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THE EFFECTS OF RETINOIC ACID ON THE PROLIFERATION AND CELL CYCLING OF MYELOID DENDRITIC CELLS IN RESPONSE TO GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR

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THE EFFECTS OF RETINOIC ACID ON THE PROLIFERATION AND CELL CYCLING OF MYELOID DENDRITIC CELLS IN RESPONSE TO GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR

By

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ABSTRACT

By

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All-trans retinoic acid (atRA) carries out most of the biological activities of vitamin A and plays a major role in embryogenesis, cell differentiation and immune functions. Vitamin A favors the differentiation of myeloid progenitors to immature myeloid dendritic cells (DC) instead of granulocytes when vitamin A is adequate in culture, suggesting that vitamin A deficiency may cause changes in adaptive immune responses that depend on myeloid DC antigen presentation. We investigated the effect of atRA on the differentiation of myeloid progenitor cells in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and cycling of the resultant cell populations. We observed a gradual decline in percent of DC obtained at culture harvest as the addition of vitamin A to the myeloid progenitors was delayed. In addition, mouse bone marrow cells cultured with GM-CSF and treated with 10 nM atRA showed increased cell proliferation. To further investigate the role of vitamin A in DC proliferation, the same concentration of atRA was used and compared to a retinoic acid receptor (RAR)-α antagonist (AGN 194301) to observe differences in cell cycle. Following addition of GM-CSF to DC cultures more cells were in S-phase when treated with atRA compared to AGN 194301. These data suggest that atRA is promoting an increase in the progression of cells from G_1 to S-phase of the cell cycle, thereby optimizing myeloid DC proliferation in response to GM-CSF.

DEDICATION

To my mom and dad,

thank you for being there.

ACKNOWLEDGEMENTS

It has been a great pleasure to have been offered an opportunity to study in the Department of Food Science and Human Nutrition at Michigan State University. I wish to say special thanks to my advisor Dr. Kathy Hoag for believing in me and granting me the opportunity to work in her lab. Many thanks to Dr. Louis King for his continued support and expertise in flow cytometry. I thank my committee members Dr. Maurice Bennink and Dr. Maija Zile for their insight and support. Last but not least, thank you to the many others that have helped me along the way, including my lab partners, friends and the Wilmot family for their continued support and motivation.

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KEY TO ABBREVIATIONS

atRA	All-*trans* retinoic acid
CD-FBS	Charcoal dextran fetal bovine serum
CDK	Cyclin dependent kinase
C/EBP	CCAAT/enhancer binding protein
CH-FBS	Characterized fetal bovine serum
cIMDM	Complete Iscove's modified Dulbecco's medium
CKI	Cyclin kinase inhibitor
CRABP-II	Cellular retinoic acid binding protein-II
DAPI	4' 6- diaminidino-2-phenylindole
DC	Dendritic cell(s)
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FITC	Fluoroscein isothiocyanate
IL-	Interleukin-
PBS	Phosphate buffered saline
PE	Phycoerythrin
9cRA	9-*cis* retinoic acid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
RAR	Retinoic acid receptor
RARE	Retinoic acid receptor element
RXR	Retinoid X receptor
SD	Standard deviation
SEM	Standard error of the mean
TTNPB	(E)-4-[2-(5, 5, 8, 8-tetramethyl-5, 6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid
Th1	T helper 1
Th2	T helper 2

INTRODUCTION

Vitamin A

Vitamin A is a fat-soluble vitamin important for cellular differentiation and proliferation, reproduction, embryonic and fetal development, vision and appropriate immune function (Vuligonda et al, 1999). Most of the functions of vitamin A are based on activation or inhibition of transcription of the genes involved in cellular proliferation and differentiation, with the exception being the role of vitamin A in vision.

Uptake and Metabolism of Vitamin A

Vitamins are micronutrients not synthesized by the human body that must be provided by food. Fat-soluble vitamins include vitamins A, D, E and K. Vitamin A, which refers to specifically retinol, was discovered in the early 20th century (Blomhoff 1994; Vogel et al, 1999). The body acquires some of its vitamin A through consumption of animal fats or liver while the remainder is synthesized from pro-vitamin A, β-carotene and possibly other carotenoids. Other natural molecular forms of vitamin A (retinol) including all-*trans* retinoic acid (atRA), 9-*cis* retinoic acid (9cRA), retinal, and retinyl esters. The hormonally active metabolite is atRA.

Vitamin A is obtained from the diet mainly in the form of fatty acid esters which are hydrolyzed by both secreted pancreatic enzymes and intestinal cell enzymes (Harrison 2005). Alternately, β-carotene can be enzymatically cleaved to retinol. The resulting retinol is taken up by intestinal cells and re-esterified by

the membrane-bound enzyme lecithin:retinol acyltransferase. Retinyl esters are then integrated into chylomicrons and are absorbed in the lymphatic system where they circulate in the lymph before entering the general circulation (Penniston and Tanumihardjo, 2006). Retinyl esters from chylomicron remnants in the vascular system are then delivered to the liver (Harrison 2005). Hepatocytes in the liver take up retinyl esters which are then transferred to hepatic stellate cells where they are stored (Harrison 2005). When there is demand for vitamin A in the tissues, retinyl esters in liver are hydrolyzed to retinol which is bound to a specific transport protein in the serum, the retinol-binding protein.

Retinoids

Vitamin A refers specifically to all-*trans* retinol. Retinoids are a group of natural derivatives and/or metabolites related to vitamin A and defined specifically by their ability to activate the retinoic acid receptors (Standeven et al, 1997; Hayes et al, 1999). Retinoids are small lipid-soluble molecules that regulate gene expression via retinoid receptors (Hayes et al, 1999; Nagpal and Chandraratna, 2000).

Retinoids are molecularly effected through two families of nuclear receptors, namely retinoic acid receptors (RARs) and retinoid X receptors (RXRs). AtRA binds and signals through the RAR family, which consists of α, β, and γ subtypes. With the binding of atRA, the RAR receptors heterodimerize with a member of the RXR family that also consists of α, β, and

γ subtypes. The 9cRA metabolite of vitamin A is able to bind both the RXR and RAR family members, which will then dimerize with another RXR. 9-cis retinoic acid binds at high affinity and is considered as a pan-agonist because it binds to all six receptors of retinoic acid (Schulman et al, 1999). The RAR/RXR and RXR/RXR heterodimers can both bind to retinoic acid response elements (RAREs) in DNA leading to the activation or inhibition of gene expression (Bastien and Rochette-Egly, 2004; Chambon 1996). The respective contributions of the three RAR subtypes to the diverse biological effects of retinoids are still being defined, but gene knockout experiments suggest that there is redundancy in function among the RARs (Mark et al, 2006).

Synthetic Retinoids

There are three classes of synthetic retinoids, namely agonists, inverse agonists and antagonists. Agonists induce expression of retinoid-responsive genes while antagonists compete with the agonists for the binding area of the receptor and inhibit agonist-dependent activation of the responsive genes (Nagpal and Chandraratna, 2000). Major synthetic RAR agonists include: (E)-4-[2-(5, 5, 8, 8-tetramethyl-5, 6, 7, 8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB), AM580 and AGN 193835. Commonly used antagonists include AGN 193109 and AGN 194301. TTNPB is a RAR-specific synthetic retinoid that binds equally to all RARs. RAR subtype selective retinoids have been synthesized by alteration of the basic retinoic acid structure (Nagpal and Chandraratna, 2000). The RAR-α specific antagonist (AGN 194301) was

designed by a combination of structural features, which provided efficient antagonism with other retinoids that had RAR-α specific binding (Johnson et al, 1996). Little has been published about this antagonist, but it is known to bind with a 25-fold higher affinity to RAR-α than some agonists (Teng et al, 1997). There is also another class of synthetic retinoids that appear to inhibit the basal level expression of retinoid-responsive genes as well as act as a classical antagonist, and these are classified as RAR inverse agonists. RAR inverse agonists have biological activities that are independent of their ability to act as RAR antagonists (Nagpal and Chandraratna, 2000). The main therapeutic use of synthetic retinoids is the treatment of dermatological diseases, including acne, psoriasis, keratinization disorders and cutaneous malignancies, and RAR-α specific retinoids are used in differentiation treatment for acute promyelocytic leukemia (Nagpal and Chandrartna, 2000).

Vitamin A Function in Vision

Vitamin A function in the visual cycle is not based on the regulation of transcription but on vitamin A acting as a prosthetic group (Zile 1998). In the eye, retinol is converted to retinal, and is isomerized to the 11-*cis* conformation, which is important in light adaptation. The metabolism of vitamin A in the retina is very distinctive (Wolf 1996). The key intermediates in the visual cycle are 11-*cis* retinal and all-*trans* retinal (Saari et al, 1999; Rucker et al, 2001; Wolf 2001). Opsin, a protonated schiff base, binds very tightly to 11-*cis* retinal forming rhodopsin (Saari et al, 1999; Rucker et al, 2001). A photon of light striking

rhodopsin causes 11-*cis* retinal to be isomerized to all-*trans* retinal (Rucker et al, 2001). There are a number of steps involved in the visual cycle; however, the final and most important step is the reduction of all-*trans* retinal to all-*trans* retinol (Saari et al, 1999).

Vitamin A Role in Reproduction and Embryogenesis

In the reproductive system, vitamin A is of major importance in spermatogenesis in the testis (Packer and Wolgemuth, 1999) and maintaining the health of the female reproductive system and preventing fetal resorption (Clagett-Dame and DeLuca, 2002). Genetic studies have indicated that atRA is the active form of vitamin A that functions in spermatogenesis and female reproductive capacity (Clagett-Dame and DeLuca, 2002); however, retinol is needed for the transport of the vitamin across the blood testis barrier (Packer and Wolgemuth, 1999).

The primary retinoids found in murine embryonic tissues are all-*trans* retinol and atRA, but avian embryonic tissues predominantly contain 3, 4-didehydroretinol and 3, 4-didehydroretinoic acid (Ross et al, 2000). However, atRA is the active agent required for the development of mammalian embryo through transcriptional regulation (Clagett-Dame and DeLuca, 2002). Besides atRA, 9cRA is another retinoid capable of transcriptional activation, but this retinoid has not been identified in developing murine embryos. Retro-retinoids are of great concern in mammalian embryogenesis, due to their ability to activate RAR-mediated transcription (Clagett-Dame and DeLuca, 2002). It has been

shown that monoclonal antibodies against atRA block normal quail embryo development by causing abnormalities in the cardiovascular system (Twal et al, 1995). During normal embryonic development, a number of developmental events are regulated in the presence of atRA (Zile 2001). Vitamin A deficiency targets the heart, central nervous system, circulatory system, urogenital and respiratory system and the development of limbs and skeleton in developing embryos (Maden et al, 1998; Zile 1998).

Vitamin A Role in Cellular Differentiation and Proliferation

Vitamin A can control gene expression through nuclear retinoic acid receptors (Mark et al, 2006; Vakiani and Buck, 1999). These receptors appear at different cell stages in development and in several cell types (Gropper et al, 2005). Retinoic acid is necessary for the differentiation and proliferation of epithelial tissues of the skin, respiratory tract, urogenital tract, gastrointestinal tract in which it maintains normal structure and function (Gropper et al, 2005). Vitamin A is critical for maintaining barrier function and epithelial tissue integrity (Ramakrishnan and Martorell, 2004). During vitamin A deficiency there are a number of alterations in the epithelial lining of vital organs (McCullough et al, 1999). The mucosal barrier found in the conjunctiva of the eye, the respiratory tract and the gastrointestinal tract are compromised due to loss of mucus producing goblet cells (Stephensen 2001).

Vitamin A has also been shown to be important in the differentiation of white blood cells, specifically lymphocytes, from stem cell progenitors in the bone

marrow (Gaines and Berliner, 2003; Garrett et al, 2000), and also directs the differentiation of keratinocytes to mature epidermal cells (Gropper et al, 2005). Retinoids have also been shown to regulate critical cell cycle genes (Harvat and Jetten, 1999) and have the ability to positively and negatively regulate growth via regulation of epidermal growth factors, transforming growth factor-α, transforming growth factor-β, interleukin (IL)-1, IL-6, insulin and interferon-γ through retinoid receptor dependent and independent pathways (Harvat and Jetten, 1999).

Vitamin A and Immune Function

The importance of vitamin A in immune function was first recognized at the beginning of the 20th century when it was deemed the "anti-infective" vitamin (Hayes et al, 1999; Rucker et al, 2001; Stephensen 2001). It has been shown that maintenance of optimal vitamin A status for several years lowers incidence of infections (Hayes et al, 1999). Thus, deficiency of this vitamin is believed to be a major cause of infant mortality associated with infectious diseases commonly observed in developing countries (Blomhoff 2004; Stephensen 2001). Children who died from vitamin A deficiency in the 1930's were examined and found to have degenerative spleen, thymus, and lymphoid tissue, and this same observation was confirmed in animal models (Semba 1998). In a community based study, vitamin A supplementation was shown to reduce childhood mortality in developing countries, especially that caused by measles and diarrhea (Villamor and Fawzi, 2000). Both the role of vitamin A in modulating disease-

specific immunity and the mechanism through which it may exert its anti-infective effects has been partly revealed (Stephensen 2001).

There are two major ways in which vitamin A promotes a normal immune function (Clagett-Dame and DeLuca, 2002). The first is through maintenance of the epithelial barriers such as the skin, gastrointestinal tract, and mucosal lining of the airways (Clagett-Dame and DeLuca, 2002; McCullough et al, 1999). Vitamin A deficiency causes changes in the mucosal barriers that are the first line of defense against infection (Stephensen 2001). The second is through observed changes in the transcriptional activity of immune cells (Clagett-Dame and DeLuca, 2002).

In mice, vitamin A deficiency causes the systemic expansion of the myeloid lineage, particularly neutrophils (Kuwata et al, 2000). Vitamin A deficiency also decreases the ability of macrophages and neutrophils to migrate to sites of infection, phagocytose, and kill bacteria (Stephensen 2001). Marginal vitamin A status decreases natural killer cell numbers and function (Dawson et al, 1999). Deficiency in vitamin A has been shown to cause a decrease in the killing ability of natural killer cells, macrophages and neutrophils in rats, and the functions can be replenished with retinoic acid supplementation (Zhao and Ross, 1995). Vitamin A deficiency causes significant changes in both innate and adaptive immune responses (Semba 1998; Hayes et al, 1999; Stephensen 2001). Sufficient vitamin A maintains normal T helper 2 (Th2) and antibody responses by suppressing IL-12 and tumor necrosis factor-α production (Stephensen 2001). Deficiency, however, causes increased production of these

cytokines leading to suppression of Th2 responses, thus causing impaired ability to defend against extracellular pathogens in vitamin A deficient individuals (Stephensen 2001; Ramakrishnan and Martorell, 2004).

Hoag et al (2002) have shown that atRA enhances naïve $CD4^+$ T cell development into Th2 cells, through action on antigen-presentation in an IL-4 dependent manner. Vitamin A supplementation increases the delayed hypersensitivity response to ovalbumin, which was used to measure Th1 function in vitamin A deficient mice (Stephensen 2001). Vitamin A supplementation also causes an increase in the serum antibody production in children after diphtheria and tetanus toxoid vaccination (Stephensen 2001). In an *in vivo* study in mice it was demonstrated that vitamin A deficiency after initial antigen exposure causes a significant increase in the development of IL-10 producing Th2 or regulatory T cells while causing a decrease in the development of Th1 memory cells (Stephensen et al, 2004). Thus, vitamin A has been shown to be able to modulate both Th1 and Th2 responses.

Myeloid Immune Cells

Cells of the myeloid lineage include neutrophils, monocytes, macrophages, and myeloid DC, and these myeloid cells are all thought to be derived from a common progenitor cell (Fogg et al, 2006). Granulocyte-macrophage colony -stimulating factor (GM-CSF) stimulates the development of myeloid progenitors (Inaba et al, 1992). DCs represent professional antigen-presenting cells, as their major function is to trigger and regulate the adaptive

immune response (Mellman and Steinman, 2001; Fogg et al, 2006). Immature myeloid DCs are located in most tissues and are able to effectively capture antigen. Activation of DC by microbial products or inflammatory mediators leads to maturation and migration to the T cell area of the lymph nodes and spleen where they are then able to stimulate and activate both $CD4^+$ and $CD8^+$ T lymphocytes (Banchereau and Steinman, 1998). Stimulation of T cells leads to a selective adaptive immune response and memory (Banchereau and Steinman, 1998; Steinman 1999). DCs play a fundamental role in controlling immunity and are a possible target of the wide effects of retinoids in immune system homeostasis (Mohty et al, 2003).

