC populations or increase variability. Particularly when studying vitamin A deficiency, it is important to analyze animals individually. Each animal has variable vitamin A status, other health parameters, and unique developmental age.

Using the protocol we developed, we show that vitamin A-deficient animals have increased lymphoid DCs (R^2 0.41, liver RAE β -0.63), lymphoid to myeloid DC ratio (R^2 0.52, liver RAE β -0.68), neutrophils (R^2 0.20, liver RAE β -0.44), memory $CD8^+$ T lymphocytes (R^2 0.38, liver RAE β -0.62), and $CD4^+$ T lymphocytes (R^2 0.36, liver RAE β 0.62) in their spleen. The increased lymphoid DC percentage was unexpected, but may be inducing memory $CD8^+$ T cells to proliferate and produce interferon- (IFN) γ. The cytokine environment is therefore Th1 and memory $CD8^+$ T cell biased and the increased Th1 environment would be expected to down-regulate the Th2 antibody-mediated immune response. Therefore, the slight decrease in myeloid DCs (R^2 0.22, liver RAE β 0.32) combined with the increase in lymphoid DCs and memory $CD8^+$ T cells provides mechanistic evidence for the depressed antibody responses observed in vitamin A-deficient populations.

The data we present here confirms reports of others, provides novel evidence of the role of vitamin A on DC homeostasis, and provides a comprehensive overview of the immune system in vitamin A-deficient mice. We confirm that vitamin A does not affect splenic B lymphocyte (R^2 0.08, liver RAE β -0.00) and $CD8^+$ T lymphocyte numbers (R^2 0.10, liver RAE β -0.07), and show that vitamin A deficiency also does not alter precursor DC (R^2 0.09, liver RAE β 0.10) or plasmacytoid DC numbers (R^2 0.02, liver RAE β 0.08). We show that the immune response bias previously reported in vitamin A deficiency correlates with an increased ratio of lymphoid to myeloid DCs in vitamin A-deficient animals. We confirm neutrophils are increased in vitamin A-deficient populations, but not to the dramatic extent previously reported.

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KEY TO SYMBOLS & ABBREVIATIONS

Allophycocyanin (APC)

American Institute of Nutrition (AIN)

Analysis of variance (ANOVA)

Antigen presenting cell (APC)

Cluster of differentiation (CD)

Cyan (Cy)

Cytotoxic T lymphocyte (CTL)

Dendritic cell (DC)

Fluorescein isothiocyanate (FITC)

Fluorescence activated cell sorting (FACS)

Forward scatter (FSC)

Granulocyte/macrophage-colony stimulating factor (GM-CSF)

Hank's balanced salts solution (HBSS)

High performance liquid chromatography (HPLC)

Immunoglobulin (Ig)

Interferon (IFN)

Interleukin (IL)

Major histocompatibility complex (MHC)

Matrix metalloproteinase (MMP)

Michigan State University (MSU)

Monoclonal antibody (mAb)

Monocyte-derived dendritic cell (mDC)

Natural killer (NK)

Negative (NEG)

Not Applicable (N/A)

Peridinin chlorophyll protein (PerCP)

Peyer's patch (PP)

Phycoerythrin (PE)

Polymorphonuclear neutrophil (PMN)

Positive (POS)

Precursor DC (preDC)

Promyelocytic leukemia (PML)

Protein-energy malnutrition (PEM)

Retinoic acid receptor (RAR)

Retinoic acid response element (RARE)

Retinoid X receptor (RXR)

Retinol activity equivalents (RAE)

Retinol binding protein (RBP)

Side scatter (SSC)

T helper (Th)

Transthyretin (TTR)

T regulatory (Treg)

Tumor necrosis factor (TNF)

Vitamin A-deficient (VAD)

Vitamin A-sufficient (VAS)

INTRODUCTION

Each year approximately 125 million children suffer from vitamin A deficiency. Vitamin A deficiency increases the risk of infection as well as the morbidity and mortality of infection. The immune response of vitamin A-deficient populations is biased to T helper 1 (Th1) cell-mediated responses with decreased Th2 antibody responses. Previous research by others has focused on lymphocyte number and function of vitamin A-deficient populations, but vitamin A has important roles in hematopoiesis, particularly myelopoiesis. Acute promyelocytic leukemia results from a fusion of retinoic acid receptor (RAR) α to the PML gene leading to an increase in immature neutrophil numbers. In addition, previously our laboratory demonstrated that murine bone marrow cells cultured in media containing granulocyte-macrophage colony stimulating factor (GM-CSF) and retinoic acid develop into dendritic cells, while bone marrow cells cultured with depleted retinoic acid or retinoic acid antagonists develop into neutrophils. Furthermore, others have shown systemic myeloid cell, specifically neutrophil, expansion in mice depleted of vitamin A.

Dendritic cells (DCs) are bone marrow derived antigen presenting cells responsible for the stimulation of naïve T cells in response to infection. In the mouse, $CD11b^+$ myeloid DCs stimulate Th2 immune responses, while $CD8\alpha^+$ lymphoid DCs stimulate Th1 immune responses. Plasmacytoid DCs secrete large amounts of type 1 intereferons (IFN), IFN-α/β. Myeloid, lymphoid and plasmacytoid DCs are mature DCs that arise from one common precursor cell circulating in blood and resident in secondary lymphoid tissues. DCs are sentinels of the body, resident in sparse numbers in every

tissue, scanning the body for danger signals. Immature DCs are phagocytic cells, sampling antigens for danger signals. Upon recognition of a danger signal, DCs undergo maturation through the upregulation of antigen-presenting, T cell costimulatory, and homing receptors to stimulate naïve T cells in secondary lymphoid tissues.

The impaired Th2 immune responses of vitamin A-deficient populations may be the result of skewed DC subsets. In addition, the requirement of retinoic acid for DC development from bone marrow progenitors and the expansion of neutrophils of vitamin A-deficient mice provide convincing evidence of skewed DC subsets of vitamin A-deficient mice. Therefore, it is hypothesized that myeloid DCs would be decreased and neutrophils would be increased in the spleens of vitamin A-deficient mice. Multicolor flow cytometry was used to identify and compare the DC, neutrophil, and lymphocyte populations of mice with varying levels of liver stores of vitamin A.

CHAPTER 1: LITERATURE REVIEW

Immune System Overview

The immune system is comprised of innate and adaptive arms that protect the body from infection. The innate arm consists of non-specific, ubiquitous, and immediate protection from pathogens. The adaptive arm consists of specific, localized, and delayed protection against "danger" signals. "Danger" signals are foreign substances such as pathogenic microorganisms and viruses, but also consist of altered "self" signals such as tumor cells. The innate and adaptive arms of the immune system communicate through various signals to provide the most effective protection from infection (1).

The innate immune system consists of epithelial barriers, non-specific phagocytic and lytic cells, and other soluble and circulating biological factors. The epithelial barriers include both skin and mucosal tissue. Phagocytic and lytic cells include macrophages, neutrophils, and natural killer (NK) cells. Other biological factors include protease enzymes, salts, pH differences, and other proteins such as complement. These biological factors inhibit microbe proliferation or directly induce microbial cell death. Biological factors work in concert with barriers and cells to provide optimal protection, such as the antimicrobial products present in mucus. Bridging the innate and adaptive arms are the phagocytic cells. The phagocytic cells, particularly dendritic cells, take up microbes, digest them, and then present antigens to T lymphocytes. Presentation of antigen leads to the initiation of an adaptive immune response through the production and secretion of cytokines creating an environment for the most effective response to a given pathogen. Other antigen-presenting cells (APCs) in addition to dendritic cells include macrophages and B lymphocytes (1).

B and T lymphocytes are cells of the adaptive immune response that recirculate through secondary lymphoid tissues, but are capable of homing to sites of infection following stimulation by an APC. The processes of antigen uptake and presentation as well as cell proliferation and differentiation that occur in an initial adaptive immune response lead to a delay in the B and T cell responses. However, previously encountered pathogens have more rapid subsequent responses due to the development of memory B and T cells. B lymphocytes produce and secrete antibody to various antigens present on a microbial cell or soluble microbial products such as a toxins. There are numerous subsets of T lymphocytes distinguished by their T cell co-receptor expression and/or function. Cluster of differentiation (CD) 4^+ T cells recognize peptide antigens presented in major histocompatibility complex (MHC) - II proteins on APCs and develop T helper (Th) responses. There are 2 classes of Th effector cell responses; Th1 which participate in cell-mediated immunity, and Th2 which aid B lymphocyte antibody immunity. T cells expressing the CD8 T cell co-receptor, also called cytotoxic T lymphocytes, recognize antigen in the context of MHC-I proteins and receive help from a $CD4^+$ Th1 cell to accomplish a cell-mediated response. T cells expressing the CD4 T cell co-receptor are also responsible for inducing tolerance to non-dangerous or self antigens, and this subset of T lymphocytes is called T regulatory (Treg) cells. Thus, the adaptive immune system is a highly complex and coordinated multi-cellular response designed to defend against specific danger signals while at the same time being tolerant to self antigens (1).

Cooperation between the innate and adaptive arms of the immune system is critical for optimal immune responses to danger signals as well as maintaining tolerance to self signals. APCs are the critical link between the innate and adaptive immune

responses through non-specific phagocytosis of antigens, antigen processing and presentation, and cytokine secretion directing the subsequent adaptive immune response. Antigen presentation and cytokine secretion communicate the most effective T lymphocyte response to a specific pathogen that has evaded the innate immune system. Due to the cooperation and coordination of signals, any perturbation in the system can lead to malfunctions of the other components of the immune system.

Dendritic Cells

Dendritic cells (DCs) are hematopoietic-derived APCs that efficiently stimulate naïve T lymphocytes (2-4). DCs migrate from the bone marrow and reside in peripheral tissues in an immature state (5). Under stead-state conditions, a reservoir of precursor and mature DCs are maintained in secondary lymphoid tissues, but DCs are sparse in other tissues where they function as sentinels (5, 6). DCs comprise about 1-3% of the cells of the spleen (7). Upon phagocytosis of pathogen molecules in tissues, the DCs mature and migrate to secondary lymphoid tissues, such as the spleen and lymph nodes, to stimulate T cells and induce an adaptive immune response. Maturation of DCs involves upregulation of homing receptors and other surface proteins necessary to efficiently stimulate T cells, including CD40, CD80, and CD86 (8, 9).

Various DC subpopulations exist, and each has a bias to stimulate a particular adaptive immune response. In the mouse, myeloid DCs preferentially stimulate antibody-mediated (Th2) responses, while lymphoid DCs stimulate cell-mediated (Th1) responses (10, 11). Plasmacytoid DCs produce large amounts of type 1 interferons (IFN), IFN-α and -β, to augment antiviral immune responses (12-15). Myeloid DCs express CD11c, MHC-II, and CD11b, but do not express CD8α. Lymphoid DCs express CD11c,

MHC-II, and CD8α, but do not express CD11b (10, 11). Plasmacytoid DCs express CD11c, MHC-II, Gr-1, and B220 (CD45RB) (12, 16). Although controversial at one point, it is now known that these DC subpopulations transcribe and translate the various surface protein markers and do not acquire the surface proteins through cell-cell contact interactions with other cell types, such as CD8 T lymphocytes (17, 18). The respective DCs present antigens to Th cells and secrete cytokines to stimulate the appropriate adaptive immune response.

DCs arise from both common myeloid or common lymphoid progenitors in the bone marrow (4, 19). Precursor DCs (preDCs) migrate from the bone marrow to the bloodstream and reside in peripheral tissues in an immature state (20-22). Upon recognition of antigens through pattern recognition receptors, such as toll-like receptors, the preDCs mature and home to secondary lymphoid tissues to stimulate naïve T cells (23). Myeloid and lymphoid DCs have half-lives of 1.5 to about 3 days, while plasmacytoid DCs have a half-life of about 9 days (19, 24-26). During an infection, cytokines stimulate DC differentiation of bone marrow progenitors, DC turnover (proliferation and apoptosis), and migration from sites of infection through the lymphatic system to the draining lymph node or through blood vasculature to the spleen during a systemic infection.

Humans have a slightly different repertoire of DCs compared to mice, but mouse models can be extrapolated to the human immune system (8). Human DCs may arise from common myeloid progenitors, common lymphoid progenitors, or differentiate from other cell types including blood monocytes (27). Human DCs are classified into mDC-1, mDC-2, and plasmacytoid DCs. Human plasmacytoid DCs are similar to mouse

plasmacytoid DCs (15). Human mDC-1 preferentially stimulate Th1 responses similar to mouse lymphoid DCs, while human mDC-2 stimulate Th2 responses similar to mouse myeloid DCs (28). Human DC research has been limited due to available tissues for analysis, and most research has been extrapolated from *in vitro* differentiation of immature DCs and monocytes obtained from the blood. In both humans and mice, DCs possess a high level of marker expression and functional plasticity. The DC plasticity, low percentages in a particular tissue, and lack of clear maturation processes make DC research difficult and complex.

Vitamin A Metabolism

Vitamin A is a generic term that encompasses retinoid and carotenoid compounds that supply humans with a biological need. The best characterized physiological forms of vitamin A are all-*trans*-retinoic acid, the carboxylic acid form, retinal, the aldehyde form, and retinol, the alcohol form, depending on specific physiological function and tissue (29).

Mammals can not synthesize vitamin A *de novo*. Vitamin A is a fat-soluble vitamin obtained by humans in two different dietary forms, i.e. retinyl esters or pro-vitamin A compounds. The physiological form of vitamin A circulating in the bloodstream, retinol, can be obtained in association with the fat of animal products as retinyl esters, or retinol esterified to fatty acids. Pro-vitamin A compounds can be consumed from yellow, orange, and some dark green plants. Pro-vitamin A compounds, including α- and β-carotenes and β-cryptoxanthin, can be metabolized to retinoids through enzymatic cleavage. The enzyme 15, 15'-monooxygenase is expressed in a multitude of human tissues and converts pro-vitamin A compounds to biologically active

forms of vitamin A (30). The tissues expressing 15, 15'-monooxygenase include cells of the gastrointestinal tract, reproductive organs, skin, liver, and skeletal muscle (30). The highest expression of 15,15'-monooxygenase is in the gastrointestinal tract. Retinoids, and a fraction of consumed carotenoids, are absorbed in the small intestine. Retinoid absorption is described below, and carotenoids are absorbed intact via passive diffusion and associate directly with chylomicrons (31).

Retinyl esters are hydrolyzed to retinol in the gastrointestinal tract by the pancreatic and enterocyte enzymes retinyl ester hydrolases and non-specific lipases (32-34). Retinol is absorbed by the enterocyte and esterified to a fatty acid, normally palmitate or stearate, by the enterocyte enzymes lecithin:retinol acyltransferase and acyl-CoA:retinol acyltransferase (35, 36). The retinyl ester is then complexed with cholesterol, phospholipids, other fatty acids, and binding proteins and excreted from the enterocyte into the lymphatic system within a chylomicron (37). The chylomicron circulates through the lymphatic and vascular system delivering retinyl esters and other chylomicron components to peripheral tissues (38). The depleted chylomicron, now a chylomicron remnant, will deposit the remaining retinyl esters into liver stellate cells for storage (39). Upon demand, liver reserves of retinyl esters are hydrolyzed to retinol which binds to retinol binding protein (RBP). Retinol-RBP complex next binds to transthyretin (TTR) in the liver prior to release into the bloodstream to maintain a relatively constant level of circulating retinol (35). The circulating retinol-RBP-TTR complex, in a 1:1:1 ratio, increases molecular mass and decreases loss through glomerular filtration (40-42). In a healthy state, vitamin A is circulating in the blood at a

consistent level of retinol:RBP:TTR at approximately 1-2 micromolar (μM) concentration, but during acute infections the concentration may decrease (43).

The membrane receptor Stra6 binds RBP and mediates the uptake of retinol into the cell, but RBP remains extracellular (44). Extracellular RBP returns to circulation and is filtered through the kidney glomerulus. A cell can store limited amounts of retinol complexed with cellular retinol binding protein or will oxidize retinol to retinal, a reversible reaction catalyzed by the enzyme retinol dehydrogenase. The retinal can then be irreversibly oxidized to retinoic acid by retinal dehydrogenase (45). The cell can store limited amounts of retinoic acid complexed with cellular retinoic acid binding protein (46). In addition, a cell can also synthesize retinyl esters as well as metabolize retinoids to polar derivatives to increase cellular reserves of retinoids (35).

Vitamin A is a hydrophobic molecule. Therefore, vitamin A is soluble in fat and requires a protein carrier to be soluble in the bloodstream or cell cytosol. Liver is the main storage site of vitamin A, but adipose and other tissues can store limited amounts of vitamin A (47). Adipose and other tissues storing vitamin A can liberate the stores in a mechanism similar to that in liver (48). Retinoic acid can be carried in the bloodstream and delivered to peripheral tissues by albumin (38). However, adipose storage and albumin delivery of retinoic acid are believed to be minor contributions to the overall metabolism and transport of vitamin A.

Vitamin A is excreted in both the feces and urine (49, 50). Vitamin A can be serially oxidized to increasingly polar metabolites; from retinyl-esters to retinyl-glucoronides (51). All-*trans*-retinyl β-glucuronide and other polar metabolites are secreted into bile and, if not reabsorbed, excreted in the feces (49). The glomerulus of

the kidney filters apo-RBP, but holo-RBP complexed with TTR is retained due to the size of the complex, recycling retinol-protein complex back to circulation (52). However, the kidney also expresses RBP and TTR binding proteins, specifically megalin, to aid in the reabsorption of vitamin A (53). Vitamin A, in various forms, is removed from the body through biliary excretion and urinary filtration.

Vitamin A Function

There are three general physiological functions of vitamin A requiring different structural forms. Retinol and all-*trans*-retinoic acid are required for reproductive processes in both males and females (54). Retinal, specifically 11-*cis*-retinal, is critical in vision (55). All-*trans*-retinoic acid in complex with its nuclear receptor functions as a steroid hormone family transcription factor (56, 57). All-*trans*-retinoic acid is a ligand for a family transcription factors involved in the regulation of cell differentiation, proliferation, maturation and apoptosis. The function of all-*trans*-retinoic acid as a ligand for transcription factors has been extensively reviewed and will be explained in greater detail below (58-60).

High intakes of vitamin A during pregnancy are teratogenic, but vitamin A, specifically retinol and all-*trans*-retinoic acid, are critical in reproduction (61-63). The role of all-*trans*-retinoic acid in reproduction is through gene regulation via nuclear receptors. However, retinol may have unique functions in reproduction. All-*trans*-retinoic acid alone can not support normal reproduction, supplemental retinyl esters or all-*trans*-retinol are required to prevent fetal resorption (64, 65). The specific role of retinol in late gestation is not clear, but is required for normal heart, brain, and eye development. The temporal-spatial distribution of vitamin A metabolites is critical in

reproduction to allow for all stages of fetal development and prevention of fetal resorption (54, 63).

Rhodopsin, a light sensing protein of the rod cells in the eye, requires 11-*cis*-retinal as a cofactor (66). Ultraviolet light causes 11-*cis*-retinal to isomerize to all-*trans*-retinal, leading to a conformational change in the rhodopsin protein (67). The conformational change in rhodopsin signals light detection to the brain (68). All-*trans*-retinal can be recycled and converted back to 11-*cis*-retinal in the eye (69). In the retina, 11-*cis*-retinal functions in light adaptation. In the cone cells, 11-*cis*-retinal functions in color vision (70). The function of 11-*cis* retinal is restricted to the eye.

All-*trans*-retinoic acid and 9-*cis*-retinoic acid are both ligands for the transcription factor family retinoic acid receptors (RARs). However, only 9-*cis*-retinoic acid has been characterized as the ligand for retinoid X receptors (RXRs). There are three isoforms of RAR (α, β, γ) and RXR (α, β, γ) (59). All-*trans*-retinoic acid translocates from the cytosol to the nucleus and binds to RARs (59). Upon ligand binding, RARs heterodimerize with RXRs and bind to a retinoic acid response element (RARE) of target genes for regulatory function (59). The RAR/RXR heterodimer will recruit other gene promoting or suppressing transcription factors to regulate the transcription of the candidate gene (59). More than 532 genes have been described as responsive to retinoic acid (71). However, only about 30% of these genes have promoters possessing classic RAREs. Classical RAREs are six nucleotide base pairs of guanine and cytosine direct repeats separated by one, two, or five nucleotides (59, 72, 73). However, non-classical RAREs exist in which guanine and cytosine rich half-site regions are separated by any number of base pairs (59). RAR/RXR transcriptional regulation is a complex and

multistep process of ligand binding, translocation to RARE sites, and association with other transcription factors. Retinoid-mediated gene transcriptional control in the absence of a classic RARE is commonly documented, but the molecular mechanism(s) involved is largely unknown (59).

The RXR proteins may heterodimerize with other transcription factor partners or may also homodimerize (59). *In vitro*, 9-*cis*-retinoic acid will bind to RXRs leading to homodimerization and transcriptional regulation. However, 9-*cis*-retinoic acid has not been measurable in tissue samples, and therefore the physiological relevance of 9-*cis*-retinoic acid and RXR homodimer transcriptional regulation remains controversial. RXRs also heterodimerize with other nuclear receptor superfamily transcription factor partners, including vitamin D receptor, peroxisome proliferator activation receptors, liver X receptors, and others (59). The alternate dimerization partners lead to binding to different response elements and modulation of other genes regulated at the transcriptional level.

In the immune system, the role of vitamin A is generally confined to a steroid hormone family transcription factor through all-*trans*-retinoic acid-RAR-RXR gene regulation. Acute promyelocytic leukemia (PML) is a hematopoietic cell cancer in which the RARα gene on chromosome 15 reciprocally translocates and fuses to the PML gene on chromosome 17 (74). The fusion of RARα to PML blocks the normal maturation of granulocytes, including neutrophils. Prescription of high dose all-*trans*-retinoic acid has been an effective treatment option through the induction of differentiation of the cancer cells into mature granulocytes which then have a finite half-life (75). Therefore, at the

transcriptional level, all-*trans*-retinoic acid is well-established to be important in the differentiation of myeloid progenitors in the bone marrow.

Vitamin A Deficiency

Vitamin A deficiency is a global nutrient concern affecting approximately 125 million children each year (76). In the U.S., the prevalence of vitamin A deficiency estimated from National Health and Nutrition Examination Survey data may be as high as 25% depending on the cutoff of vitamin A deficiency and the subpopulation of interest (77). Incidence of vitamin A deficiency is consistently higher in children, pregnant women, and patients with fat malabsorption diseases. Vitamin A deficiency can result from inadequate intakes of vitamin A rich foods or poor absorption of fat-soluble molecules. Numerous strategies have been employed and studied to ameliorate vitamin A deficiency including supplementation programs, crop distribution, and bioengineering of crops to contain pro-vitamin A compounds, specifically β-carotene (78, 79). Vitamin A supplementation efforts are a cost effective means to improve health of populations, especially of young children. Supplementing vitamin A costs cents per dose and can reduce childhood mortality on average by 30% (80, 81). Despite these efforts, to date vitamin A deficiency remains a factor in childhood morbidity and mortality around the world.

Detection of vitamin A deficiency is difficult. The high liver stores and constant release of vitamin A maintains circulating serum retinol constant at about 1-2μM until chronic undernutrition depletes liver stores. Serum retinol can also be decreased during the acute phase response of an infection (43). Therefore, a lower serum retinol (the most readily available sample for analysis) may not be indicative of vitamin A status, and other

methods must be used to evaluate vitamin A status. There are other methods of assessing vitamin A status including serum RBP concentrations, plasma RBP to TTR ratios, liver vitamin A concentrations, relative dose response tests, stable isotope tracers, and early symptoms of deficiency (82). Each method of analysis has advantages and disadvantages. The role of vitamin A in vision leads to symptoms of deficiency involving the eye, such as kertinization of the eye layers, Bitot spots and xerophthalmia. Night blindness, or poor adaptation to levels of low light, is the first sign of vitamin A deficiency but is commonly underreported and undiagnosed. As vitamin A deficiency progresses, more severe eye symptoms develop that can cause permanent visual impairment. Keratinization and Bitot spots are reversible by vitamin A supplementation, but xerophthalmia is irreversible (82).

Vitamin A and Innate Immunity

Vitamin A helps to maintain an effective innate immune system. Vitamin A is essential for maintenance of mucosal lining and skin integrity. Vitamin A is also required for maintaining optimal innate immune cell numbers and/or lytic activity. Through transcriptional regulation, vitamin A regulates immune cell differentiation, maturation, apoptosis, and function as well as maintains effective barriers.

