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MECHANISMS OF ALL-TRANS RETINOIC ACID EFFECTS ON MURINE MYELOID DENDRITIC CELL ADHESION IN VITRO

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# MECHANISMS OF ALL-TRANS RETINOIC ACID EFFECTS ON MURINE MYELOID DENDRITIC CELL ADHESION IN VITRO

By

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## ABSTRACT

MECHANISMS OF ALL-TRANS RETINOIC ACID EFFECTS ON MURINE MYELOID DENDRITIC CELL ADHESION IN VITRO

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Myeloid dendritic cells (DC) are professional antigen presenting cells (APC) that migrate to secondary lymphoid tissues where they activate naïve T cells to proliferate and differentiate to T helper 2 cells. These cells help initiate an antibody mediated immune response. Vitamin A is known to be essential for normal immune function. All-trans retinoic acid (atRA), a bioactive metabolite of vitamin A, is necessary for DC development from myeloid progenitors in culture. An integral part of DC development in culture is the loss of adherence. We investigated the ability of atRA to modulate DC adhesion in culture. The percentage of developing DC that remained adherent was higher in the presence of a specific antagonist of retinoic acid receptor (RAR)-$\alpha$ compared to cells treated with atRA. AtRA treatment decreased DC surface expression of the adhesion molecule CD11a, but not the gene expression. AtRA treatment also dramatically increased gene and protein expression of the gelatinase pro-matrix metalloproteinase (pro-MMP)-9. However, gene expression and protein production of MMP-9's primary biological inhibitor, tissue inhibitor of metalloproteinase (TIMP)-1, was unaffected by atRA treatment, altering the molar ratio of secreted pro-MMP-9:TIMP-1. Thus, atRA treatment resulted in a molecular excess of pro-MMP-9 to its primary inhibitor, indicating the possibility for increased MMP-9 enzymatic activity in atRA treated DC cultures.

MMP-9 is a gelatinase essential for DC migration. We wanted to determine whether atRA treatment increases gelatinase activity in mouse myeloid DC cultures compared to cells cultured with RARα antagonist. Adding MMP-9 inhibitor significantly blocked DC gelatinase activity and increased adherence of DC in a dose dependent manner. AtRA-induced *Mmp-9* expression in DC could be blocked by a transcriptional inhibitor. Hence, atRA up-regulation of *Mmp-9* is primarily controlled at the transcriptional level. Since the *Mmp-9* promoter contains no classic retinoic acid response element (RARE), we performed additional studies to determine how atRA regulates *Mmp-9* transcription in DC. Electrophoretic mobility shift assays for the consensus Sp1, AP-1 and NF-κB binding sites located in the *Mmp-9* promoter did not increase binding in response to atRA. Chromatin immunoprecipitation assays indicated that atRA increases recruitment of RARα and histone acetyltransferase p300 to, and histone H3 acetylation of, the *Mmp-9* promoter. These data suggest that atRA regulates DC adhesion *in vitro* through MMP-9 gelatinase activity. AtRA increases *Mmp-9* expression through a transcriptional mechanism involving enhancement of RARα promoter binding, recruitment of p300, and subsequent acetylation of histone H3, despite the absence of a consensus RARE.

This dissertation is dedicated to my parents for their loving support and to my husband Tom for his love and daily encouragement.

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## LIST OF ABBREVIATIONS

AGN: RARα antagonist

AP: Activating protein

Apo: Apolipoprotein

APC: Antigen presenting cell

atRA: all-*trans* retinoic acid

AU: Arbitrary units

BALT: Bronchial associated lymphoid tissue

CBP: CREB-binding protein

CCL: Chemokine C-C ligand

CD: Charcoal-dextran filtered

CH: Characterized

ChIP: Chromatin immunoprecipitation

COUP-TF: Chicken ovalbumin upstream promoter-transcription factor

CRBP: Cellular retinol binding protein

DC: Dendritic cell

DMSO: Dimethyl sulfoxide

DR: Direct repeat

ECM: Extracellular matrix

ELISA: Enzyme-linked immunosorbent assay

EMSA: Electrophoretic mobility shift assay

ERK: Extracellular signal-regulated kinase

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

Foxp3: Forkhead box p3

GALT: Gut associated lymphoid tissue

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GM-CSF: Granulocyte-macrophage colony-stimulating factor

HAT: Histone acetyltransferase

ICAM: Intercellular adhesion molecule

ICE: Interleukin-1$\beta$ converting enzyme

IFN: Interferon

Ii: Invariant chain

Ig: Immunoglobulin

IL: Interleukin

IMDM: Iscove's modified Dulbecco's medium

Itgal: Integrin $\alpha_L$

KLF: Krüppel-like factor

LCAT: Lecithin:retinol acyltransferase

LFA: Lymphocyte function-associated antigen

LPS: Lipopolysaccharide

MALT: Mucosal associated lymphoid tissue

MCP: Monocyte chemoattractant protein

MFI: Mean fluorescence intensity

MHC: Major histocompatability complex

MMP: Matrix metalloproteinase

NCoA: Nuclear receptor coactivator

NCoR: Nuclear receptor corepressor

NF-κB: Nuclear factor kappa B

NK: Natural killer

PAF: Platelet activating factor

PBS: Phosphate buffered saline

P/CAF: p300/CBP associated factor

PCR: Polymerase chain reaction

PE: Phycoerythrin

PEA3: Polyoma virus enhancer A-binding protein 3

PerCP: Peridinin chlorophyll protein

PGE: Prostaglandin E

PI3K: Phosphoinositide-3-kinase

RA: Retinoic acid

RAE: Retinol activity equivalents

RAR: Retinoic acid receptor

RARE: Retinoic acid response element

RBP: Retinol binding protein

RDA: Recommended dietary allowance

RE: Retinyl ester

Ral: Retinaldehyde

Rol: Retinol

RXR: Retinoid X receptor

RXRE: Retinoid X response element

SMRT: Silencing mediator of RAR and thyroid hormone receptor

SOCS: Suppressor of cytokine signaling

Sp: Specificity protein

STAT: Signal transducer and activator of transcription

STRA: Stimulated by retinoic acid

TGF: Transforming growth factor

Th: T helper cell

TIMP: Tissue inhibitors of matrix metalloproteinases

TLR: Toll-like receptor

TNF: Tumor necrosis factor

$T_{reg}$: Regulatory T cell

TTR: Transthyretin

UL: Upper limit

# CHAPTER 1

# LITERATURE REVIEW

## Introduction

Vitamin A deficiency is a public health problem in developing countries, especially Africa, Southern Asia, and the Middle East. Deficiency mainly affects pre-school age children and pregnant women due to its importance in growth and development. It is estimated that 190 million pre-school aged children and 19.1 million pregnant women are deficient in vitamin A, with plasma retinol levels less than 0.7 μg/mL (WHO 2009). Vitamin A deficiency is associated with increased morbidity and mortality due to increased susceptibility to infection, especially those of the mucosal sites, including diarrheal and respiratory infections (Fawzi et al. 1993; Stephensen 2001; Villamor and Fawzi 2005).

Vitamin A deficiency is known to compromise immune system function. Vitamin A deficiency impairs neutrophil and macrophage migration, phagocytosis, and bacterial killing ability (Twining et al. 1997; Stephensen 2001). At the same time, vitamin A deficiency leads to increased expansion of neutrophil precursors with impaired differentiation (Walkley et al. 2002). Vitamin A deficiency decreases natural killer cell number and function in rats, especially in aging animals (Dawson et al. 1999). Vitamin A deficiency also affects the adaptive immune system. Vitamin A deficiency decreases B cell production of the immunoglobulin (Ig)$G_1$, while addition of vitamin A sufficient T cells restores this production (Carman et al. 1989). $IgG_1$ production requires a T helper (Th) 2 polarized response. Addition of all-*trans* retinoic acid (atRA), the bioactive metabolite of vitamin A to cell cultures enhances naïve T cell development to Th2 through an antigen presenting cell (APC) intermediate (Hoag et al. 2002).

Myeloid dendritic cells (DC), whose development from myeloid precursor cells requires retinoic acid (Hengesbach and Hoag 2004), are a likely cell type that helps polarize naïve T cells to T helper 2 cells.

Immature myeloid DC are resident in the peripheral tissues and capture, process, and present antigen. Maturation in response to antigen induces migration to lymphoid tissues where myeloid DC present antigen in their major histocompatibility complex (MHC) class I and II molecules to naïve T cells. This results in polarization of the naïve $CD4^+$ T cell to Th2 cell, augmenting development of humoral immunity (Bastien and Rochette-Egly 2004; Hochrein and O'Keeffe 2008). DC migration through the extracellular matrix is dependent on the activity of the gelatinase matrix metalloproteinase (MMP)-9 (Ichiyasu et al. 2004). In the studies undertaken here, we attempted to determine how atRA regulates the myeloid DC adhesive phenotype and migration potential, with particular focus on retinoic acid-induced matrix metalloproteinase-9 expression by these cells.

## Vitamin A

### *Vitamin A chemistry*

Retinoids, natural and synthetic molecules of the vitamin A family, consist of a β-ionone ring, a polyunsaturated isoprenoid chain, and a polar end group. In naturally occurring retinoids, the polar end group can be an alcohol (retinol), an aldehyde (retinal), or a carboxylic acid (retinoic acid). The various domains of the molecules in the vitamin A family make the compounds hydrophobic and susceptible to oxidation, photodegradation, and isomerization (Barua and Furr 1998; Noy 1999). The hydroxyl group on retinol can be esterified by long chain fatty acids and is more stable in this form. Both retinol and retinyl esters have a characteristic UV maximum of 325 nm (Ross 1999).

### *Vitamin A discovery, digestion, storage, and transport*

Vitamin A, first identified in 1913 as a fat-soluble factor by Osborne and Mendel (1913) and McCollum and Davis (1913), is found in the form of retinyl esters in a variety of animal-derived foods such as egg yolk, fatty fish, dairy/cheese, and meat. In addition, pro-vitamin A β-carotene is obtained from orange and yellow fruits and vegetables and dark green leafy vegetables (Gibson 2007). Retinyl esters are cleaved in the lumen of the small intestine to retinol by pancreatic triglyceride lipase, cholesterol ester hydrolase, pancreatic lipase related proteins 1 and 2, as well as phospholipase B on the brush border membrane. Retinol is then absorbed by enterocytes. β-carotene is cleaved by β-carotene-15,15'-monooxygenase and converted to all-*trans*-retinol via enzymatic

reduction from all-*trans*-retinal in the intestinal epithelium (von Lintig and Vogt 2004; Harrison 2005). Carotenoids can also be absorbed into the enterocyte intact and cleaved to all-*trans*-retinol inside the enterocyte (Vogel et al. 1999). Once inside the enterocytes, retinol, associated with cellular retinol binding protein (CRBP)-II, is re-esterified by lecithin:retinol acyltransferase (LRAT) and incorporated into nascent chylomicrons containing apolipoproteins (Apo) $B_{48}$, E, and C (O'Byrne et al. 2005). These chylomicrons are absorbed by the lymphatics, enter the blood, and circulate through the body (Quadro et al. 2003). After triglycerides are broken down by lipoprotein lipase and the chylomicrons have lost ApoC, the resulting chylomicron remnants containing retinyl esters are taken up by the hepatocytes through recognition of ApoE (Vogel et al. 1999; Harrison 2005). The retinyl esters are hydrolyzed to retinol within the hepatocytes and cellular retinol binding protein (CRBP)-I aids in the transfer of retinol to the hepatic stellate cells. Retinol is again esterified within the hepatic stellate cells for storage purposes (Quadro et al. 2003). For retinol mobilization to the plasma, retinyl esters stored in the stellate cells are hydrolyzed to all-*trans*-retinol and packaged with retinol binding protein (RBP, synthesized by the hepatocytes) for secretion (Harrison 2005). RBP binds retinol in a hydrophobic pocket to solubilize it in the aqueous blood environment as well as to protect it from oxidation (Noy 1999). The physiological concentration of retinol in the plasma is $1\text{-}2 \times 10^{-6}$ M (Ross and Hammerling 1994). In the circulation, the RBP-retinol complex further associates with transthyretin (TTR, synthesized in and secreted from the liver) to prevent retinol excretion by the kidney (van Bennekum et al. 2001). Retinyl esters

present in chylomicrons can also serve as a source of retinol to non-hepatic tissues (Quadro et al. 2003, **Figure 1.1**). Alternatively, because of the importance of vitamin A in the maintenance of epithelial tissues, it has been shown that epithelial cells can locally store retinyl esters for periods where retinol is in high demand and RBP synthesis cannot occur quickly enough to supply a continuous plasma retinol concentration (Biesalski and Nohr 2004).