Neutrophils are innate immune cells of the myeloid lineage. They are the most prevalent white blood cells in adult human peripheral blood circulation and are the first immune cell to respond to invading microorganisms. Retinoids in general, and atRA in particular, play a critical role in the differentiation of neutrophils. AtRA is known to modulate gene expression by binding to its nuclear receptors, which in turn can activate transcription of genes that are essential for the differentiation of immature myeloid cells to neutrophils (Maun et al, 2004). This was implied by studies involving retinoic acid and acute promyelocytic leukemia (PML) cells. It was shown that PML cells have a chromosomal translocation targeting the RAR-α (Maun et al, 2004). Retinoic acid *in vitro* is a potent inducer of neutrophil cell fate suggesting that retinoic acid may play a role in the commitment of pluripotent hematopoietic progenitors to the neutrophil lineage (Purton et al, 2000). Vitamin A, present as serum retinol, is

necessary for myeloid DC development from bone marrow cells *in vitro*. Vitamin A, added as the active metabolite atRA, also promotes the development of myeloid DC *in vitro* in response to GM-CSF, but when vitamin A is inadequate, neutrophils are generated as the predominant cell type over DC (Hengesbach and Hoag, 2004). Neutrophils have been shown to strongly cluster with immature DCs, and activated neutrophils induce maturation of DC triggering strong T cell proliferation and Th1 polarization of T cells (Klaas et al, 2005).

Cell Cycle and Cyclins

The cell cycle is functionally divided into several phases, G_0, G_1, S, G_2 and M. The S-phase of the cell cycle is where DNA synthesis occurs, while the M-phase is mitosis, where chromosomes separate and the cells divide into two daughter cells. Following cell division, the period between M and S-phase is called G_1 where growth and preparation of the chromosomes for replication occur or cells are preparing to start another cycle (Givan 2001). The gap between S and M-phase is called G_2 where preparation for mitosis occurs or cells have already finished DNA synthesis and have double the normal amount of DNA (Givan, 2001).

Cell cycle progression is regulated by three protein classes, cyclins, cyclin-dependent kinases (CDKs), and cyclin kinase inhibitors (CKIs) (Barrera et al, 2005). Cyclins are essential for the progression of cells through the different phases of the cell cycle. Cyclins can be classified into groups based on their functions. The cell cycle regulators are cyclins A, B, D, and E. Cyclin D, along

with CDK4/6, controls the exit of cells from G_1. Cyclin A is active during S-phase of the cell cycle, but then is degraded by M-phase. Cyclin E is present in G_1, with peak activity seen at the G_1/S boundary of the cell cycle. Cyclin E is a critical molecule that dictates cell entry into the S-phase and is considered the master regulator of the S-phase. The activities of the CDKs are regulated at the level of their expression and by phosphorylation and the complexes they form with activated cyclins and CKIs where phosphorylation increases their activity and the binding to CKIs causes inhibition (Harvat and Jetten, 1999).

During cell cycle progression, in order for cells to enter S-phase, they have to pass through a restriction point in G_1. Regulation of the progression of cell cycle in mammalian cells by retinoids generally occurs in G_1 of the cell cycle, most commonly by preventing G_1 to S-phase progression (Harvat and Jetten, 1999). However, most studies have failed to carry out a careful examination of whether retinoids directly affect cell cycle regulators and thereby cause growth arrest or if they indirectly affect the growth proteins (Harvat and Jetten, 1999). The main event in G_1 to S-phase progression is phosphorylation of the retinoblastoma protein, which binds to E2F transcription factors, signaling the activation and elevation of specific cyclins (Blomhoff 2004; Harvat and Jetten, 1999). Progression from G_1 to S-phase of the cell cycle is promoted by the activities of cyclin D1, cyclin D3 and cyclin E. In myeloid cells, these cyclins are inhibited by $p21^{CIP1}$ or $p27^{KIP1}$ (Dimberg and Oberg, 2003a). Retinoic acid was shown to inhibit proliferation of myeloid cells, mainly by inhibiting progression from G_1 to S-phase (Dimberg and Oberg, 2003a); however, the concentration of

retinoic acid used by these investigators was a pharmacological dose and not physiological. In a study using normal human peripheral blood B lymphocytes, it was shown that retinoic acid prevented phosphorylation of the retinoblastoma protein, even at doses as low as 10 nM (Naderi and Blomhoff, 1999). These studies suggest that retinoic acid inhibited the transcription of genes needed for S-phase progression, thus causing cells to be trapped in G_1.

In HL-60 promyelocytic leukemia cells (which represents a precursor population of macrophages, DC, and neutrophils), cyclin D3 was reported to interact with both RAR and cellular retinoic acid binding protein II (CRABP-II) to form a ternary complex and up-regulate retinoic acid mediated transcription (Despouy et al, 2003). Cyclin D3 is found in the G_0/G_1 phases of the cell cycle but will only interact with the CRABP-II protein in the presence of retinoic acid. Cyclin D3 does not function in S-phase of the cell cycle. Progression from G_1 into S-phase of the cell cycle is promoted by the activity of cyclin D and CDK4/6 while cyclins A and E pair with CDK2 for G_1/S progression and continued progression through the S-phase (Sanchez and Dynlacht, 2005). Cells normally differentiate in G_1, and the induction of differentiation is believed to require cell cycle arrest (Chen and Ross, 2004). There is a general understanding that retinoids often induce cells to cease or slow their rate of proliferation by arresting the cells in G_1.

AIMS OF THESIS

To further explore the properties of vitamin A in myeloid DC development, we attempted to identify the myeloid progenitor stage that required vitamin A to commit to a myeloid DC versus a neutrophil. We utilized an *in vitro* culture system in which mouse bone marrow cells are stimulated with GM-CSF to cause their differentiation into myeloid DC (Inaba et al, 1992; Lutz et al, 1999). A set of time-course experiments was performed with the addition of atRA to the cultures at varying days following culture initiation. Dual labeling of cells with fluorochrome-conjugated monoclonal antibodies against mouse CD11c (clone HL3) and Gr-1 (clone RB6-8C5) was performed to identify DC and neutrophils, respectively, in the resultant cell population. We observed a gradual decline in dendritic cell percentage of the cell population as the addition of vitamin A to the cultures was delayed. We also observed a significant increase in total cell culture yield when vitamin A addition was delayed until day 3 or 4 of culture. From this set of experiments, it was concluded that vitamin A increased myeloid cell proliferation, but no particular stage of myeloid development prior to DC commitment could be identified which was absolutely dependent upon vitamin A. We hypothesize that these results were obtained because vitamin A was added to bone marrow cells which were at various different stages in their development, and the cultured cells were not initiating development from a single synchronized progenitor stage. Therefore, these initial studies were inconclusive and would need repeating with cell cultures initiated with a more purified myeloid progenitor stage to be more informative.

A second series of experiments stemmed from our observation that adding vitamin A at day 3 or later of the 10 day culture in the time course experiments yielded higher DC numbers. Therefore, we hypothesized that vitamin A would increase proliferation and cell cycling of dendritic cells in response to GM-CSF. This set of experiments was performed using the RAR-α-specific antagonist (AGN 194301) as a comparison group to identify significant differences in DC proliferation in response to GM-CSF when vitamin A signaling was blocked as opposed to present.

MATERIALS AND METHODS

Animals

BALB/cJ male mice were purchased from Jackson Laboratory (Bar Harbor, ME). They were 12-16 weeks of age at the time of the experiment and were killed by carbon dioxide asphyxiation. All experimental procedures using laboratory animals were performed according to the guidelines set by University Laboratory Animal Resources under a protocol approved by Michigan State University's Institutional Animal Care and Use Committee. The mice were fed solid pellets containing all essential vitamins and minerals (Harlan Teklad 22/5 Rodent Diet #8640, Madison, WI).

Culture of Cells from Bone Marrow

Charcoal dextran-treated fetal bovine serum (CD-FBS) and characterized fetal bovine serum (CH-FBS) were purchased from HyClone (Logan, UT) and used at 10% (v/v) in the complete medium. CD-FBS is depleted of fat-soluble components and contains less than 50% percent vitamin A (retinol) compared to CH-FBS. The medium for cell culture, complete Iscove's modified Dulbecco's medium (cIMDM) consisted of IMDM (BioWhittaker, Walkersville, MD) with 10% (v/v) serum (CD-FBS or CH-FBS), 100 U/L penicillin, 100 μg/L streptomycin, 2 mM L-glutamine (BioWhittaker) and 10 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Vitamin A was used in the form of all-*trans* retinoic acid (atRA), and was prepared as a 5 mM stock in dimethyl sulfoxide (DMSO, Sigma-Aldrich)

and stored in small aliquots at -70°C under argon before being diluted to the desired concentration in medium in each experiment. The RAR-α antagonist (AGN 194301; gift from Vitae Pharmaceuticals, Irvine, CA) was prepared and stored as for atRA stocks.

Femurs and tibias were collected from mice. The bones were sterilized by placement in 70% ethanol for 2 mins then rinsed in sterile medium to remove ethanol. The cells were collected using a flush method where the ends of the bones were clipped and a 10 mL syringe with 27 gauge 5/8 inch needle was used to rinse the cells from the bones. Red blood cells were lysed with 2 mL of ACK lysis buffer (BioWhittaker) for 2 mins on ice and the volume was brought up to 20 mL with IMDM. The cell suspension was then centrifuged and the cell pellet resuspended in 5 mL of IMDM for counting. The cell count and viability were determined using trypan blue (Sigma) and a hemacytometer. The cells were plated at 2 x 10^5 cells/mL in cIMDM. Cells were stimulated with 20 ng/mL recombinant murine GM-CSF purchased from PeproTech (Rocky Hill, NJ).

Culture for Identification of atRA-dependent Myeloid DC Progenitor

Cultures in medium containing CD-FBS were divided into different treatment groups that were supplemented with atRA beginning on different days. Treatment groups received atRA at 1 nM concentration or the DMSO vehicle control. Retinoic acid was added to separate treatment groups beginning each day from day 0-8. On day 3, all cultures received an extra 10 mL of medium with GM-CSF. On days 6 and 8, 50% of spent medium volume was removed and

centrifuged for 10 mins at 4°C, 1,200 rpm. The resultant cell pellet was then resuspended in fresh cIMDM containing GM-CSF +/- atRA and the cell suspension was returned to its original culture dish. On day 10, the cell cultures were harvested and the cell count and viability were determined using trypan blue dye (Sigma) and a hemocytometer.

Flow Cytometry - Labeling of DC and Neutrophils

The monoclonal antibodies used to label the cells were: fluorescein isothiocyanate (FITC)-conjugated anti-Gr-1 (Rat IgG_{2b}, κ) and R-phycoerythrin (PE)-conjugated anti-CD11c (HL3 clone, Armenian Hamster $IgG_{1,\lambda}$). CD11c and Gr-1 are cell-surface proteins found on specific myeloid cell types. CD11c is a marker that is specific for dendritic cells in mouse while Gr-1 (Ly-6G) is specific for neutrophils. The antibodies and their appropriate isotype controls were purchased from BD Biosciences (San Diego, CA). Cells (1×10^6) were suspended in 1 mL staining buffer. The cells were centrifuged for 10 mins at 4°C, 1,200 rpm and buffer was poured off the cell pellet. Following resuspension of the cell pellet, a purified Fc block (2.4G2 clone; monoclonal antibody to FcγRII/III) was added to cell pellets (1 μg/tube) for 5 mins on ice in the dark. The primary antibodies and isotype controls conjugated to the respective fluorochromes (1 μg/tube) were added and the cells were incubated again for 30 mins in the dark on ice. The cells were washed twice with staining buffer then fixed in 2% formaldehyde (Polysciences, Warrington, PA) in PBS, pH 7.4, and stored at 4°C in the dark until run on the flow cytometer.

Cultures for atRA Influence of DC Adherence

A second set of experiments was done using the RAR-α antagonist AGN 194301. In these experiments, modifications in the method were made based on the results from previous experiments in which we observed that AGN 194301 caused tight clustering of DC and adherence to plastic culture dish, as opposed to the release of the cells into the liquid medium. Instead of using CD-FBS, cultures were all initiated in medium containing CH-FBS with the RAR-α antagonist AGN 194301 at 10 nM. Cell cultures received fresh medium containing GM-CSF (20 ng/mL) on days 3, 6, and 8 as done in previous experiments. Handling of cell cultures differed from previous protocol beginning on day 6, in that non-adherent cells were removed and discarded and only adherent cells were retained in culture. On day 6, the entire 20 mL of medium was removed from each dish. New medium (10 mL) containing GM-CSF was added to each dish. The removed 20 mL of medium was centrifuged and 10 mL of the cell-free supernatant was added back to the original dish. On day 8, all the non-adherent cells were removed from each dish and 20 mL fresh medium was added with GM-CSF and 10 nM atRA (6 dishes) or 10 nm AGN 194301 (6 dishes). One of the dishes that atRA was added to was used to observe the changes in the cell clusters under a confocal microscope at different time points following the retinoic acid treatment. The cells were observed prior to the addition of atRA, and 24 hrs and 48 hrs after atRA addition images of the cell clusters and the changes observed were taken at all these time points. The release of cells

from the dish was observed under an inverted confocal microscope (Leica, Allendale, NJ) and digitally photographed at various timepoints.

Culture of DC for Cell Cycle Analysis

A third set of experiments was performed to analyze the effect of vitamin A on DC cell cycling following GM-CSF addition (Figure 1). All cultures were initiated in cIMDM containing 10% CH-FBS, GM-CSF (20 ng/mL) and AGN 194301 or atRA at 10 nM concentration. On day 3, 10 mL fresh medium containing GM-CSF and AGN 194301 or atRA was added to each dish. On day 6, cell cultures received 10 mL fresh medium containing GM-CSF (20 ng/mL) and atRA or AGN 194301. Medium was removed from each dish, and centrifuged for 10 mins. New medium (10 mL) containing GM-CSF was added to each cell pellet that was then returned to the original dish. Cell cultures were harvested beginning on day 6 at 4, 8, 12 and 24 hrs after addition of fresh culture medium containing GM-CSF. The 0 hr timepoint was harvested before additional GM-CSF stimulation. Non-adherent and adherent cells were harvested separately at each timepoint. Adherent cells were removed by adding 5 mL of accutase (Innovative Technologies, San Diego, CA) and incubating the dishes at 37°C for 10 mins. Cell yields were determined by counting on a hemacytometer.

Cell Cycle Analysis by Flow Cytometry

Flow cytometry uses the principles of light excitation, light scattering and emission of fluorochromes to generate specific data about the physical

characteristics of cells. The forward and side scatters are usually generated on the emission spectra, which tell the size and granularity of the cells, respectively. Flow cytometry is also used to measure the DNA content in cells. By measuring the amount of DNA, this method is able to identify the proportion of cells in each phase of the cell cycle.

Combination of cell surface antigen and DNA content analysis can sometimes be very difficult because of dye compatibility issues. There are a number of widely used dyes for DNA content analysis by flow cytometry. However, because of emission spectra overlap, as well as there being differing sensitivities of monoclonal antibody epitopes to chemical fixation, every protocol must be optimized with compatible dyes to achieve adequate surface and DNA staining at the same time. The existing flow cytometric methods for intracellular staining use either alcohol or formaldehyde in combination with a detergent to permeabilize the cells. Aldehydes cross-link the proteins of the cell membranes and do not allow adequate access of dyes into the cell (Schmid and Giorgi, 1995). This suggests alcohol may be preferred for intracellular staining protocols, since alcohol does not cross-link membrane proteins. Paraformaldehyde has been shown to be superior to alcohol fixation in the staining procedure for flow cytometric analysis of cell surface proteins (Clevenger et al, 1985; Jacobberger 1989; Mann et al, 1987). In our preliminary studies we were unable to obtain adequate staining of DNA with 4, 6-diaminidino-2-phenylindole (DAPI) when using paraformaldehyde with a non-ionic detergent for the staining procedure, but were able to obtain satisfactory results with alcohol

(ethanol) fixation. Intracellular staining experiments remain complicated because there are numerous factors that influence the formation of a given antigen-antibody complex which can quickly eliminate antigen epitopes to which the antibody-fluorochrome labeling conjugate must bind (Schmid and Giorgi, 1995).

Despite these challenges, we developed a method to simultaneously label cells for CD11c and DNA content. Cells (1×10^6) were washed with 1 mL of staining buffer [1% FBS, 0.1% sodium azide in phosphate buffered saline (PBS), sterile filtered, 4°C, pH 7.4], then were incubated with anti-mouse CD11c-PE only (see flow cytometry analysis), washed with staining buffer, resuspended in 0.5 mL of 50% FBS in staining buffer followed by addition of 1.5 mL of 70% ethanol in staining buffer. Cells were incubated at 4°C overnight in the ethanol to fix and permeabilize the cells. Cells were rinsed twice in 4 mL staining buffer to remove the ethanol, and then stained for DNA content by addition of 0.5 mL staining buffer containing 1 μg/mL DAPI and 1 mM ethylene diamine tetraacetic acid followed by flow cytometric analysis. All staining procedures were performed on ice and cells incubated in the dark to prevent fluorochrome photobleaching.

