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THE EFFECT OF RETINOIC ACID ON DENDRITIC CELL DEVELOPMENT AND MATURATION

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THE EFFECT OF RETINOIC ACID ON DENDRITIC CELL DEVELOPMENT AND MATURATION

By

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ABSTRACT

THE EFFECT OF RETINOIC ACID ON DENDRITIC CELL DEVELOPMENT AND MATURATION

By

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Retinoids encompass a group of natural and synthetic molecules with structural similarities to retinol—the major form of vitamin A. Vitamin A acts through the interaction of retinoid receptors with retinoic acid response elements promoting cellular differentiation or as a prosthetic group in vision. Hence, vitamin A is essential for several biological processes including proper immune function. During vitamin A deficiency, there is a shift in the balance of T helper 1 (Th1)–cell mediated immune responses and T helper 2 (Th2)–humoral immune responses that limits T-dependent antibody responses due to inadequate Th2 cell development.

Dendritic cells (DC) are specialized antigen-presenting cells that are known to influence the balance of Th1 and Th2 responses. DC differentiation is a complex process that begins in the bone marrow and is coordinated and regulated by the expression of cytokines, colony-stimulating factors, receptors, and transcription factors. Nutritional factors may play an important role in this process. Vitamin A is essential for proper immune function and has been implicated in the development of myeloid lineage cells. The objective of this research is to determine if vitamin A is critical to the proper development of immature myeloid DCs and dendritic cell maturation in a manner that may affect the resulting immune response.

DEDICATION

To my husband Jeff,

thank you for your never-ending love and support.

ACKNOWLEDGMENTS

It has been an honor and a privilege working with the Medical Technology Program at Michigan State University. I would especially like to thank the director, Dr. Kathy Doig, for taking a chance and accepting me into the program. It is also important that I thank my mentor, Dr. Kathy Hoag, for her guidance and expertise in the field of dendritic cell biology. I thank Dr. John Gerlach and Dr. Maija Zile for their insight and perspective in immunology and vitamin A, respectively. Many others that have helped me along the way including Dr. Louis King and Dr. Pam Fraker, The MSU Histology Laboratory, Michelle Montgomery, Sara Shea and countless others. Your contributions were greatly appreciated.

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KEY TO ABBREVIATIONS

Abbreviation	Unabbreviated
9cRA	9-*cis*-retinoic acid
APL	acute promyelocytic leukemia
atRA	all-*trans*-retinoic acid
BASF	Badische Anilin-und Sod-Fabrik
C/EBP	CCAAT/enhancer binding protein
CD-FBS	charcoal dextran fetal bovine serum
CDP	CCAAT displacement protein
CHR-FBS	characterized fetal bovine serum
cIMDM	complete Iscove's modified Dulbecco's medium
c-Myb	cellular-myeloblastosis
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
DAPI	4,6-diamino-2-phenylindol
DC	dendritic cell
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DORA	down regulated by activation
DRI	daily recommended intake
ds	double stranded
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
flt-3L or FL	fms-like tyrosine kinase-3 ligand
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte/macrophage-colony stimulating factor
HIV/AIDS	human immunodeficiency virus/acquired immunodeficiency syndrome
HPLC	high pressure liquid chromatography
IFN	interferon
IL	interleukin
IU	international units
IUBMB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry
JCBN	Joint Commission on Biochemical Nomenclature
LD_{50}	median lethal dose
LPS	lipopolysaccharide
LRAT	lecithin:retinol acyltransferase
MCP	monocyte chemotactic protein
MFI	mean fluorescence intensity
MHC	major histocompatability complex
MHC II	major histocompatability complex class II
mIMDM	modified Iscove's modified Dulbecco's medium

MIP	macrophage inflammatory protein
NF-κB	nuclear factor-kappa B
NK	natural killer
NMR	nuclear magnetic resonance
PE	phycoerythrin
poly I:C	polyinosine-polycytidylic acid
PPAR	peroxisome proliferators-activated receptor
RANTES	regulation activation, normal T cell expressed and secreted
RAR	retinoic acid receptor
RARE	retinoic acid response element
REH	retinyl ester hydrolase
RDA	recommended daily allowance
RXR	retinoid X receptor
SCF	stem cell factor
SDF	stromal cell derived factor
SEA	soluble egg antigen
Th1	T helper 1
Th2	T helper 2
TLR	toll-like receptor
TNF	tumor necrosis factor
TTR	transthyretin
TGF	transforming growth factor
UV/VIS	ultraviolet/visible
VAD	vitamin A deficiency

Chapter 1: Vitamin A

History of Vitamin A

The beneficial healing effects of vitamin A have been known for thousands of years. As early as 1500 B.C., ancient Egyptians cured a form of blindness (that might be considered night blindness by modern day clinicians) by placing ox liver, the richest food source of vitamin A, on the eyes and then consuming it. The medicinal application of applying liver to the eyes and eating it to cure blindness is apparent in many cultures after the Egyptians (109, 85).

In 1913, McCullum and Davis discovered a fat-soluble factor that was necessary for the growth of rats. They named this factor "fat-soluble A"(85). This substance also prevented night blindness and xerophthalmia (99). The precursor form of the vitamin, termed carotene, was not isolated until the 1930's by Karrer and his associates (99, 85). In 1931, the structure of retinol was discovered (99). It was then that many of vitamin A's beneficial properties were revealed. One of the most surprising findings was that vitamin A had anti-infective properties. Unfortunately, the discovery of penicillin ended further research into vitamin A's anti-infective properties until recently (85).

The discovery of cellular retinoid binding proteins and retinoic acid receptors has revolutionized the study of vitamin A (8). These findings explain the biological effects of vitamin A: proliferation and differentiation (8). The fields of oncology and dermatology have advanced due to the new understanding of how vitamin A controls transcription at the molecular level (8). Yet, there are areas of vitamin A research that remain to be explored—specifically the interaction of vitamin A within immune cells.

Chemistry of Vitamin A

Structure and nomenclature

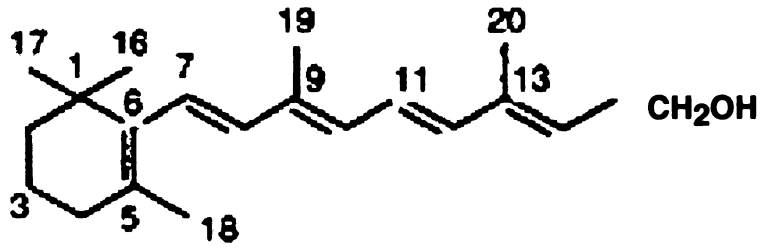
The term retinoid encompasses a large group of naturally occurring and synthetic molecules that possess the ability to elicit a specific biological response via molecular mechanism or have structural similarities with retinol. The basic structures are retinol (circulating form), retinyl esters (storage form), retinaldehyde (11-cis isomer used in vision), and retinoic acid (active form). These molecules share common structural features (Figure 1). All retinoids consists of four isoprenoid units ($H_2C=(CH_3)-CH=CH_2$) joined in a head to tail manner, a tri-methylated cyclohexane ring, a conjugated tetraene side chain, and a polar carbon-oxygen functional group (5).

The conjugated bonds of retinoids make them unstable and susceptible to rapid degradation. Retinoids are easily oxidized in the presence of air, light, and excessive heat. They are labile towards strong acids and solvents that have dissolved oxygen or peroxides (5). Therefore, these molecules must be handled with extreme care and caution at all times in order to avoid degradation.

The nomenclature of retinoids designated by the International Union of Pure and Applied Chemistry (IUPAC) and International Union of Biochemistry and Molecular Biology (IUBMB) Joint Commission on Biochemical Nomenclature (JCBN) is quite complex. For instance, all-*trans*-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)-nona-2,4,6,8-tetraen-1-oic acid is the designated IUPAC name for all-*trans*-retinoic acid (atRA) (7). In order to simplify this complex system, a conventional numbering system was given to the structure of all-*trans*-retinol. Isomers and substituted compounds can be

named unmistakably with this system (5, 85). In fact, common names are much easier for the quick identification of retinoid compounds.

A



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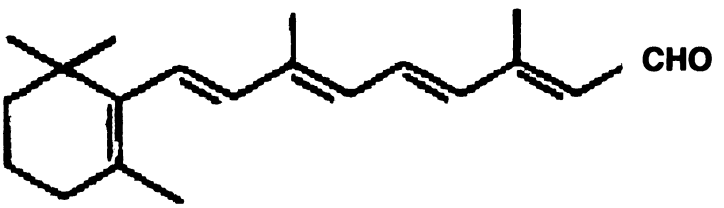

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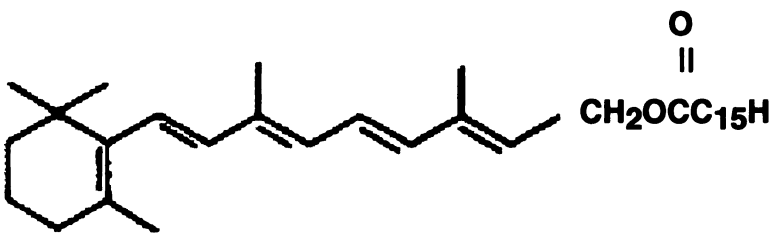

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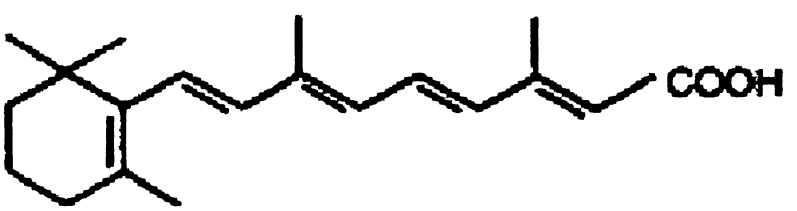


Figure 1. Structural Features of Some Naturally Occurring Retinoids.

(A) all-*trans*-retinol showing the conventional numbering system; (B) all-*trans*-retinal; (C) all-*trans*-retinyl palmitate, a major storage form of vitamin A; (D) all-*trans*-retinoic acid (28).

All-*trans*-retinoic acid and 9-*cis*-retinoic acid

All-*trans* and 9-*cis*-retinoic acid (9cRA) were chosen specifically for these experiments because they are believed to be the active forms of vitamin A functioning in the immune system. They are isoforms of one another and therefore have the same molecular weight and similar physical properties. Although both have been shown to have molecular mechanisms of action *in vitro*, atRA has been found *in vivo* while 9cRA has not.

Retinoid agonists, antagonists, and retro-retinoids

Agonists and antagonists

The development of synthetic retinoids came from breakthroughs in cancer research (22). Retinoid agonists and antagonists were developed to circumvent the toxicity and teratogenicity of atRA (4). The goal was to find an analog with a better efficacy to toxicity ratio than retinoic acid (85).

The majority of agonists and antagonists have aromatic rings in place of the tetraene bonds, allowing for conformational restriction of the molecule (22). A retinoid agonist induces the expression of the retinoid responsive gene via retinoid receptors, while a retinoid antagonist competes with the agonist for binding of the ligand and inhibits agonist-dependent activation of the responsive gene (66). Inverse agonists act as classical antagonists, but have biological activity of their own (52,66). Retinoid agonists and antagonists exert their action via the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (51, 67).

Retro-retinoids

There are three classes of active metabolites derived from retinol: aldehydes (responsible for vision), carboxylic acids (ligands for transcription factors), and retro-retinoids (signal transduction) (71). Retro-retinoids are responsible for intracellular signaling (11, 24, 47, 71) Retro-retinoids also have the ability to sustain B cell growth and activate T cells (24). The retro-retinoid that has the most physiological relevance is 14-hydroxy-4,14-retro-retinol. This molecule can be detected in the blood after ingestion of large quantities of vitamin A along with other retinoid metabolites (4).

Retro-retinoids have been implicated in the binding and activation of RARs. In F9 embryonic tetrocarcinoma cells, 4-oxo-retinol was found to activate transcription. All-*trans* and 9-*cis*-retinoic acid did not have an effect on these cells (1).

Isolation and synthesis of retinoids

Vitamin A is primarily found as an ester in animals only. The richest source is the liver of marine mammals and fish (85). The esters are isolated from liver oils via molecular distillation at low pressure (85). In addition, chloroform extraction followed by chromatography is another method of isolation, however chloroform readily interacts with vitamin A leading to isolates containing several isomers (85). Recent advances in high-pressure liquid chromatography (HPLC) have made it possible to obtain pure forms of individual retinoids (5).

Two major synthetic pathways for commercial development of vitamin A have been established. The Hoffman and LaRoche procedure involves a C14 aldehyde key intermediate and the reduction of acetylinic to olefinic bonds. The Badische Anilin-und Sod-Fabrik (BASF) method relies on the Wittig reaction (85).

Chemical and physical properties of retinoids

Commercial preparations of retinoic acid, retinyl acetate, retinyl palmitate, retinaldehyde, and retinol take the form of pale yellow to slightly reddish crystalline or amorphous solids (5, 85). The melting point of most retinoids is approximately 60°C (5). Retinoids tend to have a mild, pleasant odor (85).

Retinoids are not soluble in aqueous solution, but are miscible in most organic solvents (85, 5). Their solubility depends on the side chain terminal group. Retinol and retinoic acid, which have an alcohol and a carboxylic side chain terminal group respectively, are soluble in alcohols (5). Retinyl palmitate is not soluble in alcohol due to its esterified long chain fatty acids, however it is soluble in hexane (5). Chlorinated solvents, such as chloroform, should be avoided when dissolving retinoids because retinoids tend to isomerize, the solvent may be acidic, or the solvent may cause the formation of free radicals (5). Dimethylsulfoxide (DMSO) is a common solvent used to introduce retinoids into tissue culture (5).

Several methods have been utilized to identify retinoids. Retinoids absorb light at approximately 325 nm-375 nm in the ultraviolet (UV) visible (VIS) spectrum due to their conjugated bonds. However, several retinoids have similar maximum absorptions (Table 1) making them difficult to distinguish with UV/VIS spectrophotometry. Raman spectrophotometry has been employed to identify retinoid-protein interactions. Mass spectroscopy has been helpful in determining the mass of different retinoids and elucidating major functional groups. However, the most useful technique in determining the structure of a given retinoid is ^{1}H and ^{13}C nuclear magnetic resonance (NMR) (5).

Table 1: Light Absorbencies of Selected Retinoids.
The maximum absorption (λ_{max}) and extinction coefficient (ε=$mol^{-1}cm^{-1}$) of relevant retinoids dissolved in the specified solvent (This table was adapted from reference 101).

Retinoid	Solvent	λ_{max} (nm)	ε ($cm^{-1}mol^{-1}$)
all-*trans*-retinol	ethanol	325	52770
	hexane	325	51770
13-*cis*-retinol	ethanol	328	48305
11-*cis*-retinol	ethanol	319	34890
	hexane	318	34320
9-*cis*-retinol	ethanol	323	42300
all-*trans*-retinyl acetate	ethanol	325	51180
	hexane	325	52150
all-*trans*-retinyl palmitate	ethanol	325	49260
all-*trans*-retinal	ethanol	383	42880
	hexane	368	48000
all-*trans*-retinoic acid	ethanol	350	45300
9-*cis*-retinoic acid	ethanol	345	36900

Nutritional Aspects of Vitamin A

Vitamin A is an essential micronutrient that cannot be synthesized *de novo*. This nutrient must come from our diet and is found in animal sources as preformed vitamin A and from plant sources as provitamin A carotenoids. Preformed vitamin A includes retinyl esters, retinol, and retinoic acid. Approximately 600 carotenoids are found in nature and roughly 10 percent of the carotenoids have provitamin A activity. Of the carotenoids, β-carotene has the greatest activity, followed by α-carotene and γ-carotene. Preformed vitamin A can be found in milk, eggs, and animal tissues such as liver (the richest source of vitamin A) (29). Carotenoids are found in dark green leafy vegetables or in fruits and vegetables with orange, yellow, or red pigmentation. Excellent sources of carotenoids include spinach, broccoli, carrots, pumpkins, squash, apricots, mangos, papayas, and tomatoes (29, 28).

Dietary Reference Intakes (DRIs) set forth by the United States Federal Government encompass a group of reference values for evaluating dietary intake of nutrients. Of these reference values, the Recommended Dietary Allowance (RDA) represents the average daily intake of a nutrient that is required to meet the dietary requirements of 97-98% of the entire population. RDAs for vitamin A are reported as Retinol Equivalents (RE), which account for the differences in biological activity between retinol and provitamin A carotenoids. International Units (IU) are also used to measure vitamin A. One RE is equivalent to 1μg of retinol, 6 μg β-carotene, 12 μg of other provitamin A carotenoids, 3.3 IU from retinol or 10 IU from β-carotene (29). The current RDA values are listed in Table 2.