Retinol is taken up by cells through the multi-transmembrane domain protein stimulated by retinoic acid (STRA)6, which binds directly to the RBP portion of the RBP-retinol complex (Kawaguchi et al. 2007). Natural STRA6 human polymorphisms associated with congenital defects such as mental retardation, congenital heart defects, lung hyperplasia, and intrauterine growth retardation, result from reduced retinol uptake by the target cells (Kawaguchi et al. 2008). Within the cell, retinol can be reversibly oxidized to retinaldehyde by retinol dehydrogenase, which is then further oxidized to retinoic acid by retinaldehyde dehydrogenase in an irreversible reaction. Retinoic acid can also be found in circulation after secretion by a cell that converted retinol to retinoic acid. The brain, liver, kidney, adipose tissue, seminal vesicle, and spleen can all obtain retinoic acid from the circulation (Kurlandsky et al. 1995). Retinoic acid is protonated in most biological (slightly acidic) membranes and can thus transverse the membranes for direct cellular uptake (Noy 1999). CYP26, a member of the cytochrome P450 family, contains a retinoic acid response element (RARE) sequence in its promoter region and is transcriptionally expressed in response to atRA signaling through the retinoic acid receptor

(RAR)-retinoid X receptor (RXR) complex. CYP26 acts to oxidize atRA to less active polar metabolites 4-hydroxy-, 4-oxo-, and 18-hydroxy-RA for negative regulation of atRA signaling. The polar RA metabolites are then glucuronidated for elimination (Napoli 1999; Ross 2003). LRAT also acts to limit the pool of available retinol and is also up-regulated by atRA signaling. LRAT enzymatically converts retinol to retinyl esters. When the concentration of retinol bound CRBP is decreased with an increased concentration of apoCRBP, LRAT conversion of retinol to retinyl esters is inhibited to maintain sufficient cellular levels of retinol. Conversion of retinol to retinyl esters by LRAT functions to limit the extent of retinoic acid signaling in the cell by limiting the availability of retinol to be converted to retinoic acid (Ross 2003).

*Vitamin A requirements and deficiency*

The requirements for vitamin A vary by life cycle stage and sex and is expressed in retinol activity equivalents (RAE). One RAE is equivalent to 1 μg retinol or 12 μg β-carotene. The adequate intake of vitamin A for infants is 400-500 RAE, based on the average daily intake from breast milk. During childhood, vitamin A requirements increase based on extrapolation from adult requirements with increased allowances for growth (IOM 2001). The recommended dietary allowance (RDA) of vitamin A climbs to 700 RAE and 900 RAE by 14 years of age for females and males, respectively, based on the concentration of retinol in the liver, the catabolic rate, the proportion of liver weight to total body weight, percent of liver vitamin A that is stored, and the ratio of liver vitamin A to total body vitamin A (McLaren 1994). During pregnancy and lactation the RDA

increases to 770 RAE for fetal growth and 1300 RAE for secretion into milk, respectively (IOM 2001). Tolerable upper intake levels of vitamin A for adults occur at 3000 RAE but are as low as 600 RAE for infants and young children (**Table 1.1**).

**Table 1.1. Vitamin A requirements across the lifecycle.** RDA, recommended dietary allowance; UL, upper limit.

| Age | RDA[1,2] | UL[1,2] |
|---|---|---|
| Infants | | |
| 0-6 mo | 400 | 600 |
| 7-12 mo | 500 | 600 |
| Children | | |
| 1-3 y | 300 | 600 |
| 4-8 y | 400 | 900 |
| Males | | |
| 9-13 y | 600 | 1700 |
| 14-18 y | 900 | 2800 |
| >18 y | 900 | 3000 |
| Females | | |
| 9-13 y | 600 | 1700 |
| 14-18 y | 700 | 2800 |
| >18 y | 700 | 3000 |
| Pregnancy | | |
| ≤18 y | 750 | 2800 |
| ≥19 y | 770 | 3000 |
| Lactation | | |
| ≤18 y | 1200 | 2800 |
| ≥19 y | 1300 | 3000 |

[1]Units in μg retinol activity equivalents (RAE)/d.

[2]Values are adapted from those published by the Institute of Medicine, the National Academies, 1998.

It is estimated that 127 million pre-school aged children (between 6 months and 5 years of age) are vitamin A deficient. Of these, 4.4 million have xerophthalmia, the primary clinical manifestation of vitamin A deficiency presented as night blindness or Bitot's spots (West 2002). Xerophthalmia can progress to corneal scarring and permanent blindness if vitamin A deficiency is not treated (Rastogi and Mathers 2002). At the same time, 19.8 million pregnant women have low vitamin A status, of whom 7.2 million show frank vitamin A deficiency (West 2002). Pre-school aged children and child-bearing women are at risk for vitamin A deficiency due to the increased needs during periods of significant growth (WHO 1999).

Aside from vision impairment, vitamin A deficiency is an important public health problem because it increases mortality and morbidity due to the increased susceptibility to infection (Villamor and Fawzi 2005). Vitamin A supplementation, either at periodic high doses or more frequent lower doses, can lead to decreases in general childhood mortality in developing countries (Fawzi et al. 1993; Villamor and Fawzi 2000). In a meta-analysis of the efficacy of vitamin A supplementation to prevent early childhood mortality in hospitalized measles patients and in the community-based setting, the authors found vitamin A supplementation in addition to standard treatment in the hospital setting was significantly protective against respiratory infection mortality, even in populations not commonly found to be vitamin A deficient, with greatest effect on children less than one year of age. In the community setting, both small frequent and large infrequent doses of vitamin A reduced childhood mortality, with a 30%

reduction in diarrhea-associated mortality and a smaller reduction in measles-associated mortality (Fawzi et al. 1993). Vitamin A deficiency leads to failure of the protective mucosal epithelial barriers. Because mucosal epithelium lines the gastrointestinal, respiratory, and urogenital tracts, it is not surprising that vitamin A deficiency has been associated with increased diarrheal disease and dysentery, urinary and reproductive tract infections, and severity of measles virus lung infections (West 2003; West 2004).

*Vitamin A signaling*

Retinoic acid, in the form of atRA, is the main bioactive metabolite of vitamin A used for cell signaling. In the traditional model of retinoic acid signaling, atRA enters the cell nucleus and binds to one of the three RAR isoforms, $\alpha$, $\beta$, or $\gamma$ (Chambon 1996). Upon binding, the complex heterodimerizes with one of the three RXR isoforms, $\alpha$, $\beta$, or $\gamma$. After binding of the atRA ligand to the RAR/RXR heterodimer, the holoRAR/RXR complex binds to RARE in the promoter regions of retinoic acid responsive genes and associate with coregulatory proteins leading to promotion or inhibition of transcription and target gene expression (Bastien and Rochette-Egly 2004; Mark et al. 2006). The canonical RARE, called a direct repeat (DR)5, is the sequence PuGG/TTCA, followed by a direct repeat after five intervening nucleotides (**Figure 1.2**). The holoRAR/RXR complex is also able to bind to DR1 and DR2 RARE sites, though these are less common. Variation in the RARE structure allows for variable RAR/RXR binding characteristics, and thus variable responses to atRA (Piedrafita and Pfahl 1999). RXRs are also able to homodimerize upon binding to the appropriate ligand,

such as 9-*cis*-RA and other synthetic RXR retinoids. The holoRXR/RXR dimer binds to the retinoid X response element (RXRE) and acts similarly to effect responsive genes (Piedrafita and Pfahl 1999). However, 9-*cis*-RA, though reported in animal tissues in earlier studies (Heyman et al. 1992), has not been confirmed to exist as a natural retinoid *in vivo*. In fact, it is possible the earlier studies that detected endogenous 9-*cis*-RA actually detected 9-*cis*-RA spontaneously isomerized from atRA (Wolf 2006). The RAR molecules bind atRA with strong affinity; $K_d$ 0.2-0.7 x $10^{-9}$ M (Noy 1999). Binding of atRA to form a holoRAR/RXR complex initiates association with coactivator proteins such as CREB-binding protein (CBP)/p300, nuclear receptor coactivator (NCoA), and p300/CBP-associated factor (P/CAF), all which possess histone acetyl transferase (HAT) activity. Upon histone acetylation, transcriptional machinery is able to bind to the retinoid responsive gene for transcription initiation (Piedrafita and Pfahl 1999). Unliganded RAR binds DNA and associates with corepressors, including nuclear receptor corepressor (NCoR)-1 and NCoR-2/silencing mediator of RAR and thyroid hormone (SMRT) at the corepressor interaction domain of RAR to recruit histone deacetylases leading to chromatin condensation and transcriptional repression (Weston et al. 2003). At the same time, orphan receptors that bind RAREs as homodimers with high affinity, such as chicken ovalbumin upstream promoter-transcription factor (COUP-TF), can block binding of the holoRAR/RXR complex to the RARE and inhibit transcription of retinoic acid responsive genes (Piedrafita and Pfahl 1999).

The classical model of retinoic acid signaling explains direct transcriptional regulation by vitamin A for up to 132 genes. However, it is known that at least 532 genes are controlled by atRA, but not all are regulated through the traditional mechanism. Some are regulated by atRA via another transcription factor and some are transcriptionally regulated by atRA, but not through the classical mechanism (Balmer and Blomhoff 2002). The holoRAR-RXR complex has been shown to transrepress activating protein (AP)-1 binding in target promoters to down-regulate gene expression (Blomhoff and Blomhoff 2006). The holoRAR-RXR complex has also been shown to decrease nuclear factor-kappaB (NF-κB) promoter binding activity and vitamin A deficiency increases whole body NF-κB activity in mice (Austenaa et al. 2004; Blomhoff and Blomhoff 2006). The liganded RAR-RXR complex has been shown to interact with the transcription factor specificity protein 1 (Sp1) to augment expression of multiple genes, including urokinase plasminogen activator, transglutaminase, transforming growth factor (TGF)-β1, types I and II TGF-β receptors, and bone morphogenic protein-2, despite the lack of a traditional RARE site in the promoters (Suzuki et al. 1999; Shimada et al. 2001; Xu and Rogers 2007).