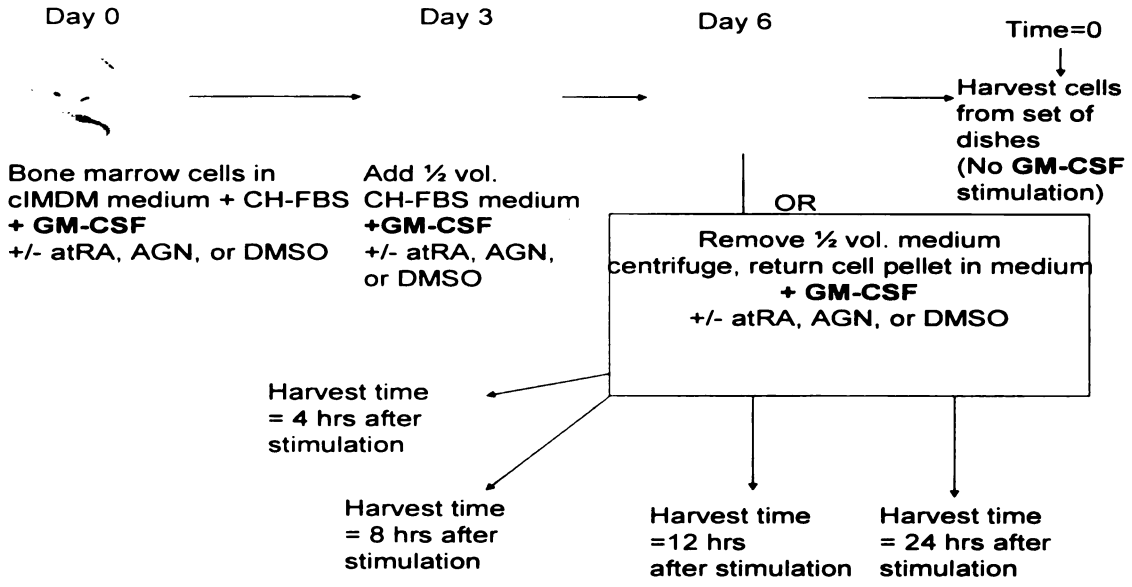


Figure 1: Outline of experiment describing the harvest timepoints for cell cycle analysis of DC following GM-CSF stimulation. Cells were cultured in atRA or AGN 194301 at 10 nM or 0.001% DMSO vehicle for 6 days. Cultures were stimulated with GM-CSF on day 6, then harvested at the above listed timepoints.

Cyclin Analysis - Intracellular Staining for Cyclin Protein

Reagents: Mouse monoclonal anti-cyclin antibodies and isotype controls were all purchased from BioSource (Camarillo, CA). The following monoclonal antibodies were used to label the cells: unconjugated mouse anti-cyclin A (clone E72, mouse IgG_1), unconjugated mouse anti-cyclin D3 (clone DCS-22, mouse IgG_1), FITC-conjugated $F(ab')_2$ goat-anti-mouse Ig's secondary, and isotype control mouse IgG_1 (clone 3B1 1C10).

Cells were washed twice with PBS without magnesium and calcium (BioWhittaker). Fixation buffer (1.5 mL, 50% FCS in 70% ethanol) was then added and cells were incubated at 4°C overnight. Cells were washed twice with PBS (2.0 mL), then incubated at 4°C for 10 mins in permeabilization buffer (50

mM PBS, pH 7.3, 2% (v/v) FBS, 0.1% (w/v) sodium azide, 0.25% (v/v) Triton-X-100). The final volume was brought up to 4 mL with permeabilization buffer, and then cells were centrifuged for 10 mins, 1,200 rpm at 4°C. The cell pellet was resuspended in the residual volume of permeabilization buffer; anti-cyclin antibodies were then added to the cells at ~0.25 μg and incubated at 4°C for 30 mins. After incubation cells were washed three times with PBS. The secondary antibody was then added at the same concentration, and cells were incubated for 30 mins. After incubation, the unbound antibody was removed from the cells by washing twice with staining buffer. After aspirating the supernatant, cells were resuspended in 1.0 mL of cold staining buffer containing DAPI (1 μg/mL).

Flow Cytometry Data Generation

Samples were transported to the Research Technology Support Facility on ice where they were ran and analyzed on BD FACS Vantage (BD Biosciences) with digital system. The sample tubes were then placed in the flow cytometer where vacuum pressure draws up the sample into a stream in air fluidics system. The cells pass through the excitation laser which emits a 488 nm wavelength to excite the fluorochromes, PE and FITC while the UV laser is used to excite DAPI. The fluorochromes then emit a longer wavelength of light that is detected by the various channels of the flow cytometer. Compensation was set up to eliminate the spectral overlap of the wavelengths emitted by the fluorochromes. This was performed using compbeads from BD Biosciences which are polystyrene beads coated with a rat IgG_{κ} antibody that captures the

specific monoclonal antibody conjugate used. For each positively labeled sample, 10,000 events were collected and analyzed based on the forward and side scatter to determine intact cells. Regions were then set based on fluorescence intensity of the positively labeled cells greater than the fluorescence intensity of the isotype control cells.

Cyclin Analysis - Gene Expression

Bone marrow cells were cultured for six days then total RNA was isolated before (0 hr) and after (1, 2, and 4 hrs) GM-CSF stimulation. The adherent and non-adherent cells were combined together so that total RNA from all cells in each dish was isolated. The entire medium was removed from each dish and the dish was then rinsed with 10 mL of PBS. The cells were centrifuged and the cell pellet resuspended in 1 mL TRIzol reagent (InVitrogen). The adherent cells were incubated with 6.2 mL TRIzol reagent (InVitrogen) for cell lysis. The adherent and non-adherent cells were then combined, final volume 7.2 mL and stored at -70°C. RNA was extracted according to the manufacturers' instructions. TaqMan reverse transcription system with MultiScribe reverse transcriptase and random hexamer primers (Applied Biosystems, Foster City, CA) was used to reverse transcribe the mRNA. PCR amplification and product detection were performed on a ABI Prism 7900HT sequence detection system (Applied Biosystems). Expression of cDNA was measured relative to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase by real-time PCR using Assays on Demand™ primer and probe sets (Applied Biosystems) for cyclins A2, D3 and

E1. Relative cDNA expression was calculated from $\Delta\Delta C_T$ values and comparison to a standard curve of multiple 10-fold dilutions using ABI 7700 software (Applied Biosystems).

Statistics

Flow cytometry data analysis for cell-surface protein expression was performed using FCS Express versions 2 and 3 (De Novo Software, Thornhill, Ontario, Canada), and statistical analysis performed using Instat version 3.05 (Graph Pad Software, San Diego, CA). Figures were created with Prism version 3.02 (Graph Pad Software). A Tukey-Kramer one-way ANOVA was used to analyze the raw data followed by Dunnett's post-test. The myeloid DC progenitor cell experiment was repeated 3 times, the DC adherence experiment was performed once and the DC cell cycle experiment was performed twice. For the myeloid progenitor experiment, data and statistics shown are 2 representative experiments with 2 or 7 technical replicates per treatment. There were 3 technical replicates for the DC adherence experiment. The cyclin mRNA analysis by real-time PCR was performed once. A significant difference was considered at $p \leq 0.05$.

Cell cycle (DNA content) analysis was performed using WinList and Mod-Fit (BD Biosciences) on the CD11c-PE positive dendritic cells. WinList was used to gate on CD11c-PE positive cells, and these gated cells were then analyzed using Mod-Fit to generate DAPI fluorescence histograms for assignment of cell cycle stages G_0/G_1 ($n=1$), S ($1>n<2$), or G_2/M ($n=2$). Data shown are the mean

and standard error of the mean (SEM) of the percent of cells for the non-adherent and adherent cell population, n = 4-6 technical replicates per timepoint. The data for the cyclin analysis experiments are one representative experiment of 2 with n=4-6 technical replicates per timepoint for the flow cytometric data and n=2-3 technical replicates for the cyclin gene expression data.

RESULTS

Retinoic Acid Dependent Myeloid Progenitors

Bone marrow cell cultures stimulated with GM-CSF and retinoic acid added on various days were used to identify a retinoic acid dependent myeloid progenitor that gave rise to DCs. In a preliminary experiment statistical analysis was not performed since there were too few replicates. Cell yields were similar among treatments, with the highest number of cells observed in cultures that received retinoic acid beginning on day 2 (Figure 2). There were approximately 70% DC and 10% neutrophils in the positive control (CH-FBS) culture, whereas the negative control (CD-FBS) had 2% DC and 80% neutrophils after 10 days of culture (Figure 3A, B). There were intermediate percentages of DC and neutrophils obtained in the other treatments that had retinoic acid added on various days following culture initiation (Figure 3A, B). There were 40% DC present in the cultures that received supplemental retinoic acid on day 0. The percent DC continued to decrease as retinoic acid addition was further delayed; on average it decreased 10% daily through day 3, and continued to slowly decrease thereafter (Figure 3A). Conversely, the percent neutrophils increased slowly with delayed retinoic acid addition (Figure 3B). Since the greatest change in the percent DC with delayed retinoic acid addition was seen prior to day 4, subsequent experiments were performed focusing on this time period.

Additional experiments were carried out under the same culture conditions but with increased number of replicates. The cell culture yields showed a similar pattern to that of the preliminary experiment with cell numbers increasing as

retinoic acid addition was delayed from day 0 to days 3 and 4 ($p \leq 0.001$; Figure 4). The lowest cell yield was obtained in the positive and negative cultures which were significantly lower than all retinoic acid treated cell cultures. The changes in percentage of DCs and neutrophils showed a similar pattern as the preliminary experiment, with DCs decreasing and neutrophils increasing, respectively, as retinoic acid addition was delayed (Figure 5A, B). The percentage DC observed in the cultures were approximately 55%, 45%, and 40% when retinoic acid was added on days 0, 2, and 3, respectively (Figure 5A). The positive control and negative control had 60% and 20% DC, respectively, with the positive control not significantly different from cultures that had retinoic acid added on day 0. The percent change in neutrophils showed similar results to the previous experiment. There were significant differences in the percent neutrophils between cultures that received retinoic acid on days 0, 2 and 3, with the highest percentage of neutrophils in the negative control (Figure 5B). Delaying the addition of retinoic acid to the cell cultures significantly decreased the percent DCs obtained, but the change was gradual and continuous, suggesting that retinoic acid is not absolutely required for commitment to DC. Additionally, retinoic acid appears to enhance the proliferation of myeloid cells in response to GM-CSF, thus significantly increasing the cell yield obtained at the end of the cultures. A further repeat of this experiment was performed and showed comparable results (data not shown).

Retinoic acid Effect on DC Adherence

Bone marrow cells that were cultured with GM-CSF in the presence of retinoid receptor antagonist (AGN 194301) for 8 days showed increased adherence of DC to culture dishes (Figure 6A, B). The cells formed tight clumps that firmly adhered to the bottom of the plastic culture dishes, instead of normally releasing by the end of the cell culture period. To identify the effect of retinoic acid on the release of these cells, retinoic acid was added to cells that had been previously cultured with the receptor antagonist for eight days. Subsequent changes in cell clusters after retinoic acid addition were observed. Although cell clusters were still present at 24 hrs (Figure 7A) and 48 hrs (Figure 7B) after retinoic acid addition, DCs that had released from the clusters could be observed floating in the medium (indicated with arrows in Figure 7A, B). As shown in Table I, approximately 50% of the cells still adhered to the dishes in the presence of retinoic acid or the antagonist, and therefore retinoic acid showed no effect on DC adherence. Surprisingly, we observed a 20-22% increase in cell yield for both the adherent and non-adherent cells that were treated with retinoic acid compared to those cultures that remained in the antagonist (Table I). Both adherent and non-adherent cells were primarily $CD11c^+$ DC (Figure 8A) and the percent were similar with retinoic acid and the antagonist. However, retinoic acid appeared to decrease the percent of non-adherent neutrophils obtained in the cultures (Figure 8B). No statistical analysis could be performed for this experiment since the dishes were pooled prior to cell counting.

Retinoic acid Effect on Cell Yield and Cell Cycling

Bone marrow cells were cultured with GM-CSF in the presence of retinoid receptor antagonist, retinoic acid, or DMSO vehicle for six days. Following addition of GM-CSF on day 6, cell yield, percent DC and cell cycling were analyzed at various 0 through 24 hr timepoints. As observed in previous experiments, addition of retinoic acid cause a gradual and continued increase in cell numbers over time (Figure 9A, B). In the first of two experiments, there were significant differences in the total cell yield between the retinoid receptor antagonist and retinoic acid at the 0 hr and 24 hr timepoints after GM-CSF stimulation. Conversely, no significant differences were observed at the 4, 8, or 12 hr timepoints after GM-CSF stimulation (Figure 9A). The cell yield remained fairly constant over time for the receptor antagonist treated cells while those treated with vitamin increased gradually. In the replicate experiment there was a significant difference in cell numbers between the receptor antagonist and retinoic acid at all the timepoints (Figure 9B). The cell cultures that were treated with retinoic acid for six days then restimulated with GM-CSF had a significantly higher cell yield compared to both the DMSO vehicle control and the receptor antagonist treated cells at the 0 hr and 24 hr timepoints. At the 12 hr timepoint, the retinoic acid and DMSO vehicle were not significantly different but both had a significantly higher cell yield than the receptor antagonist treated cells (Figure 9B). The receptor antagonist treated cell cultures did not demonstrate any appreciable increase in cell yield over the 24 hr time period following GM-CSF

addition. In summary, blocking the retinoid signaling with the receptor antagonist inhibits the proliferation of myeloid cells in response to GM-CSF.

In addition to total cell yield, the cells were also stained and analyzed for $CD11c^+$ DCs in all the treatment groups. For collecting the cells on the flow cytometer the cells were gated on all cells, to collect a total of 10,000 events that were $CD11c^+$ so that there were enough cells for the cell cycle. The percent DCs were significantly higher at all the timepoints in the cell cultures that were grown in the presence of retinoic acid and stimulated with GM-CSF when compared to the retinoid receptor antagonist (Figure 10A). Cell cultures stimulated with GM-CSF differ in total and relative DC yield when they contained retinoic acid versus the retinoid receptor antagonist. Both the DMSO vehicle control and the retinoic acid treated cell cultures were significantly different from the retinoid receptor antagonist treated cultures (Figure 10B). In conclusion, retinoic acid enhances the proliferation of myeloid cells with response to GM-CSF resulting in increased cell yield at the end of the cell culture period but also seem to cause an increase in the percent of committed DCs.

Cell cycle analysis was also performed on the $CD11c^+$ DCs at various timepoints following GM-CSF addition on day 6. The vast majority of cells in all three treatment groups were in G_0/G_1 of the cell cycle (Figure 11). DCs cultured with the retinoid receptor antagonist had significantly higher percent of cells in G_0/G_1 phase of the cell cycle compared to the retinoic acid and DMSO treated cells at all timepoints (Figure 11A, B). A significant difference was also observed between the retinoic acid and DMSO treated groups at the 0 hr and 24 hr

timepoints in the replicate experiment (Figure 11B) with no difference at 12 hr. A similar pattern of change in the percentage of cells in G_0/G_1 following GM-CSF addition was observed in the retinoic acid treated cells over time in two replicate experiments. The percentage of cells in G_0/G_1 decreased from 0 to 24 hr in cells cultured with retinoic acid or DMSO vehicle control, but appeared to remain constant for cells cultured with receptor antagonist (Figure 11).

In contrast to results for G_0/G_1, DCs cultured with retinoic acid had significantly more cells in S-phase of the cell cycle compared to DCs treated with the retinoid receptor antagonist at all the timepoints, and the percent of cells in S-phase increased from 0 to 24 hr (Figure 12A). Although some of the timepoints were excluded in the replicate experiment a similar trend was observed in the replicate with retinoic acid treated cells having an increasing percent of cells in S phase (Figure 12B). At the 0 and 24 hr timepoints there were no significant differences between the DMSO vehicle control and the retinoic acid treated cells, but the 12 hr timepoint was significantly different with retinoic acid having a higher percent of cells in S-phase compared to the DMSO control. Cells cultured with the retinoid receptor antagonist (AGN 194301) showed a significantly lower percent of cells in S-phase at all timepoints and did not increase over time. Overall, significantly more cells enter S-phase in the retinoic acid treatment group compared to the retinoid receptor antagonist.

There were only small changes observed in the percent of cells in G_2/M for both the receptor antagonist and retinoic acid treated cells following GM-CSF addition (Figure 13). Both the receptor antagonist and retinoic acid showed an

increase in cells in G_2/M at the 24 hr timepoint (Figure 13A), but in a replicate experiment the percent of cells in G_2/M failed to increase for cells cultured with the receptor antagonist (Figure 13B). This inconsistency suggests that retinoic acid has no direct effect on progression of cells through G_2/M, although these studies are inconclusive.

In summary, it appears that retinoic acid promotes exit of DCs from G_0/G_1 and entry into S-phase in response to GM-CSF addition. When retinoic acid signaling is blocked with the antagonist this proliferative response is inhibited.