Table 2: Recommended Daily Allowances and Upper Limits for Vitamin A Intake. Vitamin A is required for normal vision, gene expression, reproduction, embryonic development, and immune function. RDA's are set to meet the needs of 97-98% of individuals in a group. The UL is the maximum level of daily nutrient intake that will likely pose a risk for adverse effects. The RDA's and UL's are measured in μg/d (29).

Children			Males			Females		
Life Stage	**RDA**	**UL**	**Life Stage**	**RDA**	**UL**	**Life Stage**	**RDA**	**UL**
0-6 mo	400	600	9-13 Y	600	1700	9-13 Y	600	1700
6-12 mo	500	600	14-18 Y	900	2800	14-18 Y 19+ Y	700 700	2800 3000
1-3 Y	300	600	19-70+Y	900	3000	Pregnancy <18 Y 19+ Y	 750 770	 2800 3000
4-8 Y	400	900				Lactation <18 Y 19+ Y	 1200 1300	 2800 3000

Absorption, metabolism, transport, storage and biosynthesis of vitamin A

The bioavailability of vitamin A in a healthy individual ranges from 80-90%, while the bioavailability of carotenoids ranges from 60-70% (38, 70). As preformed vitamin A and provitamin A carotenoids pass through the stomach, the enzymatic action of pepsin removes their associated transport proteins (85). Upon entering the duodenum, bile salts emulsify fatty acids, retinol, carotenoids, and phospholipids into micelles. Pancreatic enzymes, such as triglyceride lipase and cholesteryl ester hydrolase, further digest the micelles (70). Mucosal cells of the small intestine absorb the micelles, the retinol is esterified with long chain fatty acids, and the carotenoids are cleaved into two molecules of retinaldehyde by the enzyme β-carotene 15, 15'-oxygenase (85, 99). Retinaldehyde is further reduced to retinol by retinaldehyde reductase (99). Cellular retinol binding protein II (CRBPII) aids in the reduction of retinal and retinol. CRBPII is the substrate for the action of lecithin:retinol acyltransferase (LRAT). Triacylglycerols,

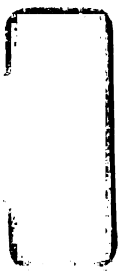
phospholipids, apolipoproteins and retinaldehyde are incorporated into chylomicrons and the resulting chylomicrons are released into the lymphatic system (85) under the direction of CRBPII (70).

Once in circulation, the chylomicron interacts with lipoprotein lipase and free fatty acids are delivered to the extrahepatic tissues including the heart, skeletal muscle, spleen, adipose tissue, and kidney (70). The resulting chylomicron remnant contains much of its original vitamin A content (approximately 75%) (70).

The liver is the major storage and retinoid metabolism organ. Chylomicron remnants are absorbed by stellate cells, which convert retinol into retinyl esters. Hepatic retinoid metabolism is mediated by LRAT and retinol ester hydrolase (REH). These enzymes esterify retinol into retinyl esters or hydrolyze retinyl esters to retinol and fatty acids, respectively (70). LRAT and REH are believed to control some aspects of vitamin A metabolism (83), however their role in the regulation and mobilization of retinol from the liver is not entirely understood (70).

The release of vitamin A by the liver is tightly regulated. Vitamin A is transported from the liver to peripheral tissues with the aid of transport proteins. The liver synthesizes retinol-binding protein (RBP) for this purpose. In order for transport of retinol to occur, retinol must first complex with RBP. Next, the retinol-RBP complex binds to transthyretin (TTR) in the plasma. The mechanism by which retinol is transported across target tissue membranes is not fully established, however it is believed that transport proteins play a potential role (summary in Figure 2).

Once retinol is inside the cell, binding proteins contributing to hormone signaling physiology are responsible for the transport of retinol. These include: cellular retinol



binding protein (CRBP), CRBPII, cellular retinoic acid binding protein (CRABP) and CRABPII (22). All vertebrates express these highly conserved, high affinity-binding proteins (22).

Retinoid binding proteins have several functions within the cell. They protect the cell from the amphipathic properties of retinoids and protect the retinoids from non-specific interactions (68, 69). Intracellular retinoid binding proteins regulate the concentration of vitamin A within the cell (69). They also restrict retinoid access to metabolic enzymes (68), which ultimately controls retinoid signaling (69).

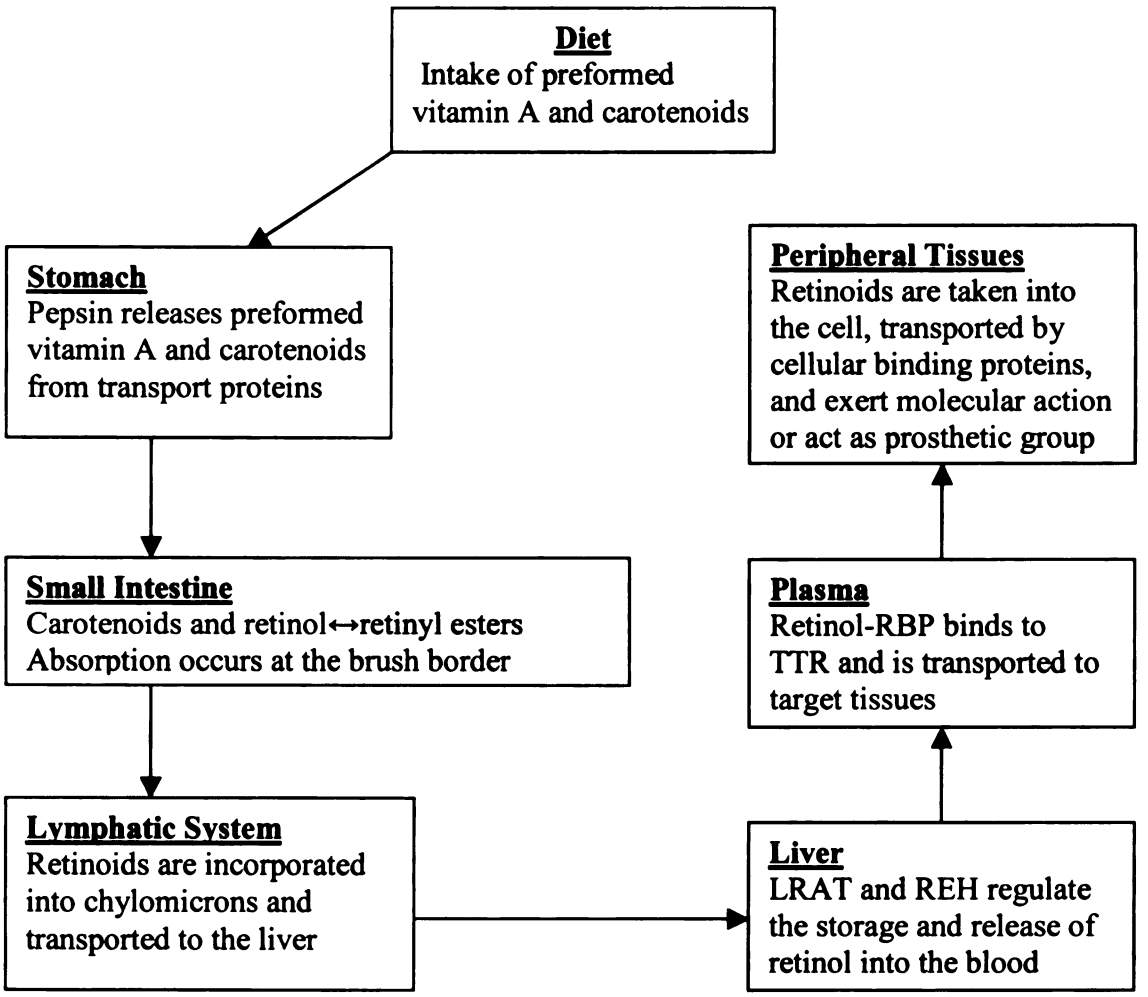


Figure 2. Overview of Vitamin A Digestion, Absorption, Storage, and Transport.

Vitamin A is taken in through the diet. Digestion begins in the stomach and continues in the intestines. Vitamin A is absorbed via the microvillus and transported across the brush border cells and into the lymphatic system. Vitamin A is incorporated into chylomicrons and transported to the liver where it is stored. The liver stores are tightly regulated, but can be mobilized to supply peripheral tissues when they are in need.

Molecular Action of Vitamin A

In 1987, a full-length cDNA encoding a 462 amino acid polypeptide similar to the DNA binding domain and the ligand-binding domain of steroid and thyroid hormones was cloned (37). The ligand for this receptor was the vitamin A related morphogen retinoic acid (37, 45). Later, it was found that the nuclear receptor for retinoic acid belonged to the nuclear sterid hormone receptor family (74). These retinoic acid receptors shared several similarities with the nuclear steroid hormone receptors including the different functional regions A through F. A year later, a second retinoic acid receptor was identified that lacked 30 amino acids in the N-terminus while sharing homology in the C region and the E region (9). Soon after, a third receptor for retinoic acid was identified (53). The discovery of a parallel regulatory system, that did not involve retinoic acid receptors, lead to the identification of the retinoid X receptors (61).

Nuclear retinoid receptors are transcription factors that act by promoting or inhibiting transcription and are the last step in retinoid signal transduction. There are two classes of retinoid receptors–retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Each class has three isoforms: α, β, and γ. The isoforms can generate multiple subtypes through differential splicing and the use of alternate promoters (70).

The amino acid structure of the receptors is divided into six regions (A-F). The transcriptional activation function lies in the A and B regions. The DNA binding domain is located in the highly conserved C region, while the hinge region (D) functions in dimerization. The ligand-binding domain is found in the highly conserved E region. Finally, the function of the carboxy terminus (F) remains unknown at this time (16).

RARs are major signal transducers of vitamin A; that is, they convert retinoid signals to transcriptional activation of a gene. The dimerization of RAR with RXR results in the most effective binding of the retinoic acid response element (RARE). Activation of the heterodimer is controlled by RAR ligation (70).

In addition to dimerization with RARs, RXRs also partner with other nuclear receptors. These receptors include the thyroid hormone receptor (70), the vitamin D receptor (70), peroxisome proliferators-activated receptor (PPAR) (16), and orphan receptors (16). The RXR can also homodimerize.

RAREs are specific DNA sequences that are located in the promoter region of the target gene. They consist of two hemimeric half sites with the consensus sequence AGGTCA. This sequence can be arranged in direct repeats, palindromes, or inverted palindromes. The variable spacing between each repeat serves as a regulatory region that mediates a retinoic acid response. Spacing between the half sites for direct repeats ranges between one and five base pairs, with five base pairs occurring most frequently. Direct repeats with one base pair tend to be promiscuous binding sites for several different nuclear hormone receptor homodimers (16).

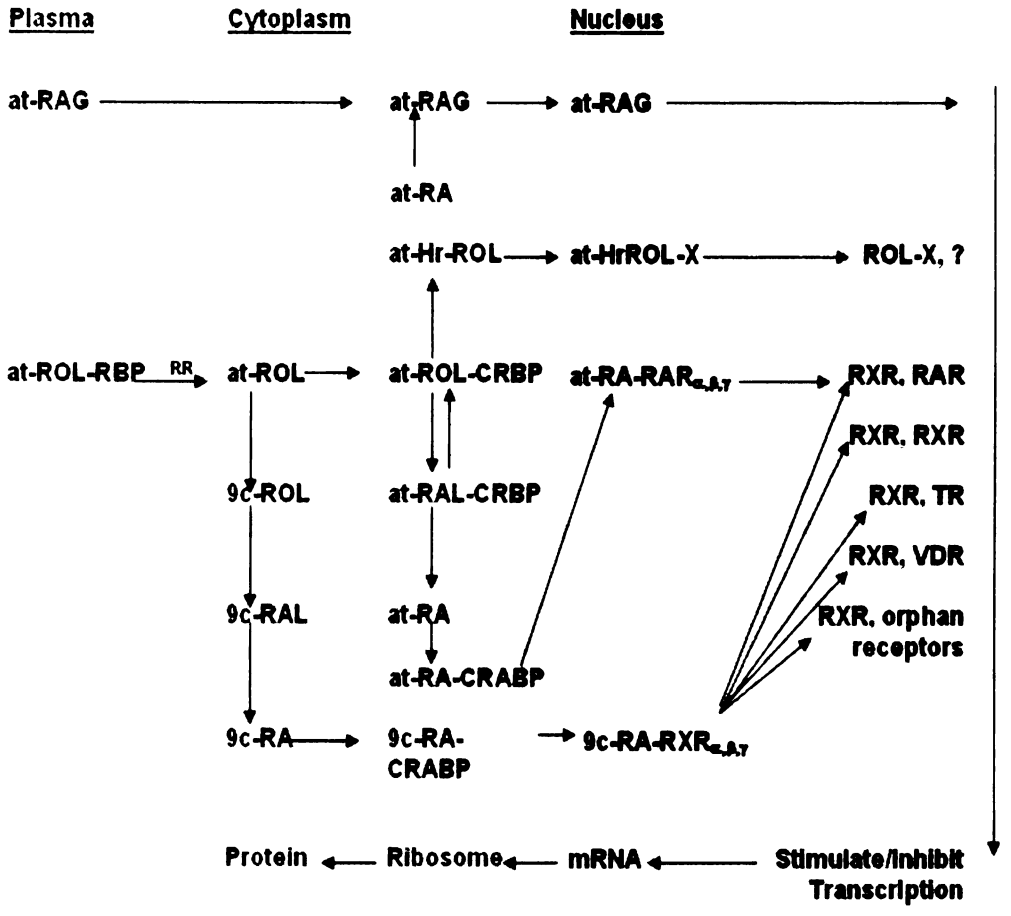


Figure 3. Retinoid Signaling and Translocation to the Nucleus.

All-trans retinol (at-ROL) bound to retinol binding protein (RBP) enters the cell and is converted to several different isoforms. These isoforms bind their respective cellular binding proteins and translocate to the nucleus where all-trans retinoic acid (atRA) and 9-cis retinoic acid (9cRA) can bind with their respective retinoid receptors, the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (85).

Functions of Vitamin A

Vitamin A is essential for vision, cellular differentiation and proliferation, growth, skeletal development, reproduction, embryonic and fetal development, epithelial maintenance, and proper immune function. Most of the functions of vitamin A rely on the activation or inhibition of transcription, of genes involved in cellular proliferation and differentiation. In fact, growth, skeletal development, embryonic and fetal development, epithelial maintenance, and immune function all rely on transcriptional regulation promoted by vitamin A. On the other hand, the role of vitamin A in the visual cycle does not rely on transcriptional regulation instead; vitamin A acts as a prosthetic group (114).

Vitamin A metabolism in the retina is unique (110, 28). Metabolic events occur in two different cell types of the retina, the cordial vasculature, and the photoreceptor cells (70). These cell types are separated by the retinal pigment epithelium (RPE). The RPE is a selectively permeable layer of cells that allow for the exchange of nutrients and wastes (70). Retinol incorporated with the TTR-RBP complex is transported across the RPE with the aid of cellular retinal binding protein (CRALBP) and interstitial retinol binding protein (IRBP) (38). Once inside the retina, retinol is stored as retinyl esters or converted to retinal for immediate use in the visual cycle (38). Both 11-*cis*-retinal and all-*trans*-retinal are key intermediates in the visual cycle (70, 85, 28, 110). 11-*cis*-retinal binds strongly with opsin via a protonated Schiff base to form rhodopsin (70, 85, 28). This secures rhodopsin in an inactive form (70). A photon of light isomerizes 11-*cis*-retinal to all-*trans*-retinal (85, 28). The conformational change produces metarhodopsin II (70), which activates a phototransduction cascade resulting in a nerve impulse to the brain (70). Several steps are involved in quelching the cascade. The final step involves the

reduction of all-*trans*-retinal to all-*trans*-retinol. All-*trans*-retinol is then committed to a regeneration pathway (Figure 4) (70, 28).

The balance between cellular differentiation, proliferation, and apoptosis is tightly regulated by vitamin A (70, 21). Vitamin A controls gene expression through nuclear retinoic acid receptors (16, 70). These receptors appear in several cell types and at many different stages of development (38). Retinoids have been shown to regulate critical cell cycle genes (70) and influence the ability to utilize both negative and positive growth factors including epidermal growth factor, transforming growth factor (TGF) α, TGFβ, insulin, interleukin (IL)-1α, IL-6, interferon (IFN)-γ, estrogen, and vitamin D_3 through retinoid dependent and independent pathways (70). Vitamin A is required for the proper differentiation and proliferation of the epithelial tissues of the lung, trachea, skin, gastrointestinal tract, urogenital tract, and several other tissues. Vitamin A maintains normal structure and function of these cells. For example, vitamin A directs the differentiation of keratinocytes to mature epithelial cells (38). Vitamin A has also been implicated in the differentiation of white blood cells from stem cell progenitors in the bone marrow (36, 33). The role vitamin A plays in cellular differentiation and proliferation is associated with its effects on growth, reproduction, epithelial maintenance, and immune function.