Vitamin A-deficient individuals have impaired mucosal barriers. Mucus is an important coating for trapping and preventing pathogens from entering or damaging the delicate mucosal tissues. Vitamin A is required for the production of mucus and mucus glycoproteins by goblet cells and maintenance of goblet cell numbers (83, 84). The skin of vitamin A-deficient populations is keratinized and increased in thickness (85). The increased thickness of skin does not directly lead to increased susceptibility to infection.

However, increased kertinization makes the skin more fragile leading to decreased skin integrity and increased susceptibility to infections caused by abrasions.

Natural killer (NK) cells are granular lymphocytes responsible for innate defenses to viral and intracellular infections. Basal NK cell numbers and activity are reduced in vitamin A-deficient animals, but are restored upon supplementation of vitamin A (86-89). Basal NK cell lytic efficiency or the ability of each NK cell to lyse a target is not impaired in vitamin A-deficient animals (90). In addition, upon stimulation with polyinosinic:polycytidylic acid there is no difference in the IFN-γ production, cell proliferation, or lytic efficiency of NK cells from vitamin A-deficient and vitamin A-sufficient animals (89). In summary, *in vivo* NK cells are depleted in vitamin A-deficient animals, but the ability of NK cells to respond upon stimulation is unaffected by vitamin A deficiency.

Macrophages are mononuclear phagocytes capable of ingesting and killing microbes within phagolyosomes through the action of lytic peptides and degradative enzymes. Macrophages also present antigens of the ingested microbes to T cells and secrete cytokines and chemokines to initiate the adaptive immune response and increase local inflammation (1). The effect of vitamin A on macrophage function is contradictory. In pathogen-free mice, vitamin A deficiency increased macrophage numbers in secondary lymphoid tissues but also impaired delayed-type hypersensitivity, or cell-mediated immune responses (91). Vitamin A supplementation has been reported to increase macrophage functions *in vivo* and *in vitro* (92-95). However, phagocytosis of antibody opsonized cells was impaired due to decreased expression of receptors for antibody on human macrophages cultured in vitamin A-deficient medium (96). Pro-inflammatory

cytokines produced by macrophages [interleukin (IL) -1, IL-12, and tumor necrosis factor (TNF) -α)] are increased and regulatory cytokines, including IL-10, are decreased in vitamin A-deficient populations (97-101). Although the cytokines that are increased in vitamin A deficiency are normally associated with induction of oxidative pathogen killing, in the case of vitamin A deficiency, they are ineffective at stimulating the pathogen killing mechanisms. Supplementation of vitamin A increases the response to these cytokines and leads to effective pathogen killing (102-105). Therefore, vitamin A deficiency increases macrophage-mediated inflammation, decreases oxidative burst killing, but phagocytic function remains controversial. There are several pathways for triggering macrophage phagocytosis, and the effect of vitamin A on each specific pathway has not been elucidated.

Vitamin A deficiency increases granulocyte numbers (106, 107). In addition, Kuwata et al. showed that vitamin A-deficient SENCAR mice had a marked significant increase in neutrophil cell numbers in the spleen, peripheral blood, and bone marrow due to impaired apoptosis (108). However, the function of granulocytes is decreased in vitamin A deficiency (109). The chemotaxic, phagocytic, and oxidative burst functions of neutrophils of vitamin A-deficient rats were significantly decreased compared to neutrophils from vitamin A-sufficient rats (110). Therefore, despite increased numbers, the ability of neutrophils to control early infections and induce a pro-inflammatory state is depressed in vitamin A deficiency.

In summary, vitamin A deficiency impairs the innate immune system through decreasing mucosal barriers and skin integrity. Interestingly, myeloid innate immune cells (neutrophils and macrophages) are increased in vitamin A deficiency and lymphoid

innate immune cells (NK cells) are decreased. Vitamin A is required for normal hematopoiesis, particularly myelopoiesis, through transcriptional regulation of genes for differentiating bone marrow progenitor cells (111). Although myeloid innate immune cells are increased in vitamin A-deficient populations, the function of innate immune cells is impaired either at baseline or in response to stimuli resulting in increased susceptibility to and severity of infections. The impaired innate immune system of vitamin A-deficient populations results in uncontrolled infections and exacerbated inflammation with potential downstream effects in the adaptive immune system.

Vitamin A and Adaptive Immunity

Vitamin A has important functions in the adaptive immune system (112). *In vitro* assays have been used to determine direct effects of vitamin A on both B and T lymphocytes. However, the *in vivo* effects of vitamin A on adaptive immune responses are the combination of direct and indirect effects on lymphocyte populations. Vitamin A, as a transcription factor ligand, affects the responses of B and T lymphocytes, but the effects of vitamin A on APCs and other cells can alter the cytokines produced and thereby alter the B and T cell responses as well. Vitamin A has effects on the proliferation, differentiation, maturation, apoptosis, and other functions of adaptive immune cells.

Vitamin A does not alter total T lymphocyte numbers of pathogen-free animals (91). However, specific subsets of T cells are altered in vitamin A-deficient populations. Cytotoxic T lymphocytes (CTLs) are antigen-specific cells that produce perforin and granzyme to induce apoptosis of cells infected with intracellular pathogens (1). Delayed-type hypersensitivity responses, CTL-mediated, are impaired in many, but not all vitamin

A-deficient populations (91, 113-115). IL-2 is an autocrine growth factor for CTLs and vitamin A upregulates IL-2 receptor mRNA and protein (116-119). The direct effect of vitamin A on CTL function has not been well established and relied primarily on experiments with Th1 cell responses.

Vitamin A deficiency skews immune responses to a Th1 bias. The Th1 cytokines IFN-γ and IL-12 are constitutively synthesized in vitamin A-deficient mice and supplementation with all-*trans* retinoic acid decreased IFN-γ synthesis (97). In addition, a severe influenza infection of vitamin A-deficient mice showed decreased influenza specific IgA levels and increased influenza specific IgG levels compared to vitamin A-sufficient control animals (120). In similar experiments, influenza infections of mice supplemented with high doses of vitamin A had increased influenza specific IgA, IL-10, decreased serum IgG, and IFN-γ (120, 121). Production of IgG augments a Th1 response through antibody-dependent cellular cytotoxicity. The depressed Th2 cytokines of vitamin A-deficient populations are the combined result of decreased Th2 cell stimulation and the cross-regulation of the increased Th1 cytokine IFN-γ (122).

Vitamin A-deficient populations have impaired antibody responses to T-dependent antigens (89, 91, 114, 120, 123-130). Antibody responses are produced through the cooperation of APCs, B cells, and Th2 cells. Vitamin A deficiency does not have direct effects on B cells; cell numbers, T-independent antibody responses, and antibody responses stimulated with Th2 cells from a vitamin A-sufficient mouse were unaltered in vitamin A-deficient populations (89, 114, 115, 131). Vitamin A supplementation increases the mRNA of IL-4 and IL-5, the classical Th2 cytokines, and decreases IFN-γ, while vitamin A deficiency decreases IL-4 (97, 122, 132). Despite

unaffected antibody production per B cell, the clonal expansion of B cells is impaired in vitamin A-deficiency (123). In addition, Th2 cells are decreased in vitamin A-deficient mice and supplementing the mice with vitamin A restored Th2 cell numbers (131). Therefore, impaired antibody responses in vitamin A-deficient populations are primarily due to impaired Th2 cell help.

Vitamin A is important for antibody class switching. At mucosal sites, IgA is important for innate protection, but vitamin A deficiency decreases mucosal IgA (120, 124, 126-130). In addition, B cell numbers are not altered in vitamin A-deficient populations (115). Therefore, the effect of vitamin A on B lymphocytes is primarily a result of impaired Th2 cell help and not directly on B cells. The depressed IgA synthesis of vitamin A-deficient populations is due to the combination of altered cytokine environment and impaired lymphocyte homing. The role of DCs in establishing the cytokine environment and directing lymphocyte homing allows for the discussion of depressed B cell function to continue in the vitamin A and DC section below.

Vitamin A deficiency increases Th1 responses with unclear CTL and macrophage defects, while decreasing Th2 responses without altering intrinsic B cell function. The altered cytokine environment and cell-cell communication skews the balance of T cells in favor of Th1, leading to aberrant antibody and cell-mediated immune responses. Vitamin A does have direct effects on T lymphocyte proliferation, but T cells also require stimulation from APCs in which vitamin A may also have direct effects.

Vitamin A and DCs

Vitamin A, as a steroid hormone transcription factor, regulates hematopoiesis (111). Hoag and Hengesbach showed that murine bone marrow cells cultured in

granulocyte/macrophage-colony stimulating factor (GM-CSF) differentiated into DCs in the presence of vitamin A or RAR agonists (133). However, the bone marrow cells cultured in medium containing GM-CSF and charcoal dextran filtered serum (depleted of vitamin A) or RAR antagonists generated greater numbers of granulocytes and fewer DCs (133). Human bone marrow cultures also increased differentiation of myeloid progenitors from $CD34^+$ stem cells in the presence of all-*trans* retinoic acid (134). Therefore, *in vitro* vitamin A induces differentiation from $CD34^+$ stem cells to myeloid progenitors to myeloid DCs. Without vitamin A, hematopoietic stem cells differentiate into myeloid progenitors at a slower rate and increase production of granulocytes instead of myeloid DCs.

In vivo, SENCAR mice consuming a vitamin A-deficient diet had dramatically increased myeloid cells in the spleen and bone marrow. The myeloid cells were primarily granulocytes, specifically polymorphonuclear neutrophils. However, the authors did not characterize the DCs of these mice (108). The effect of vitamin A on the *in vitro* differentiation of bone marrow progenitors combined with the observed *in vivo* increase in granulocytes in vitamin A deficiency leads to the hypothesis that *in vivo* vitamin A-deficient animals would have decreased myeloid DCs and increased neutrophils. Further, vitamin A-deficient populations have impaired Th2 antibody responses due to decreased myeloid DCs.

Particularly important in intestinal tissue, DCs express enzymes for the metabolism of vitamin A. Retinal dehydrogenase, the enzyme responsible for converting retinal to retinol, is expressed in mucosal tissue DCs (135). Addition of vitamin A to DCs in cultures leads to the increased expression of the vitamin A metabolic enzymes in

DCs and adoptive transfer of these DCs leads to homing of T and B lymphocytes to the gut associated lymphoid tissue (135-138). The combined effects of impaired mucosal homing of lymphocytes and decreased cytokines, specifically IL-10, in vitamin A deficiency explain the depressed IgA levels of vitamin A-deficient populations. Therefore the role of vitamin A in DC function, particularly in DCs of the gastrointestinal tract and other mucosal sites, is critically important for intestinal immune homeostasis.

Vitamin A can regulate the transcription of cytokine genes. Generally, in the presence of vitamin A, the Th1 cytokines are inhibited while the Th2 cytokines are increased. However, without vitamin A, Th1 cytokines are increased while Th2 cytokines are decreased. Thus, one may speculate that vitamin A regulates the differentiation of myeloid progenitors leading to skewed populations of APCs responsible for stimulating adaptive T lymphocyte responses.

Experiment Rationale

Vitamin A deficiency affects about 125 million children worldwide each year (76). Vitamin A deficiency is not as prevalent in the U.S. compared to other countries, but is still a major nutrient concern, especially in preschool-age children and pregnant women (77). Despite numerous supplementation programs targeting vitamin A-deficient populations, vitamin A deficiency induced morbidity and mortality remains a global concern.

Dietary depletion of vitamin A in mice leads to increased neutrophils in the spleen, bone marrow, and peripheral blood, and Th1 biased immune responses (97, 108, 120, 122). Bone marrow cell cultures stimulated with GM-CSF, in medium depleted of vitamin A or in the presence of RAR antagonist, produce increased numbers of

granulocytes with fewer myeloid DCs, while the bone marrow cultures stimulated with GM-CSF in the presence of vitamin A produced primarily myeloid dendritic cells and few granulocytes (133). Dendritic cell (DC) subsets of vitamin A-deficient animals have yet to be characterized *in vivo*.

DCs are the primary cell type responsible for stimulating naïve T cells for adaptive immune responses. In the mouse, myeloid DCs stimulate primarily Th2 responses, while lymphoid DCs stimulate primarily Th1 responses (10, 11, 28). Plasmacytoid DCs produce type 1 interferons which aid anti-viral responses by activating NK cell killing (12, 14). All mature DC subsets have been shown to arise from a common immature DC or preDC (22). In the mouse, all DC subsets express CD11c on the cell surface, and MHC-II, CD8α, CD11b, Gr-1, and CD45RB (B220) can be used in combination to distinguish the DC subsets.

We developed and employed multicolor flow cytometry to identify and quantify the DC subsets of the spleen from vitamin A-deficient (VAD) and vitamin A-sufficient (VAS) animals. **We hypothesized that vitamin A deficiency would lead to significant decreases in myeloid DCs and increases in neutrophil and preDC populations, while the depletion of vitamin A would have no effect on lymphoid or plasmacytoid DC subsets.**

The role of vitamin A in DC phenotype and function has been thus far confined to *in vitro* assays. The protocol we developed has established a reliable method for analyzing DC populations of the spleen from individual C57BL/6J mice using multicolor flow cytometry. We will provide evidence addressing the knowledge gap in the mechanism of depressed Th2 responses in vitamin A-deficient populations by quantifying

APCs responsible for stimulating naïve T cell differentiation. Our research will provide a direct *in vivo* role for vitamin A in maintenance of DC populations of the spleen. The role of vitamin A in DC population homeostasis will contribute to our understanding of the mechanism for the deficiency in Th2 and T cell-dependent antibody responses observed in vitamin A-deficient populations.

CHAPTER 2: THE IDENTIFICATION AND ENUMERATION OF DENDRITIC CELL POPULATIONS FROM INDIVIDUAL MOUSE SPLEEN AND PEYER'S PATCHES USING FLOW CYTOMETRIC ANALYSIS

Duriancik DM and Hoag KA. 2009. Cytometry, part A. 75:951-9.

Supplemental Figures and Tables published online as supporting material for this manuscript can be found in Appendix I.

ABSTRACT

Dendritic cell (DC) research currently involves pooling of tissues from multiple animals followed by enrichment techniques to obtain sufficient numbers of DCs for analysis. Enrichment techniques take advantage of DC adherence, buoyant density properties, and/or positive or negative selection of cell populations using monoclonal antibodies. However, enrichment techniques may significantly change the maturation and/or activation status of DCs or selectively eliminate one or more subpopulations of DCs. To overcome these drawbacks, we designed a multicolor flow cytometric technique for simultaneous analysis of DC populations from tissues of individual mice. The spleens and Peyer's patches were mechanically and enzymatically digested, then incubated with a panel of 6 monoclonal antibody-fluorochrome direct conjugate reagents. A BD® Biosciences LSR II flow cytometer and FCS Express® software were used to identify 3 subtypes of mature DCs (myeloid, lymphoid, and plasmacytoid), precursor DCs, polymorphonuclear neutrophils, B lymphocytes, and Gr-1^+/CD8α^+ memory T lymphocytes in the spleen. Likewise, we also identified these DC subpopulations and B lymphocytes in the Peyer's patches. The three key parameters in analysis of the DC populations were bi-exponential plotting in data analysis, collection of a minimum of 50,000 total events, and accurate color compensation. This procedure to analyze DCs from individual mice can lead to further understanding of the role of DCs in many other model systems as well as better understanding of how dietary or physiological factors may affect *in vivo* DC homeostasis.

INTRODUCTION

Dendritic cells (DCs), first described by Steinman and Cohn in 1973, are antigen presenting cells sparsely distributed throughout the body (2). The lineage development of DCs remains vague due to the plasticity of DCs. In the mouse, myeloid DCs primarily stimulate antibody-mediated T helper 2 responses and lymphoid DCs primarily stimulate cell-mediated T helper 1 responses (11, 139). Plasmacytoid DCs secrete large amounts of type 1 interferons to augment anti-viral responses (12-14). The mature DCs (myeloid, lymphoid, and plasmacytoid) have been shown to share a common DC precursor (22). However, others have reported two precursor populations, one for plasmacytoid DCs and another for both myeloid and lymphoid DCs (140, 141). At mucosal sites, yet another DC subpopulation expressing CD103 is responsible for stimulating T regulatory cell maturation (142, 143).

The lifespan among the various subpopulations varies from about 1.5 days to 3.0 days for lymphoid DCs and myeloid DCs to about 9 days for plasmacytoid DCs (8, 19). Therefore, hematopoietic precursors in the bone marrow produce new DCs to replace the dying DCs. Typically, the spleen has been reported to possess less than 1% total DCs, or 1-3 x 10^5 DC per spleen, with a ratio of 3 to 1 of myeloid to lymphoid DCs (3, 8). However, the authors' used the DC properties of low buoyant density and adherence to glass to isolate and enrich for DCs (3). A variety of factors may influence the number of DCs in a tissue or animal at a given time, including infection, chronic disease states such as diabetes, hormones such as estradiol, nutrients such as vitamins A and D, as well as others (133, 144-148). However, previous methods used to analyze DCs have used enrichment techniques from pooled samples, either multiple tissue or animal sources, to

obtain sufficient numbers of cells for analysis leading to potentially high variability and inaccurate DC number assessments.

Despite numerous publications quantifying DCs in tissues and animals, accurate analysis relies on *in vivo* expansion of DC prior to isolation, enrichment techniques, or positive or negative selection procedures (145, 149-151). Also DC identification using flow cytometry has been documented in Cytometry, Part A (152-155). However, these publications have either identified only one of the multiple DC subpopulations or focused on a specific tissue such as the lung, blood or bone marrow, not all identifiable DC populations present in the spleen or Peyer's patches. Due to the variability of immune cell populations among animals and the high cell loss associated with various enrichment techniques, we employed 6 monoclonal antibody- (mAb) fluorochrome direct conjugates to identify all resident DC subpopulations of the spleen and Peyer's patches of individual, healthy mice. The use of multiple fluorochromes to detect low percent cell populations requires digital compensation with fluorochrome-labeled compensation beads, bi-exponential scaling on analysis plots, and the collection of minimally 50,000 flow cytometric events. Here we describe the gating strategy used to detect DC populations, as well as the percent and total cell numbers observed in individual, healthy, male and female C57BL/6J mice.

METHODS

Animals

Male and female mice, C57BL/6J, were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and used as breeder pairs. Pups were weaned at 3 weeks of age and fed *ad libitum* commercial solid pellets (Harlan Teklad 22/5 Rodent Diet #8640). At 12

weeks of age, the mice were killed by CO_2 asphyxiation. All procedures were in accordance with Michigan State University Laboratory Animal Resource guidelines, and animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Tissue Isolation

The spleen and Peyer's patches were excised and placed in ice cold calcium and magnesium free Hank's balanced salts solution (HBSS, Gibco, Carlsbad, CA, USA). The tissues were mechanically minced with scissors and enzymatically digested with Spleen Dissociation Medium (Stem Cell Technologies, Vancouver, BC, Canada) following manufacturer's instructions. The tissues were filtered through 70μm mesh (Sefar America Inc, Depew, NY, USA). Cell suspensions were centrifuged at 1200 RPM, 4°C, 10 minutes to pellet. All subsequent centrifugations were at 1200 RPM, 4°C, 10 minutes. The spleen cells were resuspended and incubated for 2 minutes in PharmLyse (BD Biosciences, San Jose, CA, USA) on ice. The spleen cells were diluted with HBSS and centrifuged to pellet. The spleen and Peyer's patch cells were resuspended in 5mL fluorescence activated cell-sorting (FACS) buffer [0.1% sodium azide (Fisher Scientific, Pittsburgh, PA, USA), 1.0% fetal bovine serum (Hyclone, Logan, UT, USA) in Dulbecco's phosphate buffered saline, pH 7.4-7.6, sterile filtered]. The cells were counted on a hemacytometer using trypan blue exclusion dye (Biowhittaker, Walkersville, MD, USA). One million cells were dispensed into each tube for subsequent antibody-fluorochrome staining and analysis.

Antibody Staining

The cells were centrifuged to pellet and incubated with 5μg of anti-FcRγII/III antibody (2.4G2 hybridoma) on ice for 10 minutes. A master mix of all 6 mAb-fluorochrome conjugates (Table 1; BD Biosciences, San Jose, CA, USA) was prepared with 1μg of each mAb-fluorochrome conjugate per one million cells in 50μL total volume. The cells were incubated with the master mix at 4°C for 30-60 minutes in the dark. Cell suspensions were washed with 1mL of FACS buffer, centrifuged to pellet, resuspended in 0.5mL FACS buffer, and transported to the MSU Flow Cytometry Core Facility on ice and in the dark. The cells were run the same day and were only on ice for 1-2 hours prior to flow cytometer runs.

Flow Cytometry Acquisition & Analysis

An LSR II (BD Biosciences, San Jose, CA, USA) flow cytometer (Supplemental Figure 1) was used to collect at least 50,000 total events. The LSR II instrument parameters and general compensation matrix are shown in Supplemental Tables 1 and 2, respectively. Compensation was preformed prior to each data collection using BD Bioscience anti-rat κ CompBeads according to manufacturer's recommendations. A representative figure of the fluorochromes requiring the most compensation is shown in supplemental figure 2. Anti-CD14-PE was substituted for anti-CD11c-PE because the CompBeads do not bind a hamster antibody. We assumed an equal fluorochrome to protein ratio for both anti-CD14-PE and anti-CD11c-PE. The events were collected and post-acquisition analysis was performed using FCS Express version 3 (De Novo Software, Los Angeles, CA, USA).

Flow cytometric analysis gating strategies

Table 2 summarizes the cell surface antigens that were utilized to identify the immune cell populations. The gating strategies for spleen and Peyer's patch cells are shown in Figs. 1 and 2, respectively. The forward scatter and side scatter plot is used to distinguish the live cells from dead cells, debris, and doublet cells (Figs. 1A & 2A).

For spleen cell populations, the Gr-1-PerCp-Cy5.5 and CD8α-PE-Cy7 plot is used to distinguish the memory Gr-1$^+$/CD8α$^+$ T cells and granulocytes, since granulocytes do not express CD8α (Fig. 1B). Memory CD8$^+$ T lymphocytes have been shown to express Ly-6C, which along with Ly-6G is recognized by the anti-Gr-1 antibody (156, 157). Granulocytes (Gr-1$^+$/CD8α$^-$ gate in Fig. 1B) are confirmed as polymorphonuclear neutrophils (PMN) by the expression of CD11b in the histogram of CD11b-APC-Cy7 (Fig. 1C). The histogram of CD11c-PE (Fig. 1D) is used to identify all DC populations, and the histogram of MHC-II-FITC (Fig. 1E) is used to distinguish preDCs and mature DCs, with preDCs lacking MHC-II expression and mature DCs exhibiting MHC-II expression. In the mouse, all DC populations express CD11c (8, 11). Mature mouse DCs (CD11c$^+$/MHC-II$^+$ gate) are divided into three distinct subpopulations by the expression of Gr-1 and B220 (plasmacytoid DCs; Fig. 1F), CD8α (lymphoid DCs) and CD11b (myeloid DCs; Fig. 1G) (8, 11). The preDCs (CD11c$^+$/MHC-II$^-$ gate) are confirmed by the expression of B220 and CD11b in the plot of B220-APC and CD11b-APC-Cy7 (Fig. 1H; (22)). The histogram of Gr-1-PerCp-Cy5.5 is used to identify potential B cells, which are negative for the expression of Gr-1 (Fig. 1I). Any residual cells expressing Gr-1-PerCp-Cy5.5 not gated as PMN or plasmacytoid DC will be eliminated (Fig 1I). The B cells are further confirmed by the

expression of B220-APC and variable expression MHC-II-FITC (Fig. 1J). The expression of MHC-II on B cells is indicative of B cell antigen presentation. As cell populations are identified, exclusion gates using "not" Boolean logic are used to eliminate events from subsequent plots (Fig. 1K and supplemental figure 3). For example, granulocytes and Gr-1$^+$ memory T cells are identified and excluded using the minus granulocyte or Gr-1 T gate with the Boolean logic not (granulocyte or Gr-1 T). The mAb-fluorochrome combination we utilized is limited to the identification of the cell populations described above. Although the spleen contains other cell populations, our gates do not identify all T lymphocytes, natural killer cells or macrophages, but they are likely not contained in our final DC populations. A flow diagram of the gating strategy is shown in supplemental figure 3 for clarity of Boolean logic employed.