Cyclin Protein Analysis by Flow Cytometry

From the same cell cultures that were analyzed for cell cycle by DNA staining, we also attempted to measure cyclin A2 and D3 protein expression. Cyclin A2 and D3 were chosen because they are known to be associated with G1 to S-phase transition. Although, cyclin E1 is also associated with this same transition commercial reagents were not available for its analysis. Each sample was stained with a primary antibody against the cyclin and a secondary antibody labeled with FITC. Each treatment group was compared to control samples labeled with either an irrelevant primary antibody followed by the secondary antibody labeled with FITC, or a sample stained with CD11c-PE only. As shown in Figures 14 through 16, the CD11c-PE control stained cells had the lowest FITC intensity, as expected. However, we were unable to identify any positive population for either cyclin that was analyzed by flow cytometry, since the isotype control was nearly identical to the positive stained cells, and in certain samples

was even higher in FITC intensity. Attempts were made to increase the positive staining by adding additional blocking steps to the protocol, but this was unsuccessful in obtaining significant cyclin labeling.

Cyclin mRNA Analysis

Cells grown with receptor antagonist, atRA or DMSO vehicle and restimulated on day 6 with GM-CSF were analyzed for cyclin A2, D3 and E1 mRNA expression at 0, 1, 2, and 4 hr following GM-CSF addition. The expression of each cyclin was normalized to GAPDH. Cells cultured with retinoic acid had the lowest mRNA expression of for all cyclins analyzed (Figure 17). The DMSO and the receptor antagonist treated cells had similar expression of cyclin A2 and E1 mRNA at all timepoints (Figure 17A, C). However, cyclin D3 mRNA was expressed the greatest by receptor antagonist treated cells (Figure 17B). Cells cultured with retinoic acid had the lowest expression of all cyclin mRNAs 2 hrs post GM-CSF addition, while cyclin expression in the other two treatment groups remained relatively constant.

Cyclin mRNA expression data was also plotted as normalized mRNA expression level for each cyclin at each timepoint, in an attempt to compare relative expression of each cyclin at each timepoint (Figure 18-20). Overall, the level of expression of cyclin E1 mRNA was higher in all the treatment groups at each timepoint, and was significantly higher than cyclin D3 at all timepoints for retinoic acid and vehicle (DMSO) treated cells (Figure 19, 20). Cyclin D3 mRNA had the lowest level of expression in all treatment groups but was not

significantly different than cyclin A2. In general, the relative expression of these three cyclins in retinoic acid and DMSO treated cells was very similar, and differed from the receptor antagonist treated cells (Figure 19, 20). Specifically, cyclin D3 expression was higher in the receptor antagonist treated cells at 0, 1, and 4 hrs and became comparable in level of expression to cyclin E1 at these timepoints (Figure 18).

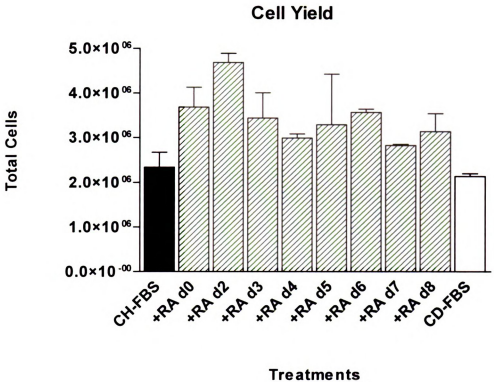


Figure 2: **Total cell yield of bone marrow cells cultured with GM-CSF, and addition of atRA (1 nM) at varying days following culture initiation.** Cells were counted on the hemocytometer to determine cell number. The cell numbers represent an average of 2 dishes; statistical analysis was not performed due to too few replicates. CH-FBS is the positive control. CD-FBS is medium containing vitamin A deficient serum and serves as the negative control. CD-FBS + atRA day 0 (+RA d0) represent cell cultures that had 1 nM of retinoic acid added to them on the day of the culture set up so they were grown with retinoic acid for 10 days. Other days noted indicate the day of initial atRA supplementation (+RA d2 through +RA d8). All cell cultures were harvested on day 10. The mean +/- SEM are shown.

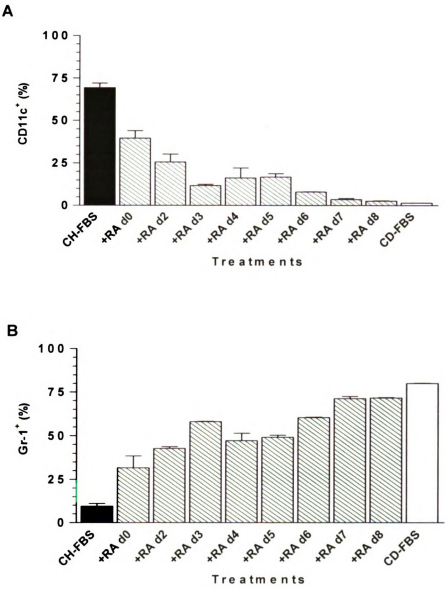


Figure 3: **Delayed atRA addition decreases percent dendritic cells and increases percent of neutrophils.** Bone marrow cells were stimulated with GM-CSF in medium containing CH-FBS, CD-FBS or CD-FBS +atRA (1 nM) as described in Figure 2 legend. Resulting cells were analyzed by flow cytometry for the percentage of cells in the cultures that were $CD11c^+$ and $Gr\text{-}1^+$. **A** and **B** represent dendritic cells and neutrophils, respectively. All cell cultures were harvested on day 10. Data shows one representative experiment of one (n=2/treatment). The mean +/- SEM are shown.

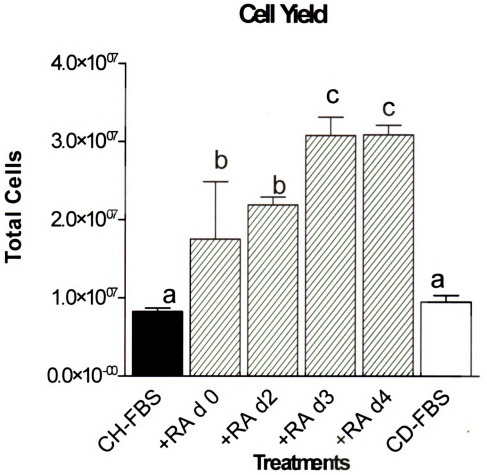


Figure 4: **Retinoic acid increases cell proliferation of myeloid progenitor cells in response to GM-CSF.** Cells were cultured as in Figure 2, with retinoic acid addition carried out only on days 0-4. CH-FBS and CD-FBS are the positive and negative controls, respectively. Data represent one experiment of 3, with n=7 technical replicates per treatment. Bars represent mean +/- SEM. Bars with different letter designations (a, b, c) are significantly different ($p \leq 0.001$). All cell cultures were harvested and analyzed on day 10.

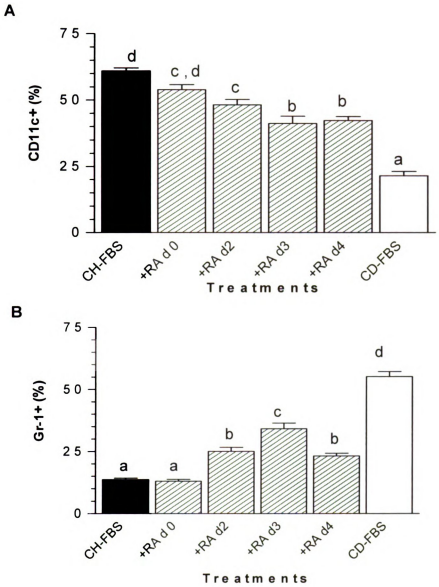


Figure 5: **Dendritic cell development is significantly decreased and neutrophil development significantly increased with delayed retinoic acid addition.** Cells were cultured with GM-CSF +/- retinoic acid (atRA) and analyzed by flow cytometry as described in Figure 3. $CD11c^+$ percent (**A**) and $Gr\text{-}1^+$ percent (**B**) represent dendritic cells and granulocytes, respectively. Data represent one experiment of 3, n=7 technical replicates per treatment. Bars represent mean +/- SEM, with a significant difference seen at $p \leq 0.001$ designated by different letters.

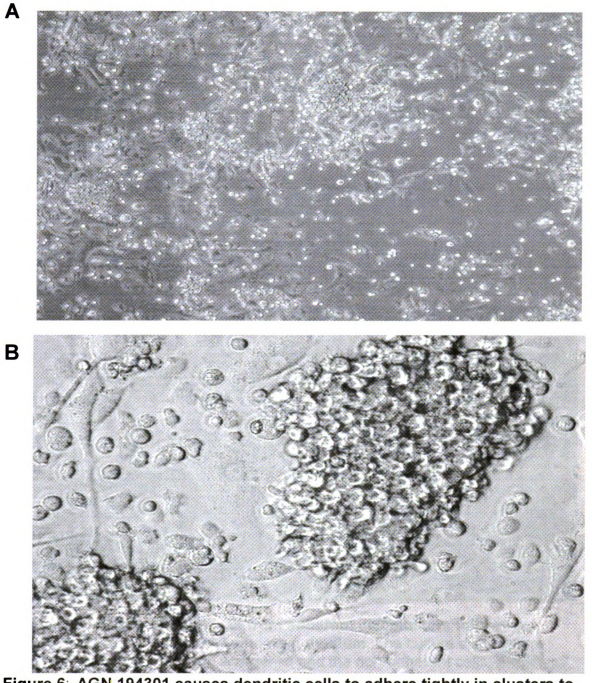


Figure 6: **AGN 194301 causes dendritic cells to adhere tightly in clusters to bottom of cell culture dishes.** Bone marrow cells were cultured with the RAR-α antagonist, AGN 194301 (10 nM), for 8 days in medium containing GM-CSF and CH-FBS. **A** and **B** were taken at 100X and 400X magnification respectively, on day 8. Cells seen on the images are tight clumps that are adhered to the bottom of the culture dishes (non-adherent cells were removed).

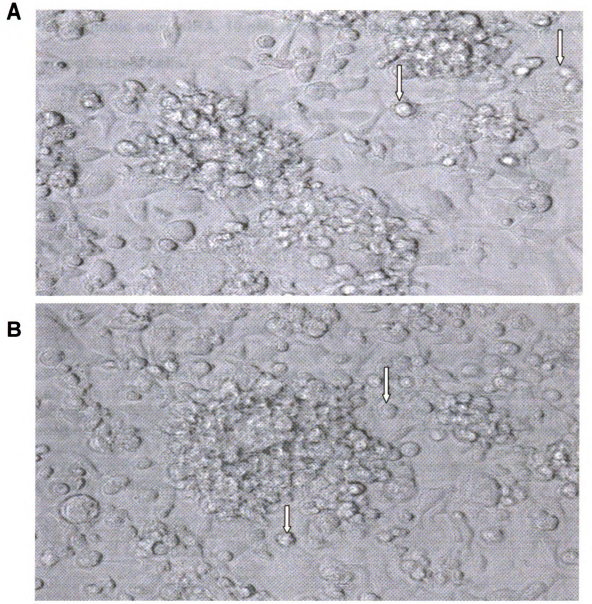


Figure 7: **Dendritic cells release from adherent cell clusters following replacement of AGN 194301 containing medium**. The culture dishes were rinsed on day 8 to remove the antagonist and the non-adherent cells. Fresh medium containing GM-CSF and atRA was added. Images were taken 24 hrs (**A**) and 48 hrs **(B)** after atRA replacement of antagonist. Both images are 400X magnification. Released dendritic cells are indicated by arrows.

Table I: Retinoic acid (atRA, 10 nM) increases proliferation of both adherent and non-adherent cells.*

Treatments	Cells/Dish			Percent Adherent
	Non-Adherent	Adherent	Total Cells	
194301	5.08×10^6	5.15×10^6	1.02×10^7	50.0 %
+ atRA (d8-d10)	6.12×10^6	6.27×10^6	1.24×10^7	50.6 %
atRA vs. 194301 (%)	120 %	122 %	122 %	

*Cells were cultured with GM-CSF and AGN 194301 (10 nM) for 8 days. On day 8, half of the cultures had medium containing AGN 194301 removed, and replaced with medium containing GM-CSF and atRA (10 nM). Adherent and non-adherent cells were harvested separately on day 10 (48 hrs after medium replacement) and pooled from 3 dishes per treatment. Cells were enumerated by hemacytometer counts. The percent cell yield of adherent and non-adherent cells from the atRA treatment group was compared with those grown in the antagonist for 10 days.

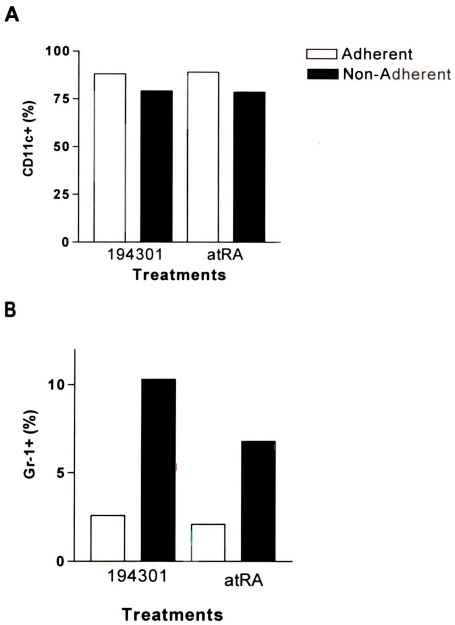


Figure 8: Adherent cells are primarily CD11c$^+$ dendritic cells. Bone marrow cells were stimulated with GM-CSF in medium containing AGN 194301 (10nM) for 8 days, then medium in half of the dishes was replaced with medium containing atRA (10 nM) and the culture was continued through day 10. Percent CD11c$^+$ dendritic cells (**A**) and Gr-1$^+$ neutrophils (**B**) were determined in both adherent and non-adherent cell populations at day 10. Results shown are from 1 experiment with 3 dishes pooled per treatment (therefore no statistical analysis was performed).

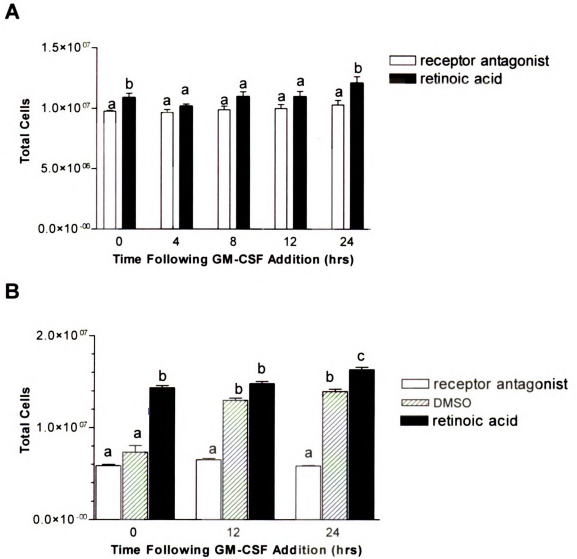
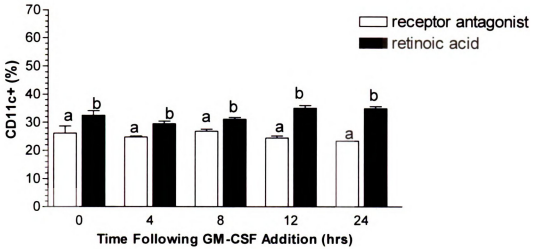


Figure 9: Cell cultures stimulated with GM-CSF have a higher cell number with retinoic acid treatment compared to DMSO and retinoid receptor antagonist treated cells. Bone marrow cells were stimulated with GM-CSF in medium containing CH-FBS and AGN 194301 (10 nM), atRA, or DMSO vehicle for 6 days. The cells were then stimulated with GM-CSF, collected and enumerated at different timepoints. The mean +/- SEM are shown as combined cell yield that was harvested and enumerated as separate adherent and non-adherent cell populations. **(B)** Replicate experiment; cells were harvested by combining adherent and non-adherent cells prior to enumeration. Bars represent mean +/- SEM of n=4-6 dishes/treatment at each timepoint. Bars with different letter designations are significantly different ($p \leq 0.05$).

A



B

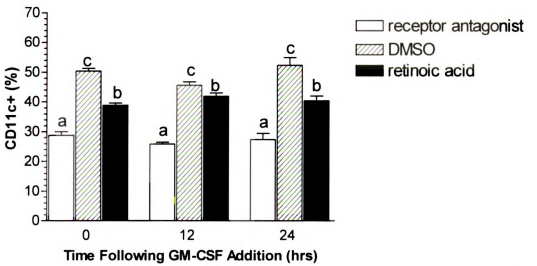
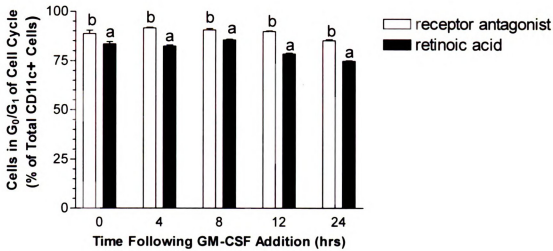


Figure 10: Retinoic acid increases the percent dendritic cells over time after GM-CSF stimulation. Bone marrow cells cultured in medium containing CH-FBS and GM-CSF, and RAR-α receptor antagonist, retinoic acid (10nM) or DMSO vehicle through day 6. All cells were harvested at timepoints listed following GM-CSF addition on day 6. Resulting cells were analyzed by flow cytometry. **A** and **B** show results from two separate experiments. Bars represent mean +/- SEM of n=4-6 dishes/treatment at each timepoint. Bars with different letter designations are significantly different ($p \leq 0.05$).