The reproductive system requires vitamin A for proper function. Vitamin A is required for spermatogenesis in the testis (70). Genetic studies indicate that atRA is the active form of vitamin A functioning in spermatogenesis, however dietary retinol is necessary for transport of the vitamin across the blood-testis barrier (70). Vitamin A is also necessary for maintaining the health of the female reproductive system and

preventing fetal resorption (18). All-*trans*-retinoic acid is the functional form of vitamin A in female reproduction and fetal development (18).

All-*trans*-retinol and atRA are the primary retinoids found in murine embryos and human embryonic tissue, yet atRA is the active moiety required for the development of the mammalian embryo via transcriptional regulation. Another retinoid capable of transcriptional activation–9cRA–has not been identified in developing murine embryos. Some *retro*-retinoids have been implicated in mammalian embryogenesis by activating RAR-mediated gene transcription (18). It has been shown that monoclonal antibodies against atRA block normal quail embryo development by causing abnormalities in the cardiovascular system (104). Vitamin A is required for the proper development of the primitive heart, circulatory system, and hindbrain formation (115). Vitamin A deficiency targets the heart, central nervous system, circulatory system, urogenital and respiratory systems, and the development of limbs and skeleton in developing embryos (60, 114).

Vitamin A is necessary for growth. Vitamin A deficiency (VAD) is characterized by impaired growth of the organism (38). Vitamin A supplementation in VAD children has been shown to modestly improve their linear growth by 0.16 cm/ 4 months (40). The resulting increase in growth is indirectly caused by increased cellular proliferation and differentiation, as well as enhanced skeletal development.

The metabolites of retinoic acid regulate skeletal development via transcriptional regulation. Retinoid signaling is crucial at distinct stages of skeletogenesis. Manipulating the retinoid signaling pathway affects the expression of skeletonogenic master regulatory factors (reviewed in 108).

Vitamin A is particularly important for maintaining epithelial barriers. Alterations in the epithelial lining of vital organs occur early in VAD (64). The epidermal barrier is an important defense against infection (70). During VAD, the mucosal barrier found in the conjunctiva of the eye, respiratory tract, gastrointestinal tract, and urogenital tract are compromised due to the loss of mucus producing goblet cells (100).

Vitamin A is essential for proper immune function and has been identified as the "anti-infective" vitamin (85, 70, 100, 91). No nutritional deficiency is more synergistic with infection than vitamin A. There are two mechanisms in which vitamin A promotes a normal immune function (18). The first is through the maintenance of epithelial barriers (discussed earlier) such as the skin and mucosal linings of the airways, gastrointestinal tract, and the urogenital tract (18, 64). The second is through transcriptional manipulation of immune cells (18). The importance of vitamin A in association with proper immune function is clearly illustrated by the abnormalities that occur on both the innate and adaptive immune systems during VAD (reviewed by 91, 100, and 70).

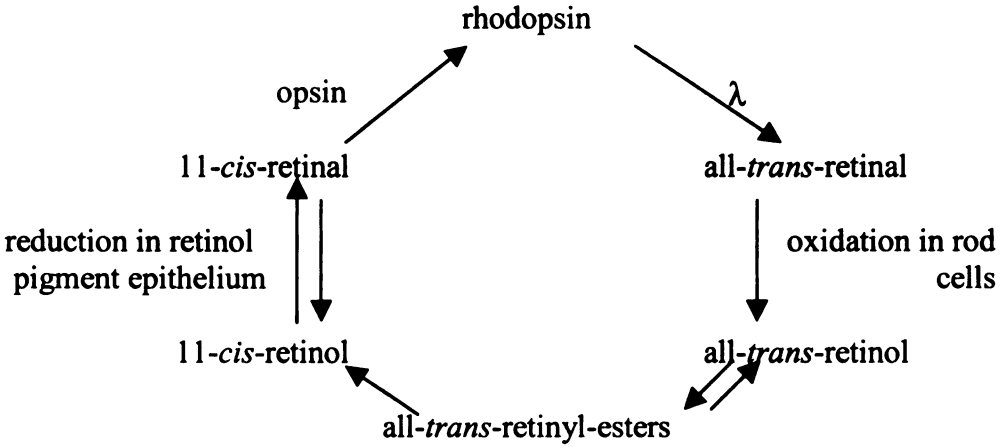


Figure 4. Vitamin A in the Visual Cycle

11-*cis*-retinal combines with opsin and forms rhodopsin. When a photon of light (λ) strikes rhodopsin, 11-*cis*-retinal is converted to all-*trans*-retinal. All-*trans*-retinal is oxidized to all-*trans*-retinol. All-*trans*-retinol is converted to all-*trans*-retinyl esters by the retinol pigment epithelium. The retinol pigment epithelium further reduces the esters into 11-*cis*-retinal (110).

Vitamin A deficiency and toxicity

Vitamin A deficiency

Vitamin A deficiency (VAD) is a disease that primarily affects preschool age children in developing countries. It manifests itself largely as xerophthalmia and keratomalacia, however anemia and decreased iron metabolism, growth cessation, epithelial changes, reproductive problems, and defects in cellular and humoral immunity are also symptoms of vitamin A deficiency (96). Xerophthalmia (greek: dry eyes) is an ocular manifestation that ranges from night blindness to severe corneal scarring resulting in blindness (95). VAD impairs iron utilization, mobilization, and transport resulting in anemia (96). Growth cessation strongly correlates with decreased levels of serum retinol while depleted liver retinol stores result in rapid weight loss (96). Keratinization of epithelium occurs in the gastrointestinal tract, respiratory tract, urogenital tract, and in other major tissues (96). VAD is also associated with an increased risk of infection and is associated with mortality from measles, respiratory infections, gastrointestinal infections, and human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (96).

Several factors contribute to the onset of VAD. Malnutrition is the main cause of VAD. Malabsorption caused by gastroenteritis has been linked to VAD. Infections such as measles, gastrointestinal infections, respiratory infections and HIV/AIDS have been shown to decrease serum retinol levels. Socioeconomic factors such as poor education, poor hygiene, and low income also contribute to the prevalence of VAD (95, 96).

Detection of vitamin A deficiency

Vitamin A status is divided into five categories based on the level of circulating retinol: deficient, marginal, satisfactory, excessive, and toxic (85). There are clinical symptoms for deficient and toxic vitamin A status, however there are no physical indicators for the other levels of vitamin A status. Vitamin A status can be assessed thought dietary, physiological, histological, and biochemical means (85).

Dietary methods for determining vitamin A status are useful in certain instances. Vitamin A is measured by charting eating habits and estimating dietary intake. This method is not suitable for young children and is not very accurate (85).

Physiological means of detecting vitamin A status include measuring the sensitivity of rod cells in the eye (85). Dark adaptation studies assess the responsiveness of the pupil to light (21). The results of this test correlate with serum retinol levels and the results from the relative dose response test (21). The dark adaptation test has several advantages. This test is low cost, non-invasive, reliable, accurate, and there are no samples to collect (21, 86). However, it is important to note that zinc deficiency and protein malnutrition interfere with normal dark adaptation and hence interfere with the assessment of vitamin A status (86).

Conjunctival impression cytology is a histological means of determining vitamin A status (85). Filter paper is applied to the temporal conjunctiva and the morphology of the cells is observed (21). Goblet cells disappear during vitamin A deficiency (85). Vitamin A deficiency is indicated by the presence of 20% or more of these abnormal cells (21). The advantages of performing this test with an experienced technician are extremely accurate, reproducible results and the test is non-invasive. The disadvantages

of this testing method are the experience and training needed to make an accurate interpretation (21).

The relative dose response test is the most common biochemical means of measuring vitamin A status (86). A direct measure of retinol or retinol binding protein can be measured in blood or tears (85). The relative dose response involves two blood tests at distinct time points—0 hours and 5 hours after a physiological dose of vitamin A. In a vitamin A deprived state, RBP accumulates in the liver. Upon vitamin A supplementation, vitamin A binds accumulated RBP and is released from the blood as a holo-RBP-retinol complex. In a vitamin A deficient state, the serum retinol-RBP levels will rise dramatically and remain elevated over the 5-hour period. In a vitamin A sufficient state, the change in RPB-retinol levels in the blood are not as dramatic and the rise is not substantial over a 5 hour period (86). This test has several advantages. It is accurate and reliable. The disadvantages of the relative dose response test are that it involves taking blood samples, and it requires complex equipment to run the test.

Vitamin A toxicity and teratogenicity

Hypervitaminosis A is the result of excessive intake if vitamin A. The symptoms include anorexia; dry, itchy, and desquamating skin; alopecia and coarsening of the hair; ataxia; headache; bone and muscle pain; and conjunctivitis (38, 86). Vitamin A toxicity particularly affects the liver. Since vitamin A is a fat-soluble vitamin, it has a long half-life and accumulates in the liver and other tissues. Changes in the liver include: fat-storing cell hyperplasia and hypertrophy; fibrogenesis; sclerosis of veins; portal hypertension; and congestion in perisinusoid cells. This damage leads to cirrhosis or cirrhosis-like hepatic disorder (38).

The potential damaging action of vitamin A can occur by acute ingestion of a single large dose or a number of large doses taken over a short period of time or chronic ingestion of ten times the RDA. The symptoms usually accompanying an acute dose will subside in a matter of days. The median lethal dose (LD_{50} value) of an intramuscular, water miscible dose of vitamin A is 500 mg for infants and 11.8 g for adults, which translates to 1.8 mM and 41 mM respectively (85). In the case of chronic intake, the effects do not disappear for weeks or months and there is ongoing damage to the liver. Alcohol ingestion, protein malnutrition, viral hepatitis, and diseases affecting the liver and the kidney are factors that increase vitamin A toxicity.

Teratogenicity, due to excess vitamin A, occurs during the first trimester of pregnancy and can cause spontaneous abortions or major fetal malformations. An acute intake (30-90 mg of retinol every day for a week) or a chronic intake (75 mg of retinol over several weeks) can cause severe damage to the fetus. Common birth defects associated with vitamin A toxicity are craniofacial defects, congenital heart disease, kidney defects, thymic abnormalities, and nervous system disorders (85). There also seems to be a link between permanent learning disabilities and the toxic effects of vitamin A on a fetus.

Vitamin A Deficiency and the Immune System

Vitamin A deficiency (VAD) has a devastating impact on immune function. Lymphoid organs undergo atrophy because of VAD. The components of the innate immune system are compromised by the penetration of pathogens through damaged mucosal barriers and the impaired function of effector cells. Because of this, the adaptive immune system suffers (reviewed in 91 and 100). During VAD, the organs of the immune system atrophy. In the 1930's, children who died with VAD were examined and found to have atrophy of the spleen, thymus, and lymphoid tissue (91). Animal models of VAD confirmed this same phenomenon (91).

Mucosal immunity

Mucosal immunity is hampered during VAD. Human as well as animal models exhibit epithelial changes. Keratinization of the mucosa (squamous metaplasia) and loss of goblet cells leads to a dramatic decrease in mucus production (91, 85, 64). In humans, it has been shown that VAD impairs the regeneration of enteric mucosa (100). This irreparable damage leads to greater susceptibility to infectious agents (91, 100). It has also been shown that secretory IgA (sIgA) production decreases during VAD (91). The decrease in sIgA and the mucosal damage cause increased severity and mortality from infection (91, 100)

Innate immunity

Many aspects of innate immunity are impaired by VAD. In mice, VAD causes the systemic expansion of the myeloid lineage (54). VAD disrupts neutrophil development, function, and numbers, and natural killer (NK) cell number and function are also affected.

Macrophage number and function are also manipulated by VAD (100). The impact of VAD on dendritic cell (DC) number and function remains to be investigated.

Neutrophil development is regulated through RARs and is vitamin A dependent (55). During VAD, neutrophil development *in vivo* goes unchecked and results in increased neutrophil numbers, but with impaired function (55, 105, 113). These neutrophils have less phagocytic capability and have a lower ability to destroy bacteria (105, 113).

NK cells are affected by VAD, but in a manner opposite of myeloid lineage cells. NK cells tend to decrease in number. They also show a decrease in lytic activity during VAD (84).

Macrophage numbers increase in the secondary lymphoid organs of mice (93). VAD causes macrophages to increase the transcription, and hence production, of interleukin (IL)-12 (12). The increased production of IL-12 directly influences the adaptive immune system by acting on T helper 1 (Th1) cells and causing increased IFN-γ production. This shifts the balance of the adaptive immune response in favor of a Th1 response.

Adaptive immunity

VAD animal models reveal a shift from T helper 2 (Th2) antibody mediated immune response to a Th1 cell mediated immune response (13). Humans show similar changes marked by decreased IL-10 production (50). VAD mice have significantly fewer Th2 cells (13). Retinol treatment before immunization of rats restored low antibody responses to that of the control level (82). Further studies indicated that atRA specifically

enhanced Th2 cell development through its effect on antigen presenting cell (APC) function (43).

Aims of this thesis

DCs are specialized APC that have the ability to control the balance of the Th1/Th2 response via antigen presentation and lineage restrictions (reviewed by 65). Myeloid DCs promote Th2 responses while lymphoid DCs promote Th1 responses (65).

VAD alters the balance of the Th1/Th2 response in favor of a Th1 response through the decreased capacity of antigen presentation to Th2 cells. Myeloid lineage development is controlled by vitamin A. Myeloid DCs are APCs. Therefore, we hypothesize that the absence of vitamin A will result in defective DC development and upon restoration of vitamin A DC development will be restored. At the same time, we propose that vitamin A does not influence DC maturation.

Chapter 2

The Role of Retinoic Acid in Dendritic Cell Differentiation

Myeloid dendritic cell development from murine bone marrow progenitor cells requires retinoic acid

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Myeloid dendritic cell development from murine bone marrow progenitor cells requires retinoic acid.

Short Title: Myeloid DC development requires retinoic acid

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Abstract

Differentiation of hematopoietic progenitor cells to dendritic cells (DCs) is a complex poorly-understood process coordinated and regulated by cytokines, colony-stimulating factors, receptors, and transcription factors. However, nutritional factors may play an important role. Vitamin A is essential for proper immune function and is implicated in the development of myeloid lineage cells. The focus of this research was to investigate the role of vitamin A in differentiation of myeloid DCs. Cultures of murine bone marrow stimulated with granulocyte/macrophage-colony stimulating factor (GM-CSF) in the absence of vitamin A significantly decreased DC development. Surprisingly, neutrophils developed in response to GM-CSF when vitamin A was depleted. Addition of all-*trans* or 9-*cis* retinoic acid significantly restored DCs and inhibited neutrophil development. The effect of vitamin A was specific to myeloid lineage development stimulated by GM-CSF, since vitamin A inhibited DC development stimulated by flt-3 ligand (FL). Vitamin A also affected DC co-stimulatory molecule and MHC class II expression. In response to increasing concentrations of vitamin A, the expression of MHC class II decreased on the DC, while the expression of co-stimulatory molecules increased, especially CD86. Our data suggests vitamin A maintains differentiation of immature myeloid DC in the steady state.

Introduction

Dendritic cells (DCs) are antigen presenting cells that link the innate and adaptive immune systems by means of antigen presentation to naïve T cells.[1] Immature DCs patrol peripheral tissues for pathogens and tissue damage. Contact with microbial products or inflammatory mediators initiates a maturation process involving migration to regional lymph nodes, antigen processing, upregulation of MHC class II surface expression, induction of co-stimulatory molecule expression, and initiation of cytokine production. Antigen presentation relies on major histocompatability complex (MHC) presentation of peptide antigens in combination with co-stimulation via CD40, CD80, and CD86.