*Vitamin A biological functions*

Vitamin A is important in a large variety of biological processes, including vision, reproduction, body patterning in embryo development, immune function (Stephensen 2001), and, in the pro-vitamin A form of β-carotene, as an antioxidant. The role of vitamin A in vision is very well characterized, where 11-*cis*-retinaldehyde, with opsin, forms rhodopsin in the rod cells of the eye for

conversion to all-*trans*-retinaldehyde by photon stimulation during the vision cycle (Wolf 2001). In embryo development, RA signaling through gradient diffusion is required for bilateral symmetry and synchronous somite formation along the left/right body axis as well as control of the position of the traveling determination front along the anteroposterior axis (Campo-Paysaa et al. 2008). RA is synthesized from retinaldehyde in different tissues in a time- and tissue-dependent manner, controlled by expression of retinaldehyde dehydrogenase enzymes (Duester 2007). It is essential for development of the neural system, including neurite outgrowth, neuron patterning, and pathfinding (Clagett-Dame et al. 2006; Duester 2007), heart morphogenesis, late-stage asymmetry, looping, and vasculature formation (Zile 2004), and forelimb buds and eyes (Duester 2007; Campo-Paysaa et al. 2008). Vitamin A is also required for proper chondrocyte formation, hypertrophy, and differentiation in long bone growth plates, probably through induction of bone morphogenic protein expression (Adams et al. 2007). Expression of RAR$\alpha$ is increased during mesenchymal cell differentiation to chondroprogenitor cell, while RAR$\gamma$ is increased in the differentiation of the chondroprogenitor cell to the chondroblast. Expression of retinaldehyde dehydrogenase, necessary for the oxidation of retinaldehyde to RA for signaling through RAR, is increased in the mesenchymal cell for commitment to chondroprogenitor cell (Weston et al. 2003).

Vitamin A deficiency decreases the number of mucus producing goblet cells in the mucosal epithelium (Stephensen 2001). Vitamin A's importance in cellular differentiation, especially in cells with a high turnover rate such as at the

epithelium, results in keratinizing metaplasia and cellular separation and distortion even with mild vitamin A deficiency (Congdon and West 2002). It has been shown that intestinal cells cultured *in vitro* supplemented with retinol have decreased apoptosis and necrosis and increased transepithelial electrical resistence, cell proliferation, and migration in the presence of *Clostridium difficile* toxin A than do intestinal cells cultured with the toxin alone (Maciel et al. 2007). *In vivo*, it was also found that vitamin A deficient mice had significantly fewer goblet cells and upon infection with rotavirus, the deficient group showed complete destruction of the small intestine villi tips. This pathologic change to the villi tips was not found in uninfected deficient mice or infected vitamin A sufficient mice (Ahmed et al. 1990). Rats consuming a vitamin A deficient diet for seven weeks have increased amount of bacteria in the ileum and the colon with decreased amounts of *Lactobacillus* spp. and increased amounts of *E. coli* in the gut. The vitamin A deficient rats also have decreased levels of *MUC2* and *defensin 6* mRNA with increased levels of toll-like receptor (TLR)2 and TLR 5 mRNA in the small intestine and the colon. Decreased *defensin 6* abundance can allow for bacterial overgrowth and penetration of the mucosal barrier (Amit-Romach et al. 2009). This dissertation will focus on the role of vitamin A in normal immune system myeloid dendritic cell function.

*Vitamin A and infection*

One very important role of vitamin A, in the form of atRA, is in the maintenance of the immune system and the protection against infection. Vitamin A was described as an anti-infective vitamin early. Rats that were maintained on

a vitamin A deficient diet were commonly found to have tongue abscesses, gastrointestinal tract infection, and kidney or bladder infection, with some examples of broncho-pneumonia (Green and Mellanby 1928). In a prospective study of children in Indonesia reported in 1983, children with Bitot's spots or night blindness had six-fold and three-fold greater mortality rate, respectively, due mainly to diarrheal and respiratory disease over an 18 month period. In children with both symptoms, the mortality rate was increased over eight-fold (Sommer et al. 1983).

Because vitamin A deficiency compromises the mucosal barriers of the eye, respiratory, and gastrointestinal tract (Stephensen 2001), the physical barriers that are the first defenses against infection, deficient individuals are more likely to suffer infections at these mucosal sites. One study has shown that children who had suffered from pneumonia, acute respiratory problems, and *Mycobacterium bovis* BCG vaccine scarring (indicating an overactivated Th1 response) had lower plasma retinol levels than children who had not experienced these problems (Jason et al. 2002). In a study of Brazilian children, low retinol concentrations were correlated with increased urinary lactulose:mannitol ratio from the lactulose-mannitol test, an indicator of intestinal permeability. The authors also measured total serum IgG in mice with varying serum concentrations of retinol due to RBP or TTR deficiency. They found that the concentration of serum IgG decreased in mice with lower serum concentrations of retinol without a change in spleen, thymus or bone marrow populations of B cells, $CD4^+$ T cells or $CD8^+$ T cells. However, the authors did not determine

whether there was a difference in $CD4^+$ Th cell polarization that could explain differences in serum IgG concentrations (Quadro et al. 2000).

Vitamin A deficient children infected with diarrheal diseases have a decreased secretory IgA response (Stephensen 2001). Vitamin A status has been shown to be related to the severity of a measles infection, a respiratory virus, with high levels of vitamin A supplementation supporting a beneficial Th2 response and significantly decreasing mortality in children (Semba 1999). Secretory IgA response in the intestines is also required to respond to and clear infections causing diarrhea. When diarrheal disease is caused by *Shigella* infection, vitamin A supplemented children had shorter duration of disease, and also showed increased secretory IgA response (Villamor and Fawzi 2000). Vitamin A supplementation has also been shown to decrease the incidence of malaria in children between the ages of 1 and 3 years (Semba 1999), and increase the antibody response to the hepatitis B vaccine in infants (Newton et al. 2007). In a mouse model of allergic airway inflammation, a Th2 response dominated disease, vitamin A deficiency has also been linked with decreased severity of symptoms by decreasing serum levels of $IgG_1$ and IgE and decreased levels of interleukin (IL)-4 and IL-5 in the bronchoalveolar lavage, while high dosage of dietary vitamin A increased serum IgE and pulmonary hyperresponsiveness (Schuster et al. 2008).

In many cases, vitamin A supplementation does not show beneficial results on immune measures in children with vitamin A deficiency because they also have other nutrient deficiencies, particularly protein-energy malnutrition. In a

study of vitamin A deficient children between 2-6 years who are otherwise nutrient sufficient, researchers found that vitamin A deficient children (serum retinol <30 μg/dl) have lower levels of serum IgA and complement factor C3, with higher levels of lysozyme. High dose vitamin A supplementation for 3 months normalized levels of serum IgA, C3, and lysozyme (Lin et al. 2007). Similarly, in children of normal height between 1 and 5 years-old, vitamin A supplementation of 200,000 IU decreased the prevalence and intensity of reinfection with the gastrointestinal nematode *Ascaris*, but not in children with stunted growth, indicating that protein-energy malnutrition complicates the effectiveness of vitamin A supplementation (Payne et al. 2007). These studies indicate that vitamin A status is an important factor in the duration and overall outcome during a pathogen infection in children and pregnant women. Understanding the mechanisms of vitamin A's effects on cells of the immune system to prevent or fight infection is crucial to further refine supplementation schedules and create new treatments for children at risk for infection due to malnutrition.

*Vitamin A and innate immunity*

Vitamin A deficient rats have been shown to sequester retinol in the bone marrow, with concentrations four-fold higher than in the bone marrow of vitamin A sufficient rats (Twining et al. 1996). Deficiency also compromises the ability of macrophages and neutrophils to migrate to sites of infection, phagocytose, and kill bacteria (Twining et al. 1997; Stephensen 2001). However, neutrophils are found in significantly increased number in the spleen, peripheral blood, and bone marrow of vitamin A deficient rodents (Kuwata et al. 2000) and *in vivo* pan-

retinoic acid receptor (RAR) antagonism leads to neutrophil precursor cell expansion with impaired differentiation (Walkley et al. 2002). Treatment of human eosinophils with high concentrations (1 μM) of RA increases cell survival through down-regulation of the apoptotic enzyme caspase-3 *in vitro* (Ueki et al. 2008).

CYP26 is an enzyme that oxidizes atRA for removal from the system. The *Cyp26* gene is induced upon atRA treatment as a negative feedback mechanism. In rats treated with lipopolysaccharide (LPS) after receiving an oral dose of atRA, liver expression of *Cyp26* is abrogated compared to rats treated only with atRA. Treatment with only LPS has no effect on baseline *Cyp26* expression. These results indicate that excess concentrations of atRA are important during inflammatory situations, so mechanisms exist to inhibit its oxidation (Zolfaghari et al. 2007).

Even marginal vitamin A status has been shown to decrease natural killer (NK) cell number and function in rats, especially with increased age (Dawson et al. 1999). In adult men, whole body vitamin A stores are positively associated with peripheral blood NK cell and NKT cell number and monocyte oxidative burst intensity (Ahmad et al. 2009). Cells treated *in vitro* with atRA augment expression of the gene for CD1d, a non-classical antigen presenting molecule that can activate NKT cells through presentation of glycolipid antigens, through holoRARα/RXRα interaction with the RARE in the CD1d promoter region. It was also found that atRA treatment enhanced cultured spleen cell proliferation in response to the glycolipid antigen α-GalCer, indicating that CD1d up-regulated by atRA may increase glycolipid presentation to NKT cells (Chen and Ross

2007). These studies all indicate the importance of vitamin A in the regulation of the anti-viral and anti-tumor activities of NK and NKT cells.

*Vitamin A and adaptive immunity*

Vitamin A deficiency has profound effects on the adaptive immune response. Antibody mediated responses are one aspect of adaptive immunity affected by vitamin A deficiency. In a number of studies reviewed by Ross and Hammerling, vitamin A deficient rats make impaired IgM and IgG responses to primary and secondary vaccination with tetanus toxoid, but the secondary response can recover if the animals are repleted with retinol prior to secondary immunization (Ross and Hammerling 1994). Overall, vitamin A deficiency decreases antibody production in response to T cell-dependent antigens and type 2 T cell-independent antigens but has not shown an effect on antibody production in response to type 1 T-cell independent antigens (Ross and Hammerling 1994). For example, vitamin A deficiency decreases the specific IgM response to the protein antigen hemocyanin despite no change in B cell number in the spleen or lymph nodes and no difference in total IgM level in more deficient animals (Smith et al. 1987). While vitamin A deficiency decreases the frequency of B and T cells in the spleen, peripheral blood, and bone marrow due to neutrophil infiltration, the actual B and T cell yields in these tissues were not changed by deficiency (Kuwata et al. 2000). Vitamin A deficient mice also make an impaired $IgG_1$ response after stimulation with hemocyanin antigen. This deficit is due to a defect in Th cells from vitamin A deficient mice. T cells from vitamin A deficient mice showed normal proliferation but decreased frequency. Addition of

T cells from vitamin A deficient mice to B cells from vitamin A sufficient mice resulted in diminished $IgG_1$ production while addition of T cells from vitamin A sufficient mice to B cells from vitamin A deficient mice resulted in a normal response. The T cell defect was reversible upon addition of retinyl acetate to cultures, indicating that vitamin A is required for Th cell stimulation of B cells to produce an appropriate humoral response to antigen (Carman et al. 1989). After oral infection with rotavirus, vitamin A deficient mice show decreased serum levels of antibody specific to rotavirus (Ahmed et al. 1991). Vitamin A deficiency can also change the predominant antibody type produced during an infection. During influenza A infection in mice, vitamin A deficiency has been shown to decrease influenza A-specific salivary IgA production while increasing the total salivary IgA concentration and the influenza A specific serum $IgG_{2a}$ concentration (Stephensen et al. 1996).

Vitamin A deficiency decreases the delayed type hypersensitivity response (a test used as a measure of Th1 function) to dinitrofluorobenzene in moderately deficient mice. However, results of the delayed type hypersensitivity response can be complicated by changes to multiple processes involved in the reaction and the decreased response seen here is likely due to impaired APC activity or development. Supplementation with vitamin A increases the delayed type hypersensitivity response to ovalbumin (Stephensen 2001), and also increases the serum antibody production in children after diphtheria and tetanus toxoid vaccination (Stephensen 2001).