A



B

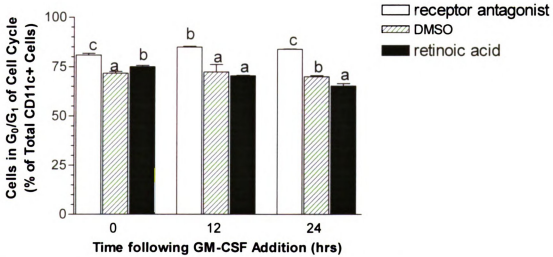


Figure 11: Retinoic acid receptor α antagonist treated cells show a retention in G_0/G_1 phase of the cell cycle after GM-CSF stimulation. The percent $CD11c^+$ cells from two replicate experiments (**A** and **B**) were analyzed using WinList and ModFit. WinList was used to gate on $CD11c^+$ cells, and these gated cells were then analyzed using Mod-fit to generate DAPI fluorescence histograms for assignment of cell cycle stages G_0/G_1 (n=1), S (1>n<2), or G_2/M (n=2). Data shown are the mean +/- SEM of the percent of total cells in culture (adherent and non-adherent cells) n= 4-6 technical replicates per timepoint. Bars with different letter designations are significantly different ($p \leq 0.05$).

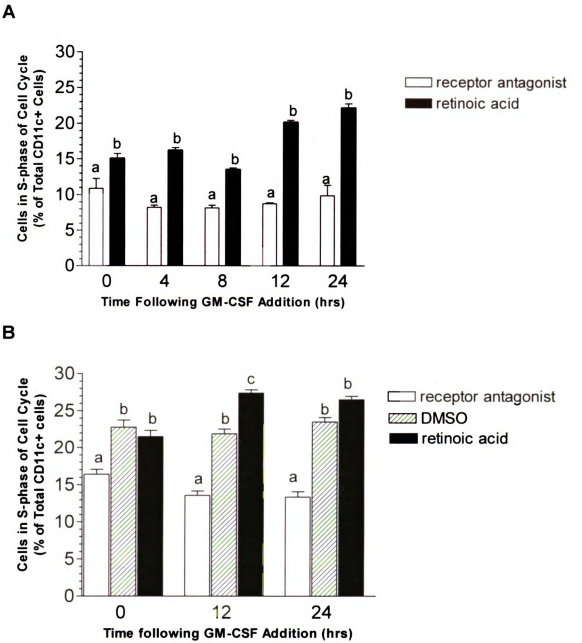


Figure 12: **Retinoic acid causes a significant increase in dendritic cells in S-phase of the cell cycle.** Two replicate experiments (**A** and **B**) showing the percent of $CD11c^+$ DC in each treatment group that were in S-phase of the cell cycle. Data were generated using WinList and ModFit as stated in Figure 11 legend. Data shown are the mean +/- SEM, n = 4-6 technical replicates per timepoint. Bars with different letter designations are significantly different ($p \leq 0.05$).

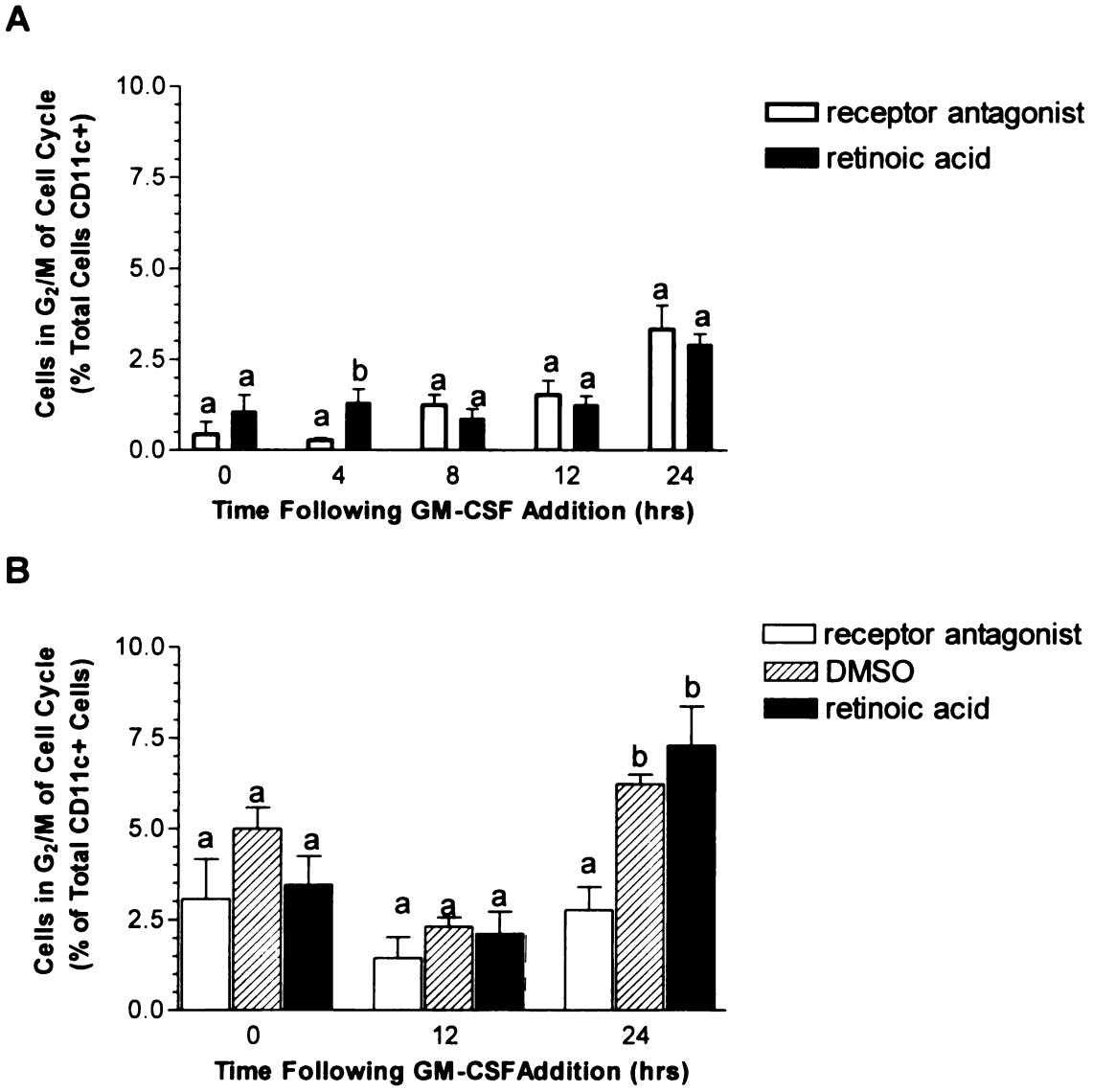


Figure 13: Retinoic acid increases the percent of CD11c+ dendritic cells in the G_2/M phase of the cell cycle 24 hrs following GM-CSF addition. Data were generated using WinList and ModFit as stated in Figure 11 legend. Two replicate experiments (**A** and **B**) are shown. Data shown are the mean +/- SEM, n = 4-6 technical replicates per timepoint. Bars with different letter designations are significantly different ($p \leq 0.05$).

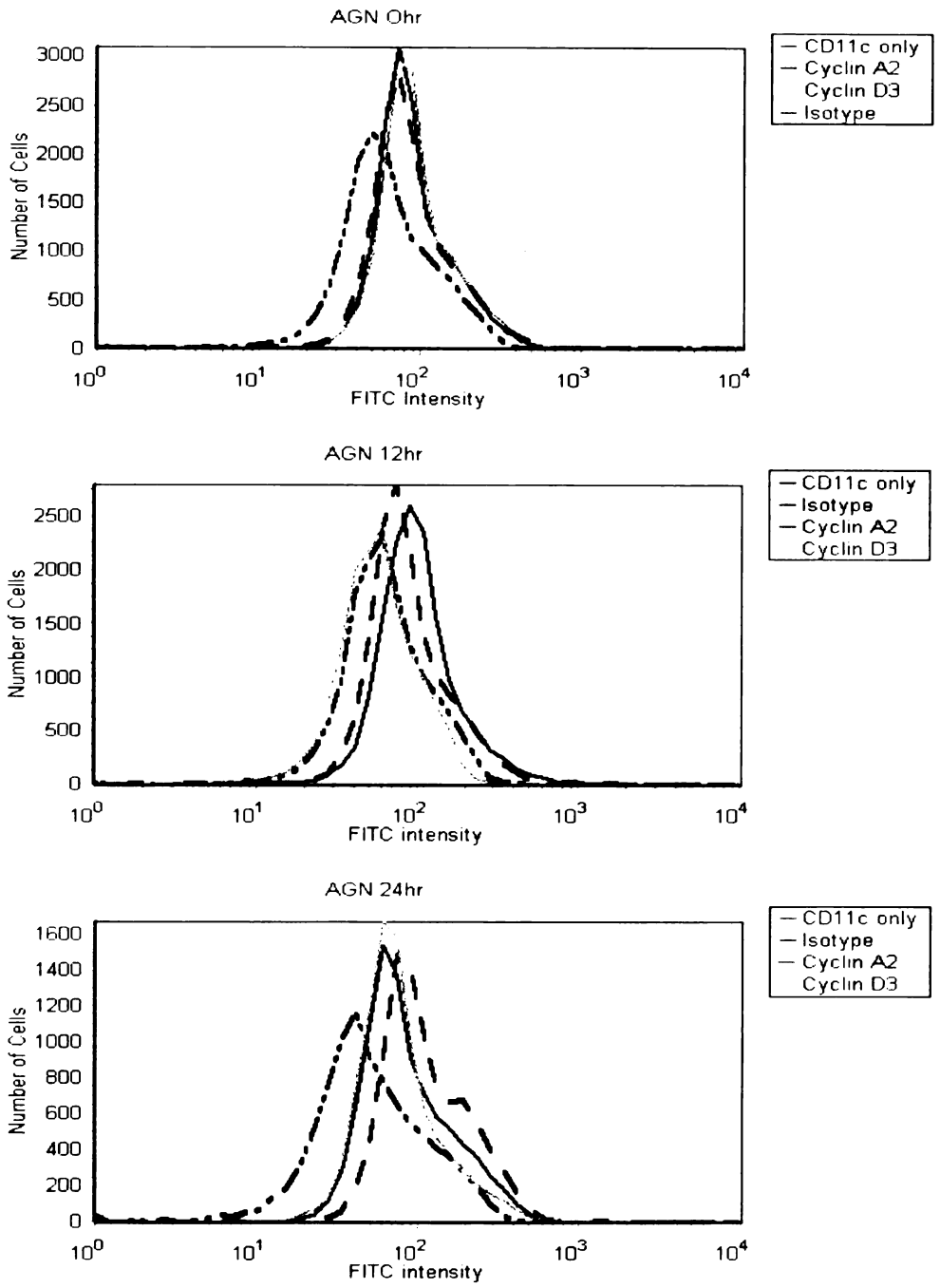


Figure 14: Flow cytometric analysis of cyclin expression in dendritic cells that were grown in AGN 194301. Bone marrow cells were stimulated with GM-CSF in medium containing CH-FBS and 10 nM AGN 194301 for 6 days. This is one representative experiment of n=4-6 dishes of cells that was harvested by combining adherent and non-adherent cells. The cells were then labeled with antibody to measure the expression of cyclin A2 or D3 by flow cytometry at each timepoint.

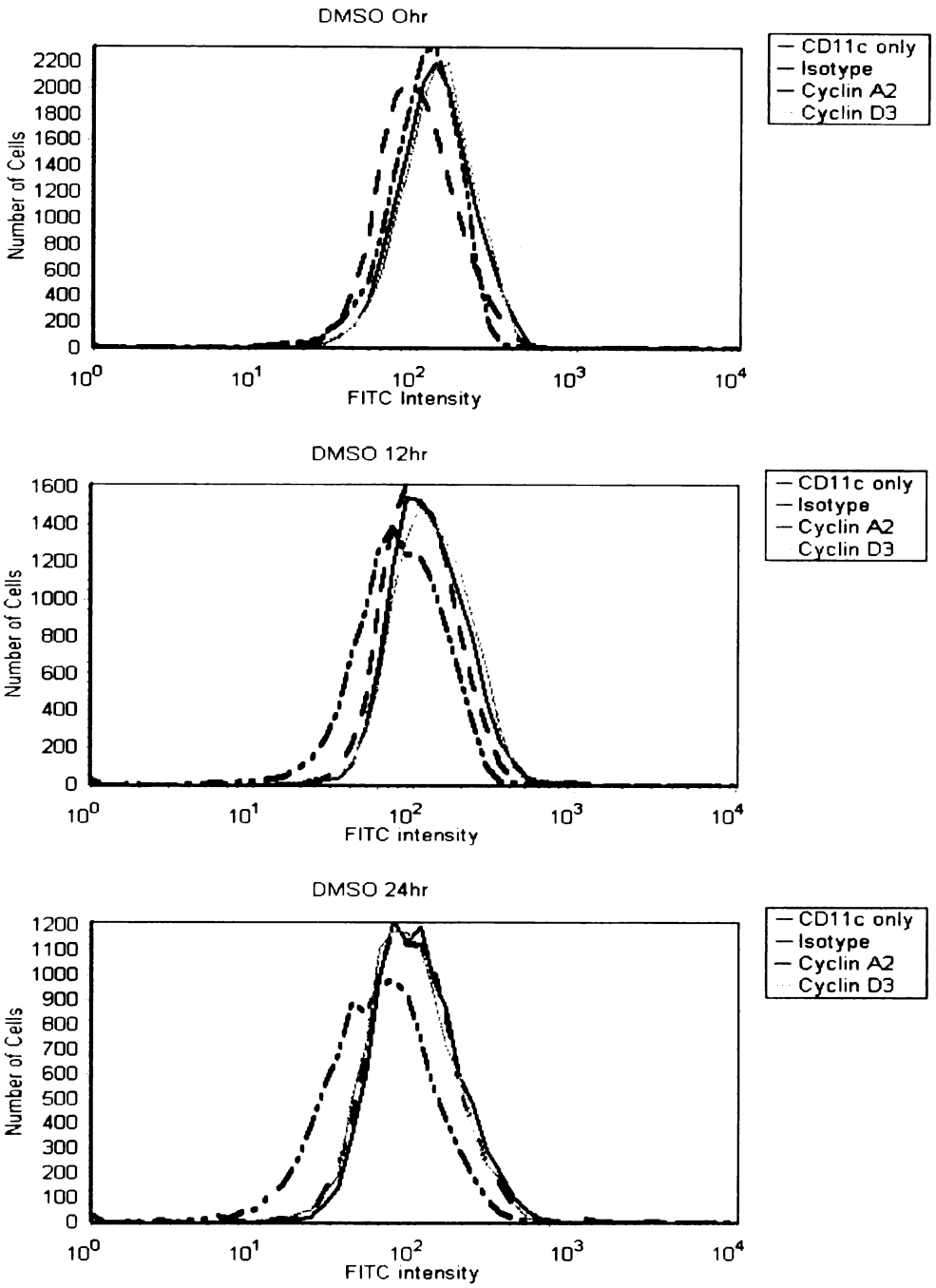


Figure 15: **Flow cytometric analysis of cyclin expression in dendritic cells that were grown in DMSO.** Bone marrow cells were stimulated with GM-CSF in medium containing CH-FBS and DMSO vehicle control for 6 days. This is one representative experiment of n=4-6 dishes of cells that was harvested by combining adherent and non-adherent cells. The cells were then labeled with antibody to measure the expression of cyclin A2 or D3 by flow cytometry at each timepoint.

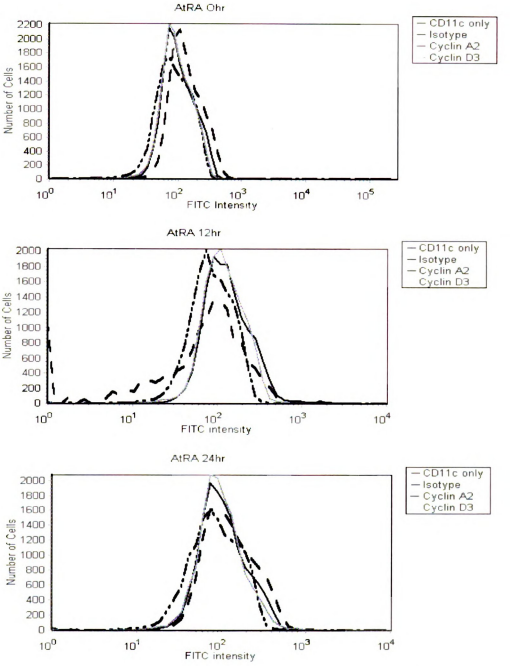


Figure 16: **Flow cytometric analysis of cyclin expression in dendritic cells that were grown in retinoic acid.** Bone marrow cells were stimulated with GM-CSF in medium containing CH-FBS and 10 nM AGN 194301 for 6 days. This is one representative experiment of n=4-6 dishes of cells that was harvested by combining adherent and non-adherent cells. The cells were then labeled with antibody to measure the expression of cyclin A2 or D3 by flow cytometry at each timepoint.