DCs are a heterogeneous mix of antigen presenting cells that may derive from separate hematopoietic lineages.[2] Certain surface markers delineate unique functional phenotypic subsets of DCS that have a propensity to cause different adaptive immune responses.[3] A subset of DCs expressing CD8α directly controls the balance of $CD4^+$ T helper 1 (Th1) and T helper 2 (Th2) responses through the preferential production of interleukin (IL)-12 which promotes a Th1 response. In contrast, CD8α negative DCs apparently play greater a role in promoting Th2 responses, although the direct mechanism is currently unclear.[4] DCs also play a role in tolerance.[1]

There are currently two main models for dendritic cell (DC) development. One model suggests that there are discrete developmental pathways resulting in separate lineages, while the second model instead delineates unique developmental stages of DC based upon differential cell surface marker expression.[5] Despite conflicting models, a pluripotent hematopoietic stem cell (lin^-, c-$kit^{<lo}$) can commit to DC development in the presence of granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-3, tumor

necrosis factor (TNF)-α, and stem cell factor (SCF).[6] It has also been shown that more mature hematopoietic progenitors (lin^-, c-kit^{lo}) differentiate into functional DCs under similar conditions.[7] The hematopoietic stem cell further develops into either a common myeloid progenitor or a common lymphoid progenitor both of which can give rise to functionally and phenotypically different subsets of DCs.[8] Hereafter, DC development becomes more complex and the requirements at each stage as well as the developmental possibilities are uncertain. It has been shown that the common lymphoid progenitor can give rise to T lymphocytes, B lymphocytes, natural killer cells and $CD8\alpha^+$ DCs.[9] The common myeloid progenitor has the potential to generate both $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets, challenging the dual lineage model.[10] Furthermore, some DCs share developmental pathways with early B lymphocyte development.[11] Additionally, two separate laboratories have identified at least two DC precursor stages that can be found in murine peripheral blood.[12,13] The DC precursors are $CD11c^{low\text{-}int}$ and MHC class II negative and can develop into both $CD8\alpha^+$ and $CD8\alpha^-$ DC, as well as a population that resembles human plasmacytoid DC. It is apparent that DC development is coordinated and regulated by the expression of cytokines, colony-stimulating factors, receptors, and transcription factors.[14] However, we hypothesize that vitamin A, an important nutritional factor, is essential for coordinating and regulating myeloid DC hematopoiesis.

Vitamin A is essential for maintaining normal immune function.[15,16] Without this micronutrient, both the innate and adaptive immune systems are compromised and the balance of the Th1/Th2 response is biased toward Th1.[15] Research studies using animal models demonstrate T lymphocyte-dependent antibody responses are markedly impaired in vitamin A deficiency,[17-19] due to inadequate stimulation of antibody-secreting B

lymphocytes by T cells.[20] Vitamin A deficiency has been shown to decrease naïve T cell stimulation for Th2 development, and the antigen presenting cells (APCs) were the target of retinoic acid in the restoration of Th2 responses.[21] However, the vitamin A deficient mice do not usually show abnormalities in the absolute numbers of B and T lymphocytes.[22]

Retinoic acid and retinoic acid receptors (RARs) are known to play a role in the regulation of myelopoiesis,[23] but disparate results exist in regards to which mature myeloid lineage cell requires vitamin A for development. Several studies indicate that RARs (and in particular RARα) are important regulators necessary for neutrophil development. Alternatively, feeding studies with retinyl acetate and 13-*cis* retinoic acid at pharmacological doses resulted in consistent enlargement of immune tissues due to an increase in macrophages and DCs.[24,25] In contrast, it has been shown that mice suffering from vitamin A deficiency exhibit an expansion of myeloid cells in the bone marrow, spleen and peripheral blood.[22] These cells express surface antigens characteristic of mature granulocytes (Gr-1 and Mac-1). Finally, it has also been hypothesized that all-*trans* retinoic acid (atRA) might regulate the proliferation and differentiation of early myeloid precursors.[23] To further understand the role that vitamin A plays in myeloid development and myeloid DC development in particular, we developed a culture system that was virtually free of retinol. We controlled the amount and form of vitamin A in the cultures by adding different concentrations of atRA or 9-*cis* retinoic acid (9cRA). The absence of retinol in the cultures resulted in a profound decrease in myeloid DC development and a dramatic increase in neutrophil commitment ($Gr\text{-}1^+$ cells). The addition of either atRA or 9cRA restored DC development significantly above the vehicle

control level. Our findings show that vitamin A is critical for the differentiation of bone marrow progenitors to immature myeloid DCs in response to GM-CSF stimulation.

Materials and methods

Mice

Male 3- to 4-week-old BALB/cJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were maintained according to institutional guidelines set by University Laboratory Animal Resources under a protocol approved by Michigan State University's Institutional Care and Use Committee. The murine diet consisted of solid pellets containing all essential macro and micronutrients (including vitamin A). The mice were killed at the age of 5-12 weeks by carbon dioxide asphyxiation.

Reagents

Characterized Fetal Bovine Serum (FBS) and Charcoal/Dextran treated FBS (CD-FBS) were purchased from HyClone (Logan, UT). The medium (cIMDM) consisted of IMDM (BioWhittaker, Walkersville, MD) supplemented with 10% serum (FBS or CD-FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (BioWhittaker) and 10^{-4} M β-mercaptoethanol (Sigma, St. Louis, MO). Vitamin A, in the form of atRA or 9cRA (Sigma), was dissolved in HYBRI-MAX ® dimethylsulfoxide (DMSO; Sigma), and was stored in single use aliquots at -70°C in the dark under an argon atmosphere. Purified recombinant GM-CSF was purchased from PeproTech (Rocky Hill, NJ). Alternately, GM-CSF containing supernatant was produced from the X-63 cell line transfected with GM-CSF cDNA.[26] The GM-CSF concentration in the X-63 supernatant was determined by ELISA (BD Biosciences, San Diego, CA). Human fms-like tyrosine kinase 3 ligand also known as flt-3 ligand (FL) was purchased from PeproTech. DAPI (4,6 diamidino-2-phenylindole; Sigma) was used to determine cell viability during flow

cytometric analysis. The following antibodies were used to label the cells: fluorescein isothiocyanate (FITC) conjugated anti-CD8α (53-6.7 clone; Rat IgG_{2a}, κ), FITC conjugated anti-CD86 (GL1 clone; Rat IgG_{2a}, κ), FITC conjugated anti-CD40 (3/23 clone; Rat IgG_{2a}, κ), FITC conjugated anti-MHC class II ($I\text{-}A^d$) (AMS-32.1 clone; Mouse IgG_{2b}, κ), FITC conjugated anti-CD80 (16-10A1 clone; Armenian Hamster IgG_{2*}, κ), FITC conjugated anti-CD11b (M1/70 clone; Rat IgG_{2b}, κ), FITC or allophycocyanin (APC) conjugated Gr-1 (RB6-8C5 clone; Rat IgG_{2b}, κ), and R-phycoerythrin (R-PE) conjugated anti-CD11c (HL3 clone; Armenian Hamster IgG_1, λ). Antibodies and the appropriate isotype controls were purchased from BD Biosciences.

GM-CSF cell cultures

Bone marrow cells obtained from the femurs and tibias of mice were cultured *in vitro* with 20 ng/ml GM-CSF using an adaptation of the protocol established by Inaba *et al.* (1998).[27] Cells were grown in 10 ml of cIMDM supplemented with either 10% FBS (positive control) or 10% CD-FBS (all other treatment groups). Cultures in medium containing CD-FBS were further divided into treatment groups (n=5/treatment) consisting of the DMSO vehicle control, 10^{-10} M atRA, 10^{-9} M atRA, 10^{-8} M atRA, 10^{-10} M 9cRA, 10^{-9} M 9cRA, or 10^{-8} M 9cRA. Cultures received one-half volume fresh medium containing GM-CSF +/- atRA or 9cRA on days 3, 6, and 8. Cells were harvested on day 10 of culture. This process involved removing the medium and forcefully washing the cells from each individual plate with staining buffer (1% FBS, 0.1% sodium azide, in phosphate buffered saline, sterile filtered, 4°C, pH 7.4). The culture medium and the wash were pooled and centrifuged (300xg, 10 minutes, 4°C) and the cells were

resuspended in staining buffer. A small volume of each cell suspension was removed to determine the cell count and viability using trypan blue dye (Sigma) and a hemocytometer. The cells were stored on ice throughout processing.

FL cell cultures

Bone marrow cells, obtained as described above, were cultured *in vitro* with 200 ng/ml FL using a protocol adapted from Brasel et al. (2000).[28] Cells were grown in 5 ml of cIMDM supplemented with either 10% FBS or 10% CD-FBS. The CD-FBS cultures were divided into the same treatment groups used in the GM-CSF experiments. The cells were harvested on day 9 of culture. Two wells were pooled per replicate to obtain enough cells for characterization (n=5 replicates per treatment group). The medium from the two wells was removed and the two wells were forcefully washed with staining buffer. The wash was added to the medium, the tubes were centrifuged, and the cells were resuspended in staining buffer. The cell number per treatment replicate was determined by manual counting with trypan blue dye and a hemocytometer.

FACS staining and analysis

Cells ($0.5\text{-}1.0\text{x}10^6$) were dispensed into 12x75mm tubes containing 1 ml of staining buffer. The tubes were centrifuged and the cells were resuspended and incubated with Fc block ™ (BD Pharmingen) or purified anti-FcγRII/III from the 2.4G2 hybridoma (ATCC; Manassas, VA) (1μg/tube, 5 minutes, on ice, in the dark). Primary antibody or isotype control antibody directly conjugated to a fluorochrome was added and the cells were incubated again (1 μg/tube, 30 minutes, on ice, in the dark). The cells were washed with staining buffer to remove excess unbound antibody and then cells were resuspended

in fresh staining buffer. DAPI (1μg/tube) was added to each sample 2-3 minutes before being run on the FACS Vantage® flow cytometer (to account for dead cells). Alternatively cells were fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS (pH 7.4) and stored at 4°C until analysis.

Immunohistochemistry

Immunohistochemistry staining was performed by the Michigan State University Histology Laboratory in the Department of Physiology; Division of Human Pathology. Thin Prep® slides were prepared and stained according to established protocol.[29] Primary antibody was rabbit anti-rat PMN antiserum (Inter-Cell Technologies, Hopewell, NJ, a gift from Dr. R.A. Roth, Michigan State University).

Statistics

Data were analyzed using Instat® version 3.05 and figures were prepared using Prism® version 3.02 (GraphPad Software, San Diego, CA). A Tukey-Kramer one-way ANOVA was used to analyze the raw data with Bartlett's post-test. The GM-CSF experiment was repeated six times (n=1-5/treatment) while the FL experiment was completed three times (n=5/treatment).

Results

Vitamin A requirement for myeloid DC development stimulated with GM-CSF

Murine bone marrow cells were cultured with GM-CSF to stimulate myeloid DC development in the presence of varying concentrations of vitamin A. Positive control cultures contained normal serum as a source of retinol. Negative control cultures contained retinol-depleted serum (CD-FBS) and the vehicle control (DMSO). Treatment groups contained CD-FBS and varying concentrations of atRA or 9cRA. Cultures were assessed for viability, total cell yield and DC content. There were no significant differences in the cell yields or the percent viability among treatment groups (data not shown; $P > .05$). Cell viability of all treatment groups was greater than 90% (data not shown). Dendritic cells were identified by CD11c expression. Positive control cultures contained an average of 62% DC while negative control cultures contained only 12% DC (Figure 1A). Dendritic cell development was significantly restored by addition of either atRA or 9cRA, with DC percent increasing to almost three times greater than the negative control. However, there is no consistent dose-dependent response observed at the concentrations of atRA and 9cRA tested.

We examined the effect of vitamin A on MHC class II (MHC-II) expression within the $CD11c^+$ DC population. The positive control cultures contained the greatest percentage of MHC-II^+ cells within the $CD11c^+$ population and were significantly higher than the negative control (Figure 1B). Addition of higher concentrations of atRA (10^{-9} M and 10^{-8} M) significantly increased the percent MHC-II^+ cells compared to the negative control, but only the highest concentration of 9cRA (10^{-8} M) had this effect. Cultures

containing lower concentrations of retinoids (10^{-10} M atRA and 10^{-9} M 9cRA) had significantly fewer MHC-II$^+$ DC than the negative control.

Effect of vitamin A on expression of co-stimulatory molecules within the CD11c$^+$ population was also analyzed. In the positive control cultures, the percentage of CD11c$^+$ cells that expressed CD80 was significantly less than the negative control (Figure 1C). However, the absolute numbers of DC in the positive control are five times greater than the negative control, therefore the positive control contains a higher absolute number of CD11c$^+$CD80$^+$ DC per culture. We found that 10^{-8}M atRA and 10^{-8}M 9cRA significantly increased the percentage of CD11c$^+$ cells that co-expressed CD80 compared to the negative control (Figure 1C). There was no significant difference in the percent of CD11c$^+$CD86$^+$ DC between the positive and negative controls (Figure 1D). The raw percentage of CD11c$^+$ cells expressing CD86 in the majority of the treatment groups did not reach levels equal to the controls. All-*trans* retinoic acid and 9cRA had opposite effects on percent of CD86$^+$ DC. Increasing the concentration of atRA increased the percent of DC expressing CD86, while increasing the concentration of 9cRA decreased the percent of cells expressing CD86.

Maturation and lineage markers were detected on only a minor percentage of the cells. The DC activation marker CD40 was present on less than 5% of the total population (data not shown). The DC lineage marker CD8α was present on less than 2% of the total population (data not shown). There were no significant differences between any of the treatment groups for either CD40 or CD8α (data not shown).

Mean fluorescence intensity (MFI) is a relative measure of the presence of a molecule on the cell surface. The MFI for CD11c, and MHC-II, CD80, and CD86 within

the $CD11c^+$ population are shown in Table 1. There is a significant relationship between MHC-II MFI and atRA or 9cRA treatment. As the concentration of atRA or 9cRA was increased, the level of MHC-II expression on the DCs decreased significantly. In the cultures treated with atRA, there was a direct relationship between concentration of atRA and the level of co-stimulatory molecules on the cell surface. The co-stimulatory molecules CD80 and CD86 increased on the DCs surface as the concentration of atRA increased, however 9cRA did not demonstrate this same effect. Only 10^{-8} M 9cRA increased the level of CD80 on the DCs surface significantly above that on the negative control and 9cRA seems to have no direct effect on the level of expression of CD86 on DCs (Table 1).

Neutrophils develop in GM-CSF stimulated cultures in the absence of vitamin A

Since the total cell yield among treatment groups did not differ even though DC percent changed dramatically, we next investigated the cell that developed in response to GM-CSF in the absence of vitamin A. Since GM-CSF stimulates myeloid development, and vitamin A has been previously shown to be important in neutrophil development, we chose markers that would identify neutrophils. The marker Gr-1 as well as immunohistochemical staining with anti-rat neutrophil antiserum were used to investigate the alternative cell population. Flow cytometric analysis for Gr-1 revealed that nearly 65% of the cells obtained in negative control cultures were $Gr\text{-}1^+$ neutrophils while <10% of the cells in the positive control cultures containing retinol were $Gr\text{-}1^+$ (Figure 2). The addition of atRA and 9cRA significantly decreased the percent of neutrophils, but atRA and 9cRA had opposite concentration-dependent effects. As the concentration of 9cRA increased, the percent of $Gr\text{-}1^+$ cells decreased. However, as the concentration of atRA

increased, the percent of Gr-1$^+$ cells increased, but remained <30% of the negative control. Immunohistochemical staining confirmed neutrophil development in the absence of vitamin A. Blinded differentials were preformed on cytospin preparations stained with rabbit anti-rat neutrophil antiserum. The results indicated that approximately 12% of cells in the positive control cultures containing retinol were neutrophils while 54% of the negative control was neutrophils (data not shown).

Vitamin A blocks DC development stimulated by FL

The DC marker CD11c was used to determine the percentage of DCs obtained following *in vitro* stimulation of mouse bone marrow cells with FL. All comparisons for DC percent in the treatment groups were done against the negative control. In cultures stimulated with FL, myeloid and lymphoid DCs are normally generated at a ratio of 1:2.[28] Cell yield data revealed that the positive control, 10^{-9} M atRA, 10^{-8} M atRA, 10^{-9} M 9cRA, and 10^{-8} M 9cRA had a significantly lower average cell yield compared to the negative control (Figure 3A). The atRA and 9cRA both appeared to inhibit FL culture cell yield in a dose-dependent fashion. Although the total cell yield was lower in the positive control cultures, the raw percent CD11c$^+$ DC obtained in the positive control cultures is 1.5 times higher than the negative control (Figure 3B). High concentrations of atRA or 9cRA (10^{-8} M) significantly decreased the percent CD11c$^+$ DC obtained in response to FL stimulation.