Vitamin A signaling is known to prevent the development of a proinflammatory environment through the regulation of naïve T cell development. In mice, vitamin A deficiency enhances the Th1 response to *Trichinella spiralis* infection through increased secretion of IFN-$\gamma$, while suppressing the Th2 response through decreased production of IL-5 and IL-10, leading to an impaired humoral immune response (Cantorna et al. 1996). AtRA, the bioactive metabolite of vitamin A, has been shown to inhibit the synthesis of the Th1 cytokine IFN-$\gamma$ in Th1 cell culture and enhance the development of $CD4^+$ T cells into Th2 cells through an APC intermediate (Hoag et al. 2002). Evidence exists that myeloid DC are the relevant APC whose function is modified by vitamin A (Hengesbach and Hoag 2004). The increased production of Th2 cytokines and decreased production of Th1 cytokines after stimulation with atRA mainly occurs through RAR$\alpha$ signaling. Peripheral blood mononuclear cells treated with atRA decrease production of the Th1 differentiation cytokine IL-12p70 (Dawson et al. 2008). It has been found that supplementing mice with atRA after a DNA vaccination leads to Th2 polarization with a greater antibody response than mice not supplemented with atRA (Yu et al. 2005). Similarly, vitamin A deficiency has been found to lessen the symptoms of ovalbumin-induced allergic airway inflammation in mice while high-dose vitamin A supplementation increased the symptoms through modification of the Th1/Th2 balance. Vitamin A deficiency led to decreased serum IgE and $IgG_1$ antibody, eosinophil, IL-4, and IL-5 concentrations in the bronchoalveolar lavage fluid, and decreased lung resistance after ovalbumin aerosol challenge. High-dose supplementation led to

the opposite responses, resulting in a response skewed toward the Th2 response (Schuster et al. 2008).

AtRA also indirectly affects the inflammatory balance during naïve $CD4^+$ T cell development through suppression of $CD4^+CD44^+$ memory T cells allowing TGF-β signaling to induce forkhead box protein 3 (Foxp3) expression (Hill et al. 2008) and decreases IL-10 and IL-17 expression in naïve $CD4^+$ T cells to produce regulatory T ($T_{reg}$) cells (Maynard et al. 2009). Treatment of $CD4^+$ T cells with TGF-β in the presence of RAR inhibitors increases IL-10 and IL-17 expression and decreases Foxp3 expression (Maynard et al. 2009). Conversely, it has also been found that vitamin A deficient mice show increased percentage of $CD4^+$ memory T cells that produce IL-10 and decreased percentage of $CD4^+$ memory T cells that produce the Th1 cytokines IFN-γ and IL-2 from the spleen and draining lymph nodes 2-5 weeks post immunization. The researchers explain that their data are not consistent with the findings of other studies because the adjuvant used for immunization creates less Th1 bias at the time of immunization than other traditionally used adjuvants. They also explain that cells cultured for cytokine analysis were cultured in the presence of fetal bovine serum containing retinol. Overall, the researchers state these factors could lead to a response that is less biased to a Th1 response in the early stages (Stephensen et al. 2004). These studies indicate that while vitamin A deficiency traditionally leads to a Th1-biased response, the situation is very complex and can vary by severity of deficiency, type of immune challenge, and the tissues and/or cells under study.

B cell proliferation and differentiation are also influenced by atRA treatment. In one model of B cell differentiation to simulate activation by Th2 cells, stimulation of the B cell receptor and CD40, with IL-4 and atRA treatment increased the subset of large, less-proliferative, differentiated splenic B cells. These cells developed cell surface $IgG_1$ expression through increased expression of activation-induced cytidine deaminase, Blimp-1, and cell surface CD138 and decreased expression of paired box gene-5 and germline transcript genes. AtRA treatment also decreased the proportion of small, proliferative splenic B cells (Chen and Ross 2005; Chen and Ross 2007). These results suggest that atRA treatment increases the B cell humoral response upon activation with Th2 cells. In another experimental model, cultures of purified early B lineage cells were treated with slightly greater concentrations of atRA than physiologically normal. This treatment decreased total cell yield but increased yields of $CD19^+$ B cells after exposure to atRA for at least 3 days and optimally for 5-7 days. It was found that RARα signaling increases gene expression of early B cell factor-1 and paired box gene-5, both required for B cell lymphopoiesis (Chen et al. 2008).

*Vitamin A and gut immunity*

A new line of research has recently emerged focusing on the role of vitamin A on gut immunity and mucosal tolerance through myeloid DCs. DC found in the gut associated lymphoid tissues (GALT) have been shown to produce atRA from retinol. Uptake of the atRA by nearby T cells leads to atRA

signaling through RAR in the T cells and increased expression of $\alpha_4\beta_7$ on the T cell surface for selective homing to the gut (Iwata et al. 2004). Similarly, atRA produced by mesenteric lymph node DC leads to $CD25^+$ $CD4^+$ $T_{reg}$ cell gut homing by increasing expression of $\alpha_4\beta_7$ for migration to mucosal tissues (Siewert et al. 2007). Stromal cells in mesenteric lymph nodes, but not the peripheral lymph nodes, recently have been found to produce atRA from retinol and induce CCR9 expression in T cells for homing to the gut (Hammerschmidt et al. 2008). Splenic DC have also been shown to produce atRA from retinol through induction of retinal dehydrogenase mRNA production upon signaling through TLR2. Increased atRA signaling induces expression of suppressor of cytokine signaling (SOCS)-3, resulting in decreased production of inflammatory cytokines and increased $T_{reg}$ cell differentiation (Manicassamy et al. 2009). AtRA produced by GALT-DC, along with IL-5, IL-6, and the GALT-DC themselves, are necessary to induce T cell-independent B cell IgA secretion in the gut. The authors also found that $\alpha_4\beta_7$ and CCR9 expression for gut homing was dependent upon atRA producing GALT-DC. They further report that vitamin A deficiency in mice leads to loss of gut homing B cells and decreased IgA production in the gut due to decreased DC atRA production (Mora et al. 2006). DC treated with atRA also directly affects the gut environment. It has been found that human peripheral blood-derived DC treated with synthetic atRA produce decreased IL-12p70 concentration and decreased Th1 polarization function. Mice with induced colitis treated with synthetic atRA had decreased colitis induced IL-

12 in the intestinal lamina propria (Wada et al. 2009). TGF-β has been shown to promote the development of $T_{reg}$ cells when alone, but with interleukin (IL)-6, it promotes the development of the inflammatory, IL-17 secreting Th17 cells. It has been found that atRA can block the Th17 promoting effects of IL-6 through blockade of signal transducer and activator of transcription (STAT)-3 signaling which normally acts to up-regulates IL-17 and through RAR interaction with STAT5, augmenting Foxp3 transcription (Mucida et al. 2007; Mucida et al. 2009). Splenic $CD8^+CD205^+$ DC are specialized DC that produce TGF-β to induce $Foxp3^+$ $T_{reg}$ cell differentiation (Yamazaki et al. 2008). Macrophages present in the lamina propria induce development of $T_{reg}$ cells through IL-10, atRA, and TGF-β, while lamina propria DC induce the development of Th17 cells. The lamina propria macrophages can interact with the DC to prevent the development of Th17 cells and promote $T_{reg}$ cell development (Denning et al. 2007).

**Dendritic cells**

DC are professional antigen presenting cells. They are the only APC able to activate naïve T cells by presenting antigen and secreting specific cytokines to yield an adaptive immune response (Iwasaki 2007). In the mouse, all DC are $CD11c^+$, but subsets can be identified by patterns of other proteins expressed on their surface. These subsets are myeloid ($CD8\alpha^-$ $CD11b^+$), lymphoid ($CD11b^-$ $CD8\alpha^+$), plasmacytoid ($CD45RB^+$ $Gr\text{-}1^+$), and Langerhans DC ($CD4^-$ $CD8\alpha^{low}$ $CD11b^+$ $CD45RB^-$ $CD205^+$) which reside in the skin epidermis. The myeloid DC and lymphoid DC are both referred to as conventional DC (cDC) (Shortman and Liu 2002; Wilson and O'Neill 2003; Heath et al. 2004).

Different subsets of DC can polarize T helper cells in the direction of either a Th1 or Th2 dominated response. Stimulation by lymphoid DC, partially through IL-12 production, influences naïve Th0 cell polarization in the direction of Th1 differentiation, where IFN-$\gamma$ and IL-2 are produced by the activated Th1 cells at high levels. When lymphoid DC are activated, they also produce high levels of IL-6 and TNF-$\alpha$. These DC can efficiently cross-present exogenous antigen on cell-surface major histocompatibility complex (MHC) class I molecules for stimulation of $CD8^+$ cytotoxic T cells and the adaptive immune response against viral infections. In contrast, stimulation by pathogen activated myeloid DC induces naïve Th0 cell polarization in the direction of Th2 response, possibly through secretion of monocyte chemoattractant protein (MCP)-1 and cell-surface OX40L,

leading to increased production of the Th2 associated cytokines IL-4 and IL-10. Myeloid DC are the most efficient stimulators of $CD4^+$ Th cells through stimulation with antigen bound to MHC class II (Moser and Murphy 2000; de Jong et al. 2005; Hochrein and O'Keeffe 2008).

Myeloid DC are derived from the bone marrow and reside in an immature stage in the peripheral tissues (Shortman and Liu 2002). Most immature DC in the bone marrow are identified as $CD11c^{int}$ MHC-$II^{low/-}$ $CD11b^+$ $CD4^-$ $CD8^-$ (Hochrein and O'Keeffe 2008). The DC precursors migrate from the bone marrow, via the blood, into the different lymphoid tissues (including the spleen, lymph nodes, and various MALT, depending on the chemokine signals expressed by these tissues) where they differentiate (Iwasaki 2007). However, the origin of DC subtypes based on CD8α expression has come into question. Researchers have shown through donor studies that common myeloid progenitor cells from donor mice can give rise to $CD8\alpha^+$ (lymphoid) DC in the spleen and thymus of lethally irradiated mice (Traver et al. 2000). It has also been argued that both $CD8\alpha^-$ (myeloid) and $CD8\alpha^+$ (lymphoid) DC can arise from a common lymphoid progenitor cell because both cell types could be found in the spleens of lethally irradiated mice after transfer of donor $CD4^{low}$ lymphoid precursor cells from the thymus (Martin et al. 2000). These researchers later discovered a $CD11c^+$ MHC $II^-$ DC precursor population which constitutes 5% of blood mononuclear cells that could give rise to all DC subtypes including $CD11b^+$ DC,

$CD8\alpha^+$ DC, and $CD45RB^+$ plasmacytoid DC. They found this precursor population was unrelated to myeloid and lymphoid lineages but carried early precursor markers (del Hoyo et al. 2002). Taken together, these studies question the traditional theories of DC ontogeny, where $CD11b^+$ DC were thought to be always myeloid lineage and $CD8\alpha^+$ DC were thought to be always lymphoid lineage.

The general belief that immature DC are only generated in the bone marrow and then migrate into the tissues has also been called into question. A recent study has shown that DC resident in the spleen and the lymph nodes can be replenished by completing a limited number of cell divisions as well as by DC precursors from the bone marrow (Liu et al. 2007). The immature myeloid DC are able to effectively capture and process antigens and present them in their MHC classes I and II (Banchereau et al. 2000). Maturation of DC through stimulation by antigen or stress signals from self-tissues leads to DC migration to the T cell areas of the secondary lymphoid tissues, where they stimulate and activate both naïve $CD4^+$ and $CD8^+$ T cells. The activation of T cells leads to adaptive immune responses and immune memory development (Banchereau and Steinman 1998; Steinman 1999).