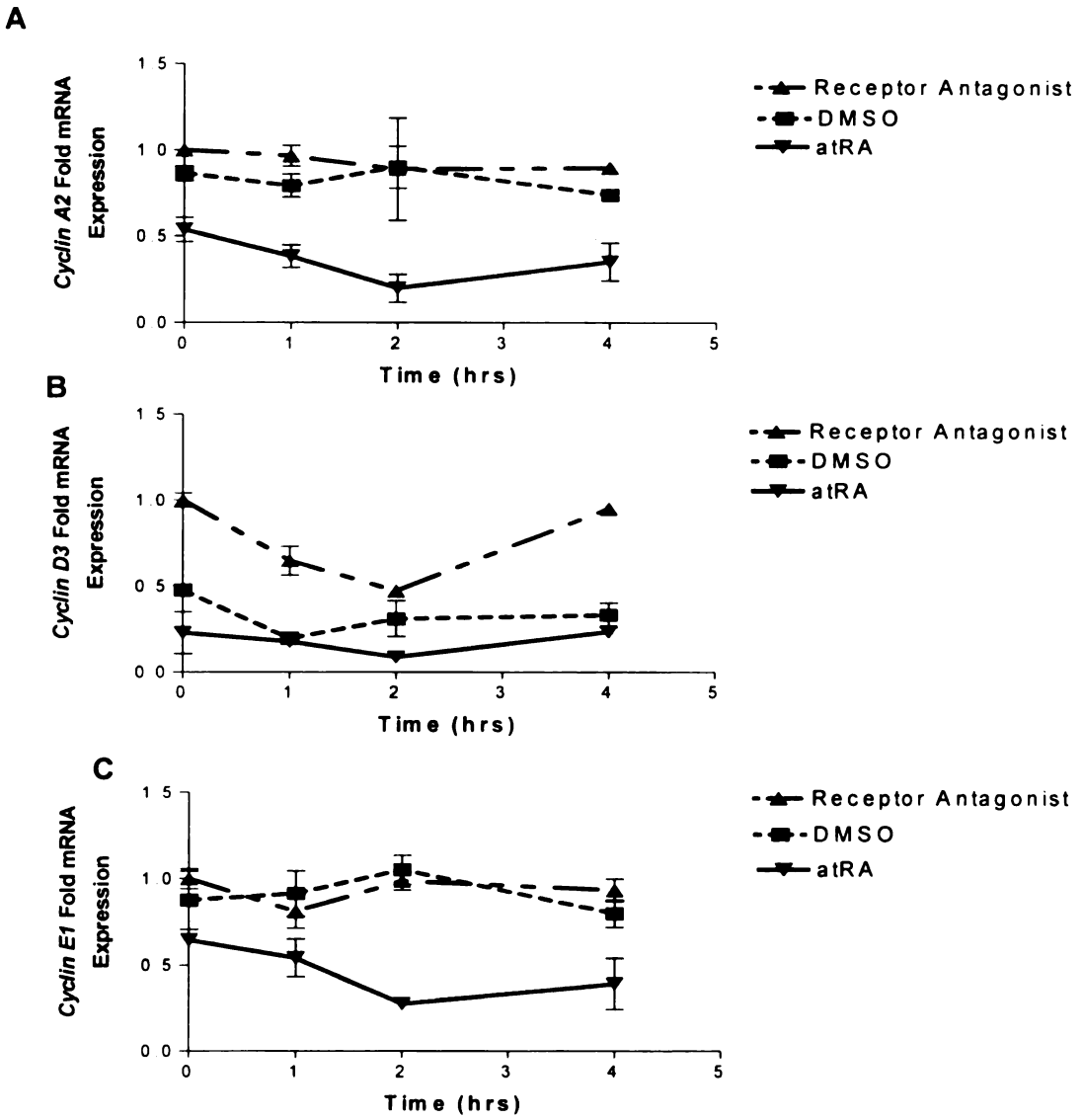


Figure 17: Cyclin mRNA expression in mouse bone marrow cells. Bone marrow cells were cultured with receptor antagonist, atRA or DMSO for six days, then stimulated with GM-CSF. Total RNA samples were prepared at times indicated following GM-CSF addition using TRIzol reagent and chloroform. Transcription of mRNA to cDNA was performed using Taqman™ reverse transcription assay kit. Expression of cDNA relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was analyzed by real-time PCR using Assays on Demand™ kits. Relative gene expression for cyclin A2 **(A)**, D3 **(B)** and E1 **(C)** was assayed at each timepoint. Data shown are the mean +/- SEM of 2 or 3 samples per treatment, calculated from the mean of triplicate PCR reactions per sample. All expression levels were normalized to the receptor antagonist at time=0. None of the treatment groups were significantly different at any timepoint.

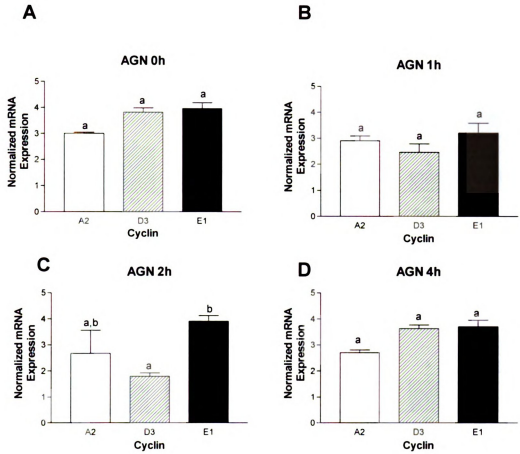


Figure 18: Cyclin mRNA expression levels from bone marrow cells cultured with AGN 194301 (receptor antagonist) following stimulation with GM-CSF. Samples were collected and analyzed as described in Figure 17. Cell mRNA was harvested following 0 **(A)**, 1 **(B)**, 2 **(C)**, or 4 **(D)** hrs following GM-CSF addition on day 6 of cell culture. Relative gene expression normalized to GAPDH for cyclin A2, D3 and E1 was done at each timepoint. Data shown are the mean +/- SEM of 2 or 3 samples per treatment, calculated from the mean of triplicate PCR reactions per sample. A significant difference was seen with $p \leq 0.05$. Bars with different letters are significantly different.

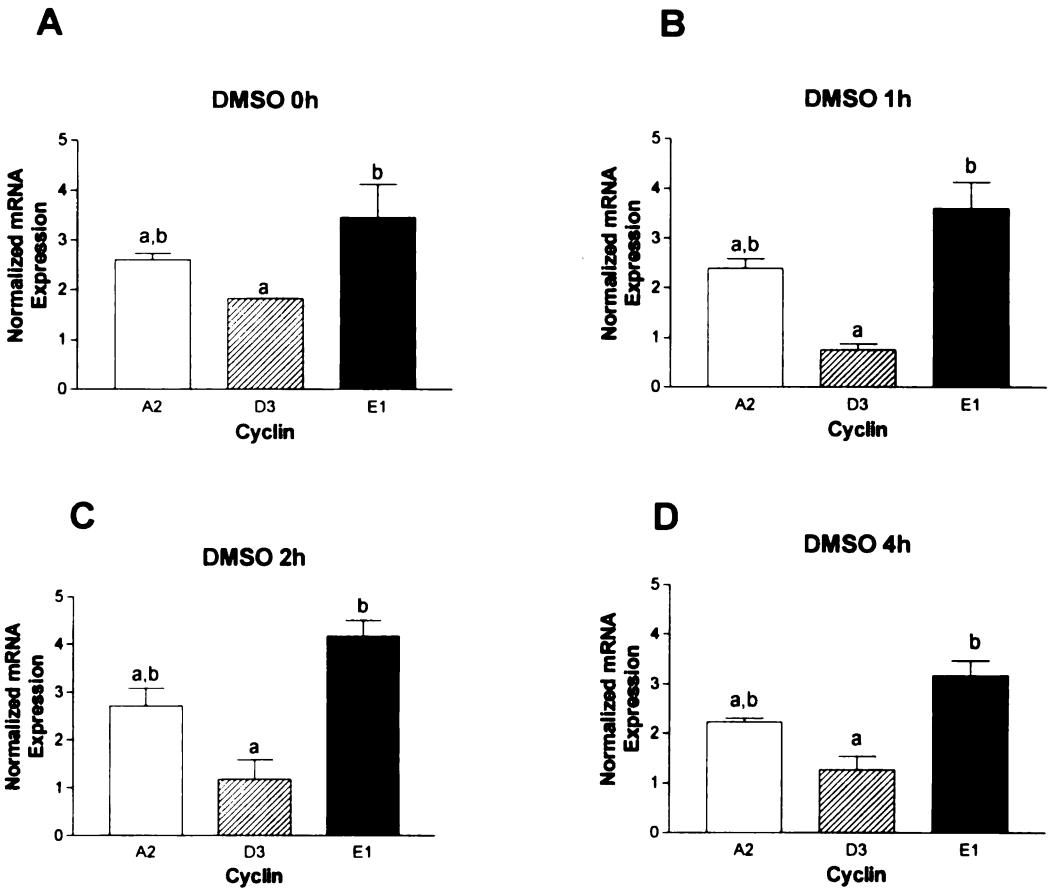


Figure 19: Cyclin mRNA expression levels from bone marrow cells cultured with DMSO following stimulation with GM-CSF. Samples were collected analysed as described in Figure 17 legend. Cell mRNA was harvested following 0 **(A)**, 1 **(B)**, 2 **(C)**, or 4 **(D)** hrs following GM-CSF addition on day 6 of cell culture. Relative gene expression normalized to GAPDH for cyclin A2, D3 and E1 was done at each timepoint. Data shown are the mean +/- SEM of 2 or 3 samples per treatment, calculated from the mean of triplicate PCR reactions per sample. A significant difference was seen with $p \leq 0.05$. Bars with different letters are significantly different.

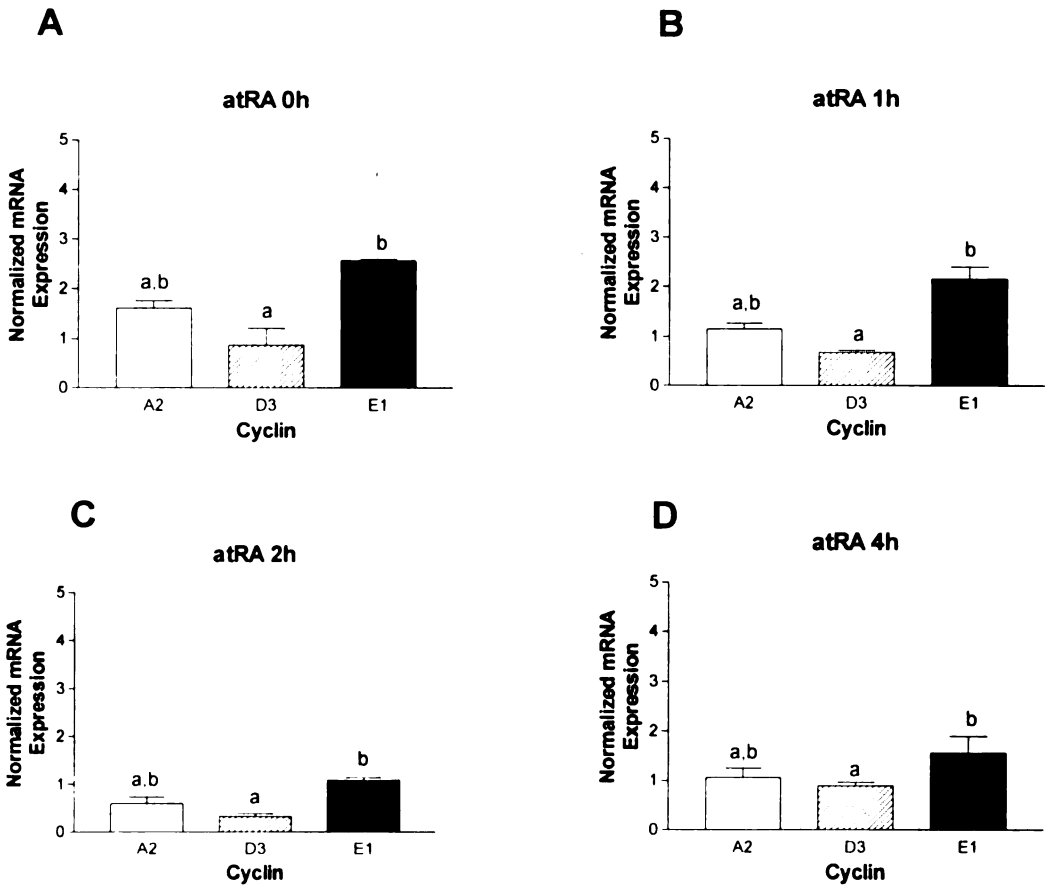


Figure 20: Cyclin mRNA expression levels from bone marrow cells cultured with retinoic acid following stimulation with GM-CSF. Samples were collected and analyzed as described in Figure 17 legend. Cell mRNA was harvested following 0 **(A)**, 1 **(B)**, 2 **(C)**, or 4 **(D)** hrs following GM-CSF addition on day 6 of cell culture. Relative gene expression normalized to GAPDH for cyclin A2, D3 and E1 was done at each timepoint. Data shown are the mean +/- SEM of 2 or 3 samples per treatment, calculated from the mean of triplicate PCR reactions per sample. A significant difference was seen with $p \leq 0.05$. Bars with different letters are significantly different.

DISCUSSION AND CONCLUSIONS

Vitamin A Dependent Myeloid Progenitor for DC

A common myeloid progenitor (CMP) exists that can give rise to neutrophils/granulocytes, monocytes/macrophages, and myeloid DCs. GM-CSF is well established as a growth factor for all three of these mature myeloid lineage cell populations (Fleetwood et al, 2005), and in particular GM-CSF *in vitro* is widely used to drive myeloid DC development from bone marrow precursors (Inaba et al, 1992; Lutz et al, 1999). It has previously been shown that GM-CSF dependent myeloid DC development *in vitro* is dependent upon vitamin A (Hengesbach and Hoag, 2004). Based upon this, we hypothesized that a particular myeloid progenitor stage may require vitamin A for commitment to DC development. To test this hypothesis, we performed cultures of murine bone marrow cells stimulated with GM-CSF, and delayed addition of retinoic acid to these cultures. We expected that there would be a timepoint where retinoic acid could no longer rescue DC development; this timepoint would be when the progenitor population committed to the granulocyte lineage instead of DC. Instead, we found that delayed retinoic acid addition resulted in a gradual decrease in DC commitment and increase in neutrophil commitment. Based upon these results, we were unable to identify a particular myeloid progenitor population that absolutely required retinoic acid for commitment to DC.

Hematopoietic Stem Cells → CMP→ Granulocytes or Monocytes or DC.

One of the primary reasons we were unable to identify a vitamin A dependent myeloid DC progenitor may have been the culture system used. The cell cultures contained a mixed bone marrow cell population which has been reported to contain approximately 45% B lymphocyte lineage cells, 50% myeloid lineage cells at various stages of development and only 5% progenitor and hematopoietic stem cells (Lagasse and Weissman 1996). The 50% myeloid cells include primarily neutrophils/granulocytes, immature neutrophil committed cells (promyelocytes, metamyelocytes), monocytes/macrophages, and DCs (in order of abundance). Recent publications have suggested that additional myeloid progenitor stages may exist between CMP and the mature myeloid effector cells. del Hoyo et al (2002) reported a DC progenitor population that could give rise to not only myeloid DC but also lymphoid and plasmacytoid DC populations. More recently, Fogg et al (2006) reported a common precursor for DC and macrophages. Since all of these myeloid precursor populations were likely present in our cultures, and all of them can likely respond to GM-CSF stimulation, our cultures represent a mixture of cells at various segments along the pathway to DC development. Therefore the commitment to DC lineage occurs over many days depending upon what stage these cells were at when the culture was initiated. Therefore, it is likely that many of the DC progenitors present in the cultures could have been beyond the stage that required vitamin A for DC development. A better experiment could have been to start with cells that were synchronized at a very early stage of development, such as lineage marker negative hematopoietic stem cells.

Retinoic Acid Addition Increases DC Culture Cell Yield

Retinoic acid supplementation in cultures of bone marrow cells stimulated with GM-CSF increased cell yield at the end of the ten day culture, with highest yield observed when retinoic acid was added between days 2 and 4. Therefore, it can be suggested that by this time in the cell cultures there may have been a high number of pre-DCs that proliferated optimally in the presence of retinoic acid thus resulting in an increased cell yield at the end of the ten day culture. After day 4, addition of retinoic acid became sub-optimal since some of these pre-DCs likely had committed to immature DCs which proliferate at a lower rate.