The gating strategy of the Peyer's patch cells is shown in Fig. 2. Since Peyer's patches do not have the blood filtering function of the spleen, PMNs should not be present in appreciable numbers and thus are excluded from the gating strategy. Therefore, the plot of Gr-1-PerCp-Cy5.5 and CD8α-PE-Cy7 used to identify granulocytes and memory T cells in the spleen is not used in the Peyer's patch cell analysis. The histogram of CD11c-PE is used to identify potential DCs (CD11c$^+$; Fig. 2B), and the histogram of MHC-II-FITC (Fig. 2C) is subsequently used to distinguish preDCs (MHC-II$^-$) and mature DCs (MHC-II$^+$). The mature DCs (CD11c$^+$/MHC-II$^+$) are divided into the 3 distinct subpopulations by the expression of Gr-1 and B220 (plasmacytoid DCs; Fig. 2D), CD8α (lymphoid DC) and CD11b (myeloid DCs; Fig. 2E). A fourth mature DC population not expressing the markers employed in the analysis is identified ("Other DC"; Fig. 2E). The preDCs (CD11c$^+$/MHC-II$^-$ gate) are confirmed by

the expression of B220 and CD11b in the plot of B220-APC and CD11b-APC-Cy7 (Fig. 2F). The histogram of Gr-1-PerCp-Cy5.5 is used to identify potential B cells (Fig. 2G). The B cells are further confirmed by the expression of B220 and variable expression of MHC-II (Fig. 2H). B cells do not express Gr-1, but do express B220. The expression of MHC-II on B cells is indicative of B cell antigen presentation. As cell populations are identified, exclusion gates using "not" Boolean logic are used to eliminate events from subsequent plots (Fig. 2I). There are other immune cell populations present in Peyer's patches (T lymphocytes), but our mAb-fluorochrome cocktail only allows for the identification of the cell populations described above.

Population calculations and statistical analysis

The total cell numbers for each population per tissue were obtained by multiplying the percentage of the respective gate from the flow cytometry analysis by the total cells (for spleens) or total cells per Peyer's patch counted on a hemacytometer. Prism (GraphPad software , San Diego, CA) was used to perform a student's t-test to detect statistical significance ($p < 0.05$) between male and female animals for each cell population percentage and total cell numbers.

RESULTS

Animal characteristics

At 12 weeks of age, the male and female mice had an average body weight of 25g and 19g, and spleens weights of 0.06g and 0.10g, respectively (Table 3). One female mouse had an unusually large spleen weighing 0.24g. Eliminating this outlier caused the average spleen weight for female mice to decrease to 0.07g. However, the female mouse

with an abnormal spleen weight contained similar number of nucleated cells, suggesting the excessive weight may have been due to red blood cell retention in the spleen. The nucleated cell number per spleen was 1.19×10^8 and 1.36×10^8 for males and females, respectively (Table 3). The average number of Peyer's patches excised was 7 for both males and females, with a minimum of 5 and a maximum of 9. The number of cells per Peyer's patch was 8.40×10^5 and 8.19×10^5 for males and females, respectively (Table 3).

Average immune cell numbers obtained from healthy C57BL/6J mouse spleen and Peyer's patches

We used the techniques described above to determine the percent and total DC subpopulation numbers (Table 4) and other non-DC immune cell populations (Table 5) present in the spleens and Peyer's patches of healthy male and female C57BL/6J mice. Due to the varying numbers of Peyer's patches excised per animal, the Peyer's patch data is shown as cells per Peyer's patch and not total cell numbers.

The total mature and preDCs represent about 4% of the spleen and 3.75% of the Peyer's patches in both male and female mice. Myeloid DCs are the predominant DC subpopulation present in spleen, representing about 2% of the total spleen cells in both male and female mice (Table 4). In the Peyer's patch, myeloid DCs compose only about 1% of the total cells in both males and females. The lymphoid DCs are about 0.7% of the spleen cells in both male and female mice, and about 0.3% and 0.4% of male and female Peyer's patch cells, respectively. Female mice have a significantly higher percentage of lymphoid DCs in the Peyer's patches than male mice ($p=0.035$). Plasmacytoid DCs are only about 0.2% of the total spleen cells in both males and females, but predominate in

the Peyer's patches, representing about 1.3% of male and 1.1% of female total Peyer's patch cells. The preDCs represent approximately 1% of total cells in both spleen and Peyer's patches of both male and female mice. "Other" DCs are not present in the spleen, but represent about 0.2% of the Peyer's patch cells.

On average, there are 2.60×10^6 myeloid DCs, 8.02×10^5 lymphoid DCs, 2.38×10^5 plasmacytoid DCs, and 1.51×10^6 preDCs in male mouse spleens (Table 4). Female mouse spleens contained an average of 2.83×10^6 myeloid DCs, 8.89×10^5 lymphoid DCs, 2.62×10^5 plasmacytoid DCs, and 1.47×10^6 preDCs. In each Peyer's patch of the males, there are an average of 8.83×10^3 myeloid DCs, 2.25×10^3 lymphoid DCs, 9.98×10^3 plasmacytoid DCs, 8.07×10^3 preDCs, and 1.89×10^3 "other" DCs. In each female mouse Peyer's patch, there are an average of 9.50×10^3 myeloid DCs, 3.20×10^3 lymphoid DCs, 8.75×10^3 plasmacytoid DCs, 8.17×10^3 preDCs, and 1.82×10^3 "other" DCs.

B cells are the majority population in both the spleen (66% in males, 62% in females) and Peyer's patches (82% in males, 78% in females; Table 5). In the Peyer's patches, male mice have a significantly higher percentage of B cells than female mice ($p=0.008$). The PMNs represent only about 2% of the spleen cells in both the male and female mice, but were not quantified in the Peyer's patches. Likewise, the Gr-1^+/CD8α^+ memory T cells were not quantified in the Peyer's patches, but represent about 3% of the cells in the spleen (Table 5). In the spleen, male mice have a significantly higher percentage of Gr-1^+/CD8α^+ memory T cells than female mice ($p=0.042$).

The spleens of male and female mice contain averages of 2.76×10^6 and 3.00×10^6 PMNs, respectively (Table 5). Memory Gr-1^+/CD8α^+ T cells average 3.71×10^6 and

3.61×10^6 in the spleens of male and female mice, respectively. The male and female mouse spleens contained 7.91×10^7 and 8.38×10^7 B lymphocytes, respectively, while there are 6.87×10^5 and 6.43×10^5 B lymphocytes per Peyer's patch in male and female mice, respectively.

DISCUSSION

Using multiple mAb-fluorochrome conjugates in flow cytometry, we were able to identify seven immune cell populations of the spleen and five immune cell populations of the Peyer's patches. Of note, all four mouse DC spleen subpopulations could be quantified, and our analysis suggests a fifth relevant mature DC population is present in the Peyer's patches. The cell populations of the spleen included myeloid DCs, lymphoid DCs, plasmacytoid DCs, preDCs, PMNs, B cells, and Gr-1^+/CD8α^+ memory T cells. There have been reports of both one common or two separate precursor DC populations present in the mouse (22, 140). Our staining and gating strategy can identify either of these preDC populations, but here we chose to identify one common precursor DC population as described by del Hoyo et al. (2002). In the Peyer's patches, the preDCs, myeloid DCs, lymphoid DCs, plasmacytoid DCs, and B cells were identified with the mAb-fluorochrome conjugates and gating strategy used. The mature DCs present in the Peyer's patches that do not stain for CD8α or CD11b and which we classify as "other" DC could be CD103$^+$ DCs, but we have not verified this (Figure 2E; (158)). The expression of CD8α and CD11b on CD103$^+$ DCs is controversial, as lung and bronchial lymph node CD103$^+$ DCs have been reported to express low levels of CD11b (159, 160) but not CD8α (159), while spleen marginal zone CD103$^+$ DC have been reported to express CD8α (161). Thus, further experimentation would be needed to identify the

"other" DC subpopulation composing approximately 6% of the Peyer's patch DCs. We suggest that DC subset analysis protocols designed for mucosal immune tissues should contain antibodies to CD103 as well as CD11b and CD8α. This could be added to our existing protocol since detection channels remained available on the LSR II flow cytometer.

The protocol employed is novel in that single tissues from individual animals were used for analysis instead of pooling multiple cell sources or use of any enrichment techniques. In the past, enrichment techniques have led to the accidental depletion of DC subsets, in particular plasmacytoid DCs. B220, or CD45RB, was used to negatively select B cells leading to the simultaneous depletion of plasmacytoid DCs (16), yet this negative selection technique is still marketed for spleen DC enrichment [BD Biosciences IMag™ cell separation system technical document (http://www.bdbiosciences.com/pdfs/brochures/04-7900030-2-A1.pdf; accessed 05/21/2009)]. Therefore, the complex expression pattern of DC subsets limits the value of negative selection protocols based on a combination of surface proteins.

The low percent of DCs, particularly in the steady-state, has led to pooling of multiple tissues or single tissues from multiple animals to obtain sufficient cell numbers for enrichment and/or analysis. However, DCs from various tissues are heterogeneous and variable depending on the tissues pooled. Lymph node analysis performed on lymph nodes pooled from various areas of the body would likely be misleading. $CD103^+$ DCs are restricted to mucosal sites, thus pooling of mucosal lymph nodes with other lymph nodes would be inadvisable. In addition, many factors can affect individual mouse

immune systems, including genetics, glucocorticoids (stress), diet, and others. Therefore, inter-animal variability can be high despite numerous controls and use of inbred strains.

Many variables can affect DC percentages and total cell numbers in a tissue. Infections cause DCs to mature, home to secondary lymphoid tissues, and proliferate. However, the maturation, homing, and proliferation of DCs are site specific to the secondary lymphoid organ of that area of the body. A respiratory infection will not cause systemic changes in DCs, but the respiratory draining lymph nodes will have vastly greater numbers of mature DCs. Due to the effect of estrogen on DCs, there may be differences in DC percentages and total cell numbers between sexes of animals (162, 163), but our data show few gender differences. In addition, animal age has been shown to modify DC populations (164, 165). Finally, the strain of the animal may also affect the percent and total DC numbers (166). BALB/cJ and C57BL/6J animals appear to have similar DC percentages and total cell numbers, but FVB/NJ animals of the same sex and age have significantly fewer spleen DCs and reduced capacity of bone marrow to generate DC *in vitro* (Duriancik, Lackey, and Hoag, unpublished data). Thus, we conclude that it is extremely important to confine DC subpopulation analysis to single tissues from individual animals, without inclusion of enrichment techniques prior to flow cytometric analysis.

The strengths of the protocol presented include the analysis of DCs from single tissues of individual animals, the lack of any cell enrichment techniques, the identification and analysis of DC subpopulations using a single cocktail of mAb-fluorochrome conjugates, and the use of direct mAb-fluorochrome conjugates. The use of direct mAb-fluorochrome conjugates decreases the incubation time because there is

only one incubation step with the antibodies. The use of indirect conjugates may increase the sensitivity of the fluorochromes, but the increased time for multiple incubations and washing steps increases the chance for cell loss through death or DC activation due to contamination. A single cocktail of mAb-fluorochrome conjugates to identify all the DCs allows the identification of all DC subpopulations in a single tube decreasing staining variability due to different cells per tube or mAb-fluorochrome conjugates per tube. Also using a single tube of cells and elimination gates decreases the possibility of counting cells as multiple populations due to expression of multiple antigens. The lack of any cell enrichment procedure decreases cell loss, the potential of DC activation, and unintentional selection or deletion of particular cell populations. The variability of DC populations associated with different tissues including the spleen, Peyer's patches and other mucosal sites, various draining lymph nodes, and other DC sources is eliminated by analyzing single tissues. Analysis of individual animals also increases statistical power by allowing for analysis of inter-animal variability within treatment groups.

The protocol described also has minor limitations including potential cell loss from multiple centrifugations, digital flow cytometric setup and analysis, sophisticated post-acquisition gating analysis and utilization of exclusion gating, and the collection of at least 50,000 events. Increased centrifugations may lead to increasing cell losses, especially because of the low buoyant density of DCs. Digital electronics of the flow cytometer are required to accurately establish the compensation matrix, and bi-exponential plotting is required due to the compensation of the fluorochromes forcing some events to fall below zero (167, 168). Bi-exponential plotting allows for better separation of populations because data falling below zero is not condensed on either axis

(168). Better separation of populations leads to more accurate gating and identification of populations. The complex compensation and low cell numbers expressing some of the mAb-fluorochromes, such as CD11c, requires the use of compensation beads to establish the compensation matrix. Without the compensation beads too many events would be required to accurately detect the positive and negative peaks of some of the fluorochromes leaving few cells left for analysis. Due to the complex expression profile of DC subpopulations, a complex gating strategy must be employed to effectively identify all of the individual DC populations. Not only must bi-exponential plotting be used to create an appropriate cell population separation, but exclusion gates should also be used to eliminate the potential of other cell populations from contaminating true populations. If exclusion gates are not used, then the potential for other events expressing a similar profile of markers could skew the population numbers. The collection of at least 50,000 events is required because of the low percent of DCs in any tissue. If the spleen contains less than 5% of cells as total DCs, collection of 50,000 events should show about 2500 total DCs, whereas collection of only 10,000 events should show only 500 total DCs. The separation of 2500 DCs compared to 500 DCs into 4 subpopulations allows for easier identification and analysis of DC subpopulations. However, the collection of 50,000 events increases the amount of time the sample must be run through the flow cytometer and requires a higher cell number to start.

In summary, our protocol for identification and enumeration of DC subpopulations from the spleen and Peyer's patches of individual uninfected C57BL/6J mice is an efficient method for analyzing DC populations. Several advancements in multicolor flow cytometry techniques were used in development of our protocol and

gating strategies (167-170). The lack of enrichment techniques decreases the associated potential cell loss and variability. The accurate identification and enumeration of DCs provides a technique that will allow for better analysis of DCs in steady-state and inflammatory conditions. Finally, we present a protocol that could be consistently adapted by multiple investigators, allowing for comparison of results from many studies performed in different laboratories.

ACKNOWLEDGEMENTS

We thank the MSU Flow Cytometry Research Technology Support Facility director, Dr. Louis King for his assistance in developing the research protocol.

Figure 2.1. Gating strategy to identify dendritic cells and other immune cell populations in mouse spleen. 1A, forward scatter (FSC) and side scatter (SSC) to identify live cells, live cell gate, based on cell size and granularity. 1B, contour plot of CD8α-PE-Cy7 and Gr-1-PerCp-Cy5.5 used to identify granulocytes, preGranulocyte gate, as Gr-1$^+$/CD8α$^-$ and memory T cells, Gr-1 T cell gate, as Gr-1$^+$/CD8α$^+$. 1C, histogram of CD11b-APC-Cy7 used to confirm preGranulocyte gated cells as PMN, Gr-1$^+$/CD8α$^-$/CD11b$^+$. 1D, histogram of CD11c-PE used to identify DCs, CD11c$^+$. 1E, histogram of MHC-II-FITC to separate DCs as preDCs, CD11c$^+$/MHC-II$^-$, and mature DCs, CD11c$^+$/MHC-II$^+$. 1F, contour plot of B220-APC and Gr-1-PerCp-Cy5.5 to identify plasmacytoid DCs, CD11c$^+$/MHC-II$^+$/B220$^+$/Gr-1$^+$. 1G, contour plot of CD8α-PE-Cy7 and CD11b-APC-Cy7 to identify non-plasmacytoid DCs as lymphoid DCs, CD11c$^+$/MHC-II$^+$/B220$^-$/Gr-1$^-$ /CD8α$^+$/CD11b$^-$, and myeloid DCs, CD11c$^+$/MHC-II$^+$/B220$^-$/Gr-1$^-$/CD8α$^-$/CD11b$^+$. 1H, contour plot of CD11b-APC-Cy7 and B220-APC to confirm preDCs, CD11c$^+$/MHC-II$^-$, as CD11b^{variable}/B220$^+$. 1I, histogram of Gr-1-PerCp-Cy5.5 to identify potential B cells as Gr-1$^-$, eliminating cells expressing Gr-1-PerCp-Cy5.5 not gated as a PMN or plasmacytoid DC. 1J, dot plot of B220-APC and MHC-II-FITC to confirm potential B cells, Gr-1$^-$, as B220$^+$/MHC-II^{variable}. 1K, Boolean logic used for hierarchical gating, including exclusion gates such as minus granulocytes or Gr-1 T. Numbers in parentheses indicate cell population percentage that falls within the gate. Representative data from one animal is shown.

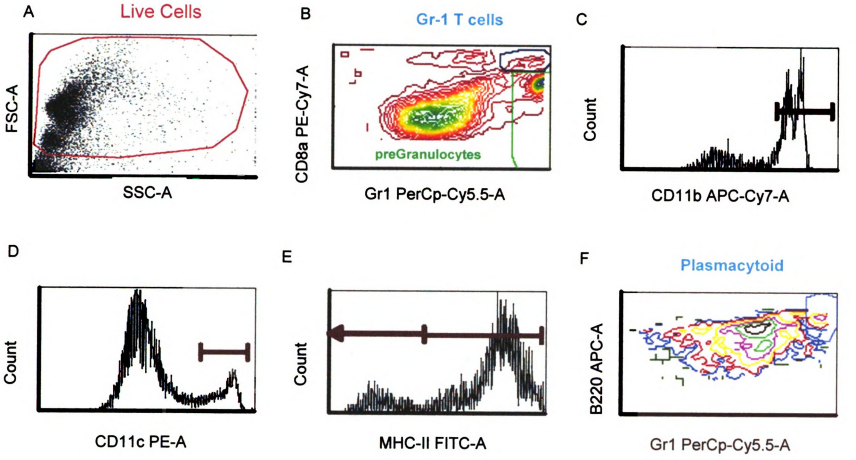
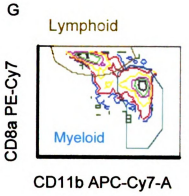
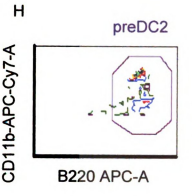
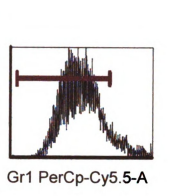
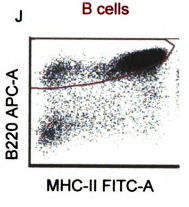

A
Live Cells
FSC-A
SSC-A
B
Gr-1 T cells
CD8a PE-Cy7-A
preGranulocytes
Gr1 PerCp-Cy5.5-A
C
Count
CD11b APC-Cy7-A
D
Count
CD11c PE-A
E
Count
MHC-II FITC-A
F
Plasmacytoid
B220 APC-A
Gr1 PerCp-Cy5.5-A

Figure 2.1 Continued. Gating strategy to identify dendritic cells and other immune cell populations in mouse spleen. 1A, forward scatter (FSC) and side scatter (SSC) to identify live cells, live cell gate, based on cell size and granularity. 1B, contour plot of CD8α-PE-Cy7 and Gr-1-PerCp-Cy5.5 used to identify granulocytes, preGranulocyte gate, as Gr-1$^+$/CD8α$^-$ and memory T cells, Gr-1 T cell gate, as Gr-1$^+$/CD8α$^+$. 1C, histogram of CD11b-APC-Cy7 used to confirm preGranulocyte gated cells as PMN, Gr-1$^+$/CD8α$^-$/CD11b$^+$. 1D, histogram of CD11c-PE used to identify DCs, CD11c$^+$. 1E, histogram of MHC-II-FITC to separate DCs as preDCs, CD11c$^+$/MHC-II$^-$, and mature DCs, CD11c$^+$/MHC-II$^+$. 1F, contour plot of B220-APC and Gr-1-PerCp-Cy5.5 to identify plasmacytoid DCs, CD11c$^+$/MHC-II$^+$/B220$^+$/Gr-1$^+$. 1G, contour plot of CD8α-PE-Cy7 and CD11b-APC-Cy7 to identify non-plasmacytoid DCs as lymphoid DCs, CD11c$^+$/MHC-II$^+$/B220$^-$/Gr-1$^-$/CD8α$^+$/CD11b$^-$, and myeloid DCs, CD11c$^+$/MHC-II$^+$/B220$^-$/Gr-1$^-$/CD8α$^-$/CD11b$^+$. 1H, contour plot of CD11b-APC-Cy7 and B220-APC to confirm preDCs, CD11c$^+$/MHC-II$^-$, as CD11b^{variable}/B220$^+$. 1I, histogram of Gr-1-PerCp-Cy5.5 to identify potential B cells as Gr-1$^-$, eliminating cells expressing Gr-1-PerCp-Cy5.5 not gated as a PMN or plasmacytoid DC. 1J, dot plot of B220-APC and MHC-II-FITC to confirm potential B cells, Gr-1$^-$, as B220$^+$/MHC-II^{variable}. 1K, Boolean logic used for hierarchical gating, including exclusion gates such as minus granulocytes or Gr-1 T. Numbers in parentheses indicate cell population percentage that falls within the gate. Representative data from one animal is shown.









K

- Live Cells (73.14%)
  - Gr-1 T cells (2.42%)
  - preGranulocytes
    - Granulocytes (8.69%)
  - Minus granulocytes and Gr-1 T
    - CD11c pos (14.32%)
      - Mature DCs
        - Plasmacytoid (0.68%)
        - Minus Plasmacytoid
          - Myeloid DCs (5.59%)
          - Lymphoid DCs (4.31%)
      - preDCs
        - preDC2 (1.66 %)
      - Minus all DCs
        - preB cells
          - B cells (55.55%)

Figure 2.2. Gating strategy to identify dendritic cells and other immune cell populations in mouse Peyer's patches. 2A, forward scatter (FSC) and side scatter (SSC) to identify live cells, live cell gate, based on cell size and granularity. 2B, histogram of CD11c-PE used to identify DCs, $CD11c^{+}$. 2C, histogram of MHC-II-FITC to separate DCs as preDCs, $CD11c^{+}$/MHC-II^{-}, and mature DCs, $CD11c^{+}$/MHC-II^{+}. 2D, contour plot of B220-APC and Gr-1-PerCp-Cy5.5 to identify plasmacytoid DCs, $CD11c^{+}$/MHC-II^{+}/$B220^{+}$/Gr-1^{+}. 2E, contour plot of CD8α-PE-Cy7 and CD11b-APC-Cy7 to identify non-plasmacytoid DCs as lymphoid DCs, $CD11c^{+}$/MHC-II^{+}/$B220^{-}$/Gr-1^{-}/$CD8\alpha^{+}$/$CD11b^{-}$, myeloid DCs, $CD11c^{+}$/MHC-II^{+}/$B220^{-}$/Gr-1^{-}/$CD8\alpha^{-}$/$CD11b^{+}$, and "Other" DCs, $CD11c^{+}$/MHC-II^{+}/$B220^{-}$/Gr-1^{-}/$CD8\alpha^{-}$/$CD11b^{-}$. 2F, contour plot of CD11b-APC-Cy7 and B220-APC to confirm preDCs, $CD11c^{+}$/MHC-II^{-}, as $CD11b^{variable}$/$B220^{+}$. 2G, histogram of Gr-1-PerCp-Cy5.5 to identify potential B cells as Gr-1^{-}. 2H, dot plot of B220-APC and MHC-II-FITC to confirm potential B cells, Gr-1^{-}, as $B220^{+}$/MHC-$II^{variable}$. 2I, Boolean logic used for hierarchal gating, including exclusion gates. Numbers in parentheses indicate cell population percentage that falls within the gate. Representative data from one animal is shown.