DC are activated through interaction of toll like receptor (TLR) with antigen. In mice, all cDC express TLR 2, 4, and 6 which can recognize peptidoglycans, lipoproteins, zymosan from yeast, protozoan glycosylphosphatidylinositol, and LPS from Gram-negative bacteria. Only

lymphoid cDC express levels of the internal TLR 3 high enough to detect viral double-stranded RNA and respond with the production of cytokines and enhance cross-presentation. Plasmacytoid DC express high levels of the internal TLR 7, 8, and 9 for detection of intracellular viruses and produce very high levels of type I interferon in response to recognition (Hochrein and O'Keeffe 2008).

Upon activation by antigen within peripheral tissues, mature DC must migrate to lymphoid tissues containing naïve T cells to present antigen. It has recently been demonstrated that DC antigen presentation and cell motility are coupled through interaction of MHC class II-associated invariant chain (Ii, CD74), involved in peptide loading, with the motor protein myosin II. DC deficient in cathepsin S which cleaves the cytosolic tail of Ii in the MHC-II complex at the endolysosome show reduced migration, while DC deficient in Ii showed continuous motility compared to wild type DC which showed both motile and stagnant phases. Inhibition of myosin II in DC leads to motility similar to that of cathepsin S-deficient DC. In early stages of DC activation by LPS, there is greater association of Ii with myosin II compared to untreated DC, leading to increased change in DC directionality and decreased DC velocity. LPS-stimulated DC deficient in Ii do not show changes in directionality or velocity compared to unstimulated wild type DC (Faure-Andre et al. 2008).

## Matrix metalloproteinase-9

*Matrix metalloproteinase structure and function*

The matrix metalloproteinase (MMP) family is composed of twenty cell surface and soluble proteins that are dependent on $Ca^{2+}$ and $Zn^{2+}$. All the members have a pre-peptide and pro-domain containing a cysteine switch and the catalytic domain. The $Zn^{2+}$ ion is located in the substrate-binding site found in the catalytic domain. The proteins are secreted as pro-enzymes and act to degrade molecules composing the extracellular matrix (ECM). The MMPs are important during embryonic implantation and development, tissue morphogenesis, including bone ossification and the formation of neural cell connections, as well as wound healing because they allow cell migration through the ECM and create space for cell proliferation (Vu and Werb 2000).

The tissue inhibitors of metalloproteinases (TIMP) family of proteins is a group of endogenous inhibitors of the MMP family. The family consists of four members that are highly conserved. TIMP proteins form 1:1 molecular complexes with soluble MMPs to inhibit MMP function. TIMPs bind to the active site of MMPs through the conserved VIRAK sequence to inhibit MMP activity. The expression of TIMP-1 and -3 is inducible, while TIMP-2 expression is constitutive. TIMP-4 is expressed in a limited number of non-immune tissues (Chirco et al. 2006; Verstappen and Von den Hoff 2006).

MMP-9 is secreted as a pro-enzyme that must be cleaved in order for full activity to be realized. MMP-2 (gelatinase A), MMP-3 (stromelysin 1), MMP-7

(matrilysin), and MMP-13 (collagenase-3) have the ability to activate latent pro-MMP-9 to active MMP-9 through enzymatic cleavage and removal of the cysteine sulfhydryl group in the pro-domain. MMP-3 is the most potent MMP activator of MMP-9. However, chemical activation of pro-MMP-9 is also possible and activation by neutrophil-produced hypochlorous acid is one of these mechanisms (Opdenakker et al. 2001; Fridman et al. 2003). MMP-9 acts to enzymatically degrade the ECM by cleaving gelatin, laminin, and types I and IV collagen. MMP-9 has been associated with the immune system in general by aiding leukocytes in entering and leaving the blood and lymphatic vessels (Faveeuw et al. 2001; Opdenakker et al. 2001). For example, MMP-9 deficiency has been implicated in insufficient prevention of *Escherichia coli* induced peritonitis due to decreased immune cell recruitment (Renckens et al. 2006). MMP-9 has been shown to be crucial for DC migration. Blocking monoclonal antibody against MMP-9 inhibits the release of hematopoietic progenitor stem cells in response to IL-8 injection in rhesus monkeys (Pruijt et al. 1999). In MMP-$9^{-/-}$ mice, recruitment of DC into the airway lumen in response to allergen (Vermaelen et al. 2003) and migration of cultured bone marrow DC through tracheal epithelial tight junctions is impaired by two-fold and four-fold, respectively, compared to wild type animals (Ichiyasu et al. 2004).

*MMP-9 and DC migration*

It has been reported that MMP-9 and TIMP expression in DC is regulated by maturation stage. Expression of TIMP-1, -2, and -3 is decreased when DC are activated by TNF-$\alpha$ or modified vaccinia virus *Ankara*, while MMP-9 activity is

increased under these conditions (Osman et al. 2002). The proinflammatory chemokine CC ligand (CCL)-5 is a potent inducer of both MMP-9 expression and secretion in immature DC, leading to increased DC migration through basement membranes (Chabot et al. 2006). Pro-MMP-9 can be found in association with cell surface associated α2(IV) chain of collagen IV, the hyaluronan receptor CD44, and CD11b (Fridman et al. 2003; Hu and Ivashkiv 2006). Recently, it has also been found that pro-MMP-9 can associate with integrin β-1 (CD29) on the cell surface and that thrombin can increase transcription of both genes in osteosarcoma cells to induce invasion of the ECM leading to increased cell proliferation and metastasis (Radjabi et al. 2008). It has been found that IFN-α activated DC produce high levels of cell surface associated MMP-9 that is necessary for the DC to migrate through the ECM in response to chemokine signaling (Hu and Ivashkiv 2006). TIMP expression impedes DC migration. $PGE_2$-induced TIMP-1 has previously been shown to inhibit the migration of monocyte-derived DC, while incubation with monoclonal anti-TIMP-1 antibody restores the ability of DC to migrate (Baratelli et al. 2004). Finally, MMP-9 has been shown to cleave and activate IL-1β [independently of caspase-1/IL-1β-converting enzyme (Schonbeck et al. 1998)], TGF-β (Yu and Stamenkovic 2000), and plasminogen to angiostatin (Pozzi et al. 2000).

*Regulation of MMP-9 expression*

The upstream region of the *Mmp-9* gene in the mouse contains four AP-1, one AP-2, one NF-κB, four polyoma virus enhancer A-binding protein (PEA)3,

and three Sp1 transcription factor binding sites within 2.8 kilobases for regulation of *Mmp-9* expression. This promoter region also contains a TATA-like box transcription start site (Munaut et al. 1999). The mouse *Mmp-9* promoter is shown in **Figure 1.3**. In comparison, the human *Mmp-9* promoter region 500 bases upstream of the *Mmp-9* gene contains two AP-1, one Sp1, and one TGF-β1 inhibitory element transcription factor binding sites along with a TATA-like box (Huhtala et al. 1991). Together, AP-1 and NF-κB binding to sites in the *Mmp-9* promoter account for the majority of the studied regulation of *Mmp-9* production, followed by Sp1 binding. In human monocytes, activation of extracellular signal-regulated kinase (ERK)1/2, through AP-1 binding, and NF-κB pathways are critical for the thrombin-induced production of MMP-9 (Chang et al. 2009). In the mouse epidermal cell line JB6 P+, TNFα induces MMP-9 production through the phosphoinositide 3-kinase (PI3K)/Akt pathway, which activates the NF-κB pathway, and subsequent binding of AP-1 and NF-κB DNA binding sites (Hwang et al. 2009). Epidermal growth factor has also been shown to induce NF-κB nuclear translocation and c-jun N-terminal kinase (JNK) signaling in prostate carcinoma cells (Kuo et al. 2009).

Transcription factors can also work alone to regulate expression of *Mmp-9*. In SKBR3 cells, it was found that epidermal growth factor signaling through the ERK pathway (an AP-1 DNA binding protein) induces *Mmp-9* expression but signaling through the STAT3 pathway negatively regulates *Mmp-9* expression (Kim et al. 2009). In human skin keratinocytes, dermal fibroblasts, and rat hepatic stellate cells, TNFα signals through p21-activated kinase-1, involving JNK, to

increase binding of the AP-1 element in the *Mmp-9* promoter. NF-κB signaling does not appear to be important in this process (Zhou et al. 2009). However, AP-1 binding in the *Mmp-9* promoter does not consistently lead to increased *Mmp-9* transcription. In the Raw 264.7 macrophage cell line, inhibition of JNK signaling, through the use of inhibitors or siRNA knock-down, induces *Mmp-9* production (Lee et al. 2009).

NF-κB is also an important transcription factor in the regulation of *Mmp-9* production. MMP-9 activity is up-regulated in the neuroblastoma cell line SK-N-SH by p50 and p65 binding to the NF-κB site in the MMP-9 promoter, but not by Sp1 or AP-1 binding, in the spontaneous conversion from epithelial cell to neuroblast cell to allow for basement membrane invasion (Farina et al. 1999). By a similar mechanism in retinoic acid differentiation-resistant SK-N-BE neuroblastoma cells, 72 h of 1-10 μM atRA treatment induces p50/p65 NF-κB activation and binding to the *Mmp-9* promoter with minimal activation of AP-1 and Sp1, resulting in augmented expression of MMP-9 and greater basement membrane invasive capacity (Farina et al. 2002). This finding is unexpected because atRA treatment has been found to decrease NF-κB binding to its consensus sequence in promoters in many previous studies. Signaling of relaxin through the NF-κB proteins p50 and p65 increases *Mmp-9* production in the THP-1 human monocyte cell line within 30 minutes of treatment (Ho et al. 2007). The *KiSS-1* gene, found to suppress cancer cell metastasis, is associated with decreased levels of MMP-9 expression. *KiSS-1* expression reduces NF-κB binding to the *Mmp-9* promoter through increased levels of IκBα resulting in

decreased translocation of p50/p65 to the nucleus. Thus, this indicates that NF-κB signaling is a major mechanism of MMP-9 expression during metastasis. *KiSS-1* expression has no effect on AP-1 or Sp1 binding (Yan et al. 2001).

Signaling through binding of the Sp1 site in the *Mmp-9* promoter has also been shown to regulate MMP-9 production. The anti-metastatic compound norcantharidin acts to down-regulate MMP-9 expression through inhibition of Sp1 DNA binding activity but not AP-1 or NF-κB DNA binding activity in colon cancer CT26 cell line, indicating that Sp1 binding is required to up-regulate *Mmp-9* expression in these cells (Chen et al. 2008). However, it has also been shown that Sp1 binding to the *Mmp-9* promoter can inhibit *Mmp-9* expression. In human corneal epithelial cells, platelet-activating factor (PAF) induces *Mmp-9* production that interferes with normal wound reepithelialization. PAF induces DNA binding for AP-1, NF-κB, and Sp1, but only AP-1 and NF-κB consensus binding sequences are required for PAF to induce *Mmp-9* transcription. Binding to Sp1 consensus sequence in the *Mmp-9* promoter inhibited PAF induced MMP-9 production in this model (Taheri and Bazan 2007). Sp1 also inhibits *Mmp-9* expression during hepatic injury. During the activation of hepatic stellate cells upon chronic liver injury, type IV collagen is broken down while cells secrete type I/III collagen and MMP-9 and develop a myofibroblast phenotype. Activated hepatic stellate cells up-regulate the expression of AP-1 and NF-κB proteins and the binding of their consensus sequences in the *Mmp-9* promoter was required for production of *Mmp-9*. Overexpression of Sp1/Sp3 proteins resulted in reduced *Mmp-9* expression and removal of the Sp1 binding site from the *Mmp-9*

promoter increased promoter activity indicating that Sp1/Sp3 activity represses *Mmp-9* transcription in these cells (Takahra et al. 2004). Collectively, these studies indicate that regulation of *Mmp-9* transcription is complex and is dependent on cell type and multiple signal inputs.