Another observation in the timecourse experiment was that adding retinoic acid on day 4 caused a slight decrease in the percent granulocytes/neutrophils that was obtained at the end of the ten day culture. This observation could be related to the increased cell yield that corresponds to retinoic acid addition at the same day. If a pre-DC is proliferating optimally when retinoic acid is added one would expect a corresponding drop in differentiated cells, since differentiation generally occurs while cells are in G_1. Another possible explanation for the drop in percent granulocytes/neutrophils may be that retinoic acid is triggering neutrophil apoptosis. It was suggested by Kuwata et al (2000) that retinoic acid deficiency results in excessive neutrophil accumulation due to a decrease in senescent neutrophil apoptosis. Therefore, it could be that adding retinoic acid on day 4 causes apoptotic death of neutrophils that matured during days 0-3 of the culture period.

Insensitivity of CD11c Staining for DC Identification

Our attempt to identify a vitamin A-dependent myeloid DC progenitor population could also have been hampered by our ability to reliably detect and quantify myeloid DC using the CD11c antibody. Although CD11c has been clearly identified as a pan-DC marker in mouse, our lab observes an overlap in fluorescence intensity for the isotype control stained cells and the CD11c positive cells (data not shown). This is due to the low intensity of fluorescence for the CD11c positive cells. Attempts to optimize the staining have not resulted in any brighter fluorescence. The overlapping fluorescence of the isotype control and the positive cells leads to an underestimation of the actual number of DCs present. This results in inconsistency in percent DC from one experiment to another preventing statistical analysis across experiments.

Vitamin A Content in Cell Culture Medium

The protocol used in culturing the DCs was developed by Inaba et al (1992) and modified by Lutz et al (1999). The culture medium contains 10% (v/v) FBS which has 100 times more the amount of serum retinol necessary to maintain cellular levels of all-*trans* retinoic acid (Cullum and Zile, 1985). Therefore, the original culture conditions contained enough serum retinol to support DC development in response to GM-CSF. Our vitamin A deficient (negative control) culture medium contained CD-FBS, which is truly not retinol deficient, but only reduced in retinol concentration by about 50%. Even though this decreased retinol concentration significantly decreases DC development, it does not prevent DC development completely. This suggests that there is enough vitamin A available to maintain the DC progenitor population and be

permissive for low level DC differentiation. This could hinder our ability to identify a defined point where vitamin A is essential for DC development.

Retinoic Acid Receptor Antagonist Influences DC Adherence

An alternative approach for controlling vitamin A availability would be to add an RAR antagonist to the cell cultures in an attempt to block vitamin A signaling at the receptor level. Preliminary experiments performed in medium containing CH-FBS with and without addition the receptor antagonist for the entire ten day culture period showed a significant decrease in the percent of DC obtained and a significant increase in the percent of neutrophils obtained in cultures that had the receptor antagonist, as expected (data not shown). However, unexpectedly, the cell yield obtained at the end of the culture period for cultures containing receptor antagonist was decreased about 75% (data not shown). After microscopic inspection of the culture dishes, it was found that there were a large number of cells adhering in tight clusters to the culture dish surface in the presence of receptor antagonist. When these adherent cells were removed with enzyme treatment and analyzed by flow cytometry, to our surprise, they were found to be ~ 80% CD11c positive. Additionally, when adherent and non-adherent cells were pooled together there was no longer a difference in cell yield between cultures with and without the receptor antagonist. These findings led us to conclude that there likely is not a vitamin A-dependent pre-DC population. Instead, the DCs were developing in the absence of vitamin A but were adhered to the culture dishes and were therefore not being analyzed.

After this observation, we next hypothesized that vitamin A was necessary for the release of DCs from the culture dishes, and designed an experiment to test this hypothesis. Cultures of bone marrow cells stimulated with GM-CSF were initiated in medium containing CH-FBS and the receptor antagonist. At day 8 of this culture period the non-adherent cells and medium were completely removed (non-adherent cells were ~80% neutrophils; data not shown). On day 8, the adherent cells were in clusters as shown in Figure 6. Fresh medium containing retinoic acid or receptor antagonist was added for the remaining two days of the cell culture. Surprisingly, we observed an equal percent of these cells released from the culture dishes whether retinoic acid or the receptor antagonist was present (Table 1). This suggests that the neutrophils present under vitamin A deficient conditions (cultures containing the antagonist) are acting on the DCs to cause them to remain adherent. Although studies focusing on the influence of neutrophils on DC are limited, there is some recent evidence suggesting that neutrophils can modify DC function. It has been shown that LPS-activated neutrophils can change the antigen presenting cell function of myeloid DC, causing them to drive Th1 development instead of Th2 development (van Gisbergen et al, 2005). This suggests that the neutrophils may be releasing factors that are having an effect on DC adherence. When neutrophils are present vitamin A allows DC to overcome the influence of the neutrophils and release from the dish. However, when neutrophils are removed vitamin A is no longer necessary for the DC to release from the dish.

Retinoic Acid Effect on DC Proliferation in Response to GM-CSF

In previous DC adherence experiments, cultures that received retinoic acid treatment for the final two days of culture showed an increase in cell yield compared to those cultures that remained in the receptor antagonist (Table 1). We hypothesized that this increase in cell yield in the presence of retinoic acid was due to increased DC cycling in response to GM-CSF. A set of experiments were designed to identify the changes in the cell cycle of DCs over time in response to GM-CSF. These cultures contained the receptor antagonist, retinoic acid or DMSO vehicle control from day 0. The cell cultures for cell cycle analysis were all harvested on day 6 because evidence suggest that between days 4 and 6 of culture, DC develop in aggregates that are loosely adherent to stromal cells (Young and Steinman 1996). This was also shown in our lab that by day 6 of culture 80% of the adherent cell population were positive for the CD11c marker (data not shown). The cells were labeled for CD11c expression and the DNA was labeled to determine the cell cycle stages. Ethanol fixation required for DNA labeling destroyed the Gr-1 conjugate labeling so emphasis was placed on DC analysis only.

Retinoic Acid Effect on DC Cycling

Our cell cycle studies demonstrated that a large proportion of cells in all three treatment groups were in G_0/G_1 at all timepoints tested. However, cells cultured with retinoic acid or the DMSO vehicle control had a significant increase of cells in S-phase following GM-CSF addition (Figure 11, 12). However, cells cultured with the receptor antagonist were retained in G_0/G_1 and were prevented

from entering S-phase, despite the addition of the growth factor. This observation correlates well with the total cells obtained from these cultures in that the cell yield from cultures containing receptor antagonist remained constant over the 24 hr testing period (Figure 9, 12).

Retinoic Acid Effect on DC Cyclin Expression

Researchers identified cyclins as one the key regulators in cell cycle. Therefore, studies were performed based on the cell cycle results to assess the changes in cyclin protein expression. Emphasis was placed on cyclin A2, D3 and E1, because the greatest differences in the treatment groups were observed in the S phase and there was a constant percent of cells in G_0/G_1 in the receptor antagonist treated cells suggesting a block in the G_1 to S transition in the presence of the receptor antagonist. Commercial reagents were only available from a single vendor and only for the study of cyclin A2 and D3. The data obtained from the analysis of cyclins by flow cytometry was far from successful or what was expected. As shown in Figures 14-16, the specific labeling for cyclins A2 and D3 resulted only in a minor shift in fluorescence intensity for the whole cell population, and negative and positive staining cells could not be identified as separate populations. Analysis of cyclins by flow cytometry is possible for human cells but at this time the proper reagents in mice are not yet available. In addition, flow cytometry is designed for detecting large changes in expression on the order of a log increase or more, and is not sensitive enough to detect 2 or 3-fold changes. It might be more reliable to test by western blot where a 3-fold difference in expression could be reasonably detected.

Further cyclin studies were performed by looking at mRNA expression; again the above named cyclins were analyzed by real time PCR. In general, expression of cyclin A2, D3 and E1 mRNA remained relatively constant during the 4 hr period following GM-CSF addition. Cells cultured with retinoic acid had the lower expression of cyclin A2 and E1 mRNA compared to the other two treatment groups. Cells cultured with the receptor antagonist expressed cyclin D3 mRNA at higher levels compared to cells cultured in retinoic acid or DMSO vehicle. Most interestingly, cells cultured with retinoic acid and the and DMSO vehicle control consistently had low level of cyclin D3 mRNA in relation to A2 and E1 mRNA, while in receptor antagonist treated cells, cyclin D3 mRNA was one of the most abundant mRNA's present (Figures 18-20). Since previous studies had shown retinoic acid and the DMSO vehicle both showed enhanced entry into S-phase following GM-CSF addition (Figure 12B), we expected retinoic acid and the DMSO vehicle results to be similar for the cyclin(s) that is/are important in G_1 to S-phase transition. Since retinoic acid and the DMSO vehicle differed in cyclin A2 and E1 mRNA abundance (with DMSO vehicle showing comparable expression to receptor antagonist), we concluded that these two cyclins were less important in G_1 to S-phase transition. However, retinoic acid and DMSO vehicle treated cells both showed about 50% lower expression of cyclin D3 at all timepoints compared to receptor antagonist treated cells. In summary, our data suggests that lower levels of cyclin D3 mRNA would enhance myeloid DC proliferation in response to GM-CSF and that retinoic acid may promote myeloid DC proliferative response to GM-CSF via regulation of cyclin D3 transcription.

However, recent studies of cyclin mRNA levels at various stages of the cell cycle suggest that cyclin mRNA transcription is relatively stable, and that cyclin protein activity is likely modulated more at the translational and post-translational level (Penelova et al, 2005). In particular cyclin D1 and D3 mRNA levels remain unchanged throughout the cell cycle. In another study performed with murine NIH-3T3 cells that were coinfected with human cyclin E it was shown that cyclin E protein levels are not necessarily reflected by its mRNA level suggesting that it is post translationally controlled. The same study also illustrated that increased free E2F (released from pRB) increases the stabilization and accumulation of cyclin E (Pajalunga and Crescenzi, 2004). Therefore the differences observed in cyclin D3 mRNA in our experiments may not relate to actual function of cyclin D3 protein in these cells and must be interpreted with caution.

Cyclin D3 and Retinoic Acid Interaction in Myeloid Cell Lines

There have been several publications implicating a role for retinoic acid in induction of cell cycle arrest in myeloid cell lines, and evidence that this is achieved through inhibition of cyclin activity. In studies using B lymphocytes and U-937 cells, retinoic acid caused cell cycle arrest in G_0/G_1 due to upregulation of CKIs $p21^{WAF/CIP1}$ and $p27^{KIP1}$, respectively (Naderi and Blomhoff, 1999; Dimberg et al, 2003b). Retinoic acid has also been shown to cause accumulation of $p27^{KIP1}$ in the nucleus of neuroblastoma cells (Borriello et al, 2006). Further studies by other researchers showed that over expression of $p27^{KIP1}$ arrests myeloid cells at G_1-S transition specifically, while $p21^{CIP1}$ can arrest cells in G_1 and G_2 (Munoz-Alonso et al, 2005). Since we see a block at G_1-S transition in

the presence of the retinoid receptor antagonist, it is possible that $p27^{KIP1}$ may be involved in retinoid regulation of myeloid DC proliferation in response to GM-CSF.

The data presented in the published literature consistently shows that retinoic acid causes cell cycle arrest at G_0/G_1. Our data contrast with this, demonstrating that myeloid DC cell cycling is enhanced in the presence of retinoic acid and lower in the presence of retinoid receptor antagonist. Our studies differ from the published literature in two important ways. First, the studies in the published literature used retinoic acid at a very high concentration (1-5 μM) compared to 10 nM concentration that we used to study the cell cycling of DCs. Second, the DCs in our studies were differentiated primary cells as opposed to progenitor cell populations such as U-937 and HL-60 promyelocytic leukemia cells. Therefore, it is very difficult to correlate our data with that published by other researchers. Nonetheless, we propose a hypothetical model by which physiological levels of retinoic acid can enhance myeloid DC cycling in response to GM-CSF, and which may also explain why pharmacologic levels of retinoic acid can cause cell cycle arrest in promyelocytic leukemia cell lines (Figure 22).

Hypothetical Model For Retinoic Acid Enhancement of Myeloid DC Proliferation

A number of studies and reviews focus on the importance of CDKs and how they form complexes with different cyclins to enable cell cycle progression (Hwang and Clurman, 2005; Malumbres and Barbacid, 2005). These reviews also suggest that the study of cyclins and their importance in cell cycle

progression is far beyond mRNA expression and protein analysis. CDK2 also poses difficulty because it has the ability of activating more than one cyclin. It is known to activate cyclin A1, A2, E1 and E2. Some of the major substrates listed also include CBP/300 (which interacts with the retinoic acid receptors) and $p21^{CIP1}$ and $p27^{KIP1}$, which are inhibitors of cyclin E (Malumbres and Barbacid, 2005). Cyclin A is also a very interesting cyclin because it binds both CDK1 and CDK2 and plays a role in both S phase and mitosis (Yam et al, 2002).

Cyclin D3 data have already been published demonstrating that this cyclin binds to RARα and CRABP-II causing upregulation of retinoid mediated transcription but there is little evidence available about the interaction of retinoic acid receptors with cyclin A and E. The E2F family of transcription factors is known to be important in the expression of genes for G_1/S transition and DNA synthesis (Cam and Dynlacht, 2003). There is numerous evidence that has already been published about the control of E2F by the pRB protein; however, the pRB protein can become inactivated by activation of cyclinE/cdk2. This is normally due to the increase expression of cyclin E which is increased by E2F when pRB is phosphorylated (Bartek and Lucas, 2001; Hinds 2003). Therefore, from deep analysis of published data and reviews a hypothetical model was developed suggesting what might be happening in the dendritic cells in the presence of the different treatments and GM-CSF (Figure 22).

Suggested Future Research

There are a number of limitations that were identified in our studies. However, with the observations and conclusions made from these studies future

studies can be performed based on these limitations. To avoid the problem of having a mixed cell population for these cell cultures, it would have been best to use bone marrow cells in which lineage positive cells were depleted. However, this would have been a very expensive method since more animals would be needed per experiment and the cell yield would have been much lower.

The central dogma is DNA→ RNA → protein which is a continuous cycle. In some cell types retinoids have been known to induce growth arrest causing an increase in the total amount of the Rb protein without affecting phosphorylation (Harvat and Jetten, 1999). To clearly explain our data more experiments will have to be performed with emphasis on the key cell cycle regulators. Cell cycle is regulated by cyclins, the Rb protein, p53 gene (in the presence of DNA damage), c-myc expression, and AP-1 growth mediated signals (Harvat and Jetten, 1999). In our experiments the greatest differences were seen in the G_0/G_1 phase and the S-phase. Since the cells were retained in G_0/G_1 in the receptor antagonist, the first step would be to change the protocol of the experiment: by growing the cells all in the receptor antagonist up to day 6, then remove all the cells and medium, restimulate with GM-CSF and retinoic acid then harvest at different timepoints. Secondly, synchronize the cells by using a chemical such as indole-3-carbinol which has been used by other researchers to synchronize cells in G_0/G_1, then adding retinoic acid on day 6 and restimulating with GM-CSF. The commitment of myeloid to specific lineages is controlled by transcription factors that regulate differentiation in response to specific colony-stimulating factors (Kawamoto and Minato, 2000). Therefore to understand what

retinoic acid is doing in increasing the commitment and progression of DCs through the cell cycle more cyclin studies would have to be performed. These experiments would better explain the hypothetical model and would provide more evidence of what is happening in retinoic acid treated cells versus those with the receptor antagonist. Since in both suggested studies all the floating cells and medium would be removed, this would minimize the influence of neutrophils on the DCs. Even though, it would be very good to know how the neutrophils are cycling versus the dendritic cells. Cyclins E1 and E2 are known to be expressed during late G_1 phase and their expression continues through the end of the S-phase but work in conjunction with kinase subunit CDK2 (Möröy and Geisen, 2004). One of the most important factors in observing cyclin E levels is that in different cell cycle phases both the degradation of the protein and its stability are post-translationally modified (Moroy and Geisen, 2004). Some of the modifications and instability of the cyclin E were not taken into consideration before the DC cyclin studies were performed, however a better way to analyse post-translational regulation is by western blot analysis, paying particular attention to phosphorylation status of the cyclin proteins.

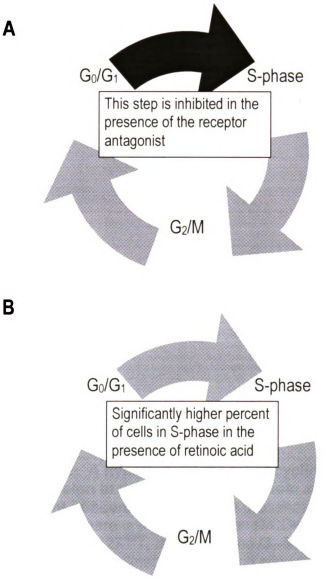


Figure 21: **Outline of the progression of dendritic cells through the cell cycle phases.** Model depicts cell cycling in the presence of the receptor antagonist (**A**) and in the presence of retinoic acid (**B**) at 10 nM concentration.