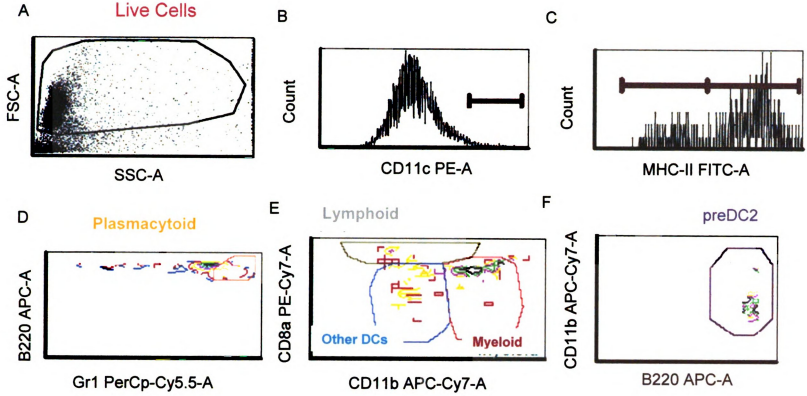
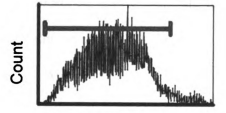
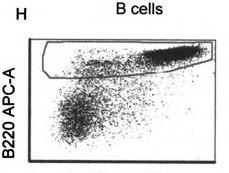

A
Live Cells
FSC-A
SSC-A
B
Count
CD11c PE-A
C
Count
MHC-II FITC-A
D
Plasmacytoid
B220 APC-A
Gr1 PerCp-Cy5.5-A
E
Lymphoid
CD8a PE-Cy7-A
Other DCs
Myeloid
CD11b APC-Cy7-A
F
preDC2
CD11b APC-Cy7-A
B220 APC-A

Figure 2.2 Continued. Gating strategy to identify dendritic cells and other immune cell populations in mouse Peyer's patches. 2A, forward scatter (FSC) and side scatter (SSC) to identify live cells, live cell gate, based on cell size and granularity. 2B, histogram of CD11c-PE used to identify DCs, $CD11c^+$. 2C, histogram of MHC-II-FITC to separate DCs as preDCs, $CD11c^+$/$MHC\text{-}II^-$, and mature DCs, $CD11c^+$/$MHC\text{-}II^+$. 2D, contour plot of B220-APC and Gr-1-PerCp-Cy5.5 to identify plasmacytoid DCs, $CD11c^+$/$MHC\text{-}II^+$/$B220^+$/$Gr\text{-}1^+$. 2E, contour plot of CD8α-PE-Cy7 and CD11b-APC-Cy7 to identify non-plasmacytoid DCs as lymphoid DCs, $CD11c^+$/$MHC\text{-}II^+$/$B220^-$/$Gr\text{-}1^-$/$CD8\alpha^+$/$CD11b^-$, myeloid DCs, $CD11c^+$/$MHC\text{-}II^+$/$B220^-$/$Gr\text{-}1^-$/$CD8\alpha^-$/$CD11b^+$, and "Other" DCs, $CD11c^+$/$MHC\text{-}II^+$/$B220^-$/$Gr\text{-}1^-$/$CD8\alpha^-$/$CD11b^-$. 2F, contour plot of CD11b-APC-Cy7 and B220-APC to confirm preDCs, $CD11c^+$/$MHC\text{-}II^-$, as $CD11b^{variable}$/$B220^+$. 2G, histogram of Gr-1-PerCp-Cy5.5 to identify potential B cells as $Gr\text{-}1^-$. 2H, dot plot of B220-APC and MHC-II-FITC to confirm potential B cells, $Gr\text{-}1^-$, as $B220^+$/$MHC\text{-}II^{variable}$. 2I, Boolean logic used for hierarchal gating, including exclusion gates. Numbers in parentheses indicate cell population percentage that falls within the gate. Representative data from one animal is shown.

G


Count
Gr1 PerCp-Cy5.5-A

H


B cells
B220 APC-A
MHC-II FITC-A

I

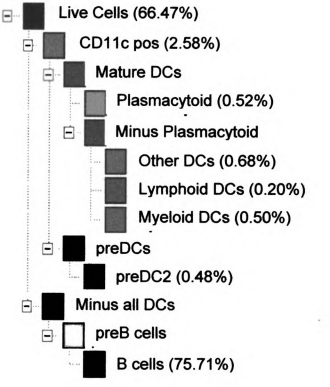

Live Cells (66.47%)
CD11c pos (2.58%)
Mature DCs
Plasmacytoid (0.52%)
Minus Plasmacytoid
Other DCs (0.68%)
Lymphoid DCs (0.20%)
Myeloid DCs (0.50%)
preDCs
preDC2 (0.48%)
Minus all DCs
preB cells
B cells (75.71%)

Table 2.1. Antibody-fluorochrome conjugate reagents employed in labeling cell suspensions for flow cytometric analysis. The fluorochromes were selected for maximum sensitivity (171).

Antigen	Clone	Isotype	Fluorochrome
MHC-II (I-A/I-E)	2G9	Rat IgG_{2a}, κ	FITC
CD11c	HL3	Arm. Hamster IgG_1, λ_2	PE
CD8α	53-6.7	Rat IgG_{2a}, κ	PE-Cy7
CD11b	M1/70	Rat IgG_{2b}, κ	APC-Cy7
CD45RB (B220)	RA3-6B2	Rat IgG_{2a}, κ	APC
Gr-1	RB6-8C5	Rat IgG_{2b}, κ	PerCp-Cy5.5

Table 2.2. Antigen expression of immune cell populations in mouse spleen and Peyer's patches used in gating strategy.

	Cell Population			
	Dendritic cells			
Antigen	Myeloid	Lymphoid	Plasmacytoid	PreDC
CD11c	Pos[b]	Pos	Pos	Pos
MHC-II	Pos	Pos	Pos	Neg[d]
CD11b	Pos	Neg	N/A	Pos/Neg
CD8α	Neg	Pos	N/A	N/A
Gr-1	Neg	Neg	Pos	N/A
B220	Neg	Neg	Pos	Pos

Table 2.2

and Peyer

Antigen	
CD11c	
MHC-II	
CD11b	
CD8α	
Gr-1	
B220	

[a]PMN is

[b]Pos ind

[c]N A ind

strategy.

[d]Neg ind

Table 2.2 Continued. Antigen expression of immune cell populations in mouse spleen and Peyer's patches used in gating strategy.

	Cell Population		
Antigen	PMN[a]	B Cells	Gr-1^{+}CD8α^{+} T Cells
CD11c	N/A[c]	N/A	N/A
MHC-II	N/A	Pos/Neg	N/A
CD11b	Pos	N/A	N/A
CD8α	Neg	N/A	Pos
Gr-1	Pos	Neg	Pos
B220	N/A	Pos	N/A

[a]PMN is polymorphonuclear neutrophil.

[b]Pos indicates that the cell population is positive for the antigen.

[c]N/A indicates that the antigen is not used to distinguish the cell population in our gating strategy.

[d]Neg indicates that the cell population is negative for the antigen.

Table 2.3. Physiological characteristics of C57BL/6J mice used in the studies[a].

	Body Weight (g)	**Spleen Weight (g)**	**Cells/Spleen**	**Number of PP[b]**	**Cells/PP[c]**
Male (n=7)	24.77 ± 1.06	0.06 ± 0.01	$1.19 \times 10^8 \pm 2.94 \times 10^7$	6.9 ± 1.4	$8.40 \times 10^5 \pm 3.33 \times 10^5$
Female (n=6)	19.03 ± 0.58	0.10 ± 0.07	$1.36 \times 10^8 \pm 1.77 \times 10^7$	6.5 ± 1.1	$8.19 \times 10^5 \pm 2.27 \times 10^5$

[a]All data presented as mean ± 1 SD.

[b]PP is Peyer's patches.

[c]Total cells counted for all Peyer's patches from one animal divided by the total number of Peyer's patches harvested from the animal.

Table 2.4. Percentage and total numbers of dendritic cell populations of C57BL/6J spleen and Peyer's patches.

	Spleen				
Mouse Gender	**Myeloid DCs**	**Lymphoid DCs**	**Plasmacytoid DCs**	**Precursor DCs**	**Other DCs**
Male	2.18 ± 0.16[a] ($2.60 \times 10^6 \pm 6.89 \times 10^5$)[b]	0.68 ± 0.07 ($8.02 \times 10^5 \pm 1.85 \times 10^5$)	0.20 ± 0.04 ($2.38 \times 10^5 \pm 6.84 \times 10^4$)	1.22 ± 0.38 ($1.51 \times 10^6 \pm 8.08 \times 10^5$)	N/A[c]
Female	2.08 ± 0.28 ($2.83 \times 10^6 \pm 5.84 \times 10^5$)	0.66 ± 0.13 ($8.89 \times 10^5 \pm 1.99 \times 10^5$)	0.19 ± 0.07 ($2.62 \times 10^5 \pm 1.05 \times 10^5$)	1.07 ± 0.31 ($1.47 \times 10^6 \pm 4.82 \times 10^5$)	N/A

[a]Average +/- standard deviation of spleen cell population percentage.

[b]Average +/- standard deviation of total cells for each population in the spleen.

[c]N/A indicates that Other DCs were not detected in the spleen.

Table 2.4 Continued. Percentage and total numbers of dendritic cell populations of C57BL/6J spleen and Peyer's patches.

	Peyer's patches				
Mouse Gender	**Myeloid DCs**	**Lymphoid DCs**	**Plasmacytoid DCs**	**Precursor DCs**	**Other DCs**
Male	0.99 ± 0.59[d] ($8.83 \times 10^3 \pm 7.40 \times 10^3$)[e]	0.26 ± 0.09* ($2.25 \times 10^3 \pm 1.19 \times 10^3$)	1.27 ± 0.34 ($9.98 \times 10^3 \pm 2.10 \times 10^3$)	1.01 ± 0.49 ($8.07 \times 10^3 \pm 3.47 \times 10^3$)	0.20 ± 0.12 ($1.89 \times 10^3 \pm 1.82 \times 10^3$)
Female	1.05 ± 0.54 ($9.50 \times 10^3 \pm 6.45 \times 10^3$)	0.38 ± 0.09* ($3.20 \times 10^3 \pm 1.19 \times 10^3$)	1.10 ± 0.21 ($8.57 \times 10^3 \pm 1.70 \times 10^3$)	1.00 ± 0.27 ($8.17 \times 10^3 \pm 3.23 \times 10^3$)	0.22 ± 0.03 ($1.82 \times 10^3 \pm 6.05 \times 10^2$)

[d]Average +/- standard deviation of Peyer's patch cell population percentage.

[e]Average +/- standard deviation of total cells for each cell population in each Peyer's patch.

*Statistically significant difference between male and female mice ($p<0.05$).

Table 2.5. Percentage and total cell numbers of non-dendritic immune cell populations enumerated in C57BL/6J spleen and Peyer's patches.

	Spleen		
Mouse Sex	**Neutrophils**	**B lymphocytes**	**CD8α$^+$/Gr-1$^+$ T lymphocytes**
Male	2.26 ± 0.39[a] (2.76 x 10^6 ± 1.14 x 10^6)[b]	66.22 ± 1.24 (7.91 x 10^7 ± 2.10 x 10^7)	3.10 ± 0.39* (3.71 x 10^6 ± 1.19 x 10^6)
Female	2.22 ± 0.35 (3.00 x 10^6 ± 5.52 x 10^5)	62.07 ± 5.86 (8.38 x 10^7 ± 1.02 x 10^7)	2.65 ± 0.30* (3.61 x 10^6 ± 7.08 x 10^5)

Table 2.5 Continued. Percentage and total cell numbers of non-dendritic immune cell populations enumerated in C57BL/6J spleen and Peyer's patches.

	Peyer's patches		
Mouse Sex	**Neutrophils**	**B lymphocytes**	**CD8α$^+$/Gr-1$^+$ T lymphocytes**
Male	N/A[c]	81.58 ± 2.05[d]* (6.87 x 10^5 ± 2.75 x 10^5)[e]	N/A
Female	N/A	78.18 ± 1.64* (6.43 x 10^5 ± 1.87 x 10^5)	N/A

[a]Average +/- standard deviation of spleen cell population percentages.

[b]Average +/- standard deviation of total cells for each population in the spleen.

[c]N/A indicates cell populations not detected in Peyer's patches.

[d]Average +/- standard deviation of Peyer's patch cell population percentages.

[e]Average +/- standard deviation of total cells for each cell population in each Peyer's patch.

*Statistically significant difference between male and female mice.

CHAPTER 3: VITAMIN A DEFICIENCY ALTERS SPLENIC DENDRITIC CELL SUBSETS AND INCREASES $CD8^+GR\text{-}1^+$ MEMORY T LYMPHOCYTES IN C57BL/6J MICE.

Duriancik DM and Hoag KA. In preparation for submission to *Journal of Nutrition.*

Abstract

Vitamin A-deficient populations have impaired T cell-dependent antibody responses. Dendritic cells (DCs) are the most proficient antigen presenting cells to naïve T cells. In the mouse, $CD11b^+$ myeloid DCs stimulate T helper (Th) 2 antibody immune responses, while $CD8\alpha^+$ lymphoid DCs stimulate Th1 cell-mediated immune responses. Therefore, we hypothesized that vitamin A-deficient animals would have decreased numbers of myeloid DCs and unaffected numbers of lymphoid DCs. We performed dietary depletion of vitamin A in C57BL/6J male and female mice and used multicolor flow cytometry to quantify immune cell populations of the spleen, with particular focus on DC subpopulations. We show that vitamin A-depleted animals have increased polymorphonuclear neutrophils, lymphoid DCs, and memory $CD8^+$ T cells and decreased $CD4^+$ T lymphocytes. Therefore, vitamin A deficiency alters splenic DC subpopulations, which may contribute to skewed immune responses of vitamin A-deficient populations.

Introduction

Vitamin A has numerous roles in the immune system. Populations deficient in vitamin A have impaired innate immunity, including loss of mucosal barrier integrity and decreased numbers and/or function of innate immune phagocytic and natural killer cells (112). The adaptive immune response is also altered by vitamin A deficiency. Lymphocyte homing, immunoglobulin A (IgA) responses in the mucosa, and T-dependent antibody responses are decreased in vitamin A-deficient individuals (112, 121, 130, 135, 172, 173). Vitamin A is also required for the balance of T regulatory (Treg) and T helper (Th) 17 cells in mucosal tissues (174). Therefore, vitamin A is required in the maintenance of the immune system to adequately respond to danger signals and maintain tolerance to self.

Dendritic cells (DCs) are the most proficient stimulators of naïve T lymphocytes and link the innate and adaptive arms of the immune system (20, 175). DCs derive from hematopoietic progenitors and reside in peripheral tissues and circulate in blood in an immature state. DC half-lives range from 3 to 9 days (24). Multiple DC subsets have been distinguished by differential surface protein expression, and these subsets preferentially stimulate varying adaptive immune functions. In the mouse, $CD11b^+$ myeloid DCs stimulate Th2 responses, while $CD8\alpha^+$ lymphoid DCs stimulate Th1 responses (10, 11). Plasmacytoid DCs are mature DCs that secrete type 1 interferons to augment anti-viral immune responses (12-14). All mature mouse DC populations appear to arise from one common precursor DC population (22).

Vitamin A plays a role in maintaining DCs, but the reports have been limited primarily to *in vitro* studies. Hengesbach and Hoag (2004) have shown that bone marrow

precursors stimulated with GM-CSF differentiate into DCs in the presence of medium containing vitamin A. However, if the medium was depleted of vitamin A or contained a retinoic acid receptor antagonist, the precursors generated increased numbers of neutrophils (133). *In vivo*, vitamin A-deficient SENCAR mice had increased neutrophils and decreased lymphocyte populations compared to vitamin A-sufficient controls (108). However, the authors did not characterize the DC populations of these animals. DCs are hematopoietic-derived cells, but also reside and proliferate in secondary lymphoid tissues (5). In addition, DCs are motile cells traveling between sites of infection and secondary lymphoid tissues. The chemokine receptor for DCs to migrate to intestinal tissues is depressed in vitamin A deficiency (147). The expression of matrix metalloproteinases (MMPs) are also skewed in DCs of vitamin A deficient origin (176, 177). The combination of depression of homing receptors and matrix degrading enzymes leads to impaired trafficking of DCs to stimulate immune responses. Therefore, in specific-pathogen free vitamin A-deficient animals, the DC populations may be skewed, even in the absence of an infectious challenge.

Since vitamin A deficiency is known to impair Th2 responses and myeloid DCs stimulate the Th2 response, we hypothesized that vitamin A-deficient (VAD) animals would have decreased myeloid DCs compared to vitamin A-sufficient (VAS) control animals. To address this hypothesis, we used multicolor flow cytometry to quantify the DC populations in the spleen of C57BL/6J mice and compared these populations in animals that were depleted of vitamin A or were vitamin A-sufficient. The combination of surface markers used led to a comprehensive overview of the effect of vitamin A

depletion on many cells of the immune system over various degrees of vitamin A deficiency in both male and female mice.

Methods

Animals

Male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Vitamin A-deficient mice were obtained following an established protocol (91). Briefly, breeder pairs were allowed to mate and upon indication of pregnancy the female was placed on a vitamin A-deficient AIN-93G diet. The pups were weaned into one of four groups based on diet and gender. Weaned mice were fed *ad libitum* pelletized AIN-93M diets free of vitamin A, or a control diet containing 8 mg retinyl-palmitate/Kg diet. The control diet had added red dye to aid in preventing accidental feeding of incorrect diet. All diets were purchased from Dyets, Inc. (New Bethlehem, PA) and stored frozen in vacuum-sealed bags to maintain freshness. Mice were weighed once per week starting at 3 weeks of age to monitor general animal health. At 6 weeks of age, and every 2 weeks after, serum was obtained from saphenous vein punctures and analyzed for serum retinol. Mice were sacrificed via carbon dioxide asphyxiation at 8, 10, and 12 weeks of age and spleen, liver, and serum were collected. All animal procedures were performed in accordance with protocols approved by the Michigan State University Animal Care and Use Committee.

Vitamin A analysis

Vitamin A status was assessed using an adapted high performance liquid chromatography (HPLC) protocol (178). A 75% acetonitrile, 25% water mobile phase

was used over a ten minute run time through a Prism RP column (Part #32103-153030) purchased from Thermo Electron Corp (Waltham, MA). A 1μM retinyl-acetate (Sigma, St. Louis, MO) internal standard was used to assess retinol concentrations. Retinol was quantified in serum and following saponification of liver retinyl esters. The retinol was extracted into hexane, dried under argon stream, and redissolved in acetonitrile. Liver retinol is expressed as nanomole (nMole) retinol activity equivalents (RAE) per gram of liver.

Tissue Processing

Spleens were processed for flow cytometric analysis as previously described . Briefly, tissues were harvested, weighed and placed in ice cold calcium and magnesium free Hank's Balanced Salts Solution (Lonza, Basel, Switzerland). Tissues were digested into single cell suspensions using enzymatic dissociation medium from Stem Cell Technologies (Vancouver, BC, Canada) per manufacturer's instructions. The digest was filtered, red blood cells lysed, and cell suspensions resuspended in FACS Buffer [0.1% sodium azide (Fisher Scientific, Pittsburgh, PA), 1% fetal bovine serum (Hyclone, Logan, UT), in Dulbecco's phosphate buffered saline, pH 7.4-7.6, sterile filtered].

Flow Cytometry

Spleen cell suspensions were blocked with 5 μg of anti-FcRγII/III antibody (2.4G2 hybridoma) on ice for 10 minutes and then incubated with two separate cocktails of monoclonal-antibody (mAb) fluorochrome conjugate reagents. A cocktail of 6 monoclonal antibodies (mAb) was used for DC analysis, as previously described by Duriancik and Hoag (179), and a 3 mAb cocktail for T lymphocyte analysis [hamster anti-mouse CD3ε-PE (clone 500A2), rat anti-mouse CD4-APC (clone RM4-5), and rat

anti-mouse CD8α-PE-Cy7 (clone 53-6.7)]. All mAb-fluorochrome conjugates were purchased from BD Biosciences (San Jose, CA). Flow cytometry data was collected on a BD Biosciences LSR II flow cytometer and analyzed with FCS Express (version 3) software to identify DC (179) and T lymphocyte cell populations.

Statistical Analysis

Statistical analysis was performed using SPSS statistics 17. All figures were designed using Prism (GraphPad Software). Three factorial (age, gender, diet) analysis of variance (ANOVA) and Bonferroni post-test was performed on spleen and liver weights, liver RAE data, and total spleen cellularity. Body weight and serum retinol were analyzed using repeated measures ANOVA at 12 weeks due to missing data at 8 and 10 week time points. Multiple linear regression analysis was performed to determine the effects of vitamin A (nMole RAE/g liver) depletion on each spleen cell population.

Results

The VAD altered the growth kinetics of the mice (Figure 1). Male and female mice gained weight through 11 weeks of age and maintained weight from 11 to 12 weeks. There was a significant effect of diet from 8 weeks through 11 weeks of age. Gender had a significant effect on weight from 3 weeks through 9 weeks, with an exception from 7 to 8 weeks of age. The weight, diet, and gender interaction was significant starting at 9 weeks of age and continued through 12 weeks of age. Diet and gender both significantly affected the weight of the animals.

As expected, livers of male mice weighed significantly more than livers of female mice due to body size, but diet and age had no effect on liver weight of the mice (Table 1). Serum retinol significantly differed from previous measurement within a group only

from 6 to 8 weeks of age (Figure 2A). Diet, gender, and the diet-gender interaction significantly affected serum retinol. Male animals on control vitamin A-sufficient diet had higher serum retinol values than females, but lower liver RAE. Despite other factors, diet, age, and gender each significantly affected liver RAE (Figure 2B). As expected, the interaction of age and diet on liver RAE was significant, indicating that time consuming the vitamin A-deficient diet affected liver stores. However, surprisingly, the interaction of diet and gender was significant indicating that consuming a VAD diet affects male and female mice differently.

Spleens of VAD animals weighed significantly more than spleens from VAS animals, regardless of age or gender (Table 2). There was a diet-independent significant interaction of age and gender for spleen weight, because as males aged the spleen weight decreased, but as females aged the spleen weight increased. The total cellularity of the spleen was unaffected by vitamin A depletion (Figure 3). The age and gender of the animal significantly affected the total spleen cell counts. In addition, the interaction of age and diet and age and gender significantly affected the cell counts.

Multiple linear regression analyses were performed on each cell population identified in the spleen (Figures 4, 5, and 6). The total spleen cells were not different between VAD and VAS groups; therefore any difference in percent cell populations would also be reflected in cell population total numbers. Therefore, only percent of each cell type is shown. The liver RAE was log transformed and the logarithmic regression line was interpolated. The table insets indicate the β coefficients and their p values as well as the R^2 value and the ANOVA significance of the model which was adjusted for

age and gender variables. Significant and strong associations were β coefficients and R^2 values greater than 0.4 and p-values less than 0.05.

Myeloid DCs were only slightly decreased by VAD. Even though the model and the β coefficient were significant, the β coefficient for liver RAE was small at 0.32 (Figure 4A). Lymphoid DCs increased as severity of VAD increased (Figure 4B). The β coefficient for liver RAE and lymphoid DCs was -0.63. Since lymphoid and myeloid DCs preferentially stimulate Th1 and Th2 responses, respectively, we also assessed the lymphoid to myeloid DC ratio, as this would be assumed to alter the Th1/Th2 bias in immune responses. The ratio of lymphoid DCs to myeloid DC significantly increased as severity of VAD increased with a β coefficient of -0.68 (Figure 4C). Plasmacytoid and preDCs were unaffected by VAD (Figures 4D and 4E).

Some, but not all, spleen lymphocyte populations were altered by VAD (Figure 5). B lymphocytes ($B220^+$/MHC-$II^{variable}$) , $CD3^+/CD4^-/CD8^-$ lymphocytes, and total $CD3^+/CD8^+$ T lymphocytes were unaffected by vitamin A deficiency (Figure 5A, B, and D). However, $CD3^+/CD4^+$ T lymphocytes were decreased as severity of VAD increased (Figure 5C). The β coefficient of liver RAE and $CD4^+$ T cells was 0.62. Interestingly, memory $CD8^+$ T lymphocytes ($Gr\text{-}1^+/CD8^+$) were increased as severity of VAD increased (Figure 5D). The β coefficient of liver RAE and memory $CD8^+$ T cells was -0.62.

As hypothesized and previously reported by others, polymorphonuclear neutrophils (PMN) percentage increased as severity of VAD increased (Figure 6). The β coefficient of liver RAE and PMN was -0.44.

Discussion

The data presented here indicate the C57BL/6J animals depleted of vitamin A have skewed splenic DC subpopulations that could contribute to the observed Th1 bias in vitamin A-deficient populations. Vitamin A-deficient populations have impaired Th2-dependent antibody immune responses, and enhanced Th1 cell-mediated immune responses (114, 121, 122). In the mouse, $CD11b^+$ myeloid DCs stimulate Th2 responses, while $CD8\alpha^+$ lymphoid DCs stimulate Th1 responses (10, 11). Previously, *in vitro* work has established that vitamin A is important for the differentiation of bone marrow progenitor cells into myeloid DCs and blocking vitamin A signaling leads to greater numbers of neutrophils (133). We hypothesized that myeloid DCs would be decreased in the spleens of vitamin A-deficient mice. Interestingly, our data indicate that *in vivo*, lymphoid DCs are increased in VAD mice and myeloid DCs are only modestly affected. The skewed DC proportion was contradictory to our original hypothesis of decreased myeloid DCs and unaffected lymphoid DCs. However, our data demonstrating an alteration in lymphoid to myeloid DC ratio in VAD could directly contribute to insufficient Th2 responses associated with VAD.

We used the dietary depletion protocol of Smith et al (1987) to obtain VAD animals (91). We monitored feed consumption (data not shown) and body weights (Figure 1) during the study to determine the point of inanition which would lead to protein-energy malnutrition (PEM). Detection of inanition was established to be a loss of greater than 10% of body weight. Interestingly, depletion of vitamin A in female mice had slower kinetics than depletion of male mice (Figure 2). Few male mice reached the 12 week time point without losing 10% of their body weight, hence only four VAD males

at 12 weeks of age were analyzed. Therefore, the protocol produces marginally vitamin A-deficient mice at 8-10 weeks of age and severe vitamin A deficiency in male animals at 12 weeks.