Within the CD11c$^+$ population, the percentage of cells expressing MHC-II and CD11b (a myeloid lineage marker) were assessed. Because the cell yields for several treatment groups were significantly different from the negative control, the absolute number of DCs expressing these molecules was calculated (Figure 4B & 4D). The

percentage of MHC-II$^+$ DC was slightly higher in the positive control compared to negative control (Figure 4A). MHC-II expression remained relatively consistent throughout the treatment groups, and was only significantly lower with 10^{-9} M atRA and 10^{-8} M 9cRA. Addition of higher concentrations of vitamin A (10^{-9} M atRA, 10^{-8} M atRA, 10^{-9} M 9cRA, and 10^{-8} M 9cRA) dramatically reduced the absolute number of DCs expressing MHC-II compared to the negative control (Figure 4B). The percentage of cells that expressed CD11b increased significantly in the presence of retinoic acid (Figure 4C). Since the total cell yield was adversely affected by high concentrations of vitamin A, the absolute number of DCs expressing CD11b was significantly lower than the negative control in cultures containing 10^{-8} M atRA and 10^{-8} M 9cRA (Figure 4D).

The co-stimulatory molecules CD80 and CD86 were examined in the CD11c$^+$ population. For both CD80 and CD86, the positive control cultures had a greater percentage of cells expressing the co-stimulatory molecules compared to the negative control (Figure 4E & 4G), as well as a significantly greater absolute number of DC expressing these molecules (Figure 4F & 4H). Both 10^{-8} M atRA and 10^{-8} M 9cRA treatment significantly increased the percent of DC expressing CD80 compared to the negative control. Only higher concentrations of atRA (10^{-9} M and 10^{-8} M) were able to significantly increase the percent of CD86$^+$ DC above the negative control. However, the addition of retinoic acid did not increase the absolute number of DCs expressing CD80 or CD86 when compared to the negative control (Figure 4 F and H).

The affect of vitamin A on the MFI of CD11c, MHC-II, CD11b, CD80, and CD86 was assessed for DC obtained by FL stimulation of mouse bone marrow cells. The MFI of CD11c was significantly lower in 10^{-8} M 9cRA compared to the negative control while

the positive control was significantly higher than the negative control (Table 2). The addition of vitamin A significantly enhanced the MFI of MHC-II above the negative control for all concentrations of atRA and 10^{-9} M 9cRA. Compared to the negative control, CD11b MFI was significantly higher in the positive control culture and all vitamin A treatment groups except 10^{-10} M 9cRA. There were no significant differences in the CD80 MFI among any of the treatment groups. The MFI for CD86 was significantly enhanced by 10^{-10} M atRA and 10^{-9} M atRA, but was slightly decreased by 10^{-8} M atRA. Addition of 9cRA did not affect CD86 MFI on the DC.

Discussion

Our data demonstrates that vitamin A is necessary for myeloid DC development from bone marrow hematopoietic progenitors. In the absence of serum retinol, the development of myeloid DC resulting from GM-CSF stimulation of primary bone marrow cells decreases more than 80%. However, the total cell yield in these cultures is not significantly different, indicating that some other cell fate is chosen in the absence of vitamin A. Further investigation reveals that neutrophils are composing a significant proportion of the final cell population when serum retinol is depleted. Both atRA and 9cRA could significantly restore the DC development and significantly inhibit neutrophil development. Since DC developmental stages remain largely uncharacterized, it is difficult to pinpoint exactly what stage of development requires vitamin A. However, our data indicates that vitamin A is essential for DC commitment and is crucial to development of immature myeloid DCs.

The bulk of the literature concerning the role of retinoic acid in myeloid lineage development comes primarily from experiments using cell lines or human acute promyelocytic leukemia cells.[23] The data published from these experiments suggests retinoic acid plays a role in the development and maturation of neutrophils. Our data directly contradicts these published reports. We believe the dramatic difference may stem from the cell populations being studied. The majority of published results are based on cell lines known to have defective RARs, which we believe alters the signaling mechanisms substantially during development and led to the currently held belief that vitamin A promotes neutrophil development. However, our culture system uses primary

cells taken from normal animals, and therefore is not expected to yield identical results to those models that rely on immortal cell lines.

Although retinoic acid enhances myeloid dendritic cell differentiation, it dramatically inhibits lymphoid dendritic cell differentiation in bone marrow cell cultures stimulated with FL. Generation of DCs by FL normally results in a mixture of myeloid and lymphoid DCs at a ratio of 1:2.[28] Our data demonstrates that retinoic acid decreases the total culture cell yield to only about one-third of the negative control. However, the percent $CD11b^+$ cells increase in the presence of vitamin A, implying that retinoic acid inhibits the lymphoid DC development in response to FL much greater than it inhibits myeloid DC development in response to FL.

The effect of RA on the expression of MHC-II and co-stimulatory molecules on DC's suggest that RA may favor development of a Th2 response. A previous study by Cantorna et al. (1994) shows that RA down-regulates the ability of antigen presenting cells to stimulate IFN-γ secretion, although the target cell(s) was not identified.[30] We find the phenotype of the DCs exposed to increasing concentrations of vitamin A have not only a decreased percentage of $CD11c^+MHC\text{-}II^+$ DCs but also a decreased level of expression of MHC-II on the cell. At the same time, these dendritic cells are upregulating co-stimulatory molecules on the surface – especially CD86. This antigen-presenting cell phenotype is reminiscent of $CD8\alpha^-$ DC pulsed with the soluble egg antigen (SEA) from *Schistosoma mansoni*, a Th2 antigen.[31]

Careful analysis of the phenotype of DCs obtained in culture also suggests that the DCs present in negative control cultures stimulated with GM-CSF may not have resulted from *in vitro* development. Since we did not select for progenitor cells from the

bone marrow cell population, we believe that these DCs represent mature dendritic cells that were present in the unseparated bone marrow and were simply maintained in the culture system. This hypothesis is supported by the fact that the DCs in the negative control had a high level of expression of MHC-II and co-stimulatory molecules, which leads us to believe that they were more mature than the DCs in the other treatment groups and were merely sustained in the presence of GM-CSF.

Although we have not yet investigated the molecular mechanism of action of retinoic acid in promotion of myeloid DC development, it is likely that nuclear receptor modification of gene expression must be involved. Both atRA and 9cRA are able to act as ligands for RAR, while only 9cRA can act as a ligand for retinoid X receptors (RXR). Since atRA and 9cRA had similar (but not identical) effects on myeloid DC development, this suggests that RARs must be the primary target of vitamin A action in our culture system. Studies of acute promyelocytic leukemia clearly demonstrate that RARα is necessary for terminal myeloid differentiation, and the presence of a mutated RARα caused by gene translocation blocks cells in the immature promyelocyte stage. Since GM-CSF is known to upregulate RARα expression and signaling activity,[32,33] our studies suggest that holo-RARα promotes final differentiation to myeloid DC in the presence of GM-CSF, while apo-RARα favors neutrophil development in the presence of GM-CSF. We have developed a model hypothesizing that in the steady-state in response to GM-CSF, retinoic acid would promote development of immature myeloid DCs that seed peripheral tissues (Figure 5). However, since serum retinol is precipitously lost in the urine during an acute phase response stimulated by infection,[16,34] we hypothesize that the decrease in available retinoic acid would promote rapid development of neutrophils

instead of myeloid DC. This model correlates with the need to release large numbers of neutrophils during an acute infection, since these cells are short-lived and are rapidly depleted if not replaced. At the same time, existing immature myeloid DC in the peripheral tissue are available to pick up foreign antigen, migrate to draining lymph nodes, and mature into fully-activated antigen-presenting cells that can stimulate an adaptive T lymphocyte response. However, in prolonged vitamin A deficiency, the immature myeloid DC would not be produced and these cells would be expected to be significantly reduced in peripheral tissues. Their absence would result in diminished antibody responses that require Th2 help. This model correlates with the general observation that vitamin A deficiency compromises T lymphocyte dependent antibody responses.

Acknowledgments

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Table 1: Mean Fluorescence Intensity (MFI) of Cell Surface Markers on Dendritic Cells Generated with GM-CSF and varying concentrations of vitamin A.

Culture Treatments	CD11c	MHC class II	CD80	CD86
Positive control	179 ± 18	289 ± 28	54 ± 2[a]	44 ± 0.5[a]
Negative control	182 ± 19	286 ± 20	38 ± 1	87 ± 9
+ 10^{-10} M atRA	198 ± 16	203 ± 28[a]	33 ± 0.8[c]	63 ± 5[a]
+ 10^{-9} M atRA	186 ± 7	112 ± 3[a]	50 ± 1[a]	73 ± 5[a]
+ 10^{-8} M atRA	175 ± 7	91 ± 15[a]	56 ± 4[a]	86 ± 3
+ 10^{-10} M 9cRA	227 ± 13[a]	276 ± 43	36 ± 0.9	73 ± 4[b]
+ 10^{-9} M 9cRA	177 ± 15	250 ± 25	37 ± 0.6	61 ± 3[a]
+ 10^{-8} M 9cRA	188 ± 12	88 ± 3[a]	49 ± 4[a]	68 ± 6[a]

Cell cultures were grown in cIMDM + 10% serum. The positive control contains 10% characterized fetal bovine serum (FBS) which has retinol as the source of vitamin A. All other cultures contain 10% charcoal dextran FBS plus different concentrations of all-*trans* retinoic acid (atRA) or 9-*cis* retinoic acid (9cRA) as indicated. The negative control contains dimethylsulfoxide (the vehicle control). MFI from flow cytometric analysis is expressed as mean ± SEM (n = 5/treatment). Significantly different from negative control ($^{a}P < .001$; $^{b}P < .01$; $^{c}P < .05$).

Table 2: Mean Fluorescence Intensity (MFI) of Cell Surface Markers on Dendritic Cells Generated with Flt-3 ligand (FL) and varying concentrations of vitamin A.

Culture Treatments	CD11c	MHC class II	CD11b	CD80	CD86
Positive control	41 ± 2^{b}	242 ± 12^{a}	151 ± 21^{b}	37 ± 5	29 ± 0.6
Negative control	34 ± 3	130 ± 8	93 ± 6	38 ± 3	26 ± 0.9
+ 10^{-10} M atRA	36 ± 1	227 ± 28^{a}	139 ± 15^{c}	38 ± 3	30 ± 0.7^{a}
+ 10^{-9} M atRA	35 ± 2	241 ± 38^{a}	178 ± 46^{a}	38 ± 3	30 ± 0.9^{a}
+ 10^{-8} M atRA	31 ± 3	201 ± 9^{a}	230 ± 13^{a}	41 ± 3	24 ± 0.5^{c}
+ 10^{-10} M 9cRA	32 ± 1	165 ± 9	76 ± 6	48 ± 12	28 ± 2
+ 10^{-9} M 9cRA	37 ± 3	250 ± 26^{a}	199 ± 11^{a}	40 ± 3	27 ± 0.7
+ 10^{-8} M 9cRA	28 ± 1^{a}	140 ± 12	137 ± 18^{c}	43 ± 2	26 ± 0.4

Cell cultures were grown in cIMDM + 10% serum. The positive control contains 10% characterized fetal bovine serum (FBS) which has retinol as the source of vitamin A. All other cultures contain 10% charcoal dextran FBS plus different concentrations of all-*trans* retinoic acid (atRA) or 9-*cis* retinoic acid (9cRA) as indicated. The negative control contains dimethylsulfoxide (the vehicle control). MFI from flow cytometric analysis is expressed as mean ± SEM (n = 5/treatment). Significantly different from negative control ($^{a}P < .001$; $^{b}P < .01$; $^{c}P < .05$).

Figure 1. Retinoic acid promotes the development of GM-CSF stimulated myeloid lineage DCs and increases their expression of co-stimulatory molecules. Mouse bone marrow cells were cultured in the presence of GM-CSF in medium containing characterized (CHR) FBS, or charcoal-dextran (CD) treated FBS and DMSO vehicle or varying concentrations of either all-*trans* retinoic acid (atRA) or 9-*cis* retinoic acid (9cRA) as indicated. Resulting cells were analyzed by flow cytometry for the percent of cells expressing CD11c as a marker of total DC (A), or $CD11c^+$ DC co-expressing MHC-II (B), CD80 (C) or CD86 (D). Data represents the mean +/- SEM for one representative experiment of six (n = 5/treatment). Significantly different from the negative control ($^*P < .001$; $^{**}P < .01$).

Figure 2. Neutrophil development is significantly enhanced in the absence of retinoic acid. Mouse bone marrow cells were cultured in the presence of GM-CSF in medium containing characterized (CHR) FBS, or charcoal-dextran (CD) treated FBS and DMSO vehicle or varying concentrations of either all-*trans* retinoic acid (atRA) or 9-*cis* retinoic acid (9cRA) as indicated. Resulting cells were analyzed by flow cytometry for the percent of cells expressing Gr-1. Note the vehicle control contains five times more $Gr\text{-}1^+$ cells than the positive control and is three times higher than the highest retinoic acid treatment group. The number of neutrophils in culture are significantly different from the negative control ($^*P < .001$). Data represents the mean +/- SEM (n = 3/treatment).

Figure 3. Retinoic acid significantly decreases cell yield from bone marrow cells stimulated with FL, while the percent of DCs remains constant. Mouse bone marrow

cells were stimulated with flt-3 ligand (FL) in medium containing characterized (CHR) FBS, or charcoal-dextran (CD) treated FBS and DMSO vehicle or varying concentrations of either all-*trans* retinoic acid (atRA) or 9-*cis* retinoic acid (9cRA) as indicated. Resulting cells were analyzed by flow cytometry for the percent $CD11c^+$ DCs (A). The absolute number of $CD11c^+$ DCs obtained from each culture was calculated by multiplying the percent DCs times the cell yield/culture (B). Data represents one representative experiment of three (n = 5/treatment). The mean +/- SEM are shown. Significantly different from negative control, $*P < .001$.

Figure 4. Retinoic acid inhibits the expression of MHC-II on FL-derived DCs, but promotes the expression of co-stimulatory molecules CD80 and CD86. Mouse bone marrow cells were stimulated with flt-3 ligand (FL) in medium containing characterized (CHR) FBS, or charcoal-dextran (CD) treated FBS and DMSO vehicle or varying concentrations of either all-*trans* retinoic acid (atRA) or 9-*cis* retinoic acid (9cRA) as indicated. Resulting cells were analyzed by flow cytometry for the percent of $CD11c^+$ DCs co-expressing MHC-II (A), CD11b (C), CD80 (E), or CD86 (G). Absolute numbers of these dual positive cells obtained per culture treatment were calculated and are shown: $CD11c^+MHC\text{-}II^+$ (B); $CD11c^+CD11b^+$ (D); $CD11c^+CD80^+$ (F); and $CD11c^+CD86^+$ (H). Data represents one representative experiment of three (n = 5/treatment). The mean +/- SEM are shown. Significantly different from negative control ($*P < .001$; $**P < .01$; $***P < .05$).

Figure 5. Hypothetical model for effect of retinoic acid on GM-CSF-stimulated myeloid DC and neutrophil development during disease-free steady state versus during acute-phase response induced by infection. During a healthy steady-state (A), GM-CSF in combination with retinoic acid (RA) promotes development of immature myeloid DCs that seed the periphery and is permissive for neutrophil development at low levels. During an acute-phase response (B), loss of RA signaling does not allow for myeloid DC development and instead favors neutrophil development in response to GM-CSF stimulation.