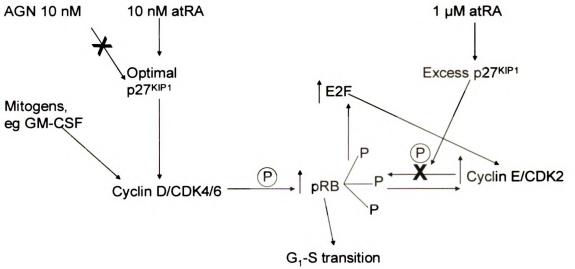


Figure 22: A hypothetical model of cyclin/CDK control of cell cycle progression in the presence of retinoic acid and the receptor antagonist at different concentrations. In the presence of GM-CSF and 10 nM receptor antagonist there is a block in cyclin D/CDK4/6 complex formation normally promoted by $p27^{KIP1}$, resulting in no G_1-S transition. In the presence of 10 nM atRA, there is optimal $p27^{KIP1}$ which allows for the formation of cyclinD/CDK4/6 leading to phosphorylation of pRB. Phosphorylation of pRB causes release of E2F by pRB, allowing E2F to increase cyclin E expression. Cyclin E binds to CDK2 to further phosphorylate pRB, leading to G_1-S transition. With 1 μM atRA there is an excess of $p27^{KIP1}$ which inhibits cyclin E/CDK2 phosphorylation of pRB resulting in a block in G_1-S transition.

References

Banchereau J and RM Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245-252.

Barrera G, S Pizmenti, S Laurora, F Briatore, C Toaldo and M Dianzani. 2005. 4-Hydroxynonenal and cell cycle. *Biofactor* 24:151-157.

Bartek J and J Lukas. 2001. Pathways governing G1/S transition and their responses to DNA damage. *FEBS Lett.* 490:117-122.

Bastien J and C Rochette-Egly. 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* 328:1-16.

Blomhoff HK. 2004. Vitamin A regulates proliferation and apoptosis of human T- and B-cells. *Biochem. Soc. Trans.* 32:982-984.

Blomhoff R. 1994. Transport and Metabolism of Vitamin A. *Nutr. Rev.* 52:S13-S23.

Borriello A, V Cucciolla, M Criscuolo, S Indaco, A Oliva, A Giovane, D Bencivenga, A Iolascon, V Zappa and FD Ragione. 2006. Retinoic acid induces $p27^{KIP1}$ nuclear accumulation by modulating its phosphorylation. *Cancer Res.* 66:4240-4248.

Cam H and BD Dynlacht. 2003. Emerging roles for E2F: Beyond the G1/S transition and DNA synthesis. *Cancer Cell* 3:311-316.

Chambon P. 1996. A decade of molecular biology of retinoic acid receptors. *FASEB. J.* 10:940-954.

Chen Q and AC Ross. 2004. Retinoic acid regulates cell cycle progression and cell differentiation in human monocytic THP-1 cells. *Exp. Cell Res.* 297:68-81.

Clagett-Dame M and HF DeLuca. 2002. The role of vitamin A in mammalian reproduction and embryonic development. *Annu. Rev. Nutr.* 22:347-381.

Clevenger CV, KD Bauer and AL Epstein. 1985. A method of simultaneous nuclear immunofluorescence DNA content using monoclonal antibodies and flow cytometry. *Cytometry* 6:208-214.

Cullum ME and MH Zile. 1985. Metabolism of all-*trans* retinoic acid and all-*trans* retinyl acetate. Demonstration of common physiological metabolites in rat small intestinal mucosa and circulation. *J. Biol. Chem.* 260:10590-10596.

Dawson HD, NQ Li, KL Decicco, JA Nibert and AC Ross. 1999. Chronic marginal vitamin A status reduces natural killer cell number and function in aging Lewis rats. *J Nutr.* 129:1510-1517.

DeKoter RP and H Singh. 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* 26:1439-1441.

del Hoyo GM, P Martin, HH Vargas, S Ruiz, CF Arlas and C Ardavin. 2002. Characterization of a common precursor population for dendritic cells. *Nature* 415:1043-1047.

Despouy G, JN Bastie, S Deshaies, N Balitrand, A Mazharian, C Rochette-Egly, C Chomienne, and L Delva. 2003. Cyclin D3 is a cofactor of retinoic acid receptors modulating their activity in the presence of CRABPII. *J. Biol. Chem* 278:6355-6362.

Dimberg A and F Oberg. 2003a. Retinoic acid-induced cell arrest of human myeloid cell lines. *Leukemia. Lymphoma.* 44:1641-1658.

Dimberg A, I Kalberg, K Nilsson and F Oberg. 2003b. Ser727/Tyr701-phosphorylated Stat1 is required for regulation of c-Myc, cyclins, and p27KIP1 associated with atRA-induced G0/G1 arrest of U-937 cells. *Blood* 102:254-261.

Fleetwood AJ, AD Cook and JA Hamilton. 2005. Functions of granulocyte-macrophage colony-stimulating factor. *Crit. Rev. Immunol.* 25:405-428.

Fogg DK, C Sibon, C Miled, S Jung, P Aucouturier, DR Littman, A Cumano and F Geissmann. 2006. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 311:83-87. (Published online 24 November 2005; 10.1126/science.1117729)

Gaines P and N Berliner. 2003. Retinoids in myelopoiesis. *J. Biol. Regul. Homeost. Agents* 17:46-65.

Garrett W, L Chen, R Kroschewski, M Ebersold, S Turley, S Trombetta, JE Galan and I Mellman. 2000. Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* 102: 325-344.

Givan AL. 2001. Flow Cytometry: First Principles. 2nd ed. Wiley-LISS.

Gropper SS, JL Smith and JL Groff. 2005. Advanced Nutrition and Metabolism. 4th ed. Belmont, CA: Thomson Wadsworth.

Harrison EH. 2005. Mechanism of Digestion and Absorption of Dietary Vitamin. *Annu. Rev. Nutr.* 25:87-103.

Harvat B and AM Jetten. 1999. "Growth control by retinoids: Regulation of cell cycle progression and apoptosis", in Handbook of Experimental Pharmacology; Retinoids, H Nau and WS Blaner, Eds Springer-Verlag, pp. 239-259.

Hayes CE, FE Nashold, FE Gomez and KA Hoag. 1999. "Retinoids and Immunity" in Handbook of Experimental Pharmacology; Retinoids, H Nau and WS Blaner, Eds Springer-Verlag, pp. 589-610.

Hengesbach LM and KA Hoag. 2004. Physiological concentrations of retinoic acid favor myeloid dendritic cell development over granulocyte development in cultures of bone marrow cells from mice. *J. Nutr.* 134: 3736-3739.

Hinds PW. 2003. Cdk2 dethroned as master of S phase entry. *Cancer Cell* 4:305-307.

Hoag KA, FE Nashold, J Governman and CE Hayes. 2002. Retinoic acid enhances the T helper 2 cell development that is essential for robust antibody responses through its action on antigen-presenting cells. *J Nutr.*132: 3736-3739.

Hwang HC and BE Clurman. 2005. Cyclin E in normal and neoplastic cell cycles. *Oncogene* 24:2776-2786.

Inaba K, M Inaba, N Romani, H Aya, M Deguchi, S Ikeharaa, S Muramatsu, and RM Steinman. 1992. Generation of large numbers of DCs from mouse bone marrow cultures supplemented with granulocyte-macrophage colony stimulating factor. *J. Exp. Med.* 176:1693-1702.

Jacobberger WJ. 1989. Cell cycle expression of nuclear proteins, In: Flow Cytometry. Advanced Research and Clinical Applications. Yen A, (ed). CRC Press. 1:305-326.

Johnson AT, ES Klein, SJ Gillet, L Wand, TK Song, ME Pino and RAS Chandraratna. 1996. Synthesis and characterization of a highly potent and effective antagonist of RARs. *J. Med. Chem.* 38: 4764-4767.

Kawamoto H and N Minato. 2003. Cells in focus: Myeloid cells. *Int. J. Biochem. Cell Bio.* 36:1374-1379.

Klaas PJM, V Gisbergen, MS Hernandez, T Geijtenbeek and Y Kooyk. 2005. Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J Exp. Med.* 201:1281-1292.

Kuwata T, IM Wang, T Tamura, RM Ponnamperuma, R Levine, KL Holmes, HC Morse, LM DeLuca and K Ozato. 2000. Vitamin A deficiency in mice causes a systemic expansion of myeloid cells. *Blood* 95:3349-3356.

Lagasse E and IL Weissman. 1996. Flow cytometric identification of murine neutrophils and monocytes. *J. Immunol. Methods* 197:139-150.

Lutz MB, N Kukutsch, ALJ Ogilvie, S RoBner, F Koch, N Romani and G Schuler. 1999. An advanced culture method for generating large quantities of highly pure DCs from mouse bone marrow. *J. Immunol. Methods* 223:77-92.

Maden M, E Gale and M Zile. 1998. The role of vitamin A in the development of the central nervous system. *J. Nutr.* 128:471S-475S.

Malumbres M and M Barbacid. 2005. Mammalian cyclin-dependent kinases. *Trends Biochem. Sci.* 30:630-641.

Mann GJ, M Dyne and EA Musgrave. 1987. Immunofluorescence quantification of ribonucleotide reductase M1 subunit and correlation with DNA content by flow cytometry. *Cytometry* 8:509-517.

Mark M, NB Ghyselinck and P Chambon. 2006. Function of Retinnoid Nuclear Receptors: Lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annu. Rev. Pharmacol. Toxicol.* 46:451-480.

Maun N, P Gaines, A Khanna-Gupta, T Zibello, L Enriquez, L Goldberg and N Berliner. 2004. G-CSF signaling can differentiate promyelocytes expressing a defective retinoic acid receptor: evidence for divergent pathways regulating neutrophil differentiation. *Blood* 103:1693-1701.

McCullough FS, CA Northhrop-Clewes and DI Thurnham. 1999. The effect of vitamin A on epithelial integrity. *Pro. Nutr.Soc.* 58:289-293.

Mellman I and RM Steinman. 2001. Dendritic cell: specialized and regulated antigen-processing machines. *Cell* 106: 255-258.

Mohty M, S Morbelli, D Isnardon, D Sainty, C Arnoulet, B Gaugler and D Olive. 2003. All-*trans* retinoic acid skews monocyte differentiation into interleukin-12 secreting dendritic-like cells. *Br. J. Haematol.* 122: 829-836.

Moroy T and C Giesen. 2004. Cyclin E. *Int. J. Biochem. Cell Biol.* 36:1424-1439.

Munoz-Alonso MJ, JC Acosta, C Richard, MD Delgado; J Sedivy and J Leon. 2005. $p21^{CIP1}$ and $p27^{KIP1}$ induce distinct cell cycle effects and differentiation programs in myeloid leukemia cells. *J.Biol.Chem.* 280:18120-18129.

Naderi S and HK Blomhoff. 1999. Retinoic Acid prevents phosphorylation of pRB in normal human B-lymphocytes: regulation of cyclin E, cyclin A, and $p21^{Cip1}$. *Blood* 94:1348-1358.

Nagpal S and RAS Chandraratna. 2000. Recent development in receptor- selective retinoid. *Curr. Pharm. Des.* 6:919-931.

O'Neill HC, HL Wilson, B Quah, JL Abbey, G Despars and K Ni. 2004. Dendritic cell development in long-term spleen stromal cultures. *Stem Cells* 22:475-486.

Packer Al and DJ Wolgemuth. 1999." Genetic and molecular approaches to understanding the role of retinoids in mammalian spermatogenesis," in Handbook of Experimental Pharmacology; Retinoids, H Nau and WS Blaner, Eds Springer-Verlag, pp. 347-363.

Pajalunga D and M Crescenzi. 2004. Regulation of cyclin E protein levels through E2F- mediated inhibition of degradation. *Cell Cycle* 3:1572-1578.

Penelova A, L Richman, B Neupert, V Simanis and LC Kühn. 2005. Analysis of the contribution of changes in mRNA stability to the changes in steady-state level of cyclin mRNA in mammalian cell cycle. *FEBS. J.* 272:5217-5229.

Penniston KL and SA Tanaumihardjo. 2006. The acute and chronic toxic effects of vitamin A. *Am. J. Clin. Nutr.* 83:191-201.

Purton LE, ID Bernstein and SJ Collins. 2000. All-trans retinoic acid enhances the long-term repopulating activity of cultured hematopoietic stem cells. *Blood* 95:470-477.

Ramakrishnan U and R Martorell. 2004 The role of vitamin A in reducing child mortality and morbidity and improving growth. Handbook of Nutrition and Immunity. ME Gershwin, W Penelope and C Keen Eds. New Jersey. Human Press pp. 93-116.

Ross, SA, PJ McCaffery, UC Drager and LM DeLuca. 2000. Retinoids in Embryonal Development. *Physiol. Rev.* 80:1021-1054.

Rucker RB, JW Suite, DB McCormick and LJ Machlin. 2001 Handbook of Vitamins. 3rd ed. New York: Marcel Dekker. pp. 11-50.

Saari JC. 1999. "Retinoids in Mammalian Vision". In H Nau and WS Blaner (eds), Handbook of Experimental Pharmacology; Retinoids. Springer-Verlag pp. 563-583.

Sanchez I and BD Dynlacht. 2005. New insights into cyclins, CDKs and cell cycle control. *Cell. Dev. Biol.* 16:311-321.

Schmid I and Giorgi JV. 1995. Preparation of Cells and Reagents for Flow Cytometry. Current Protocols in Flow Cytometry. John Wiley & Sons, New York. pp. 5.3.1-5.3.23.

Schmid I, J Ferbas, CH Uittenbogaart, and JV Giorgi. 1999. Flow cytometric analysis of live cell proliferation and phenotype populations with low viability. *Cytometry* 12: 64-74.

Schulman IG, D Crombie, RP Bissonnette, R Cesario, K Roegner G Shao and RA Heyman. 1999. "RXR-specific agonist and modulators:A new retinoid pharmacology". In H Nau and WS Blaner (eds), Handbook of Experimental Pharmacology; Retinoids. Springer-Verlag pp. 215-232.

Semba RD. 1998. The role of vitamin A and related retinoids in immune function. *Nutr. Rev.* 56:S38-48.

Standeven AM, M Teng and RAS Chandraratna. 1997. Lack of involvement of retinoic acid receptor α in retinoid-induced skin irritation in hairless mice. *Toxicol. Lett.* 92:231-240

Steinman RM. 1999. Dendritic cells. In: Fundamental Immunology, 5th Ed. WE Paul. Lippincott-Raven Publishers, Philadelphia. pp. 547-573.

Stephensen CB. 2001. Vitamin A, infection, and immune function. *Annu Rev Nutr.* 21:167-192.

Stephensen CB, X Jiang and T Freytag. 2004. Vitamin A deficiency increases the *in vivo* development of IL-10 positive Th2 cells and decreases the development of Th1 cells in mice. *J. Nutr.* 134:2660-2666.

Teng M, TT Duong, AT Johnson , ES Klein , L Wang , B Khalifa and RAS Chandraratna. 1997. Identification of highly potent retinoic acid receptor alpha-selective antagonists. *J. Med. Chem.* 40:2445-2451.

Twal W, L Roze and MH Zile. 1995. Anti-retinoic acid monoclonal antibody localizes all-trans retinoic acid in target cells and blocks normal development in early quail embryo. *Dev. Biol.* 168:225-234.

Vakiani E and J Buck. 1999." *Retro*-Retinoids: Metabolism and Action", in Handbook of Experimental Pharmacology; Retinoids, H Nau and WS Blaner, Eds Springer-Verlag. pp. 97-113.

van Gisbergen KP, TB Geijtenbeek, and Y van Kooyk. 2005. Close encounters of neutrophils with DCs. *Trends Immunol.* 26:626-631.

Villamor E and WW Fawzi. 2000. Vitamin A supplementation: Implications for morbidity and mortality in children. *J Infect Dis.* 182:S122-133.

Vogel S, MV Gamble and WS Blaner. 1999. "Biosynthesis, absorption, metabolism and transport of retinoids". In: Handbook of Experimental Pharmacology; Retinoids, H Nau and WS Blaner, Eds Springer-Verlag pp. 31-95.

Vuligonda V, Y Lin, S Thacher, A Standeven, D Kochar and RAS Chandraratna. 1999. A new class of RAR subtype selective retinoids: Correlation of pharmacological effects with receptor activity. *Biorg. Med. Chem.* 7:263-270.

Wolfe G. 1996. A history of vitamin A and retinoids. *FASEB. J.* 10:1102-1106.

Yam CH, TH Fung and RYC Poon. 2002. Cyclin A in cell cycle control and cancer. *Cell. Mol. Life Sci.* 59:1317-1326.

Young JW and RM Steinman. 1996. The hematopoietic development of dendritic cells: A distinct pathway for myeloid differentiation. *Stem Cells* 14:376-387.

Zhao Z and AC Ross. 1995. Retinoic acid repletion restores the number of leukocytes and their subsets and stimulates natural cytotoxicity in vitamin A-deficient rats. *J Nutr.* 125:2064-2073.

Zile MH. 1998. Vitamin A and embryonic development: An overview. *J Nutr.* 128:S455-458.

Zile MH. 2001. Function of Vitamin A in vertebrate embryonic development. *J. Nutr.* 131:705-708.