Our data are in agreement with many of the spleen immune cell percentages of vitamin A-deficient populations reported previously by others, including B and T lymphocytes (91, 122, 131). However, the dramatic increase in neutrophils reported in VAD SENCAR mice contradicts the mild increase observed in our mice [Figure 6, (108)]. In conjunction with the large increase in neutrophils, VAD SENCAR mice also had decreased lymphocyte populations (108). Therefore, the 14 week dietary depletion of vitamin A may have resulted in PEM and/or correspondingly increased glucocorticoids. Lymphocytes decrease and neutrophils increase in response to PEM and increased levels of glucocorticoids (180-182). Chronic vitamin A deficiency leads to PEM, which complicates study of vitamin A deficiency.

In agreement with previous reports, we show that B lymphocyte and $CD8^+$ T lymphocyte numbers were unaffected and $CD4^+$ T lymphocyte numbers were decreased in vitamin A-deficient mice. However, we can not distinguish $CD4^+$ T cells as Th1, Th2, or Treg using our multicolor flow cytometric analysis. Surprisingly, our gating strategy identified a population of $CD8^+$ T lymphocytes that expressed Gr-1were increased in VAD. These $CD8\alpha^+$/Gr-1^+ cells were previously characterized as memory $CD8^+$ T lymphocytes (156, 157). Vitamin A-deficient populations have an increased reliance on memory cell immune responses and decreased reliance on naïve cell immune responses (131, 183, 184). Memory T cells, as well as natural killer (NK) and NKT cells, are maintained by the growth factor interleukin- (IL) 15. Hepatic and pancreatic stellate

cells, as well as DCs, produce IL-15 (185-187). Hepatic stellate cells and DCs can also store and metabolize vitamin A (135). Although vitamin A did not significantly alter IL-15 cytokine production *in vitro*, the dual function of vitamin A metabolism and production of IL-15 by DCs and liver stellate cells leads to a possible inter-relationship that would be expected to selectively enhance memory T cell reliance during VAD (188).

Our data indicate, for the first time, that *in vivo* spleen DC populations are altered by vitamin A deficiency. Vitamin A deficiency skews DC subset proportions in favor of Th1 responses, leading to the down-regulation of Th2 responses. The altered DC proportions provide mechanistic evidence for a role of DCs in altered immune responses of vitamin A-deficient populations. Further work should expand the DC analysis of vitamin A-deficient populations to other tissues and newly described DC subpopulations. The DC populations of the mesenteric lymph nodes and Peyer's patches should be assessed during vitamin A deficiency. The effects of vitamin A deficiency on newly identified DC populations such as $CD103^+$ DCs should also be assessed due to the roles of vitamin A and $CD103^+$ DCs in the Treg and Th17 immune cell balance in the gastrointestinal tract (137, 143, 161, 174).

Table 3.1. Liver weight of VAS and VAD male and female animals. Week 8 (n=10 mice per group), week 10 (n=10 mice per group), week 12 VAS female and VAD female (n=10 mice per group), week 12 VAS male (n=8) and week 12 VAD male (n=4 mice).

	Week 8		Week 10		Week 12	
	VAS	VAD	VAS	VAD	VAS	VAD
Male*	0.91 +/- 0.05[a]	0.81 +/- 0.05	0.88 +/- 0.04	0.85 +/- 0.04	0.93 +/- 0.02	0.98 +/- 0.03
Female	0.75 +/- 0.02	0.68 +/- 0.05	0.75 +/- 0.05	0.82 +/- 0.02	0.78 +/- 0.05	0.72 +/- 0.05

[a] Values are mean +/- standard error of the mean of weight in grams.

* Statistical significance ($p<0.05$). Male livers weighed significantly more than female livers, regardless of age or diet.

Table 3.2. Spleen weight of VAS and VAD male and female animals. Week 8 (n=10 mice per group), week 10 (n=10 mice per group), week 12 VAS female and VAD female (n=10 mice per group), week 12 VAS male (n=8) and week 12 VAD male (n=4 mice).

	Week 8		Week 10		Week 12	
	VAS*	VAD	VAS*	VAD	VAS*	VAD
Male	0.07 +/- 0.01[a]	0.08 +/- 0.01	0.06 +/- 0.00	0.07 +/- 0.01	0.06 +/- 0.00	0.07 +/- 0.01
Female	0.06 +/- 0.00	0.07 +/- 0.01	0.07 +/- 0.01	0.08 +/- 0.01	0.07 +/- 0.01	0.09 +/- 0.01

[a] Values are mean +/- standard error of the mean of weight in grams.

* Statistical significance ($p<0.05$). Spleens of VAD animals weighed significantly more than spleens of VAS animals, regardless of age or gender.

Figure 3.1. Body weights of C57BL/6J mice consuming VAS and VAD diet. VAS animals are indicated by filled symbols and VAD animals are indicated by open symbols, male mice are indicated by squares (■) while female mice are indicated by triangles (▼). *, Weight is significantly different from previous week ($p<0.05$). #, indicates a significant time*gender interaction ($p<0.05$) at weeks 3-7 and 8 to 9. $, indicates a significant time*diet interaction ($p<0.05$) at weeks 8-11. &, indicates a significant time*diet*gender interaction at weeks 9-12 ($p<0.05$). The data represents 10 animals per group, except VAD males (n=4).

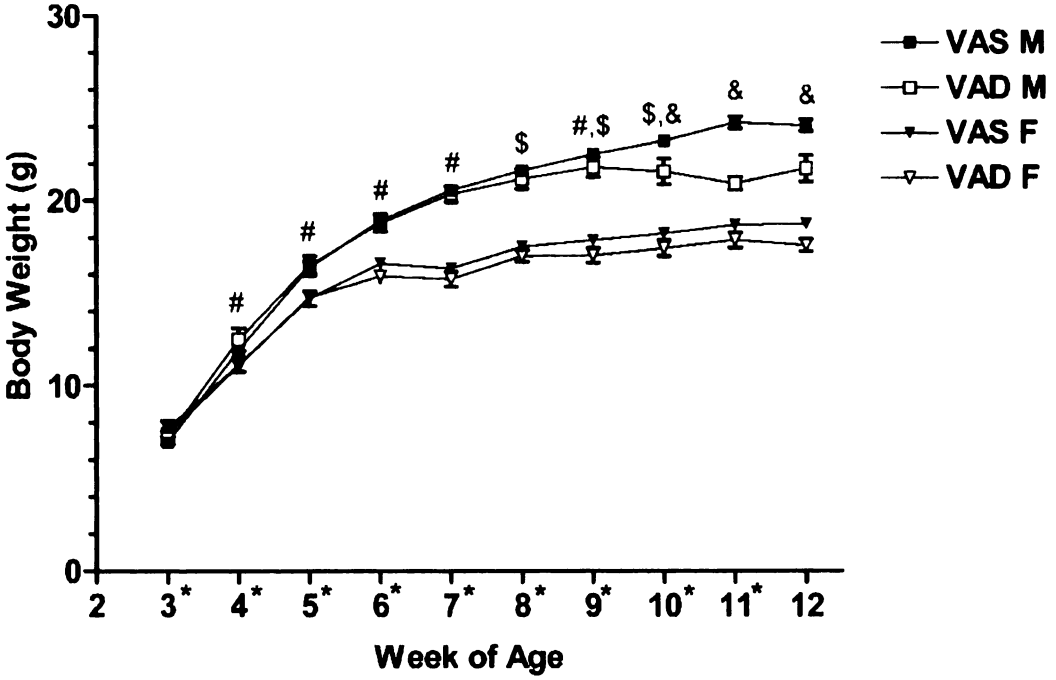

30
20
10
0
Body Weight (g)
2
3*
4*
5*
6*
7*
8*
9*
10*
11*
12
Week of Age
#
#
#
#
$
#,$
$,&
&
&
VAS M
VAD M
VAS F
VAD F

Figure 3.2. Vitamin A status of C57BL/6J mice. VAS animals are indicated by filled symbols and VAD animals are indicated by open symbols. Male mice are indicated by squares (■) and female mice are indicated by triangles (▼). *A*, serum retinol analyzed with repeated measures ANOVA. *, serum retinol is significantly different from previous week ($p<0.05$). Diet, gender, and the diet*gender interaction are significant ($p<0.05$). The data represents 2-6 animals per group for serum retinol analysis. *B*, liver retinol activity equivalents analyzed with 3-factorial ANOVA. Diet, gender, age*diet, and diet*gender are significant ($p<0.05$). The data represents 10 animals per group, except week 12 VAD males (n=4) for liver RAE analysis.

A

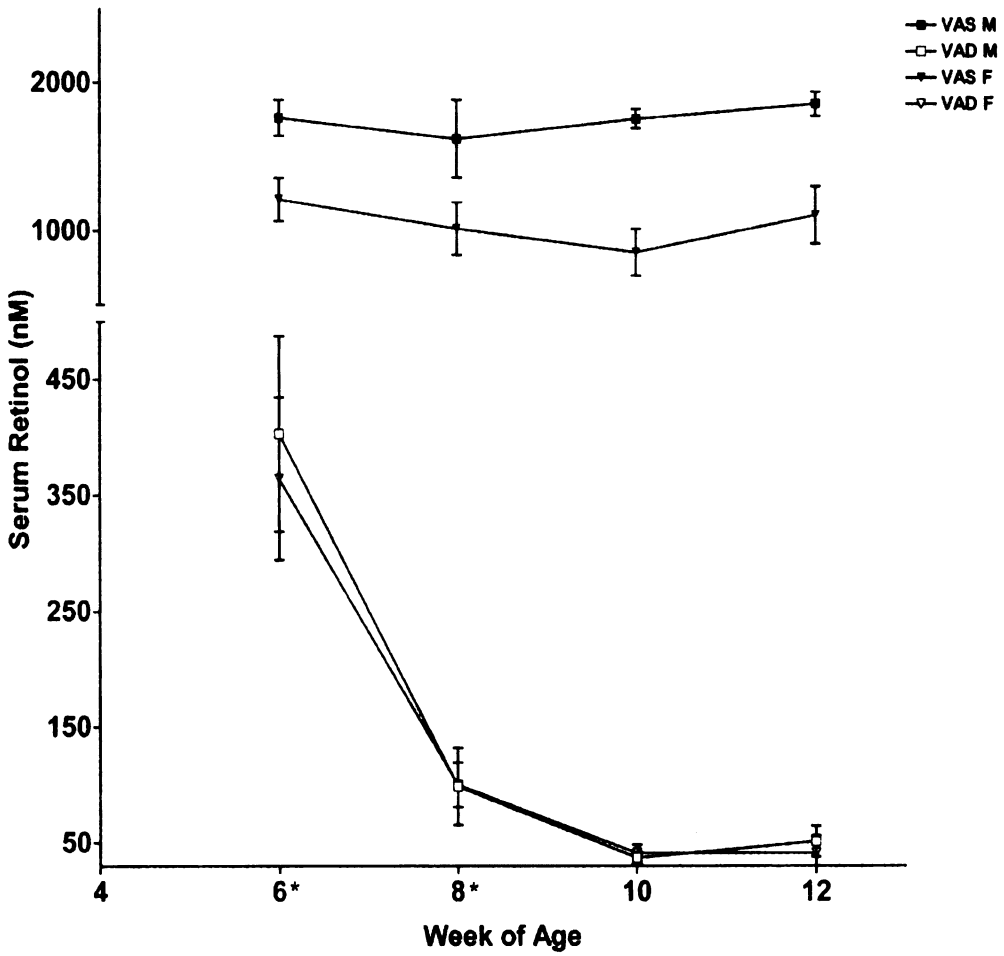

VAS M
VAD M
VAS F
VAD F
2000
1000
450
350
250
150
50
Serum Retinol (nM)
4
6*
8*
10
12
Week of Age

Figure 3.

indicated

are indic

retinol.

gender.

animals

gender.

animals

Figure 3.2 Continued. Vitamin A status of C57BL/6J mice. VAS animals are indicated by filled symbols and VAD animals are indicated by open symbols. Male mice are indicated by squares (■) and female mice are indicated by triangles (▼). *A*, serum retinol. *, serum retinol is significantly different from previous week ($p<0.05$). Diet, gender, and the diet*gender interaction are significant ($p<0.05$). The data represents 2-6 animals per group for serum retinol analysis. *B*, liver retinol activity equivalents. Diet, gender, age*diet, and diet*gender are significant ($p<0.05$). The data represents 10 animals per group, except week 12 VAD males (n=4) for liver RAE analysis.

B

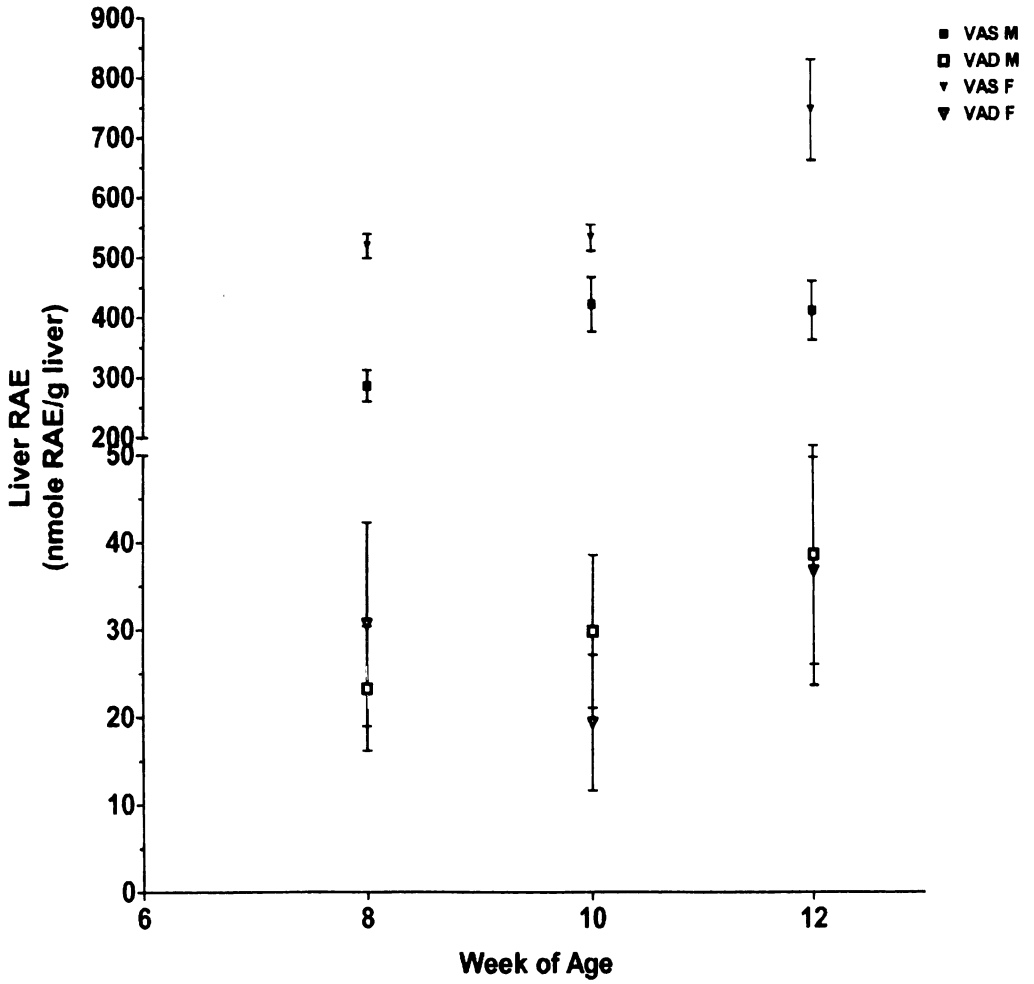

900
800
700
600
500
400
300
200
50
40
30
20
10
0
Liver RAE
(nmole RAE/g liver)
6
8
10
12
Week of Age
VAS M
VAD M
VAS F
VAD F

Figure 3.3. Total spleen cells per animal of C57BL/6J mice. Filled bars are vitamin A-sufficient animals, open bars are vitamin A-deficient animals. * indicates significant differences ($p<0.05$). Week 8 is significantly different than week 10, but week 8 and 10 are not significantly different than week 12, regardless of gender and diet. Male and female mice are significantly different regardless of time and diet. The diet*age and age*gender interactions are significant as well. The data represents 10 animals per group, except week 12 VAD males (n=4).

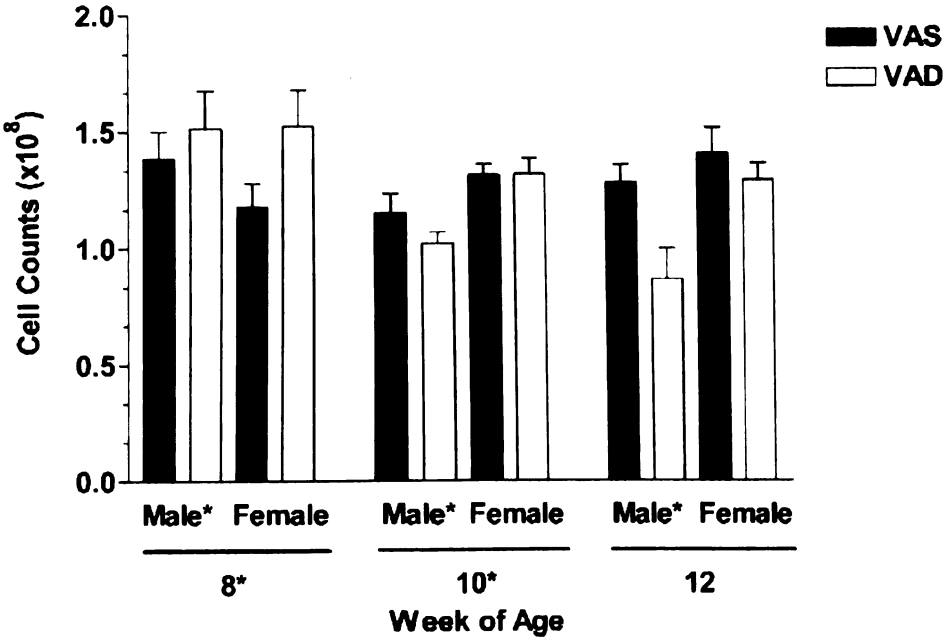

2.0
1.5
1.0
0.5
0.0
Cell Counts (x10^8)
VAS
VAD
Male* Female
Male* Female
Male* Female
8*
10*
12
Week of Age

Figure 3.4. Effe

DCs. *B*. lympho

preDCs. Table i

model.

Figure 3.4. Effects of depleted liver RAE on spleen DC populations. *A*, myeloid DCs. *B*, lymphoid DCs. *C*, ratio of myeloid to lymphoid DCs. *D*, plasmacytoid DCs. *E*, preDCs. Table insets indicate significant β coefficients, R^2 values, and significance of model.

A

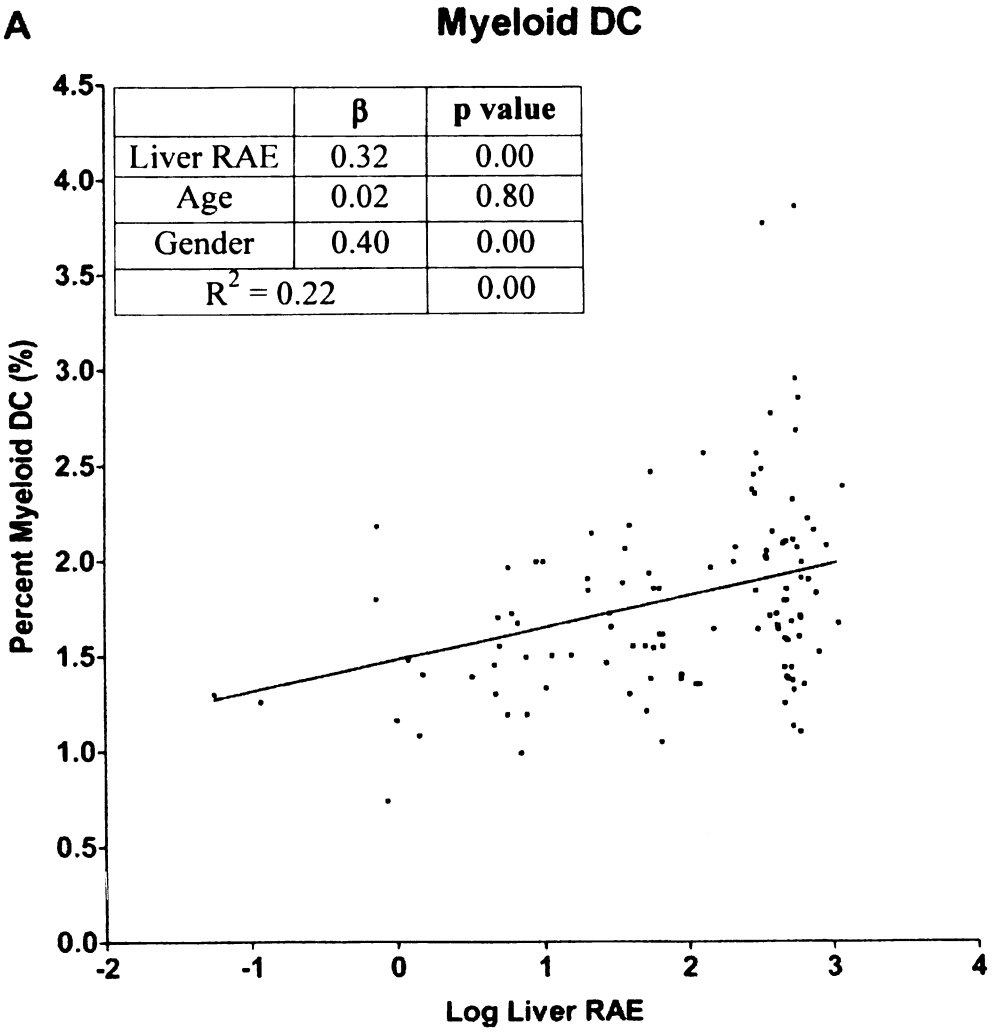

Myeloid DC
β
p value
Liver RAE
0.32
0.00
Age
0.02
0.80
Gender
0.40
0.00
R2 = 0.22
0.00
Percent Myeloid DC (%)
Log Liver RAE
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-2
-1
0
1
2
3
4

Figure 3.4 Cont

myeloid DCs. *B*

DCs. *E.* preDCs

significance of m

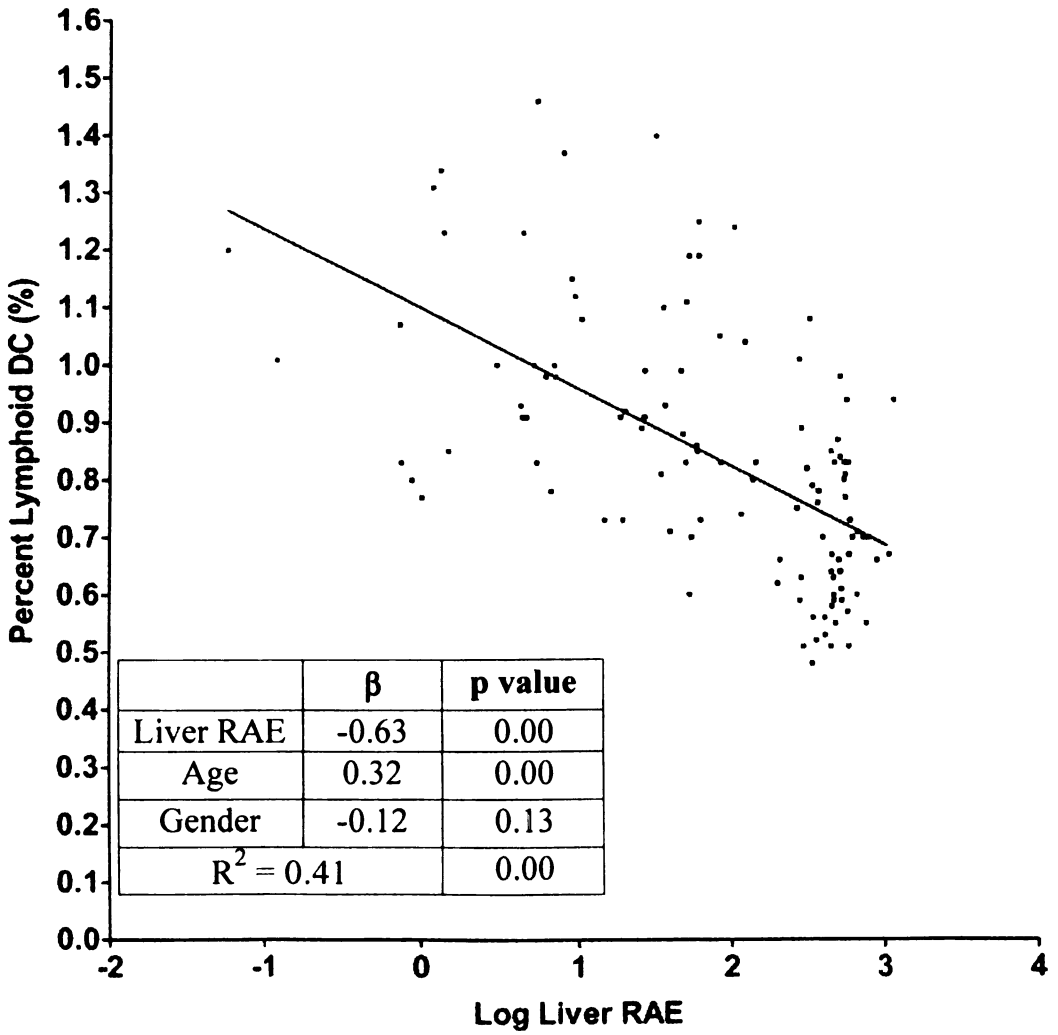
Figure 3.4 Continued. Effects of depleted liver RAE on spleen DC populations. *A*, myeloid DCs. *B*, lymphoid DCs. *C*, ratio of myeloid to lymphoid DCs. *D*, plasmacytoid DCs. *E*, preDCs. Table insets indicate significant β coefficients, R^2 values, and significance of model.