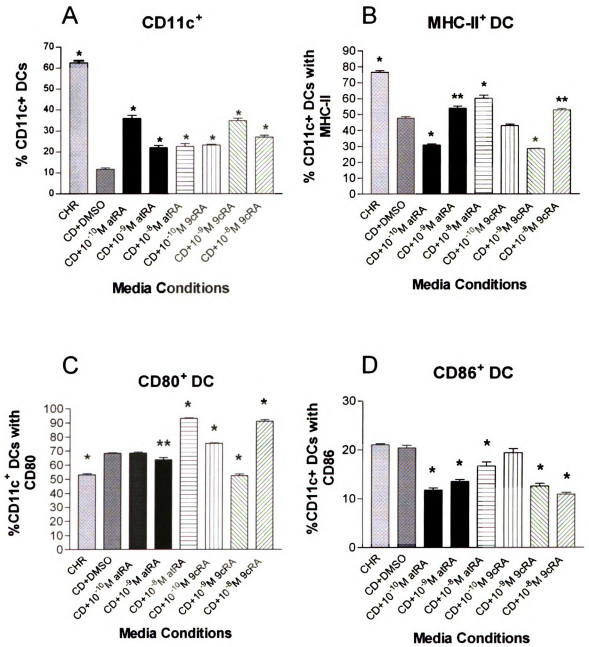

A
CD11c+
% CD11c+ DCs
Media Conditions
B
MHC-II+ DC
% CD11c+ DCs with MHC-II
Media Conditions
C
CD80+ DC
%CD11c+ DCs with CD80
Media Conditions
D
CD86+ DC
%CD11c+ DCs with CD86
Media Conditions
CHR
CD+DMSO
CD+10-10M aRA
CD+10-9M aRA
CD+10-8M aRA
CD+10-10M 9cRA
CD+10-9M 9cRA
CD+10-8M 9cRA

Figure 1

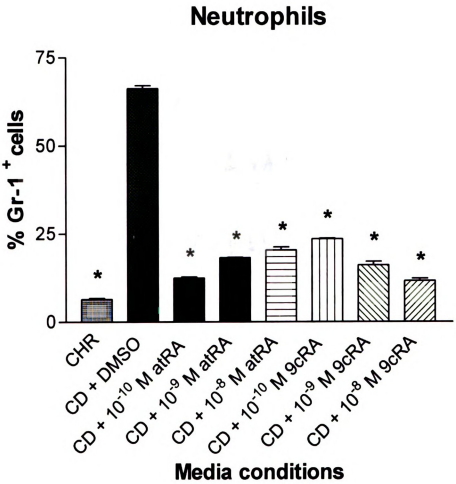
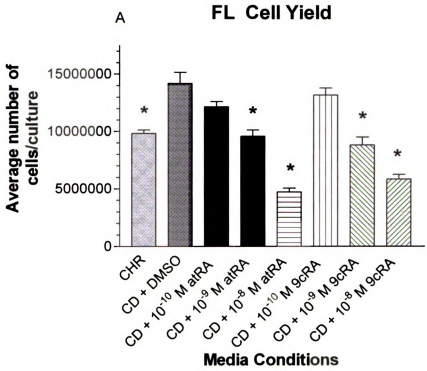
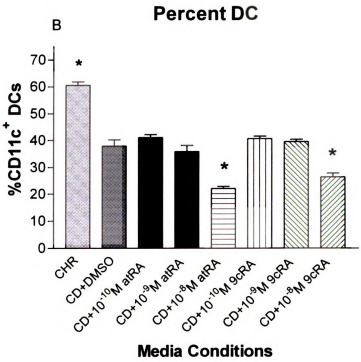

Neutrophils
% Gr-1 + cells
75
50
25
0
*
CHR
CD + DMSO
CD + 10-10 M atRA
CD + 10-9 M atRA
CD + 10-8 M atRA
CD + 10-10 M 9cRA
CD + 10-9 M 9cRA
CD + 10-8 M 9cRA
Media conditions

Figure 2


A
FL Cell Yield
Average number of cells/culture
15000000
10000000
5000000
0
CHR
CD + DMSO
CD + 10-10 M atRA
CD + 10-9 M atRA
CD + 10-8 M atRA
CD + 10-10 M 9cRA
CD + 10-9 M 9cRA
CD + 10-8 M 9cRA
Media Conditions


B
Percent DC
%CD11c+ DCs
70
60
50
40
30
20
10
0
CHR
CD+DMSO
CD+10-10M atRA
CD+10-9M atRA
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CD+10-10M 9cRA
CD+10-9M 9cRA
CD+10-8M 9cRA
Media Conditions

Figure 3

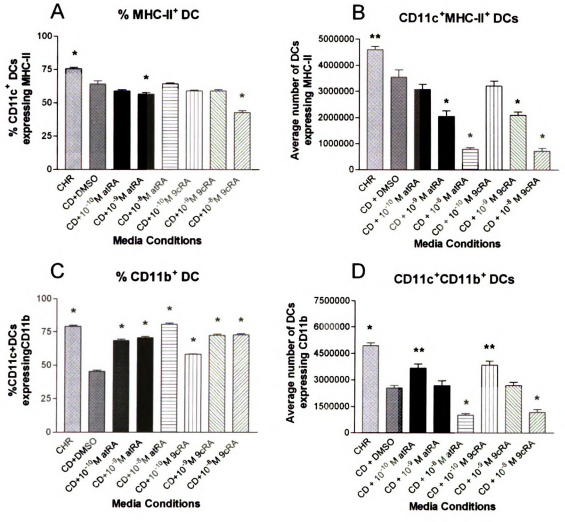
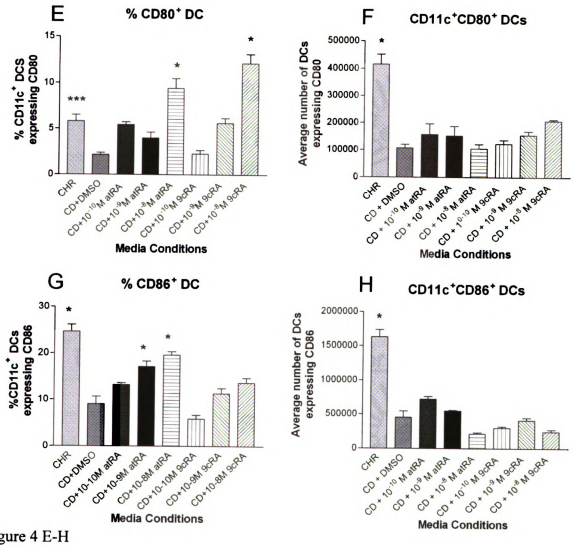

A
% MHC-II+ DC
% CD11c+ DCs expressing MHC-II
100
75
50
25
0
CHR
CD+DMSO
CD+10-10M atRA
CD+10-9M atRA
CD+10-8M atRA
CD+10-10M 9cRA
CD+10-9M 9cRA
CD+10-8M 9cRA
Media Conditions
B
CD11c+MHC-II+ DCs
Average number of DCs expressing MHC-II
5000000
4000000
3000000
2000000
1000000
0
CHR
CD + DMSO
CD + 10-10 M atRA
CD + 10-9 M atRA
CD + 10-8 M atRA
CD + 10-10 M 9cRA
CD + 10-9 M 9cRA
CD + 10-8 M 9cRA
Media Conditions
C
% CD11b+ DC
%CD11c+DCs expressingCD11b
100
75
50
25
0
CHR
CD+DMSO
CD+10-10M atRA
CD+10-9M atRA
CD+10-8M atRA
CD+10-10M 9cRA
CD+10-9M 9cRA
CD+10-8M 9cRA
Media Conditions
D
CD11c+CD11b+ DCs
Average number of DCs expressing CD11b
7500000
6000000
4500000
3000000
1500000
0
CHR
CD + DMSO
CD + 10-10 M atRA
CD + 10-9 M atRA
CD + 10-8 M atRA
CD + 10-10 M 9cRA
CD + 10-9 M 9cRA
CD + 10-8 M 9cRA
Media Conditions

Figure 4 A-D


E
% CD80+ DC
% CD11c+ DCS expressing CD80
***
*
*
CHR
CD+DMSO
CD+10-10M atRA
CD+10-9M atRA
CD+10-8M atRA
CD+10-10M 9cRA
CD+10-9M 9cRA
CD+10-8M 9cRA
Media Conditions
F
CD11c+CD80+ DCs
Average number of DCs expressing CD80
*
CHR
CD + DMSO
CD + 10-10 M atRA
CD + 10-9 M atRA
CD + 10-8 M atRA
CD + 10-10 M 9cRA
CD + 10-9 M 9cRA
CD + 10-8 M 9cRA
Media Conditions
G
% CD86+ DC
%CD11c+ DCs expressing CD86
*
*
*
CHR
CD+DMSO
CD+10-10M atRA
CD+10-9M atRA
CD+10-8M atRA
CD+10-10M 9cRA
CD+10-9M 9cRA
CD+10-8M 9cRA
Media Conditions
H
CD11c+CD86+ DCs
Average number of DCs expressing CD86
*
CHR
CD + DMSO
CD + 10-10 M atRA
CD + 10-9 M atRA
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CD + 10-10 M 9cRA
CD + 10-9 M 9cRA
CD + 10-8 M 9cRA
Media Conditions

Figure 4 E-H

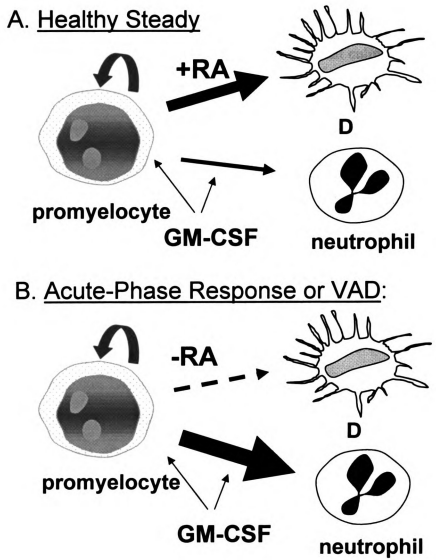
A. Healthy Steady
+RA
D
promyelocyte
GM-CSF
neutrophil
B. Acute-Phase Response or VAD:
-RA
D
promyelocyte
GM-CSF
neutrophil

Figure 5

Supplemental Data for the Role of Retinoic Acid in Dendritic Cell Development

Pertinent supplemental information regarding the role of vitamin A in DC development that was not included in the manuscript has been included in the following section. This information includes a comparison of the different culture methods used in the manuscript, representative raw data from flow cytometry for cultures stimulated with GM-CSF, and the immunohistochemistry images that went unpublished.

The culture methods in the manuscript utilizing GM-CSF and FL, published by Inaba et al. (48) and Brasel et al. (10) respectively, are very different from one another (Figure 5). Cultures stimulated with GM-CSF (20 ng/ml) were initiated with 1 million bone marrow cells in 10 ml of cIMDM plus 10% characterized or charcoal dextran FBS. Cultures stimulated with FL (200 ng/ml) were initiated with 5 million bone marrow cells in 5 ml of mIMDM plus 10% characterized or charcoal dextran serum. The charcoal dextran FBS cultures were further divided into treatment groups with atRA (10^{-8}, 10^{-9}, and 10^{-10} M) or 9cRA (10^{-8}, 10^{-9}, and 10^{-10} M) in both culture systems. Cultures stimulated with GM-CSF underwent a series of medium additions and changes over the course of the 10-day culture. These changes in medium took place on day 3, day 6 and day 8 of culture. The purpose of these medium additions and changes was to give the culture a continuous supply of GM-CSF, and nutrients including vitamin A (except in the controls which did not receive additional vitamin A). Cultures stimulated with FL did not undergo the changes in medium. FL cultures received their total allotment of cytokine and vitamin A on the first day of culture and therefore did not likely maintain a continuous supply throughout the culture period. Cultures stimulated with GM-CSF were harvested on day 10, while cultures stimulated with FL were harvested on day 9. These

subtle differences in culture procedure account for the dramatic difference in DC yield between the two published culture methods.

Several populations of DCs were present in cultures stimulated with GM-CSF. A representative example of raw flow cytometry data is in Figure 6. The positive control (characterized FBS) contains three distinct populations of dendritic cells: $CD11c^{hi}MHC\text{-}II^{lo}$, $CD11c^{int}MHC\text{-}II^{int}$ and $CD11c^{hi}MHC\text{-}II^{hi}$. The negative control contains a small population of $CD11c^{hi}MHC\text{-}II^{hi}$ DCs. We believe this population resulted from preexisting DCs in the bone marrow and was maintained in the presence of GM-CSF. A representative example of a culture treated with 10^{-9} M atRA shows a shift in populations compared to the positive control, however all three populations remain present. The same is true for the representative culture treated with 10^{-9} M 9cRA.

In the manuscript, we were unable to publish the data pertaining to the immunohistochemistry staining for neutrophils. This information is provided in Figure 7. Images in this thesis/dissertation are printed in color.

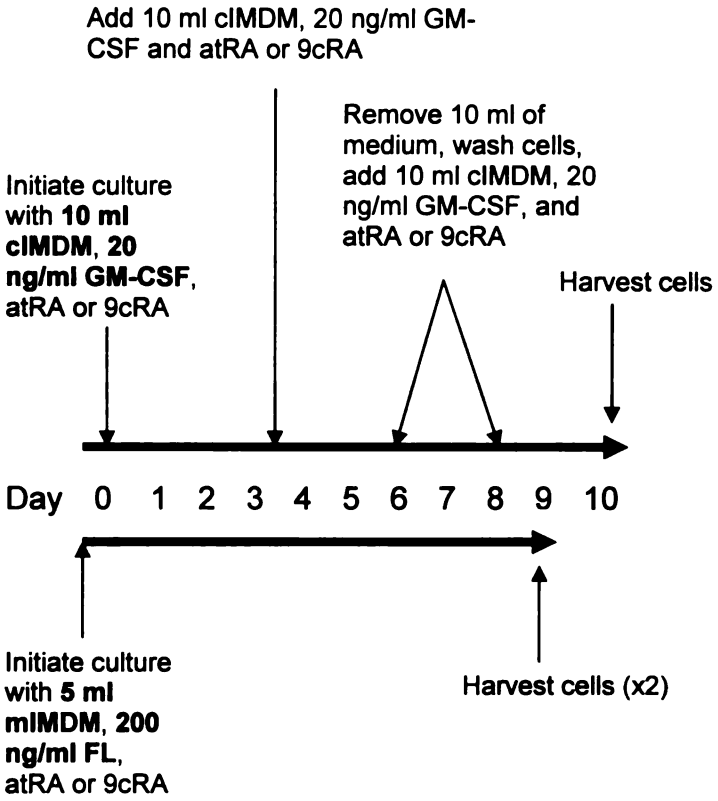

Figure 5. Bone Marrow Culture Systems: Stimulation with GM-CSF vs. FL.

One million bone marrow cells were stimulated with GM-CSF and cultured according to the protocol described for the top arrow. Five million bone marrow cells were stimulated with FL and cultured according to the protocol described for the bottom arrow.

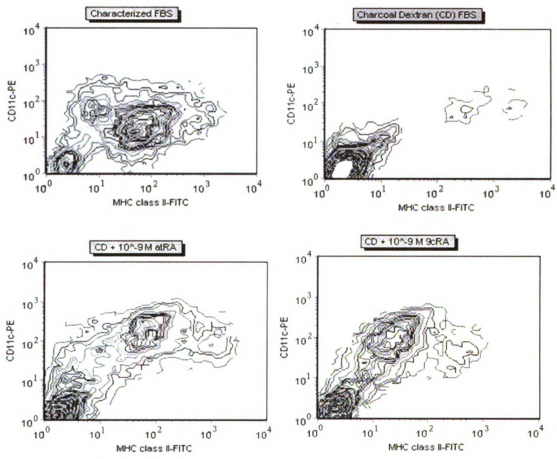

Figure 6. Representative Flow Cytometry Data from Cultures Stimulated with GM-CSF.

The contour plots show MHC class II-FITC on the X-axis and CD11c-PE on the Y-axis. The positive control (Characterized FBS, upper left panel) contains three populations of DCs with the following phenotype: $CD11c^{hi}MHC\text{-}II^{lo}$, $CD11c^{int}MHC\text{-}II^{int}$ and $CD11c^{hi}MHC\text{-}II^{hi}$. The negative control (Charcoal Dextran FBS, upper right panel) contains a small preexisting population of DCs ($CD11c^{hi}MHC\text{-}II^{hi}$) that was likely maintained by the presence of GM-CSF. Both representative cultures of 10^{-9} M atRA (lower left panel) and 10^{-9} M 9cRA (lower right panel) contain the $CD11c^{hi}MHC\text{-}II^{lo}$, $CD11c^{int}MHC\text{-}II^{int}$ and $CD11c^{hi}MHC\text{-}II^{hi}$ DC populations, although at different distributions than the positive control.

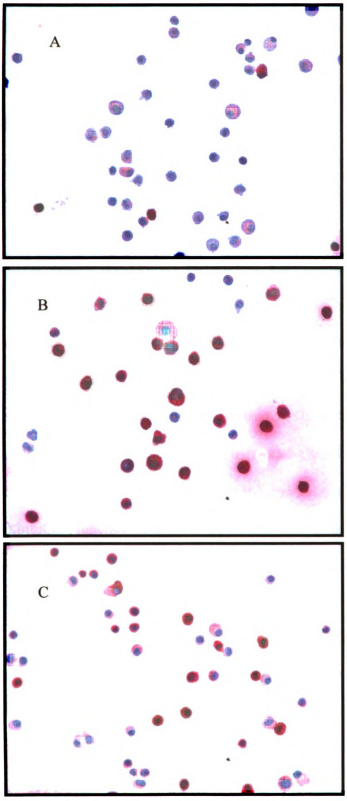

Figure 7. Immunohistochemistry Staining of Cultures Stimulated with GM-CSF Reveals Neutrophil Development in the Absence of Vitamin A.