*Vitamin A regulation of MMP-9*

AtRA has been previously linked with negative regulation of *Mmp-9* expression in diabetic skin cultures (Varani et al. 2002; Lateef et al. 2004), tumor cell invasion (Tsang and Crowe 2001; Andela and Rosier 2004), bronchoalveolar lavage cells (Frankenberger et al. 2001), and emphysema (Mao et al. 2003). In squamous cell carcinoma lines, atRA treatment displaces AP-1 coactivators from the *Mmp-9* promoter, leading to decreased promoter activity (Tsai et al. 2008). However, atRA has been shown to up-regulate *Mmp-9* expression in other cell types. In mammary epithelial cells cultured on collagen, atRA induces the cells to form colonies containing lumen through a mechanism involving MMP-9 production (Montesano and Soulie 2002). In rat mammary tissue, Zaragozá *et al.* (2007) have shown through chromatin immunoprecipitation studies that atRA acts through RARα on the *Mmp-9* promoter, in association with the coactivation molecule p300, to up-regulate gene expression during involution, despite the lack of a RARE site in the *Mmp-9* promoter. The actual mechanism of RARα interaction with the *Mmp-9* promoter to positively regulate promoter activity is currently unknown.

**Aims of the research**

The long-term goal of the research is to further elucidate the role of vitamin A in immune system function and the maintenance of optimal host immunity. The influence of vitamin A in the immune system is well studied in immune cell types such as neutrophils, macrophages, and lymphocytes, but current knowledge is limited in the area of DC biology. DC are especially important for bridging innate and adaptive immunity. When activated by antigen or microbial signals, these innate immune cells migrate from peripheral tissues to present antigen to the adaptive immune cells located in secondary lymphoid tissues. It is necessary to study the role of vitamin A's control of DC adherence because vitamin A may be necessary for:

1. release of immature DC from the bone marrow,
2. DC migration to secondary lymphoid tissues,
3. synapse formation with lymphocytes necessary for lymphocyte activation.

Preliminary studies investigated the phenomenon of increased DC adherence *in vitro* under vitamin A deficient conditions or in the presence of antagonist to the RARα receptor. It was found that adherent and floating DC previously cultured with RARα antagonist have decreased cell surface expression of integrin-$\alpha_L$ (CD11a, *Itgal*) following rescue with physiologically relevant concentrations of atRA. AtRA rescue also increased levels of *Mmp-9* expression but had no effect on gene expression of *Itgal*, as indicated by a gene array. These results were confirmed in a time course experiment by real-time

PCR and ELISA. The results from these studies were published and are presented in detail in Chapter 2 of this dissertation. Based on these preliminary findings, I have generated the following specific aims:

***Specific Aim 1:*** *Determine whether vitamin A supported MMP-9 activity is essential for decreased dendritic cell adhesion.* The working hypothesis is that murine bone marrow cells cultured *in vitro* under myeloid dendritic cell producing conditions in the presence of sufficient vitamin A lose their adhesive properties as they mature due to MMP-9 enzymatic activity. Further, it is hypothesized that loss or decrease of MMP-9 activity in the absence of vitamin A will increase dendritic cell adherence.

***Specific Aim 2:*** *Identify the signaling mechanism by which retinoic acid increases MMP-9 expression in dendritic cells.* The working hypothesis is that retinoic acid, through retinoic acid receptor (RAR)-$\alpha$ signaling, induces increased *Mmp-9* promoter activity and gene expression. Further, it is hypothesized that RAR$\alpha$ interacts with transcription factors with known binding sites on the *Mmp-9* promoter.

These research objectives are important because a critical function for DC is migration from the peripheral tissues to the spleen and lymph nodes to activate adaptive immune cells. I propose to elucidate vitamin A-dependent mechanisms of cellular adhesion modulation that are important to DC mobility. This basic research could have important implications for world health recommendations, especially in the area of vaccination. DC are vital to the ability to make a robust immune response to inoculations by delivering the vaccine to immune cells that

can develop protective immunity. The results from this research could potentially lend greater support to the role of vitamin A deficiency in infectious disease. In particular, understanding the exact mechanisms by which vitamin A modulates immune function could help to refine vitamin A supplementation guidelines by increasing recommended frequency of supplementation to ensure the maintenance of DC populations, and thus an optimal memory response to vaccinations and/or infectious insults.

**Figure 1.1. Vitamin A digestion, transport, and storage.** 15, 15'-O'ase, 15, 15'-monoxidase; $B_{48}$, apolipoprotein $B_{48}$; C, apolipoprotein C; Chm, chylomicron; CR, chylomicron remnant; CRBP, cellular retinol binding protein; E, apolipoprotein E; LRAT, lecithin:retinol acyltransferase; RBP, retinol binding protein; RE, retinyl ester; REH, retinyl ester hydrolases; Rol, retinol; SI, small intestine; TTR, Transthyretin.

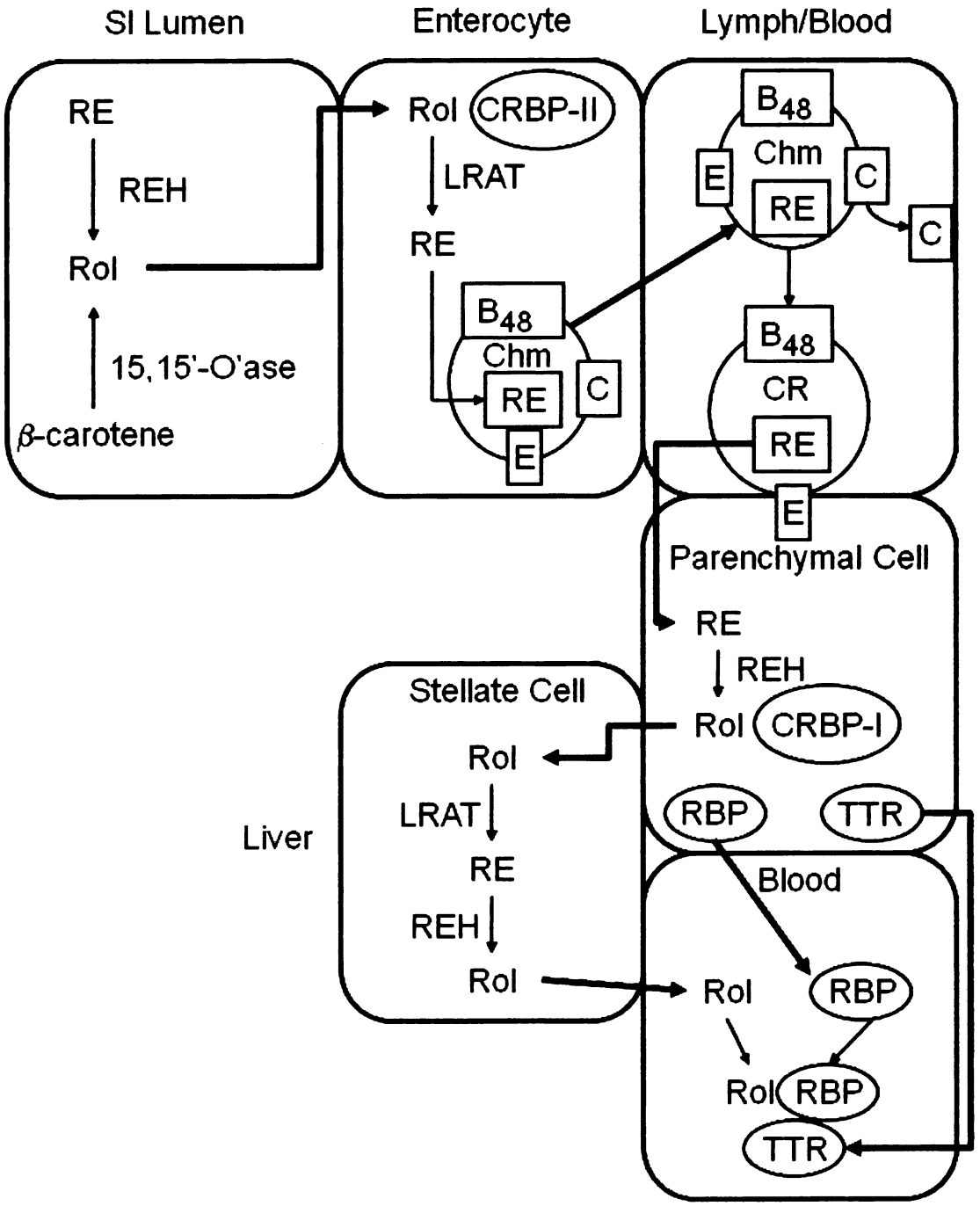

SI Lumen
Enterocyte
Lymph/Blood
RE
REH
Rol
15,15'-O'ase
β-carotene
Rol
CRBP-II
LRAT
RE
B48
Chm
RE
C
E
B48
Chm
E
C
C
B48
CR
RE
E
Parenchymal Cell
RE
REH
Rol
CRBP-I
RBP
TTR
Blood
Rol
RBP
Rol
RBP
TTR
Stellate Cell
Rol
LRAT
RE
REH
Rol
Liver

Figure 1.1

**Figure 1.2. Classic vitamin A signaling mechanism.** AtRA binds to form holoRAR/RXR which binds to RARE in a target gene promoter. A consensus DR5 RARE is depicted. AtRA, all-*trans* retinoic acid; Ral, retinal; RalDH, retinal dehydrogenase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RBP, retinol binding protein; Rol, retinol; RolDH, retinol dehydrogenase; RXR, retinoid X receptor.