B

Percent Lymphoid DC (%)

B

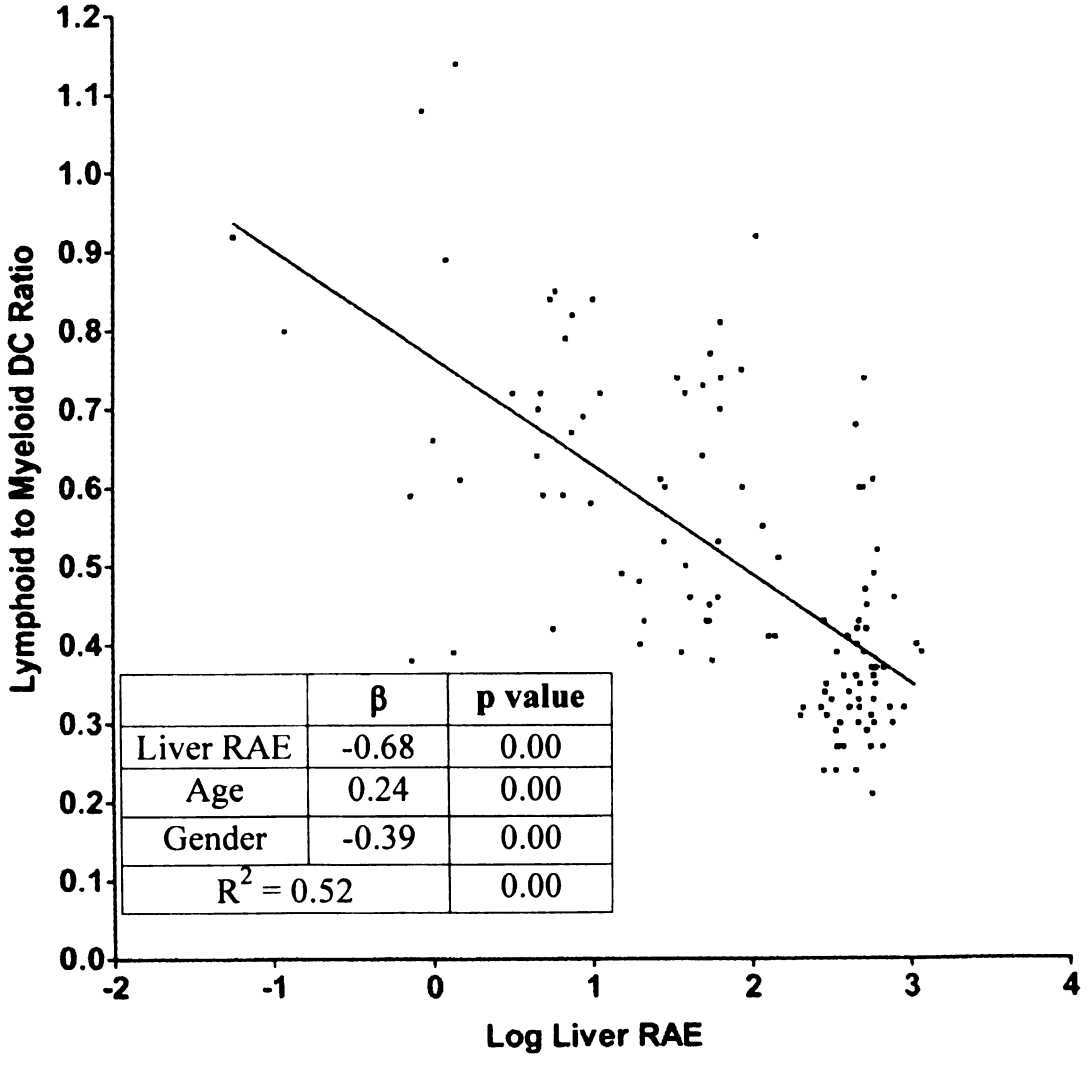
Lymphoid DC



	β	p value
Liver RAE	-0.63	0.00
Age	0.32	0.00
Gender	-0.12	0.13
$R^2 = 0.41$		0.00

Figure 3.4 Continued. Effects of depleted liver RAE on spleen DC populations. *A*, myeloid DCs. *B*, lymphoid DCs. *C*, ratio of myeloid to lymphoid DCs. *D*, plasmacytoid DCs. *E*, preDCs. Table insets indicate significant β coefficients, R^2 values, and significance of model.

C



	β	p value
Liver RAE	-0.68	0.00
Age	0.24	0.00
Gender	-0.39	0.00
$R^2 = 0.52$		0.00

Figure 3.4 Continued. Effects of depleted liver RAE on spleen DC populations. *A*, myeloid DCs. *B*, lymphoid DCs. *C*, ratio of myeloid to lymphoid DCs. *D*, plasmacytoid DCs. *E*, preDCs. Table insets indicate significant β coefficients, R^2 values, and significance of model.

D

Plasmacytoid DC

	β	p value
Liver RAE	0.08	0.40
Age	-0.03	0.77
Gender	0.12	0.21
$R^2 = 0.02$		0.57

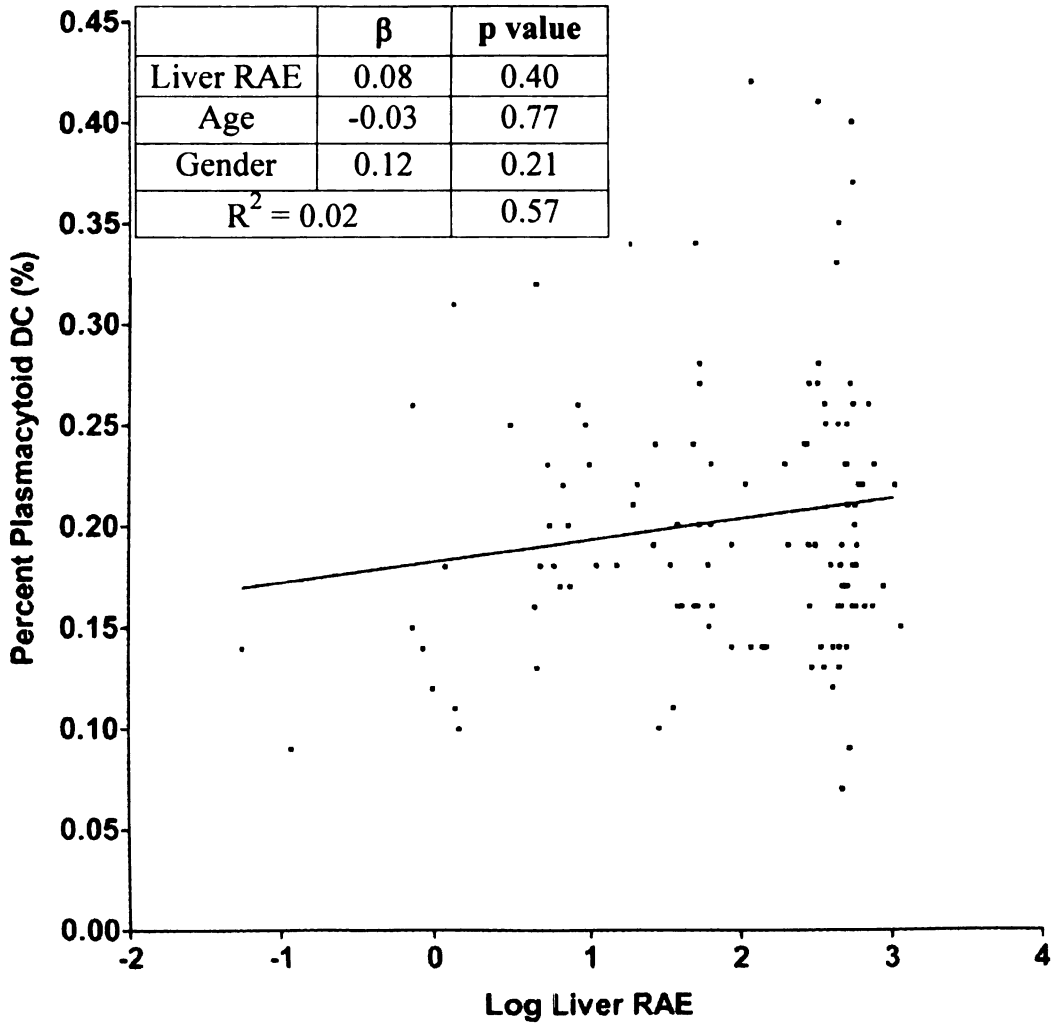


Figure 3.4 Continued. Effects of depleted liver RAE on spleen DC populations. *A*, myeloid DCs. *B*, lymphoid DCs. *C*, ratio of myeloid to lymphoid DCs. *D*, plasmacytoid DCs. *E*, preDCs. Table insets indicate significant β coefficients, R^2 values, and significance of model.

E **preDC**

	β	p value
Liver RAE	0.10	0.31
Age	-0.20	0.03
Gender	0.20	0.03
$R^2 = 0.09$		0.02

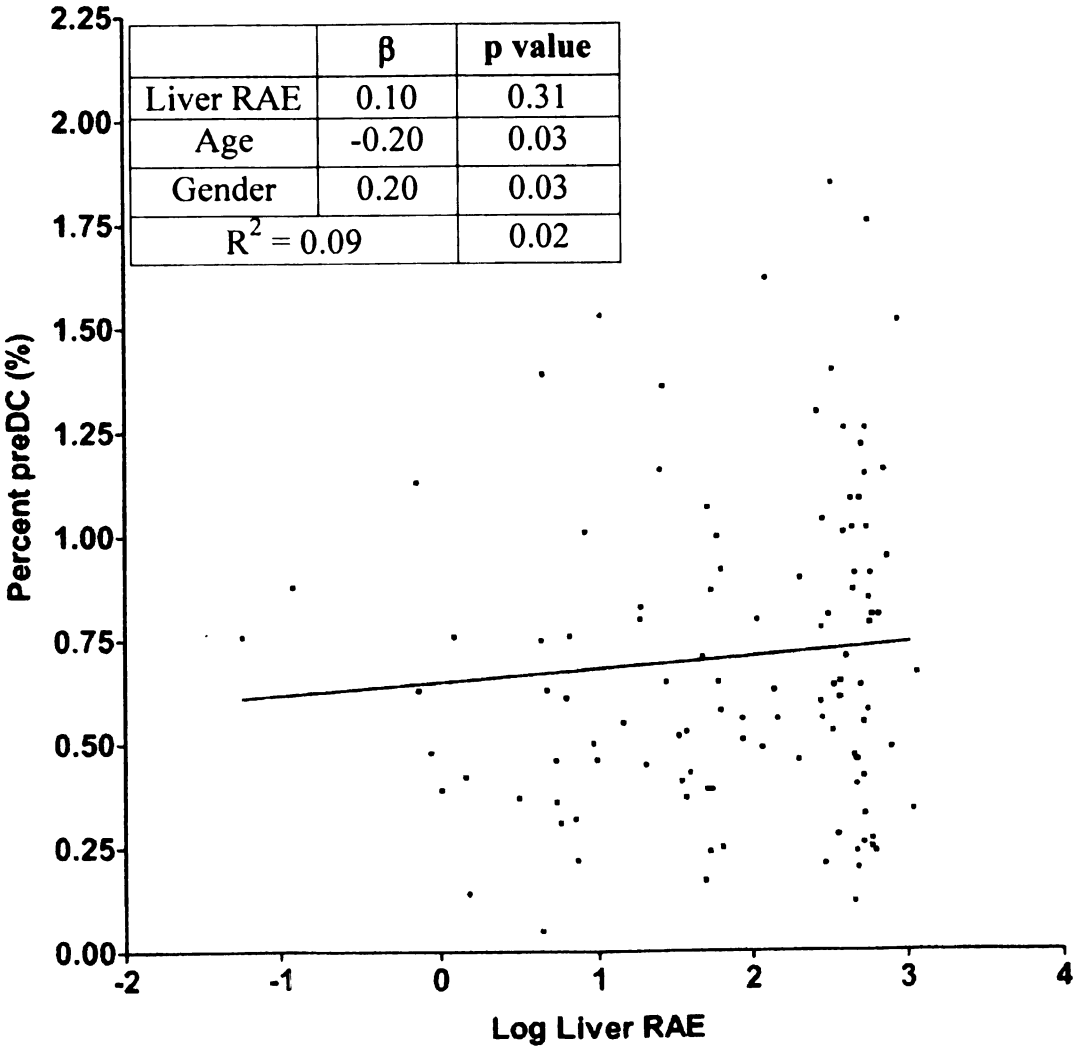
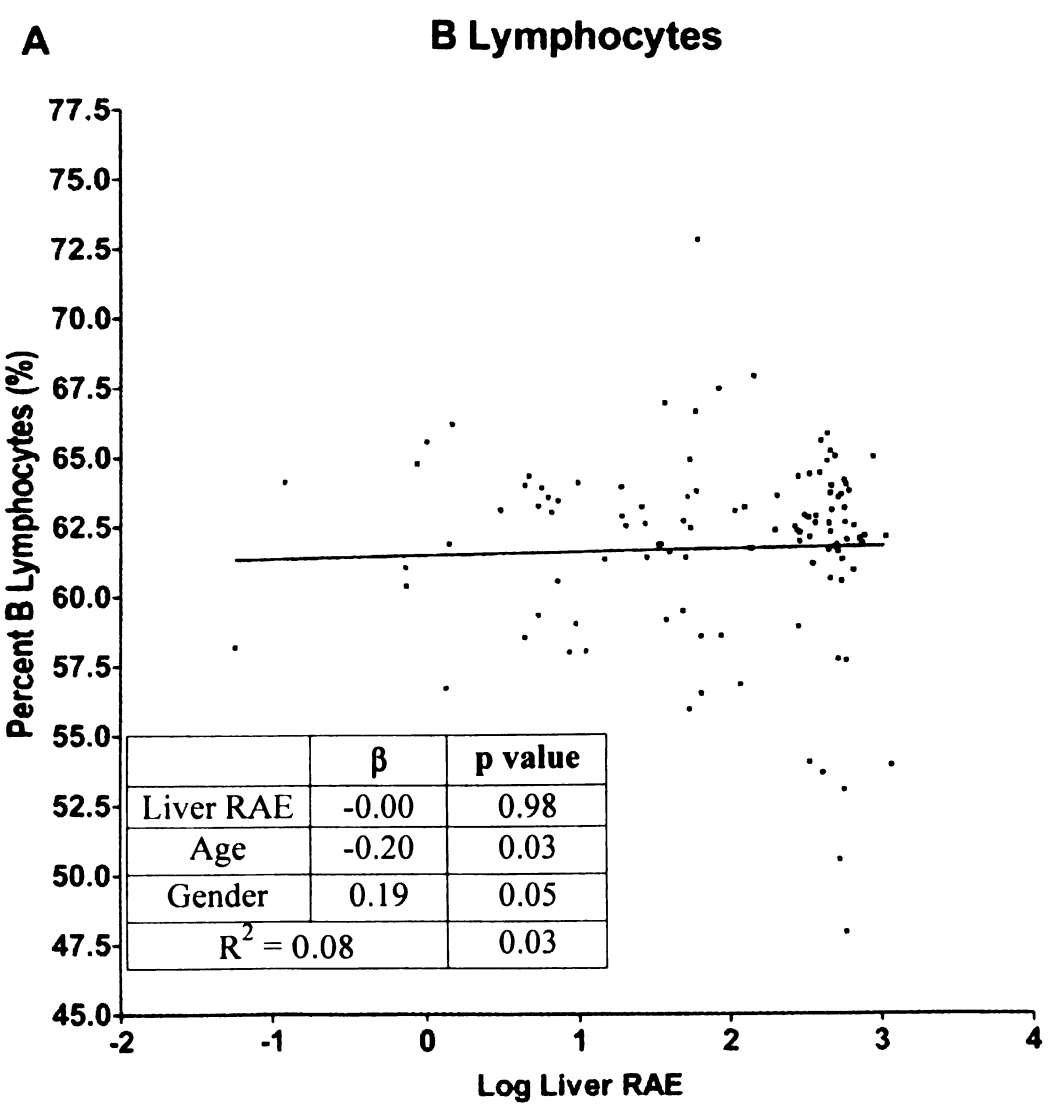


Figure 3.5. Effects of depleted liver RAE on spleen lymphocyte populations. *A*, B cells. *B*, $CD3^+/CD4^-/CD8^-$ T cells. *C*, CD4 T cell. *D*, total CD8 T cells. *E*, $Gr\text{-}1^+/CD8^+$ memory T cells. Table insets indicate significant β coefficients, R^2 values, and significance of model.

A



	β	p value
Liver RAE	-0.00	0.98
Age	-0.20	0.03
Gender	0.19	0.05
$R^2 = 0.08$		0.03

Figure 3.5 Continued. Effects of depleted liver RAE on spleen lymphocyte populations. *A*, B cells. *B*, $CD3^+/CD4^-/CD8^-$ T cells. *C*, CD4 T cell. *D*, total CD8 T cells. *E*, $Gr\text{-}1^+/CD8^+$ memory T cells. Table insets indicate significant β coefficients, R^2 values, and significance of model.

B

$CD3^+/CD4^-/CD8^-$ Lymphocytes

	β	p value
Liver RAE	0.04	0.70
Age	-0.18	0.06
Gender	-0.11	0.25
$R^2 = 0.04$		0.18

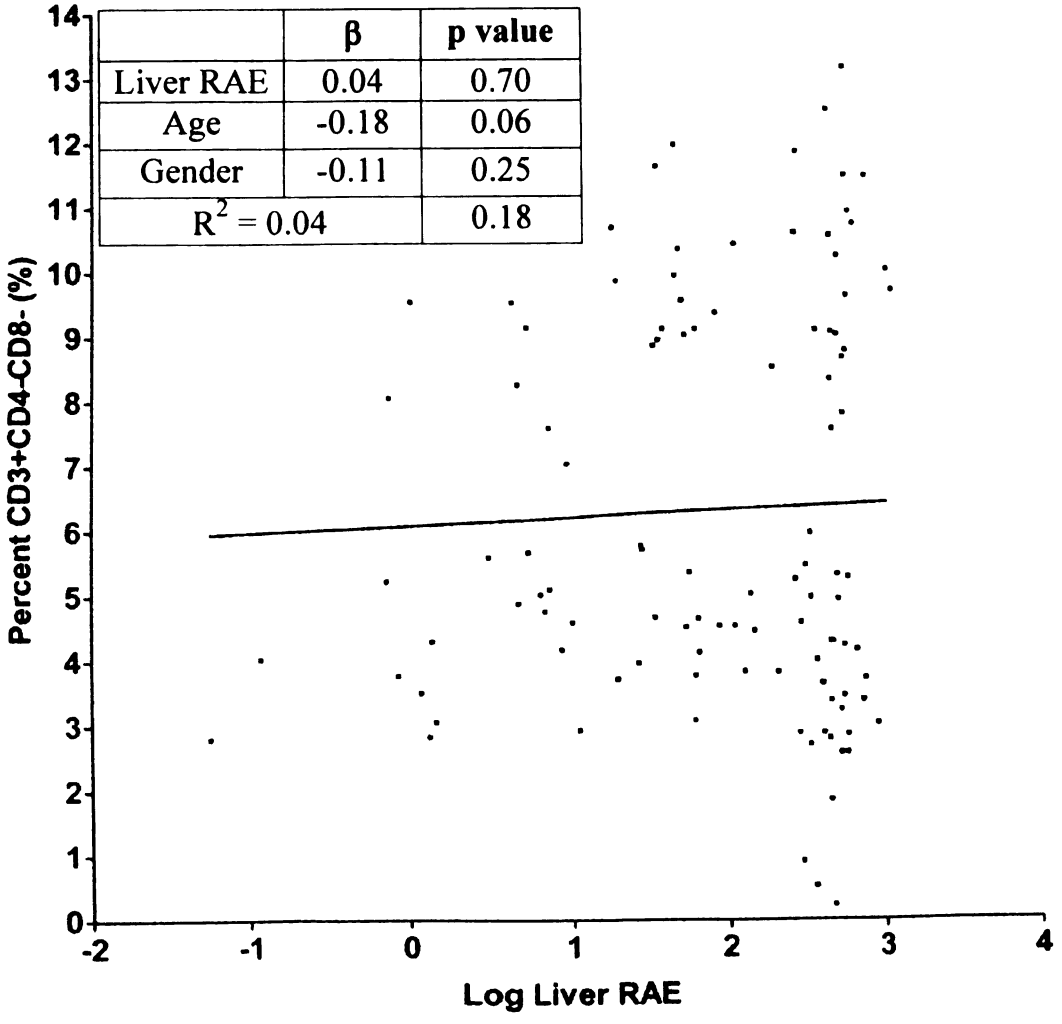


Figure 3.5 Continued. Effects of depleted liver RAE on spleen lymphocyte populations. *A*, B cells. *B*, $CD3^+/CD4^-/CD8^-$ T cells. *C*, CD4 T cell. *D*, total CD8 T cells. *E*, $Gr\text{-}1^+/CD8^+$ memory T cells. Table insets indicate significant β coefficients, R^2 values, and significance of model.