(100 x magnification) Blind differentials were preformed on cytospin preparations stained with rabbit anti-rat neutrophil antiserum (+ results are red). The results indicated that approximately 12% of cells in the positive control (A) cultures containing retinol were neutrophils while 54% of the negative control were neutrophils (B). (C) The addition of 10^{-9} M atRA to negative control cultures shows a decrease in positive-stained cells (neutrophils).

Proposed Molecular Mechanism of Retinoic Acid in Dendritic Cell Development

Our understanding of the influence of hormones and nutritional factors over gene transcription has advanced dramatically over the past two decades (81). Pluripotent hematopoietic stem cells have the capacity to differentiate into erythroid, lymphoid and myeloid precursors. Cellular differentiation is coordinated and regulated by the expression of cytokines, colony-stimulating factors, receptors, and transcription factors (102). Several transcription factors have been implicated in the differentiation of myeloid lineage cells (31,26). Of these studies, several have explored the notion that vitamin A may exert an effect on cellular differentiation through transcriptional regulation. These studies conclude that vitamin A is necessary for the development and terminal differentiation of neutrophils (reviewed in 33 and 19). However, a translocation that involves the retinoic acid receptor α gene on chromosome 17 is present in cell lines used in several experiments (reviewed in 103 and 19) and this translocation has been shown to inhibit the function of normal RARs. In addition, the concentrations of vitamin A used in several of these experiments are above 10^{-5} M. This level of vitamin A has been shown to force the expression of neutrophil development (72, 20).

Granulocytes, monocytes, and dendritic cells can be generated from a common myeloid progenitor (31, 102). The transcription factors that are involved in the differentiation of monocytes, early granulopoiesis, and terminal granulopoiesis have been characterized. Early granulopoiesis is characterized by an up regulation of CCAAT/enhancer binding protein α (C/EBPα), PU.1, retinoic acid receptors, CBF (core binding factor), and c-Myb (cellular-myeloblastosis). One or more of the following

initiates monopoiesis: Maf-B, c-Jun, or Erg-1 and monopoiesis is dependent on the upregulation of PU.1. Terminal neutrophil differentiation is characterized by the up regulation of C/EBPε, PU.1, Sp1, CDP (CCAAT-displacement protein), and HoxA10 (31). PU.1 and GATA-2 are specifically required for eosinophil differentiation in mice (39). The EML stem cell line has also been used to determine the expression of transcription factors during myelopoiesis. However, this cell line expresses a dominant negative RARα molecule and may give misleading results when it comes to the decision between DCs and neutrophils development (81). The most likely transcription factors that may interact with retinoid receptors to regulate the decision between dendritic cell and neutrophil differentiation are PU.1 and C/EBPs because they are key regulators for the development and function of myeloid cells (49).

CCAAT/enhancer binding proteins (C/EBPs) are transcription factors that function in energy metabolism, inflammation, cellular proliferation, cellular differentiation of adipocytes and other cell types, and in cellular differentiation of myeloid lineage cells (76). These transcription factors play a role in mediating the effects of hormones and nutrients by cooperating with other transcription factors, which modulate transcription (81). There are six different forms encoded by six different genes denoted by the Greek letters α, β, γ, δ, ε, and ζ (76). Eight or more isoforms can be generated from the six genes through the use of different start codons, differential splicing, and alternative promoters (76, 81). The C-terminus contains a highly conserved basic leucine zipper region for dimerization and DNA binding (76). C/EBPα, β, γ, δ, and ε can heterodimerize, and share the same DNA binding site, RTTCGCYAAY (where R is A or G and Y is C or T) (76). All forms except C/EBPζ have activation domains that

stimulate transcription. C/EBPζ does not have an activation domain and therefore represses transcription. C/EBPα is expressed at high levels in adipose tissue, liver, intestine, lung, peripheral blood mononuclear cells, and placenta. C/EBPβ is found at high levels in liver, intestine, lung, adipose tissue, spleen, kidney, and myelomonocytic cells. C/EBPδ is found in adipose tissue, lung, and intestine. C/EBPγ and C/EBPζ are ubiquitous, while C/EBPε is restricted to myeloid and lymphoid cells (76).

Myeloid cells express numerous genes with binding sites for C/EBPs. As noted above, C/EBPα, C/EBPβ, and C/EBPε are expressed in myeloid cells (76). It seems that C/EBPα expression increases in early myeloid progenitors and decreases during granulocytic differentiation. C/EBPβ is specifically upregulated during monocyte differentiation. C/EBPε is preferentially expressed during granulocyte differentiation (76).

C/EBPs play an important role in determining neutrophil differentiation over dendritic cell differentiation. C/EBPα knock-out mice have a profound block in granulocyte differentiation (20). Treatment of cells from these mice with GM-CSF, G-CSF, or atRA at high concentrations (10^{-5} M) enhanced granulocytic differentiation through a pathway independent of C/EBPα (20). Another experiment used C/EBPα$^{-/-}$ acute promyelocytic leukemia (APL) cell line. This experiment found that C/EBPα did not respond to atRA or G-CSF and induced neutrophil differentiation through the induction of C/EBPε or C/EBPβ instead of C/EBPα (112). Loss of C/EBPε results in absolute neutropenia (56). C/EBPε has been shown to have a RARE in its promoter region and can be induced through the RARα pathway (72). In U937 cells,

C/EBPε expression was forced when exposed to RA concentrations of greater than or equal to 10^{-6} M. These elevated concentrations of vitamin A forced terminal granulocyte differentiation that was not present at the lower concentrations of vitamin A (72). C/EBPε knock-out models show failure of the organism to produce normal neutrophils (56) further supporting the specific role of C/EBP in granulopoiesis. Regarding DCs, a dominant negative C/EBP blocks granulocyte-macrophage commitment in progenitors and induces Langerhan's cell commitment in the absence of TNFα (49).

PU.1 is an ets family transcription factor required for the proper development of both myeloid and lymphoid progenitors (39). Its upregulation is an early event during the differentiation of multipotent progenitors (17). PU.1 directs the differentiation of these progenitors into macrophages, neutrophils, mast cells, B lymphocytes and myeloid dendritic cells (107, 39). In fact, PU.1 is expressed in myeloid DCs and is crucial for myeloid DC development (3).

PU.1 has been shown to bind the promoters of several macrophage genes including the macrophage scavenger receptor and the cell surface molecule CD11b (17). CD11b expression is directly regulated by PU.1 (107). Furthermore, PU.$1^{-/-}$ mice fail to express CD11b and CD64 all together (56). PU.1 has also been shown to directly activate the transcription of genes encoding the subunits of the receptors for lineage-restricted cytokines including GM-CSF, G-CSF, M-CSF, and IL-7 (107).

The expression of C/EBPα blocks the function of PU.1 (77). This occurs through the leucine zipper in the DNA binding domain of C/EBPα interacting with the DNA binding domain of PU.1 (77). C/EBPα binding to PU.1 causes the displacement of the PU.1 co-activator c-Jun (77).

C/EBPα and PU.1 play reciprocal roles when it comes to DC differentiation (49). A dominant negative C/EBPα expressed in U937 cells resulted in the differentiation of DCs (49). U937 were not known to differentiate into DCs until this experiment (49). Furthermore, C/EBPα is a negative regulator when it comes to DC commitment, while PU.1 actually facilitates DC development (49).

Very few have looked at the differentiation of DCs and none have looked at the role vitamin A plays in the differentiation of DCs until now. Evidence published by others supports the roles of C/EBPs and PU.1 in the differentiation of neutrophils and DCs. This evidence combined with our findings of increased CD11b expression in response to vitamin A leads to the proposal of a hypothesis for further study. I hypothesize that the transcription of PU.1 is atRA dependent and the concentration of atRA acts as a regulator between neutrophil and DC development by controlling the ratio of C/EBP to PU.1.

Chapter 3: Aspects of Dendritic Cell Maturation

Contact with inflammatory cytokines, T cell derived signals, or microbial products cause DC maturation (27). It is hypothesized that the pattern of recognition by the DC determines the production of cytokines by individual DC subsets (27). Murine DC exposure to IL-15 causes an upregulation of co-stimulatory molecules, an increase in IFN-γ production, and an increase in stimulation of antigen specific $CD8^+$ T cell proliferation (62). Cella et al. (15) discovered the interaction of CD40 on the DC with CD40L on the T cell regulates DC function by increasing IL-12 production, co-stimulatory molecules CD80 and CD86, the adhesion molecule ICAM-1, and HLA-DQ (MHC class II) on human DCs. The release of heat shock proteins by tumor cells, as a result of injury, or by virus-induced apoptotic cell death promotes immunity through DC maturation (46, 94). Exposure to bacterial DNA (CpG oligodeoxynucleotides) upregulates CD40, CD80 and MHC class II and also increases the production of the cytokines IL-12, TNF-α and IL-6 (98). DCs phagocytize yeast and germ tubes from *Candida albicans*. Both forms of the fungus initiate DC maturation and an increase in IL-12 production (80).

Recruitment of dendritic cells to the site of inflammation by chemokines can also initiate the maturation process. Chemokines are small molecular weight proteins (8-10 kD) that regulate white blood cell migration and activation (25). In humans, immature DCs migrate in response to CC chemokines such as macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES (regulated on activation, normal T cell expressed and secreted), and monocyte chemotactic protein (MCP)-3 (58, 25). It has also been shown that the C-type lectin receptor DC-SIGN plays a role in DC migration (30).

Once in contact with antigen or other stimuli, dendritic cells respond to different chemokines and egress from the site of inflammation. After stimulation with LPS, TNF-α or IL-1β, human DCs no longer respond to MIP-1α, MIP-1β, or MCP-3 (58). Instead there is an enhanced response to MIP-3β, and stromal cell derived factor (SDF)-1α (a CXC chemokine)(58). Likewise, stimulation with CD40L inhibits response to MIP-1α, MIP-1β, and RANTES (97).

Chemokine receptors change with DCs activation state. Immature human DCs express high levels of CCR1, CCR2, CCR5, CCR6 (receptor for MIP-3α), and CXCR1 (58, 90, 25). The ligands for these chemokine receptors are produced during inflammation. LPS or CD40L stimulation results in an up regulation of CXCR4 (58) and CCR7 mRNA and surface expression (90, 25). Murine DCs also show an increase in CCR7 upon stimulation (111). CCR7 responds to MIP-3β, which is produced by lymphoid organs (90, 25). Upon ligation with CD40, there is a complete down regulation of CCR1 and CCR5 (88). These different patterns of chemokine receptor expression are consistent with primary and inflammatory phases of DC function.

Antigen capture is a crucial step in initiating an immune response and DCs perform this function using many unique routes. DCs use macropinocytosis to allow for continuous internalization of large volumes of fluid in order to detect soluble antigen (89). Receptor mediated phagocytosis of antigen-antibody complexes occurs through Fc receptors for IgG and IgE (87, 78, 63). Internalization of antigen through FcγRII/III induces DC maturation similar to the effects of LPS stimulation and allows for the promotion of efficient MHC class II and MHC class I presentation from exogenous IgG-antigen complexes (78). Internalization of IgE-antibody complexes is mediated via FcεR

(63). Lectin and lectin-like receptors on DCs are members of the calcium dependent C-type lectin family and are associated with antigen uptake of carbohydrate-bearing pathogen-derived antigen (reviewed by 30). Other molecules such as immunoglobulin-like transcript (ILT)-3 in humans, down regulated by activation (DORA) in humans, and Cdc 42 in mice have been implicated in antigen uptake and show down regulation upon DC stimulation with CD40-L or other DC maturation stimuli (14, 6, 34).

Toll-like receptors (TLR) recognize specific microbial products and play a unique role in the activation of dendritic cells. The TLR family is composed of at least ten members and is important for initiating innate immune responses against microbial pathogens. TLR-2 recognizes microbial lipopeptides including peptidoglycan and lipoteichoic acid (42). DC activation through TLR-2 induces immunomodulatory genes [particularly nuclear factor (NF)-κB] that lead to the production of pro-inflammatory cytokines (101). TLR-3 recognizes double stranded (ds) DNA and polyinosine-polycytidylic acid [poly (I:C)]. Upon TLR-3 ligation with dsDNA or poly (I:C), the transcription factor NF-κB is activated through a MyD88 dependent or a MyD88-independent pathway respectively, causing the transcription of pro-inflammatory cytokines (2). TLR-4 specifically recognizes LPS, a potent stimulator of DC maturation (79). A highly conserved site on bacterial flagellin is recognized by TLR-5 (92). Unmethylated CpG dinucleotides found in bacterial DNA mediate a DC response through TLR-9 (41).

Cell-to-cell interactions not only cause DC maturation, but also benefit other cell populations. Natural killer (NK) cell cytotoxicity is enhanced after culture with DC in the presence of *Mycobacterium tuberculosis*, IFN-α, or LPS. Culturing activated human NK

cells with immature human DCs results in mature DCs with enhanced surface marker expression of MHC class II, CD80, CD86, CD83, and CCR7 (35). The DC-NK cell interaction requires cell-to-cell contact to amplify cytokine production exponentially (75). CD1 restricted T cells (also known as γδ T cells) can promote DC maturation by recognizing CD1 in the absence of foreign antigen. Group 2 CD1 molecules in both mice and humans mediate the activation of innate or regulatory T cells independent of foreign antigen (106). The interaction of DCs with CD1 restricted T cells also requires cell-to-cell contact. When DCs were cultured with γδ T cells in the presence of anti-CD1, maturation of DCs was blocked. The DC CD1 interaction with γδ T cells is hypothesized to promote rapid activation of DCs during microbial infection (57).

Dendritic cell maturation can be inhibited by pathogens attempting to evade the immune system, tumors, nutritional and other various factors. DCs infected with measles virus and stimulated with CD40 decrease their co-stimulatory capacity and production of IL-12, forcing the DC back to an immature phenotype (32). Human DCs in contact with tumor cells expressing decoy receptor 3 (a member of the TNF-R superfamily) down regulate CD40, CD80, CD54, and MHC class II, while CD86 expression increases. The resulting DC is induced by the cancerous cell to promote a Th2 response when a Th1 response is in order (44). Pharmacological doses of vitamin D have been shown to inhibit dendritic cell maturation (73). Ligation of CD47 (an adhesion molecule) on human DCs also counteracts maturation of DCs and helps the cell to retain its endocytic activity (23). The inhibition of dendritic cell maturation may play an important role in modulating immune responses toward Th2 or induce regulatory T cells that block immune activation.

The role of retinoic acid in dendritic cell maturation

Dendritic cells (DCs) are potent antigen presenting cells present in almost every tissue in the body. They are a critical link between the innate and adaptive immune systems. Dendritic cells not only initiate immune responses, but they also help control immunity by promoting tolerance, or regulating the balance of the Th1/Th2 responses. DC development begins with bone marrow progenitors and requires cytokines, colony-stimulating factors, receptors, and transcription factors (56).

Dendritic cell maturation is a complex process that can be induced by bacteria and bacterial derived antigens, inflammatory cytokines, ligation of cell surface receptors and viral products. There are several phenotypic and functional changes that occur during DC maturation including the down regulation of endocytosis, change in cell surface molecules to prepare for interaction with T lymphocytes, alteration of the cytoskeleton and the formation of dendrites, and other possible genetic alterations. Many of the factors influencing DC maturation were detailed in the previous section. However, the nutritional aspects of DC maturation have not been fully investigated. We hypothesize that retinoic acid will not affect the ability of immature DCs to mature when stimulated with LPS. This investigation considers the role of vitamin A in modulating dendritic cell maturation.

Materials and methods

Mice

Male 3-to 4-week-old BALB/cJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were maintained according to institutional guidelines set by

University Laboratory Animal Resources under a protocol approved by Michigan State University's Institutional Care and Use Committee. The murine diet consisted of solid pellets containing all essential macro and micronutrients (including vitamin A). The mice were killed at the age of 5-12 weeks by carbon dioxide asphyxiation.