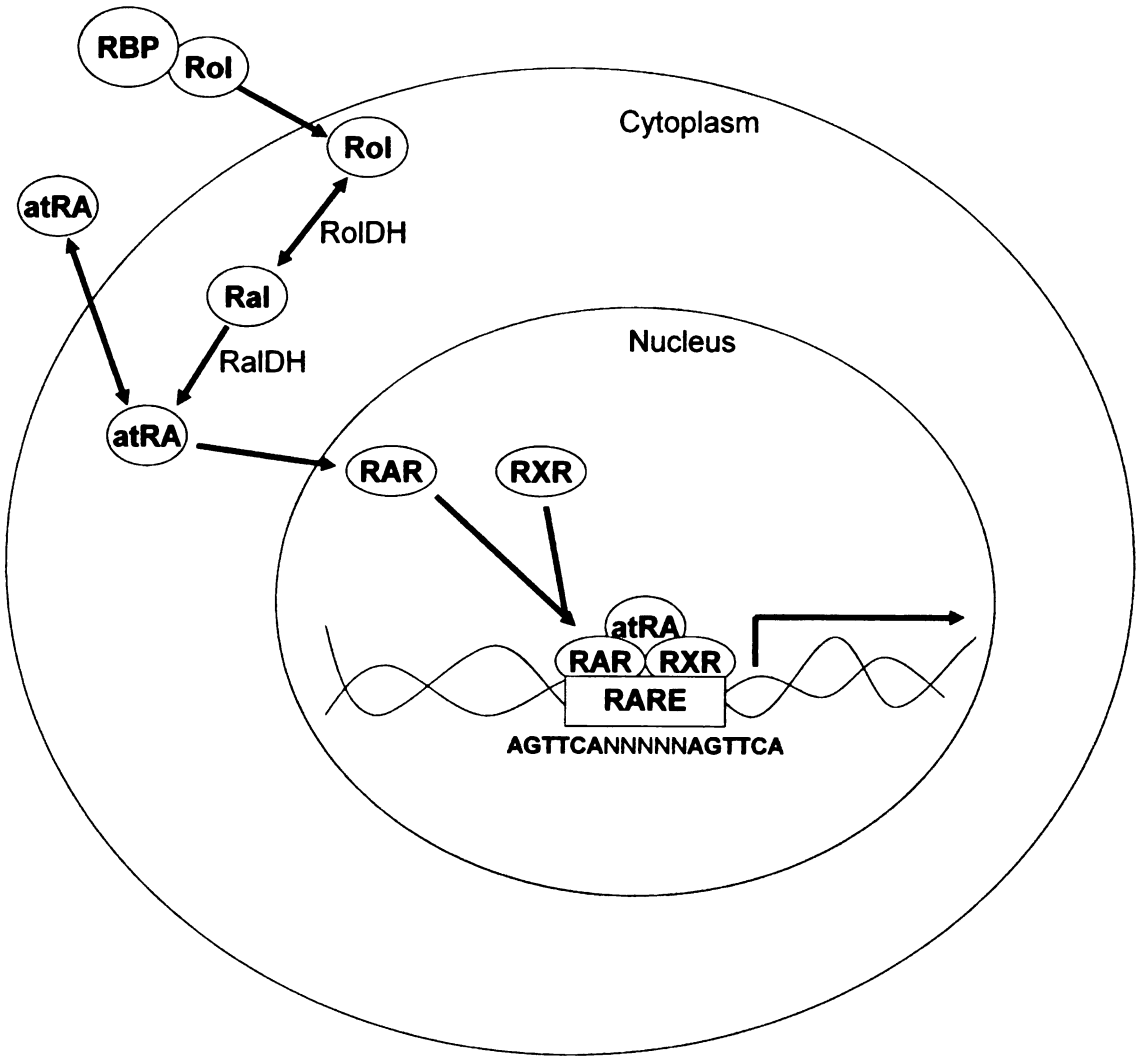
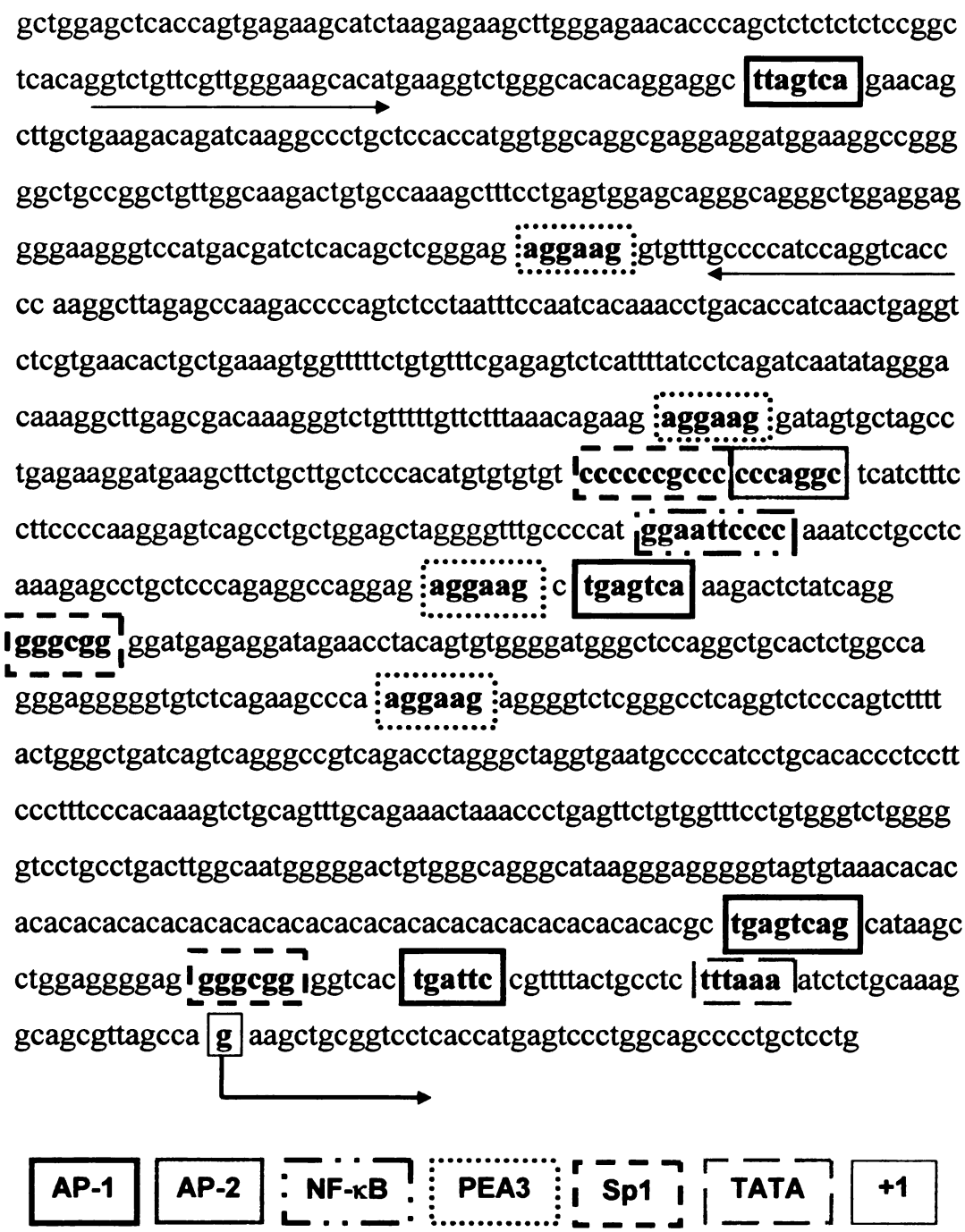

RBP
Rol
Rol
Cytoplasm
atRA
RolDH
Ral
Nucleus
RalDH
atRA
RAR
RXR
atRA
RAR
RXR
RARE
AGTTCANNNNNAGTTCA

Figure 1.2

**Figure 1.3. Mouse *Mmp-9* promoter with transcription factor binding sites noted.** AP, activating protein; NF-κB, nuclear factor kappa B; PEA3, polyoma virus enhancer A-binding protein 3; Sp1, specificity protein 1; TATA, TATA-like box. Bent arrow indicates transcription start site. Arrows beneath letters indicate primers used in PCR analysis of ChIP chromatin described in Chapter 3. From Munaut et al. 1999.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cttgctgaagacagatcaaggccctgctccaccatggtggcaggcgaggaggatggaaggccggg
ggctgccggctgttggcaagactgtgccaaagctttcctgagtggagcagggcagggctggaggag
gggaagggtccatgacgatctcacagctcgggag aggaag gtgtttgccccatccaggtcacc
cc aaggcttagagccaagaccccagtctcctaatttccaatcacaaacctgacaccatcaactgaggt
ctcgtgaacactgctgaaagtggtttttctgtgtttcgagagtctcattttatcctcagatcaatatagga
caaaggcttgagcgacaaagggtctgtttttgttctttaaacagaag aggaag gatagtgctagcc
tgagaaggatgaagcttctgcttgctcccacatgtgtgtgt ccccccgccc cccaggc tcatctttc
cttccccaaggagtcagcctgctggagctaggggtttgccccat ggaattcccc aaatcctgcctc
aaagagcctgctcccagaggccaggag aggaag c tgagtca aagactctatcagg
gggcgg ggatgagaggatagaacctacagtgtggggatgggctccaggctgcactctggcca
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actgggctgatcagtcagggccgtcagacctagggctaggtgaatgccccatcctgcacaccctcctt
ccctttcccacaaagtctgcagtttgcagaaactaaaccctgagttctgtggtttcctgtgggtctgggg
gtcctgcctgacttggcaatgggggactgtgggcagggcataagggaggggtagtgtaaacacac
acacacacacacacacacacacacacacacacacacacacacacacgc tgagtcag cataagc
ctggaggggag gggcgg ggtcac tgattc cgttttactgcctc tttaaa atctctgcaaag
gcagcgttagcca g aagctgcggtcctcaccatgagtccctggcagcccctgctcctg
AP-1
AP-2
NF-κB
PEA3
Sp1
TATA
+1

Figure 1.3

# CHAPTER 2

## RETINOIC ACID DECREASES ADHERENCE OF MURINE MYELOID DENDRITIC CELLS AND INCREASES PRODUCTION OF MATRIX METALLOPROTEINASE-9

**Lackey DE,** Ashley SA, Davis AL, Hoag KA. Retinoic acid decreases adherence of murine myeloid dendritic cells and increases production of matrix metalloproteinase-9. J Nutr. 2008;138:1512-1519.

Appendix A contains an expansion of data found in Table 2.1.

**Abstract**

Myeloid dendritic cells (DC) are professional antigen presenting cells (APC) that migrate to secondary lymphoid tissues where they activate naïve T cells to proliferate and differentiate to T helper 2 cells. These cells help initiate an antibody mediated immunie response. Vitamin A is known to be essential for normal immune function. We investigated the ability of all-*trans* retinoic acid (atRA), a bioactive metabolite of vitamin A, to modulate DC adhesion in culture. The percentage of developing DC that remained adherent was higher in the presence of a specific antagonist of retinoic acid receptor (RAR)-$\alpha$ compared to cells treated with atRA treatment from days 8-10 of culture ($P$ <0.05). AtRA treatment on day 8 of the culture period decreased DC surface expression of the adhesion molecule CD11a ($P$ <0.0001), but not the gene expression. Treatment with atRA also dramatically increased gene and protein expression of the gelatinase zymogen pro-matrix metalloproteinase (pro-MMP)-9 ($P$ <0.05). However, gene expression and protein production of MMP-9's primary biological inhibitor, tissue inhibitor of metalloproteinase (TIMP)-1, was unaffected by atRA treatment, altering the molar ratio of secreted pro-MMP-9:TIMP-1. Thus atRA treatment resulted in a molecular excess of pro-MMP-9 to its primary inhibitor ($P$ <0.05), indicating the possibility for increased MMP-9 enzymatic activity in atRA treated DC cultures. These data suggest that atRA is essential to augment MMP-9 expression in myeloid DC and can alter their surface expression of adhesion molecules.

KEY WORDS: all-*trans* retinoic acid, dendritic cells, neutrophils, vitamin A

## Introduction

Vitamin A has long been known for its role in immunity. It is currently estimated that about 127 million pre-school aged children and 20 million pregnant women in developing countries are vitamin A deficient (West 2002) leading to increased risk of night blindness and mortality (Christian et al. 2001). Vitamin A deficiency compromises the mucosal barriers of the eye, respiratory, and gastrointestinal tract, the first defenses against infection (Stephensen 2001). Deficiency also compromises the ability of macrophages and neutrophils to migrate to sites of infection, phagocytose, and kill bacteria (Twining et al. 1997; Stephensen 2001), while increasing neutrophil precursor cell expansion (Kuwata et al. 2000; Walkley et al. 2002). Marginal vitamin A status has been shown to decrease natural killer cell number and function (Dawson et al. 1999). Vitamin A is also important for development of a memory response to antigens introduced in the form of infection or vaccination.

Vitamin A supplementation increases the delayed type hypersensitivity response to ovalbumin [a measure of T helper (Th)1 function] in vitamin A deficient mice, and also increases the serum antibody production in children after diphtheria and tetanus toxoid vaccination (Stephensen 2001). In mice, vitamin A deficiency enhances the Th1 response to *Trichinella spiralis* infection through increased secretion of interferon (IFN)-$\gamma$, while suppressing the Th2 humoral response through decreased production of interleukin (IL)-5 and IL-10 (Cantorna et al. 1994). All-*trans* retinoic acid (atRA), a bioactive metabolite of vitamin A, has been shown to inhibit the synthesis of the Th1 cytokine IFN-$\gamma$ in Th1 cell culture

(Cantorna et al. 1996) and enhance the development of $CD4^+$ T cells into Th2 cells through an antigen presenting cell (APC) intermediate (Hoag et al. 2002). Evidence exists that myeloid dendritic cells (DC) are a relevant APC whose function is modified by vitamin A (Hengesbach and Hoag 2004).