C

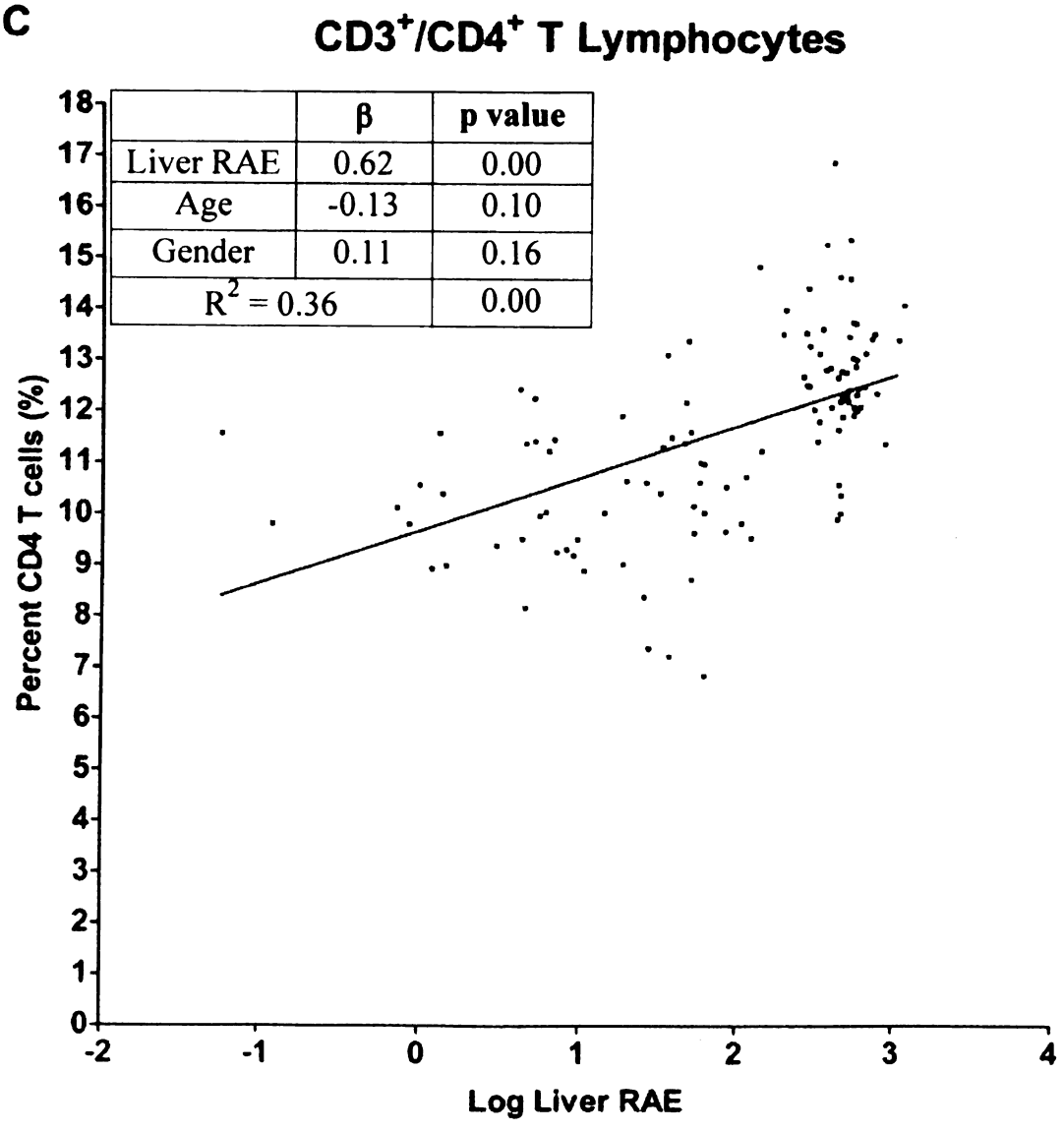
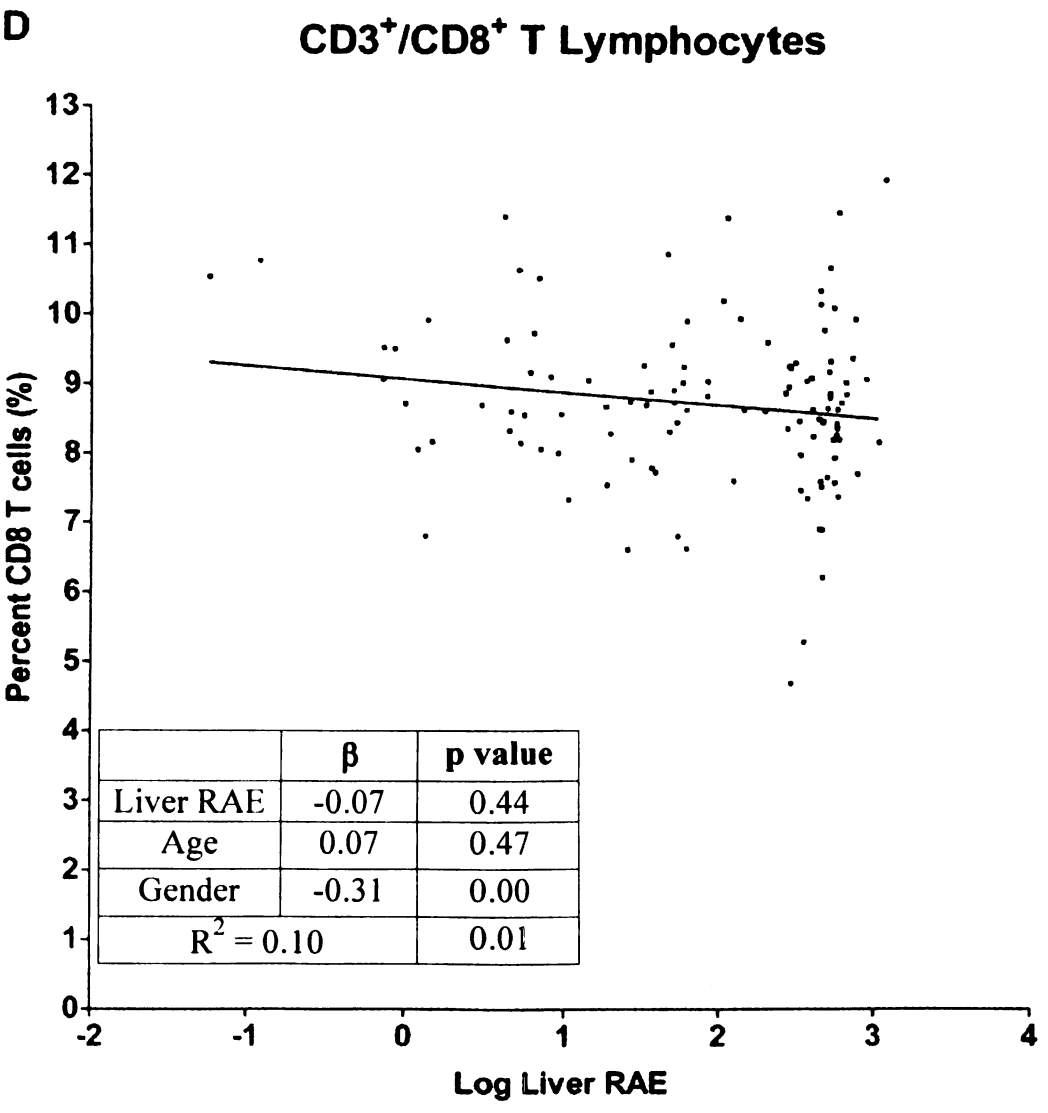

CD3+/CD4+ T Lymphocytes
β
p value
Liver RAE
0.62
0.00
Age
-0.13
0.10
Gender
0.11
0.16
R2 = 0.36
0.00
Percent CD4 T cells (%)
Log Liver RAE
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-2
-1
0
1
2
3
4

Figure 3.5 Continued. Effects of depleted liver RAE on spleen lymphocyte populations. *A*, B cells. *B*, $CD3^+/CD4^-/CD8^-$ T cells. *C*, CD4 T cell. *D*, total CD8 T cells. *E*, $Gr\text{-}1^+/CD8^+$ memory T cells. Table insets indicate significant β coefficients, R^2 values, and significance of model.

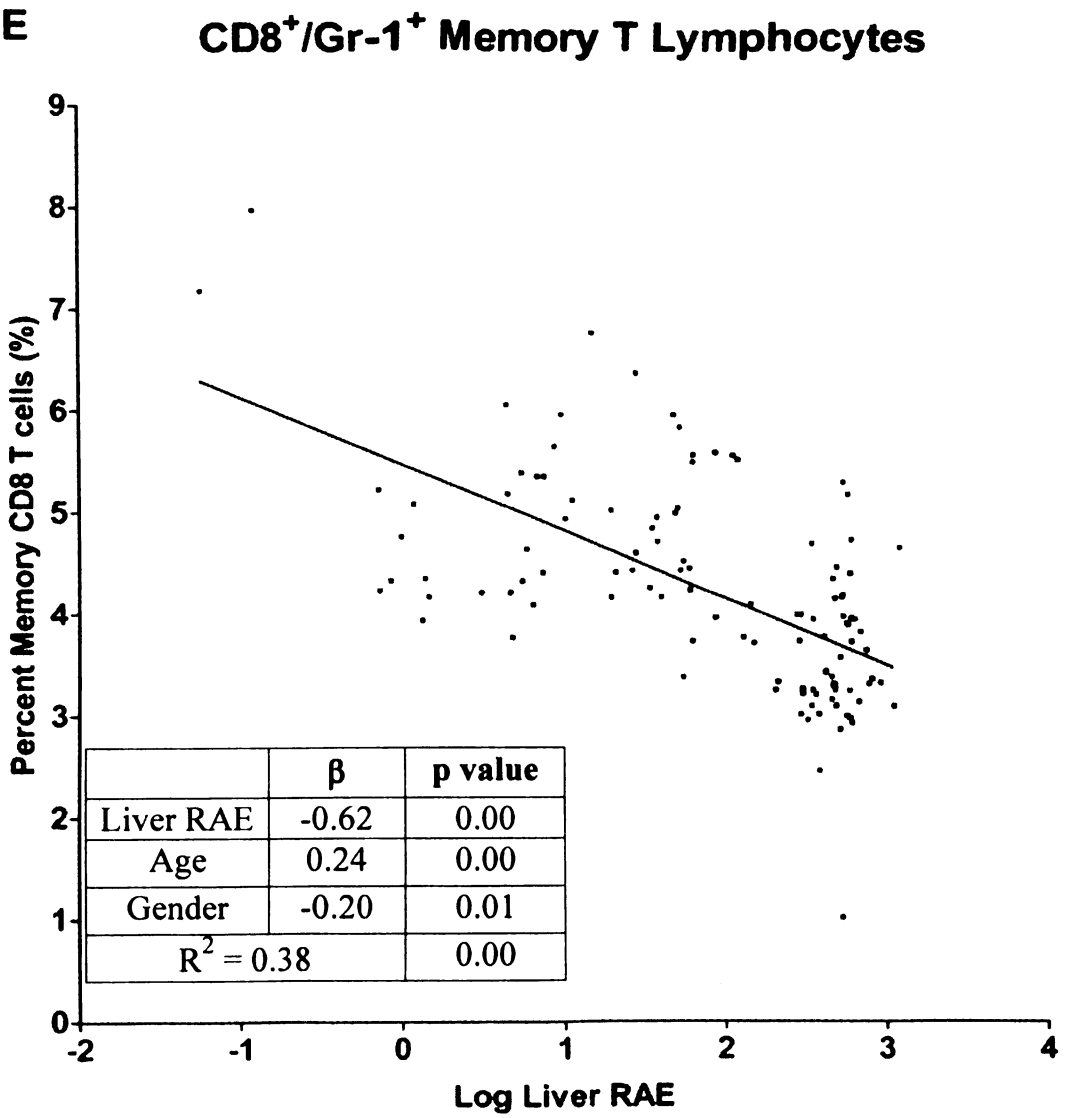
D



	β	p value
Liver RAE	-0.07	0.44
Age	0.07	0.47
Gender	-0.31	0.00
$R^2 = 0.10$		0.01

Figure 3.5 Continued. Effects of depleted liver RAE on spleen lymphocyte populations. *A*, B cells. *B*, $CD3^+/CD4^-/CD8^-$ T cells. *C*, CD4 T cell. *D*, total CD8 T cells. *E*, $Gr\text{-}1^+/CD8^+$ memory T cells. Table insets indicate significant β coefficients, R^2 values, and significance of model.

E

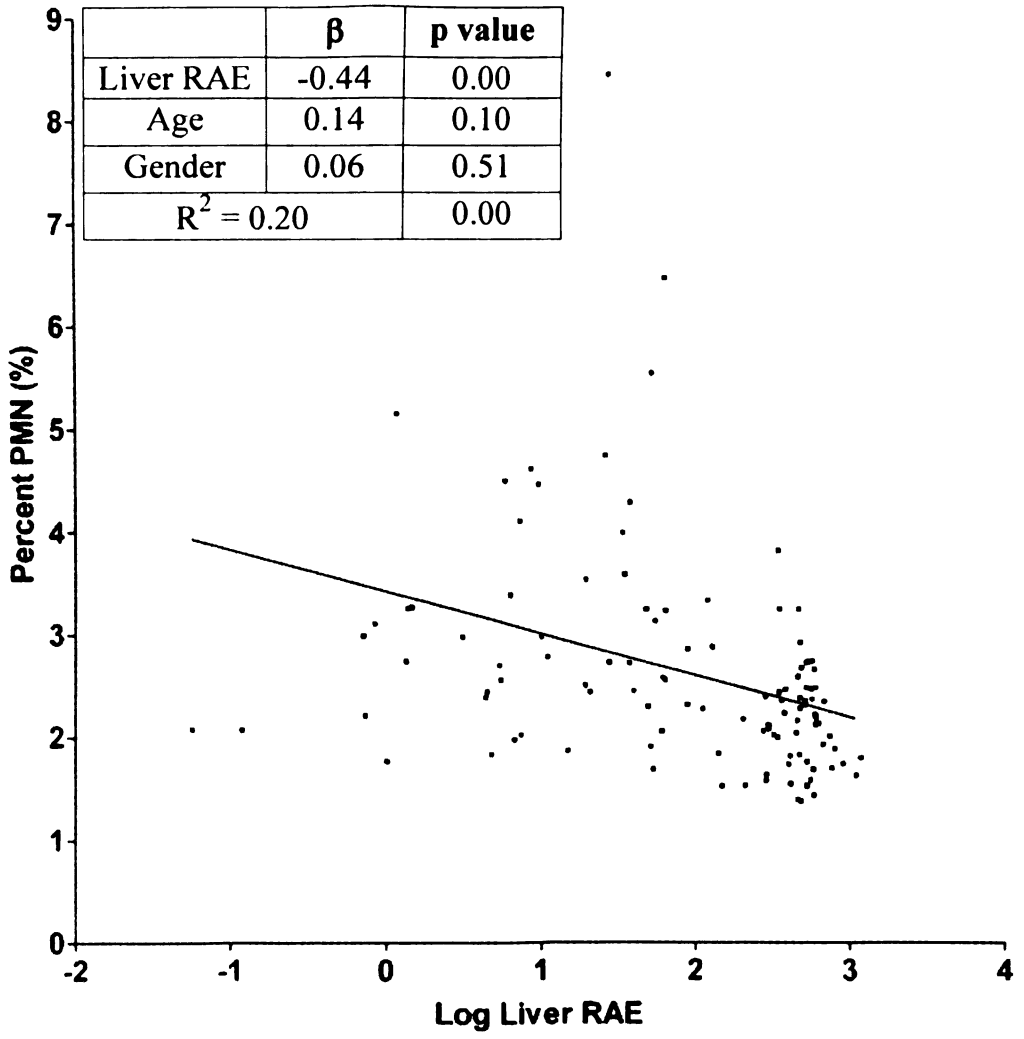


	β	p value
Liver RAE	-0.62	0.00
Age	0.24	0.00
Gender	-0.20	0.01
$R^2 = 0.38$		0.00

Figure 3.6. Effects of depleted liver RAE on spleen PMN. Table inset indicates significant β coefficients, R^2 values, and significance of model.

Neutrophils

	β	p value
Liver RAE	-0.44	0.00
Age	0.14	0.10
Gender	0.06	0.51
$R^2 = 0.20$		0.00



CHAPTER 4: CONCLUSIONS & FUTURE DIRECTIONS

Flow Cytometry of DCs

The multicolor flow cytometry protocol developed can identify relevant DC populations of the spleen and Peyer's patches of C57BL/6J mice (179). However, research continues to identify more DC subpopulations, such as $CD103^+$ DCs present in the spleen and mucosal tissues capable of antigen cross-presentation, phagocytosis and presentation of apoptotic cells, and induction of tolerance (143, 159, 161). The BD Biosciences LSR II flow cytometer is capable of distinguishing and analyzing up to 18 separate fluorochromes. Therefore, open channels remain to add more monoclonal antibody-fluorochrome conjugates to identify more antigens on the DCs and therefore identify additional DC populations. However, careful antigen choices must be performed to ensure the antigen is specific to only one DC population or can be used in combination with other markers to distinguish one cell type. For example, in the murine spleen $CD103^+$ DCs are a subpopulation of $CD8\alpha^+$ lymphoid DCs (161).

Murine Model of Vitamin A Deficiency

Others have reported methods of inducing vitamin A deficiency in mice using dietary restriction (91, 108, 120). The model employed in chapter 3 has characteristics of human vitamin A deficiency including depressed Th2 responses. We did not measure other parameters of deficiency, but the deficient animals likely suffer from impaired mucosal integrity and eventual protein-energy malnutrition if the mice were continued on the vitamin A-deficient diet. Weight, serum retinol, and liver retinol activity equivalent data provide further insight into other immunological studies of vitamin A-deficient mice. Dietary restriction of vitamin A leads to depleted serum retinol and liver retinyl-esters by

8 to 12 weeks of age (91). Continued restriction of vitamin A, over 8 to 12 weeks, leads to complications such as protein-energy malnutrition (PEM) due to inanition (112). Any animal that lost greater than or equal to 10% of total body weight was classified as suffering PEM and was excluded from our analysis. Therefore, we believe our immune cell population changes are attributable to VAD itself, and not related to PEM. The time course of analysis of the animals also demonstrates the effects of various degrees of vitamin A deficiency.

DC Populations of Vitamin A-Deficient Mice

Dietary depletion of vitamin A skews the proportion of DC populations in the spleen of mice. In accordance with our hypothesis, mice with the lowest liver retinol activity equivalents (RAE) had modestly decreased myeloid DCs and increased neutrophils. Unexpectedly, the lymphoid DCs were increased with more severe vitamin A deficiency. The increase of lymphoid DCs, responsible for stimulating Th1 responses in mice, is in accordance with reports that vitamin A deficiency increases Th1 responses at the expense of Th2 responses. The plasmacytoid and preDCs were unaffected by depletion of vitamin A. In summary, the main effect was increased lymphoid DCs which stimulate Th1 responses leading to cross-regulation of Th2 responses, but in combination with decreased myeloid DCs that stimulate Th2 responses.

Other Immune Cell Populations of Vitamin A-Deficient Mice

Vitamin A-deficient mice have skewed T lymphocyte populations, in addition to DC subpopulations. T helper $CD4^+$ cells are decreased and memory $CD8^+$ CTLs are increased in vitamin A-deficient animals compared to vitamin A-sufficient animals. The increased CTL population correlates with increased lymphoid DCs and Th1 responses of

vitamin A-deficient populations. B lymphocytes are unaffected by dietary depletion in mice. Other reports have shown vitamin A deficiency does not alter B lymphocyte numbers despite depressed Th2 antibody responses. *In vivo*, vitamin A deficiency has only minor affects on PMNs. Kuwata et al reported a marked significant increase in PMN populations in vitamin A-deficient SENCAR mice (108). However, the SENCAR mice were depleted of vitamin A for 14 weeks and therefore severely depleted of vitamin A and likely PEM. Glucocorticoid responses induced by PEM lead to increased PMN and decreased lymphocytes, both of which were observed in SENCAR vitamin A-deficient mice (181).

Future Directions

Vitamin A may have differential effects on other immune cell populations, DC populations, and cell populations of various tissues. Others have demonstrated the effects of vitamin A on various T cell populations. *In vivo*, we demonstrate the effects of vitamin A depletion on total $CD4^+$, $CD8^+$, memory $CD8^+$, and $CD3^+/CD4^-/CD8^-$ T cell populations. However, there are other T lymphocyte populations dependent on vitamin A. As DC research continues to identify new DC populations, the effects of vitamin A deficiency on these populations can lead to more thorough understanding of the cooperation and coordination of the immune response in vitamin A-deficient populations. The effects of vitamin A are most dramatic in mucosal tissues, particularly the mucosal tissues of the gastrointestinal tract. The skewed cell numbers of various immune cell populations is interesting, but the findings are limited in that we could not describe any functional changes in these studies. Therefore, various infection models could be employed in vitamin A-deficient animals to determine if the decreased cell populations

observed have the expected significant effects on the immune response to the various pathogens. Also it is important to determine the effects of supplementing vitamin A-deficient populations to determine if restoring adequate vitamin A status can return the immune cell populations to similar levels of vitamin A-sufficient animals and the kinetics of this restoration. Future directions therefore include expanding the research to include other immune cell populations and other secondary lymphoid tissues, determining DC function during vitamin A deficiency using infection models, and supplementing deficient animals to determine if the period of deficiency permanently depresses immune cell populations.

We characterized many immune cell populations in these studies. However, Treg and Th17 cells are also dependent on vitamin A (172, 174). *In vitro* T cells differentiate into Th17 cells in media lacking vitamin A. However, in media containing vitamin A the T cells differentiate into Treg cells (174). Th17 cells are T lymphocytes that produce IL-17 and have been shown to be responsible for development of autoimmune disease. Therefore, not only is vitamin A responsible for stimulating adequate Th2 antibody responses, but vitamin A may also be critical in maintaining tolerance to self antigens. $CD103^+$ DCs are present in mucosal tissues and the germinal center marginal zones of the spleen and induce tolerance to self antigens (142, 143, 159, 161). Therefore, in combination with the Treg and Th17 balance, a role of vitamin A in $CD103^+$ DCs may provide insight into the observed imbalance of Treg and Th17 cell populations in vitamin A-deficiency. Comparison of Treg, Th17 and $CD103^+$ DCs of vitamin A-deficient to vitamin A-sufficient animals using similar *in vivo* methods would provide evidence of the role of vitamin A in development of tolerance.

It is of particular interest that lymphoid DCs and memory $CD8^+$ T lymphocytes are both increased in vitamin A-deficient animals. Memory T cells, as well as natural killer (NK) and NKT cells, are maintained by the growth factor IL-15. Hepatic and pancreatic stellate cells, as well as DCs, produce IL-15 (185-187). Hepatic stellate cells and DCs can also store and metabolize vitamin A (135). Although vitamin A did not significantly alter IL-15 cytokine production by human T lymphocytes *in vitro*, the dual function of vitamin A metabolism and production of IL-15 by DCs and liver stellate cells leads to a possible inter-relationship that would be expected to selectively enhance memory T cell reliance during VAD (188). Therefore, the source of IL-15, plasmacytoid or lymphoid DCs, should be further investigated as well as any increase in IL-15 in VAD animals.

Vitamin A has ubiquitous functions, but focused and major roles in the eye and mucosal tissues. The role of vitamin A in the eye employs the 11-*cis*-retinal form of vitamin A and not the all-*trans*-retinoic acid form functioning in the immune system. Therefore, characterization of DC populations and other immune cells of mucosal tissues in vitamin A-deficient animal may be significantly altered compared to vitamin A-sufficient populations. Homing of gut B and T lymphocytes and development of mucosal $CD103^+$ DCs relies on the presence of vitamin A (135, 136, 143, 172, 189). Therefore, the cell populations may be decreased in the whole animal or in specific tissues due to the lack of homing responses. Since the spleen is a filtering organ for the entire body, our findings are representative of the immune system as a whole, but can not be extrapolated to specific body sites. The differences between vitamin A-sufficient and vitamin A-deficient animals may be more pronounced in the intestinal mucosa due to the added

homing function of vitamin A. In addition, the intestine is the first encounter immune cells have with dietary vitamin A and DCs possess the enzymes to convert vitamin A to the biologically active form. So in combination, the homing of immune cells, the enzymes to metabolize vitamin A, and the decreased immune cell populations observed in the spleen indicate that the intestinal mucosa is an important site to characterize immune cell populations of vitamin A-deficient animals. We show preliminary data for the effects of VAD of immune cells of the Peyer's patches is Appendix II, but the data are unadjusted for age and gender and also lack statistical analysis.

Skewed immune cell numbers of vitamin A-deficient animals does not directly indicate an increased susceptibility to infection or mortality from infection. Therefore, the vitamin A-deficient animals and vitamin A-sufficient control animals should be exposed to various pathogens eliciting various immune responses and self peptides leading to tolerance to determine if the changes in immune cell numbers leads to changes in disease susceptibility. A pathogen that leads to primarily a Th2 response is hypothesized to lead to increased susceptibility and mortality in vitamin A-deficient animals while a pathogen requiring primarily a Th1 response should survive the infection better than vitamin A-sufficient controls. Exposing the animals to a "self" antigen that leads to a Th17 autoimmune reaction is hypothesized to cause a more severe immune reaction in vitamin A-deficient animals compared to control, vitamin A-sufficient, animals. The models described could highlight the role of vitamin A, specifically the role of vitamin A in DCs, in clearing infections or establishing an autoimmune prone environment. Using the flow cytometry methodology described in chapter 2, a comprehensive overview of the immune response to various pathogen challenges in

vitamin A-deficient animals could establish a mechanism of vitamin A in the immune system during an immune challenge.

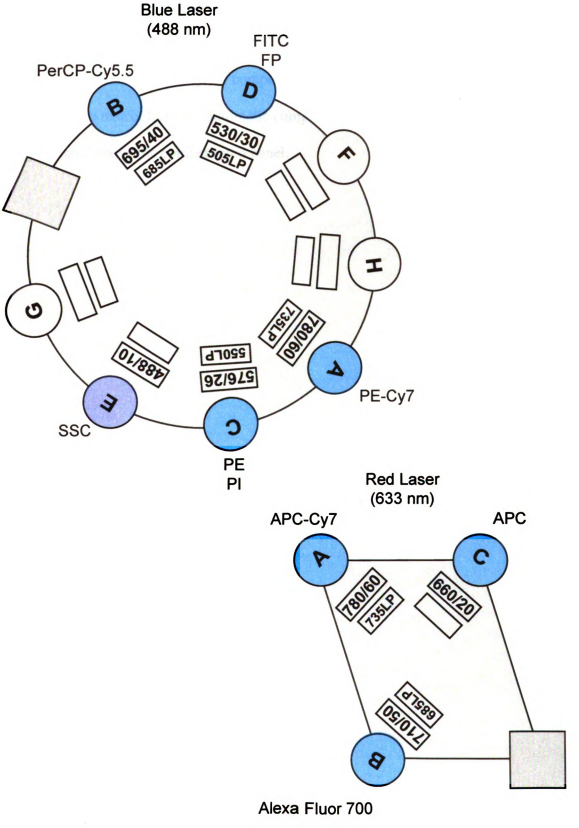
The changes in immune cell numbers during vitamin A deficiency may be permanent changes. The deficient animals may not restore the immune cell numbers comparable to the sufficient animals even after supplementation. Animals depleted of vitamin A could be supplemented after various degrees of deficiency or after various times after deficiency has been established to determine appropriate supplementation strategies.