Reagents

Characterized Fetal Bovine Serum (FBS) and Charcoal/Dextran treated FBS (CD-FBS) were purchased from HyClone (Logan, UT). The medium (cIMDM) consisted of IMDM (BioWhittaker, Walkersville, MD) supplemented with 10% serum (FBS or CD-FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (BioWhittaker) and 10^{-4} M β-mercaptoethanol (Sigma, St. Louis, MO). Vitamin A, in the form of atRA or 9cRA (Sigma), was dissolved in HYBRI-MAX ® dimethylsulfoxide (DMSO; Sigma), and was stored in single use aliquots at -70°C in the dark under an argon atmosphere. Purified recombinant GM-CSF was purchased from PeproTech (Rocky Hill, NJ) or GM-CSF containing supernatant was produced from the X-63 cell line transfected with GM-CSF cDNA (Zal et al. 1994). The concentration of GM-CSF was determined by ELISA (BD Biosciences, San Diego, CA). Lipopolysaccharide (LPS; Sigma) was used to induce the maturation of DCs. The following antibodies were used to label the cells: fluorescein isothiocyanate (FITC) conjugated anti-CD8α (53-6.7 clone; Rat IgG_{2a}, κ), FITC conjugated anti-CD86 (GL1 clone; Rat IgG_{2a}, κ), FITC conjugated anti-CD40 (3/23 clone; Rat IgG_{2a}, κ), FITC conjugated anti-MHC class II ($I\text{-}A^d$) (AMS-32.1 clone; Mouse IgG_{2b}, κ), FITC conjugated anti-CD80 (16-10A1 clone; Armenian Hamster IgG_{2*}, κ), FITC conjugated anti-CD11b (M1/70 clone; Rat IgG_{2b}, κ), R-

Phycoerythrin (R-PE) conjugated anti-CD11c (HL3 clone; Armenian Hamster IgG_1, λ). Antibodies and the appropriate isotype controls were purchased from BD Biosciences.

GM-CSF culture and stimulation

Bone marrow cells, obtained from the femurs and tibias of mice, were cultured *in vitro* with 20 ng/ml GM-CSF using an adaptation of the protocol established by Inaba *et al*, (48). Cells were grown in 10 ml of cIMDM supplemented with 10% FBS (positive control) until day eight. On the eighth day, the medium was replaced and cultures were divided into four treatment groups (n = 5). The positive control contained characterized serum as the source of vitamin A. The remaining treatment groups were placed in medium containing charcoal dextran FBS and supplemented with concentrations of retinoic acid including no retinoic acid (the DMSO vehicle control), 10^{-9} M atRA, or 10^{-9} M 9cRA. On the ninth day of culture, the dendritic cells were stimulated with 100 ng/ml LPS for a period of 24 hours. Two cultures (in addition to the 5 replicates) in the positive control did not receive LPS and were used as an unstimulated control.

Cells were harvested on day 10 of culture. This process involved removing the medium and forcefully washing the cells from each individual plate with staining buffer (1% FBS, 0.1% sodium azide, in phosphate buffered saline, sterile filtered, 4°C, pH 7.4). The culture medium and the wash were pooled and centrifuged (300xg, 10 minutes, 4°C), the supernatant was discarded, and the pellet was resuspended in 4 ml staining buffer. A small amount (10 µl) of the cells were removed to determine the cell count and viability using trypan blue dye (Sigma) and a hemocytometer. The resuspended cells were stored on ice.

FACS staining and analysis

Cells ($0.5\text{-}1.0\text{x}10^6$) were dispensed into 12x75mm tubes containing 1 ml of staining buffer. The tubes were centrifuged, the supernatant was discarded, and the cells were resuspended, and incubated with Fc block ™ (BD Pharmingen) or purified anti-FcγRII/III from the 2.4G2 hybridoma (ATCC; Manassas, VA) (1μg/tube, 5 minutes, on ice, in the dark). The cells were incubated with a primary antibody or the corresponding isotype control directly conjugated to a fluorochrome (1 μg/tube, 30 minutes, on ice, in the dark). The cells were washed with staining buffer to remove the excess antibodies and 1 ml of staining buffer was added to each tube. DAPI (1μg/tube) was added approximately 2-3 minutes before each tube was run on the FACS Vantage® flow cytometer to account for dead cells. Alternatively, cells were fixed in 2% para-formaldehyde in PBS, pH 7.4 (Electron Microscopy, Fort Washington, PA) and stored at 4°C until analysis.

Statistics

Data were analyzed using Instat® and figures were prepared using Prism® (Graphpad). A Tukey-Kramer one-way ANOVA was used to analyze the raw data (n = 5).

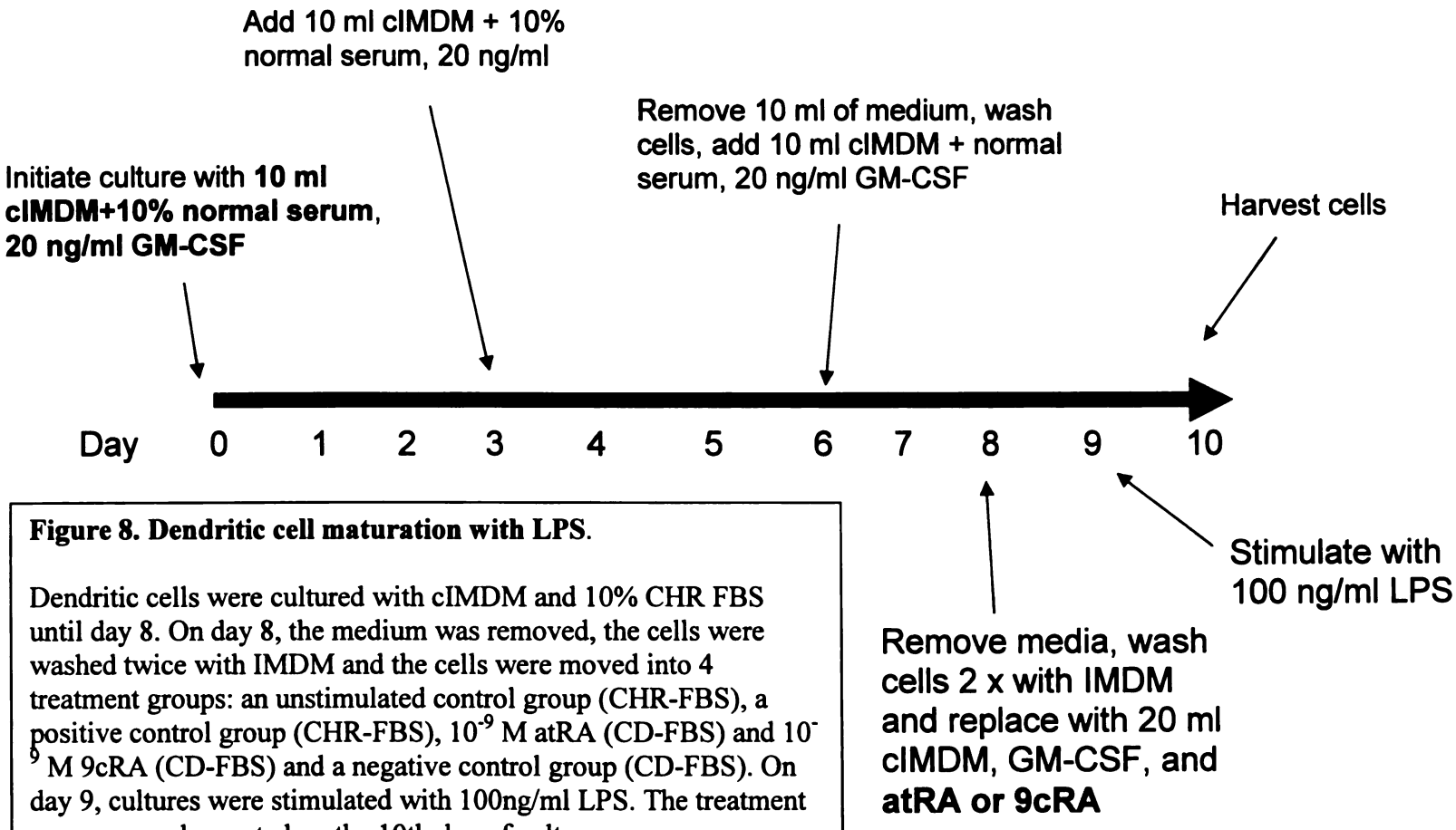

Figure 8. Dendritic cell maturation with LPS.

Dendritic cells were cultured with cIMDM and 10% CHR FBS until day 8. On day 8, the medium was removed, the cells were washed twice with IMDM and the cells were moved into 4 treatment groups: an unstimulated control group (CHR-FBS), a positive control group (CHR-FBS), 10^{-9} M atRA (CD-FBS) and 10^{-9} M 9cRA (CD-FBS) and a negative control group (CD-FBS). On day 9, cultures were stimulated with 100ng/ml LPS. The treatment groups were harvested on the 10th day of culture.

Results

DCs generated in the presence of vitamin A were placed in medium with or without atRA or 9cRA and then stimulated with LPS to assess the effect of vitamin A on DC maturation. A representative contour plot of cells dual stained for CD11c and MHC-II is shown in Figure 9. The DC markers CD11c and MHC-II were analyzed within the cultures. The percent of cells expressing CD11c and MHC-II were not significantly different among treatment groups. (Figure 10 and Figure 11). The mean fluorescence intensity (MFI), a measure of the relative amount of a molecule on a cell's surface, was also assessed (Figure 10 B). The MFI for CD11c was not significantly different among treatment groups. The MFI for MHC-II was significantly lower in cultures treated with either atRA or 9cRA when compared to the negative control (Figure 11 B).

The expression of co-stimulatory molecules CD40, CD80, and CD86 were also analyzed on the DCs. In unstimulated control cultures, less than 5 percent of the cells expressed CD40 and the percentage of CD80 and CD86 were similar to those listed in Figure 1. The stimulated cultures varied in the percentage of CD40 positive cells. The positive control, atRA and 9cRA treatment groups expressed a significantly lower percent of mature DCs with CD40 compared to the negative control and these cells also had significantly less CD40 on the cells surface (MFI) (Figure 12). Following LPS stimulation, the percentage of the co-stimulatory molecules CD80 and CD86 were found to be significantly different in cultures treated with vitamin A when compared to both the positive and negative control (Figure 13). Addition of atRA during LPS stimulation significantly reduced the MFI for both CD80 and CD86, while 9cRA treatment significantly decreased the MFI of CD86.

Conclusions

This data demonstrates vitamin A is not necessary for DC maturation, however it has the ability to modulate mature DC phenotype. The presence of either atRA or 9cRA prevented a significant portion of the DC population from reaching full maturation. The decreased cell surface expression of MHC-II, CD40, and CD86 in the cultures treated with atRA and 9cRA clearly indicates an intermediate phenotype that correlates with DC phenotype necessary for the promotion of a Th2 response (59).

CD40 is normally upregulated on DCs stimulated with LPS. Negative control cultures as well as treatments with atRA or 9cRA all demonstrate a down regulation of CD40 expression with LPS treatment. This could indicate that retinol and its potential metabolites are necessary for the proper maturation of DCs and that atRA or 9cRA alone cannot support CD40 expression. It is also possible that some other fat-soluble organic molecule is removed during the charcoal dextran treatment of the serum and vitamin A has no role in the control of CD40 expression.

Vitamin A has been shown to have molecular effects on cellular activity through the interaction of retinoid receptors and retinoic acid response elements in the DNA. Both atRA and 9cRA interact with the RAR. However, 9cRA can also bind the ligand-binding domain of the RXR. Since both atRA and 9cRA are exerting similar affects on DC maturation, it is possible that the RAR is playing a major role in inhibiting DC maturation by competing with other transcription factors or blocking transcription all together.

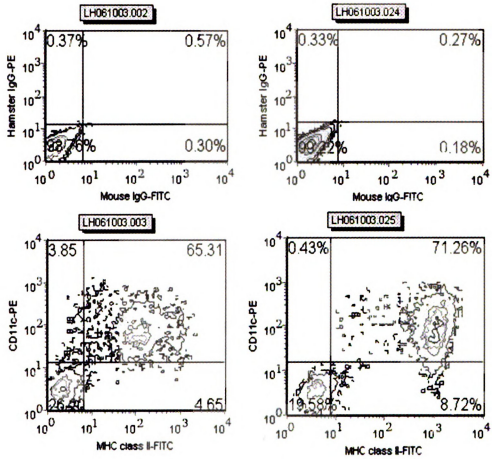

Figure 9: A Representative Contour Plot of DC Maturation. Cells were cultured for 9 days in cIMDM with 10% characterized FBS. On day 9, the cultures were stimulated with LPS (100 ng/ml) for 24 hours. The cultures were harvested at the end of this period. The isotype controls for MHC class II and CD11c are shown for both the untreated culture (A) and the culture exposed to LPS (B). Immature DCs not exposed to LPS (C). Mature DCs exposed to 100 ng/ml LPS for 24 hours (D).

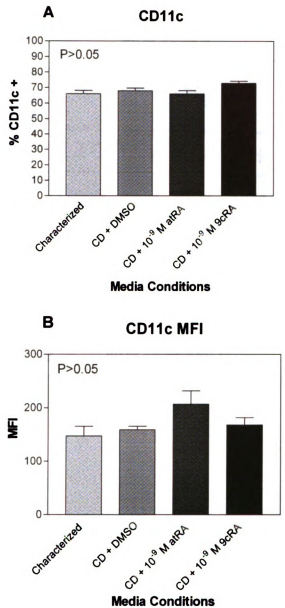

Figure 10. Retinoic Acid does not affect the Expression of CD11c on DCs Stimulated with LPS. Cultures of mouse bone marrow cells were grown in cIMDM with characterized (CHR) FBS for 8 days. On the 8th day the medium was removed, the cells were washed and the cultures were divided into four treatment groups: a positive control (characterized), a negative control (CD+DMSO), 10^{-9} M atRA, and 10^{-9} M 9cRA. The cultures rested for 24 h, and then were stimulated with 100 ng/ml LPS for 24 h. FACS analysis revealed there were no significant differences among treatment groups in (A) the percentage of $CD11c^{+}$ DCs or (B) the MFI for CD11c.

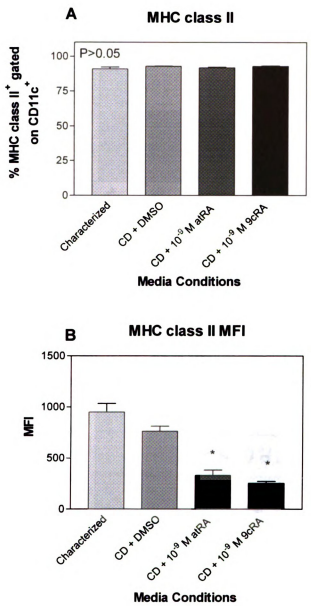

Figure 11: Retinoic Acid Significantly Decreases the Cell Surface Expression of MHC class II on DCs Stimulated with LPS. Cultures were performed as in Figure 10. (A) FACS analysis revealed that the percentage of DCs expressing MHC class II was not significantly different among treatment groups, however (B) the MFI for MHC class II dramatically decreased in cultures treated with atRA or 9cRA (* = $P < 0.001$ when compared to the positive and negative control).

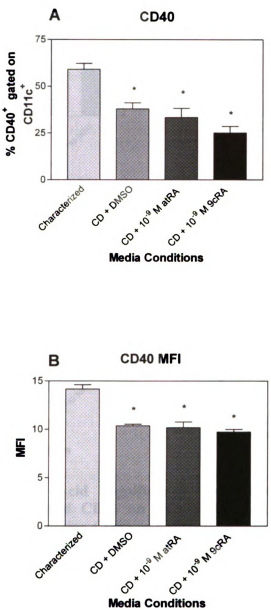

Figure 12: Retinoic Acid does not exert an Effect on the Level of CD40. Cultures were performed as in Figure 10. Cultures were assessed for percent $CD11c^{+}CD40^{+}$ (A) and MFI for CD40 (B). (* = $P < 0.001$ when compared to the positive control cultures supplemented with characterized FBS).

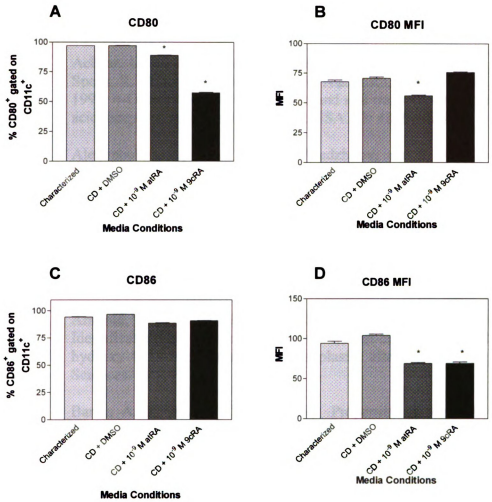

Figure 13: Retinoic Acid Significantly Decreases the Cell Surface Expression of CD80 and CD86 on DCs Stimulated with LPS. (A) The percentage of DCs expressing CD80 was significantly lower in cultures treated with atRA or 9cRA. (B) CD80 MFI was significantly lower for cultures treated with atRA than the control. (C) All treatment groups were significantly different from one another for the percentage of DCs that were CD86 positive. (D) The MFI for CD86 is also significantly lower than both the positive and negative control in cultures treated with atRA or 9cRA (* = $P < 0.001$ when compared to the positive and negative control).

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