Analysis of immune cell populations using the multicolor flow cytometry elucidated a role of vitamin A in maintaining DC subpopulations during homeostasis. However, more research needs to be conducted to further describe the increased susceptibility to infections in vitamin A-deficient populations. In addition, supplementation strategies may be improved by focusing on improved immune cell populations instead of circulating serum retinol levels.

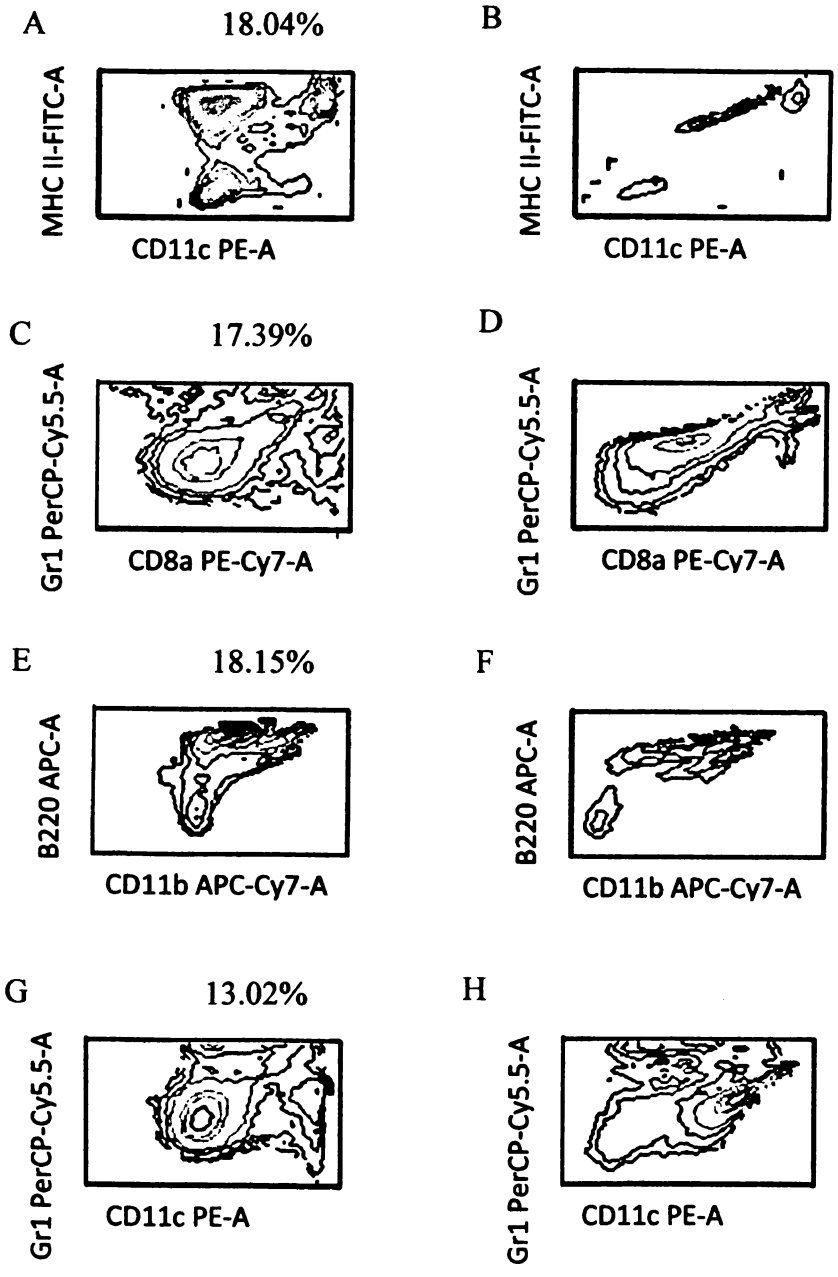
APPENDIX I

Supplemental data from the identification and enumeration of dendritic cell populations from individual mouse spleen and Peyer's patches using flow cytometric analysis (Chapter 2).

Supplemental Figure 1.1. LSR II optics block and filter scheme of the fluorescent channels utilized for data collection.


Blue Laser
(488 nm)
FITC
FP
PerCP-Cy5.5
B
D
F
H
G
A
E
C
695/40
685LP
530/30
505LP
780/60
735LP
550LP
576/26
488/10
PE-Cy7
SSC
PE
PI
Red Laser
(633 nm)
APC-Cy7
APC
A
C
780/60
735LP
660/20
B
710/50
685LP
Alexa Fluor 700

Supplemental Figure 1.2. Comparison of compensated and uncompensated data. Left panels (A, C, E, G) are compensated contour plots. Right panels (B, D, F, H) are uncompensated contour plots. Percentage of compensation employed, calculated from CompBead fluorescence matrix from the automatic compensation feature of the DIVA software, is represented above the compensated panel.


A
18.04%
MHC II-FITC-A
CD11c PE-A
B
MHC II-FITC-A
CD11c PE-A
C
17.39%
Gr1 PerCP-Cy5.5-A
CD8a PE-Cy7-A
D
Gr1 PerCP-Cy5.5-A
CD8a PE-Cy7-A
E
18.15%
B220 APC-A
CD11b APC-Cy7-A
F
B220 APC-A
CD11b APC-Cy7-A
G
13.02%
Gr1 PerCP-Cy5.5-A
CD11c PE-A
H
Gr1 PerCP-Cy5.5-A
CD11c PE-A

Supplemental Figure 1.3. Flow diagram of Boolean logic used in sequential gating of spleen cell populations. Final populations gated and enumerated are shown in red boxes.

Live Cells
Figure 1A
Contour Plot of
Gr-1-PerCp-Cy5.5 and CD8a-PE-Cy7
Figure 1B
preGranulocytes
Gr-1+/CD8a+ T cells
CD11b Histogram
Figure 1C
Polymorphonuclear Neutrophils
CD11c Histogram
Subtracted out PMN and Gr-1+/CD8a+ T cells
Using Boolean Logic not (PMN or Gr-1 T cells) gate out of Live Cells
Figure 1D
Eliminate all DC populations
Using Boolean Logic
not (Plasmacytoid DC or Myeloid DC or Lymphoid DC or preDCs
out of the not (PMN or Gr-1 T cell) gate which is
out of the Live Cell Gate
Histogram of Gr-1-PerCp-Cy5.5
Figure 1I
Dot Plot of
B220-APC and MHC-II-FITC
Figure 1J
B Lymphocytes
Potential B Lymphocytes
Gr-1- Cells
Contour Plot of
CD11b-APC-Cy7 and B220-APC
Figure 1H
Confirmed preDCs
MHC-II- preDCs
CD11c+ DCs
Histogram of MHC-II-FITC
Figure 1E
MHC-II+ Mature DCs
Contour Plot of
B220-APC and Gr-1-PerCp-Cy5.5
Figure 1F
Plasmacytoid DCs
Contour Plot of
CD11b-APC-Cy7 and CD8a-PE-Cy7
Figure 1G
Lymphoid DCs
Myeloid DCs

Supplemental Table 1.1. BD® Biosciences LSR II flow cytometer laser parameters used in flow cytometry data collection.

Parameters (Channel)	Voltage	Scale
FSC	415	Linear
SSC	382	Linear
FITC (FL1)	484	Bi-exponential
PE (FL2)	415	Bi-exponential
PerCP-Cy5.5 (FL3)	725	Bi-exponential
PE-Cy7 (FL4)	525	Bi-exponential
APC (FL5)	516	Bi-exponential
APC-Cy7 (FL6)	535	Bi-exponential

Supplemental Table 1.2. BD® Biosciences LSR II general compensation matrix.

Fluorochromes	Value (%)
PE – FITC	18.04
PerCp-Cy5.5 – FITC	1.91
PE-Cy7 – FITC	0.19
APC – FITC	0.28
APC-Cy7 – FITC	0.00
FITC – PE	0.46
PerCp-Cy5.5 – PE	13.02
PE-Cy7 – PE	1.26
APC – PE	0.10
APC-Cy7 – PE	0.00
FITC – PerCp-Cy5.5	0.00
PE – PerCp-Cy5.5	0.00
PE-Cy7 – PerCp-Cy5.5	17.39
APC – PerCp-Cy5.5	1.94
APC-Cy7 – PerCp-Cy5.5	1.67
FITC – PE-Cy7	0.16
PE – PE-Cy7	3.27
PerCp-Cy5.5 – Pe-Cy7	0.84

Supplemental Table 1.2 Continued. BD® Biosciences LSR II general compensation matrix.

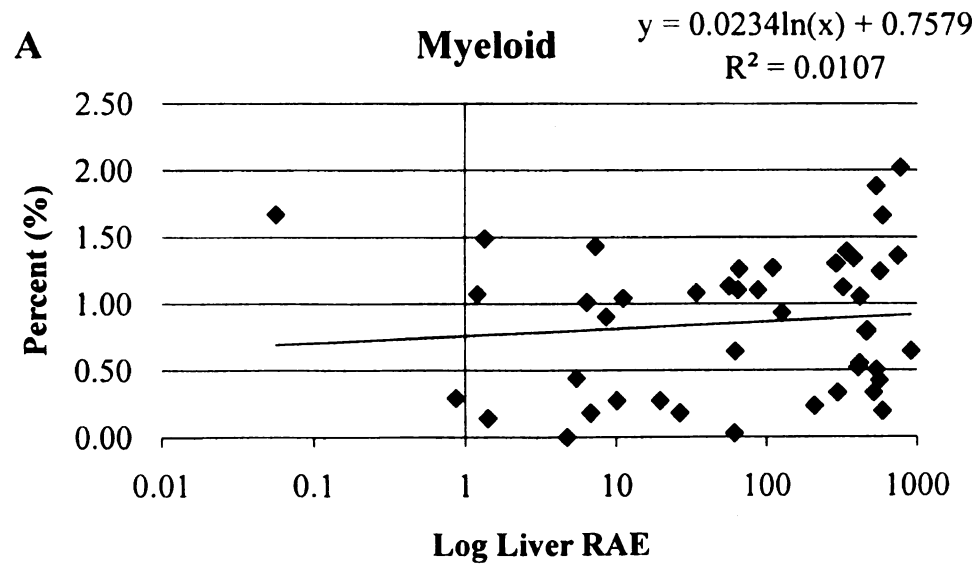
APC – PE-Cy7	0.14
APC-Cy7 – PE-Cy7	2.24
FITC – APC	0.00
PE – APC	0.00
PerCP-Cy5.5 – APC	0.84
PE-Cy7 – APC	0.14
APC-Cy7 – APC	3.09
FITC – APC-Cy7	0.00
PE – APC-Cy7	0.00
PerCp-Cy5.5 – APC-Cy7	0.11
PE-Cy7 – APC-Cy7	4.37
APC – APC-Cy7	18.15

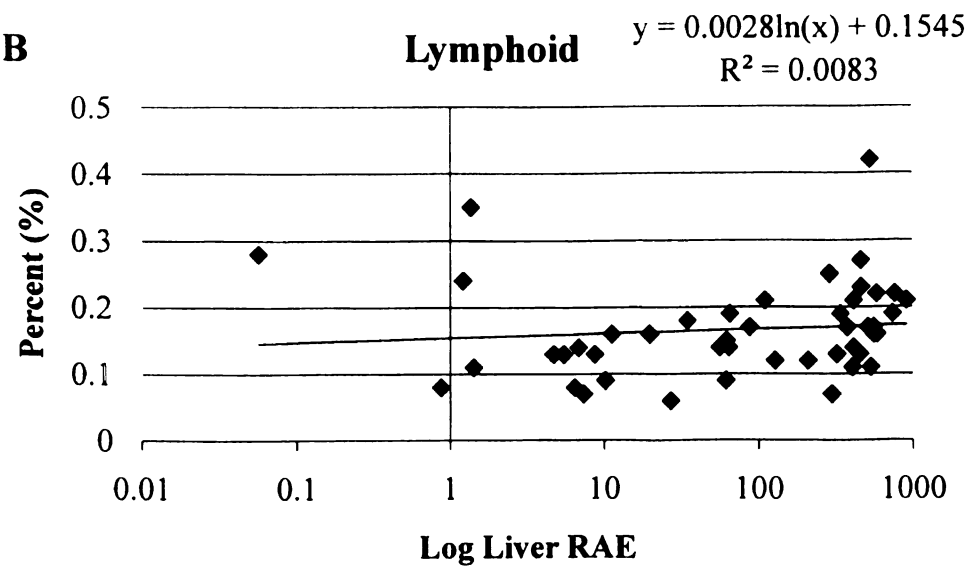
APPENDIX II

Preliminary data on the effects of vitamin A deficiency on immune cells of the Peyer's patches.

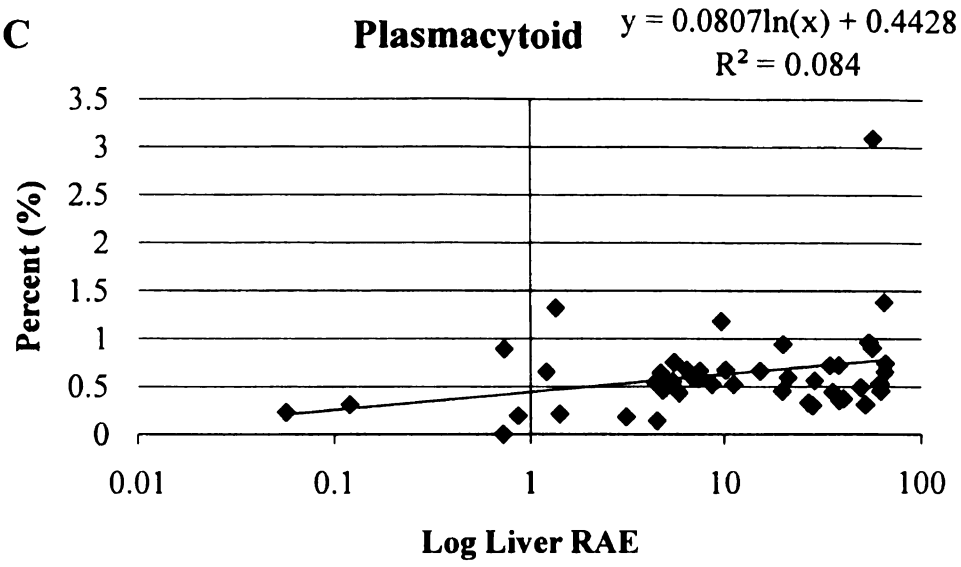
This data is not adjusted for gender or age effects, and has not been statistically analyzed. In addition this data has not been adjusted for total cell numbers per Peyer's patch to decrease variability of numbers of Peyer's patches excised from each animal.

Supplemental Figure 2.1. Immune cell percentages of vitamin A-deficient mice.

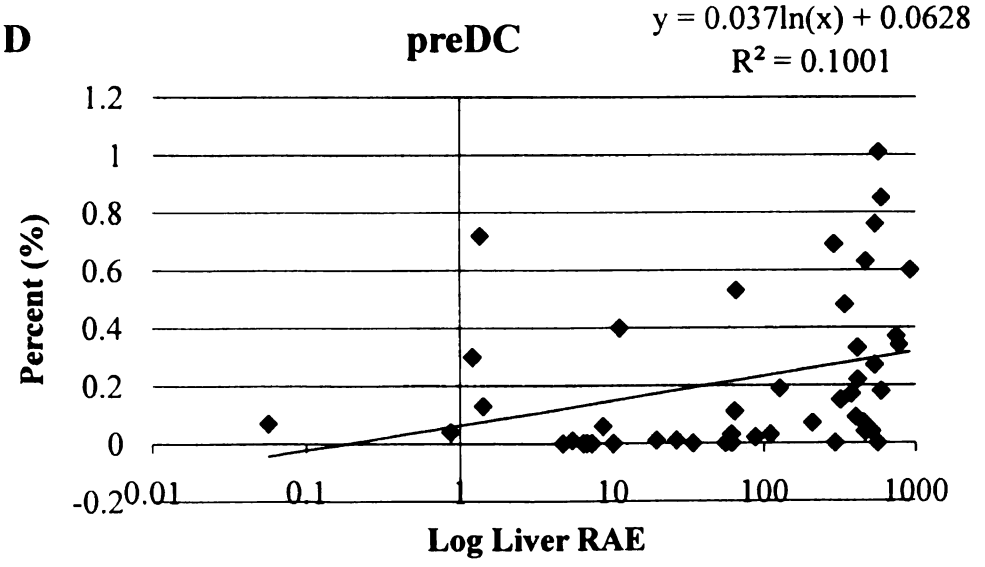




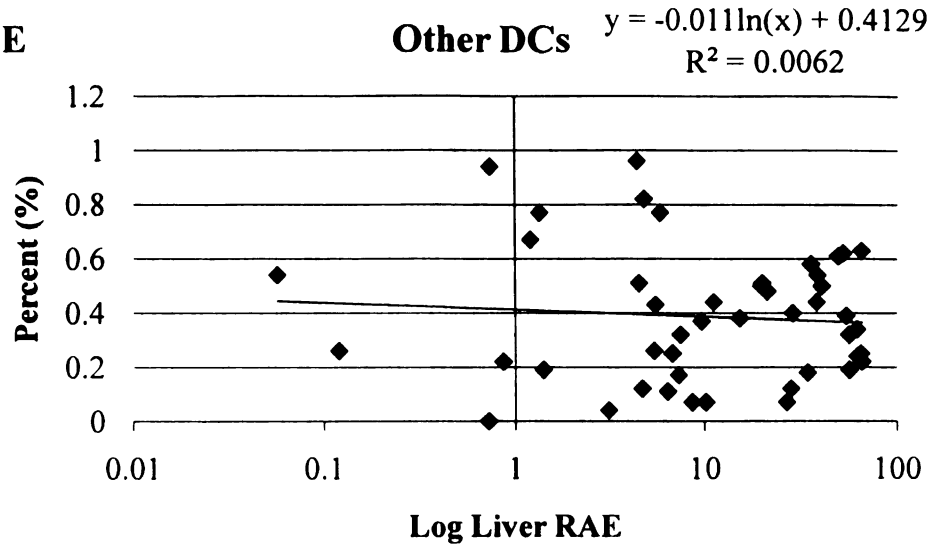
C


Plasmacytoid
y = 0.0807ln(x) + 0.4428
R² = 0.084
Percent (%)
3.5
3
2.5
2
1.5
1
0.5
0
0.01
0.1
1
10
100
Log Liver RAE

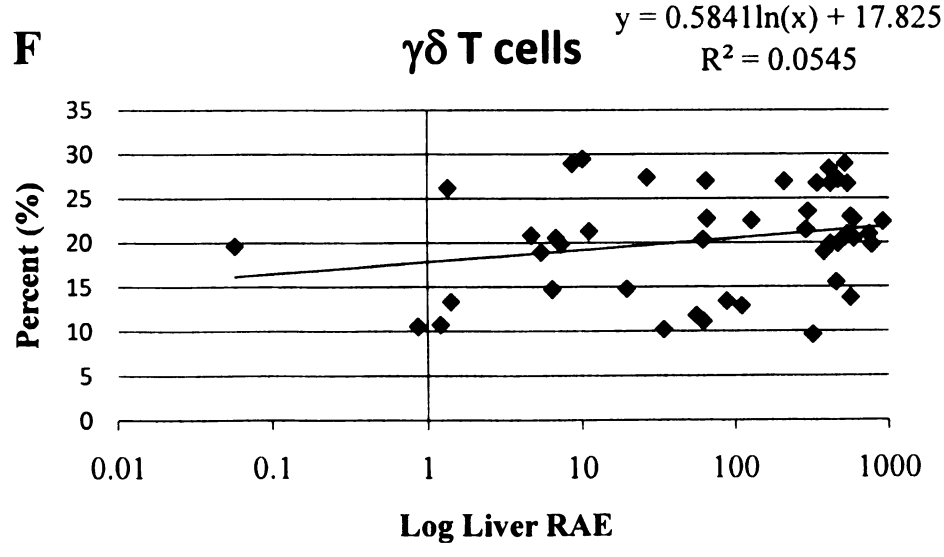
D


preDC
y = 0.037ln(x) + 0.0628
R² = 0.1001
Percent (%)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0.01
0.1
1
10
100
1000
Log Liver RAE

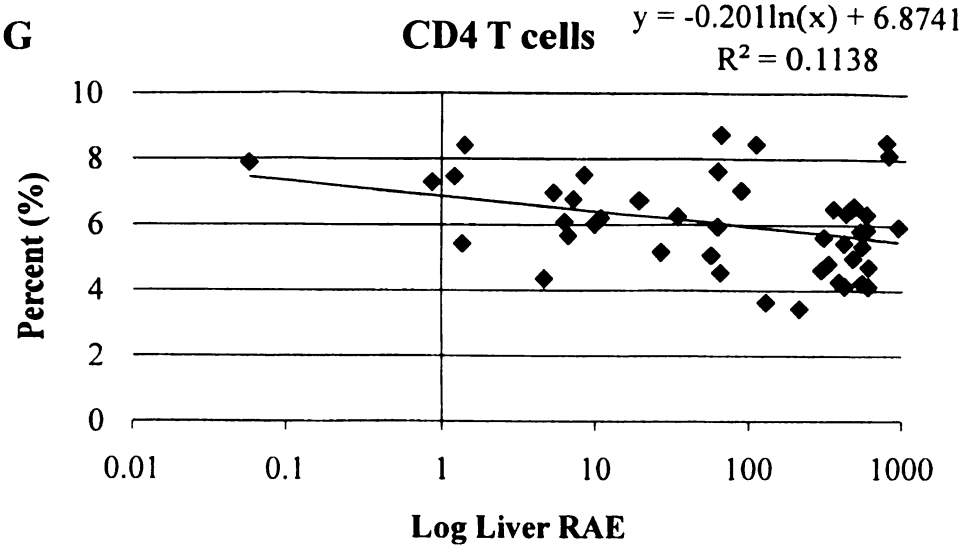
E


Other DCs
y = -0.011ln(x) + 0.4129
R² = 0.0062
Percent (%)
1.2
1
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
100
Log Liver RAE

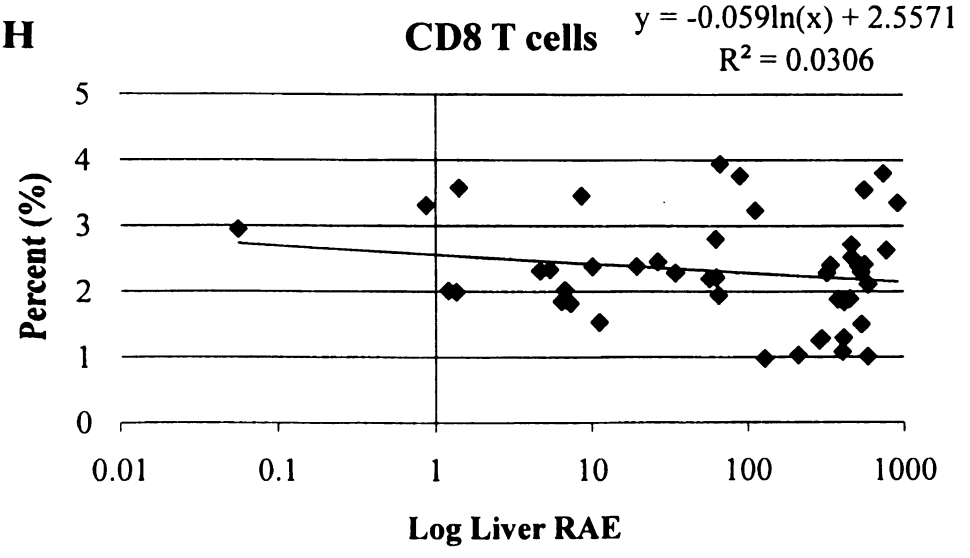
F


γδ T cells
y = 0.5841ln(x) + 17.825
R² = 0.0545
Percent (%)
35
30
25
20
15
10
5
0
0.01
0.1
1
10
100
1000
Log Liver RAE

G


CD4 T cells
y = -0.201ln(x) + 6.8741
R² = 0.1138
Percent (%)
10
8
6
4
2
0
0.01
0.1
1
10
100
1000
Log Liver RAE

H


CD8 T cells
y = -0.059ln(x) + 2.5571
R² = 0.0306
Percent (%)
5
4
3
2
1
0
0.01
0.1
1
10
100
1000
Log Liver RAE

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