Myeloid DC are professional APCs derived from a common myeloid progenitor cell and in mice are characterized as $CD11b^+$ $CD11c^+$ $CD8\alpha^-$ (Shortman and Liu 2002). There is evidence that a lineage marker negative $CX_3CR1^+$ $CD117^+$ common progenitor cell in the mouse gives rise to both myeloid DC ($CD11b^{hi}$ $CD11c^{+/hi}$) and macrophages ($CD11b^{+/mid}$ $CD11c^{variable}$ or $CD11b^-$ $CD11c^{+/hi}$ for alveolar macrophages), but not other mature myeloid cell types (Gonzalez-Juarrero et al. 2003; Fogg et al. 2006). Immature myeloid DC from the bone marrow reside in the peripheral tissues and are able to efficiently capture and process antigens and present them in the major histocompatibility class I and II molecules (Banchereau et al. 2000). DC maturation through activation by antigen or stress signals from self tissues leads to migration to the T cell areas of the lymph nodes and spleen where they stimulate and activate both cytotoxic and helper naïve T cells. The activation of T cells leads to adaptive immune responses and memory (Banchereau and Steinman 1998; Steinman 1999).

AtRA signals through the retinoic acid receptor (RAR) family, composed of $\alpha$, $\beta$, and $\gamma$ isotypes in the class II family of nuclear receptors (Chambon 1996). This family also includes the thyroid hormone receptor, vitamin D receptor, and

peroxisome proliferator-activated receptor (Chawla et al. 2001; Olefsky 2001). Upon atRA ligand binding, RAR heterodimerizes with a retinoid X receptor (RXR) family member, also consisting of $\alpha$, $\beta$, and $\gamma$ isotypes. The RAR/RXR dimer then binds to retinoic acid response elements within the promoter regions of retinoid responsive genes and associates with co-regulating proteins, eventually leading to the promotion or inhibition of transcription and target gene expression (Bastien and Rochette-Egly 2004; Mark et al. 2006).

RARs are important in the regulation of hematopoiesis (Evans 2005). Deletion studies indicate that RAR$\gamma$ is necessary for maintaining the hematopoietic stem cell population, while overexpression of RAR$\alpha$ in bone marrow cells indicate RAR$\alpha$ activation favors neutrophil development (Purton et al. 2006). We originally observed that treating BALB/cJ mouse bone marrow-derived myeloid DC cultures with the RAR$\alpha$-specific antagonist AGN 194301 throughout the culture period led to a decreased yield of floating DC, while a large number of DC remained adherent on the dish surface. AGN 194301 competes with atRA for binding on the ligand binding domain of RAR$\alpha$ and blocks atRA activity (Nagpal and Chandraratna 2000). Based on these initial observations, experiments were designed to assess the ability of atRA to rescue DC development after culture initiation with the RAR$\alpha$-specific antagonist.

## Materials and Methods

### *Materials*

Stock solutions of atRA (Sigma, St. Louis, MO) were prepared in dimethyl sulfoxide (Sigma) and stored at –70°C under an argon atmosphere in the dark. Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from PeproTech (Rocky Hill, NJ) and dissolved in molecular biology grade water. Iscove's modified Dulbecco's medium (IMDM, BioWhittaker, Walkersville, MD) was supplemented with 10% (v/v) serum [characterized (CH) or charcoal/dextran treated (CD) FBS, HyClone, Logan, UT], 2 mmol/L L-glutamine, 100,000 U/L penicillin, 100 mg/L streptomycin (BioWhittaker), and 10 μmol/L β-mercaptoethanol (Sigma) to make complete IMDM (cIMDM).

### *Animals*

Male BALB/cJ mice (Jackson Laboratories, Bar Harbor, ME) were maintained according to institutional guidelines set by University Laboratory Animal Resources under a protocol approved by Michigan State University's Institutional Care and Use Committee. The animals were fed commercial solid pellets (22/5 Rodent Diet #8640, wt/wt composition: 22.58% protein, 5.23% fat, 3.94% fiber, 7.06% ash, 51.15% nitrogen-free extract; metabolizable energy content, 16.00 kJ/g; Harlan Teklad, Madison, WI) and killed between 6 and 12 weeks of age by carbon dioxide asphyxiation.

### *Cell Culture*

Femurs and tibiae were collected from male BALB/cJ mice and kept on ice. Bones were sterilized in 70% ethanol for 2 min and transferred to RPMI-1640 medium (BioWhittaker) where the bone ends were clipped and the bone marrow was flushed by syringe. Cells were filtered and pelleted, then resuspended in ACK lysing buffer (BioWhittaker) to lyse red blood cells. Bone marrow cells were then pelleted and resuspended in RPMI-1640 medium. A portion of cells were mixed with trypan blue dye solution and counted to determine cell number and viability. Cells were cultured for DC development using a method based on that of Inaba *et al.* (1998) and modified by Lutz *et al.* (1999).

For the atRA and receptor antagonist full-culture experiments, bone marrow cells were plated 2 x $10^6$ per dish containing 10 mL cIMDM supplemented with 10% CH-FBS and 20 μg/L GM-CSF in the presence or absence of 1 or 10 nmol/L AGN 194301 (provided by Dr. R.A.S. Chandraratna, Vitae Pharmaceuticals, Irvine, CA), an RARα-specific antagonist, or 10 mL cIMDM supplemented with 10% CD-FBS and 20 μg/L GM-CSF in the presence or absence of 1 nmol/L atRA for 10 d, with fresh medium and treatment given on d 3, 6, and 8 of culture. For atRA rescue experiments, bone marrow cells were plated 2 x $10^6$ per dish containing 10 mL cIMDM supplemented with 10% CH-FBS and 20 μg/L GM-CSF with 10 nmol/L AGN 194301. Cells were given 10 mL fresh cIMDM containing 20 μg/L GM-CSF and 10 nmol/L AGN 194301 on d 3 and 6 of culture. On d 8 of culture, all dishes had a complete medium replacement with cIMDM containing 20 μg/L GM-CSF and either 10 nmol/L of

atRA or AGN 194301. Cells were harvested for RNA extraction and flow cytometry staining at various time points between d 8 and d 10 of culture.

***Flow Cytometric Analysis***

Floating cells were collected by rinsing culture dishes with staining buffer [1% FBS, 0.1% (w/v) sodium azide in PBS, pH 7.4] and pelleted by centrifugation. Adherent cells were removed with Accutase (Innovative Cell Technologies, Inc., San Diego, CA) according to manufacturer instructions, collected, and pelleted by centrifugation. The cells were washed with staining buffer and a portion of cells from each sample were mixed with trypan blue dye to determine cell viability and count. One million cells from each sample were incubated with purified anti-$F_c\gamma$RII/III monoclonal antibody from the 2.4G2 hybridoma to block non-specific binding. Each sample was incubated in a specific antibody cocktail to label a variety of individual cell surface molecules or to control for the isotype. Monoclonal antibodies used were phycoerythrin (PE)-conjugated hamster anti-mouse CD11c, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Ly-6C and Ly-6G (Gr-1), biotin conjugated rat anti-mouse CD11a, PE-conjugated hamster $IgG_{1/\lambda}$, FITC-conjugated rat $IgG_{2b/\kappa}$, biotin-conjugated rat $IgG_{2a/\kappa}$, and allophycocyanin-conjugated streptavidin (BD Biosciences Pharmingen, San Jose, CA). Cells were washed and fixed in 2% paraformaldehyde in PBS. Samples were run at the MSU flow cytometry facility using a BD FACS Vantage equipped with DiVA digital system and analyzed using FCS Express v. 3.0 software (DeNovo Software, Ontario, CA).

***Real-Time PCR***

Total RNA was isolated from adherent cells at 0, 4, 8, 12, and 24 h post atRA rescue during the *in vitro* culture period. Medium was removed from the culture dishes and the cells were incubated with TRIzol reagent (InVitrogen, Carlsbad, CA) for cell lysis. RNA extraction was completed according to the manufacturer's instructions. Chloroform was added to solubilize lipids in the samples and for phase separation. RNA was precipitated from the aqueous phase with isopropanol and washed with 75% ethanol. RNA pellets were air-dried, resuspended in molecular biology grade water (Sigma), and stored at –70°C. RNA concentration was measured at 260 and 280 nm using the CellQuant spectrophotometer (Amersham, Piscataway, NJ).

Total RNA was reverse transcribed using the TaqMan Reverse Transcription System with MultiScribe reverse transcriptase and random hexamer primers (Applied Biosystems, Foster City, CA). Expression of cDNA relative to the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) housekeeping gene was analyzed using Assays-on-Demand™ Gene Expression primer/probe sets (Applied Biosystems) for *Itgal* (Mm00801807_m1), *Mmp-9* (Mm00600163_m1), and *Timp-1* (Mm00441818_m1). PCR was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Monitoring of PCR occurred in real-time by detection of FAM fluorescence on the 5' end of the probe, quenched with a non-fluorescent quencher on the 3' end of the probe. 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).

***Microarray Analysis***

Total RNA was isolated from adherent cells and $CD11c^+$ purified floating cells on d 10 of culture. AtRA-treated floating cells were purified for $CD11c^+$ cells by labeling with biotin-conjugated hamster anti-mouse CD11c, secondary incubation with BD Streptavidin Particles Plus-DM, and separated using the BD IMagnet (BD Biosciences Pharmingen). Medium was removed from the culture dishes and RNA extraction was completed as described above. Differential RNA expression was analyzed using the GEArray Q Series Mouse Extracellular Matrix and Adhesion Molecules Gene Array by SuperArray (Frederick, MD) by the manufacturer.

***ELISA***

Quantikine mouse ELISA kits for detection of secreted pro-MMP-9 and TIMP-1 from cell culture supernatants were purchased from R & D Systems (Minneapolis, MN). The manufacturer protocol was followed. Briefly, standard dilutions, internal controls, and sample dilutions were incubated in the appropriate pre-coated ELISA plate with assay diluent in technical duplicate. After washing, the wells were incubated with the appropriate conjugate. Finally, the wells were incubated with substrate and then the reaction was stopped with hydrochloric acid. The well absorbances were read on a plate reader at 450 nm, using a correction wavelength of 570 nm. SOFTmax Pro for Life Sciences software version 4.0 (Molecular Devices, Sunnyvale, CA) was used for concentration calculations from the standard curve.

***Statistical Analysis***

Data were analyzed using InStat software version 3.05 (GraphPad Software, Inc., San Diego, CA). Changes in percentages of $CD11c^+$ and $Gr\text{-}1^+$ cells in the atRA and receptor antagonist full culture experiments were determined using one-way ANOVA with a post-hoc Tukey-Kramer multiple comparisons test for the floating cells group and the Kruskal-Wallis nonparametric ANOVA with a post-hoc Dunn's multiple comparisons test for the all cells group. Comparisons between two groups were made using Student's unpaired *t* test, except for ELISA experiments where the non-parametric Mann-Whitney test was employed. Data are presented as means ± SEM and differences between groups of $P < 0.05$ were considered significant.

## Results

### *RARα antagonism increases adherence of $CD11c^+$ DC*

Previous *in vitro* studies from our laboratory have shown that insufficient concentrations of vitamin A result in decreased DC development and increased neutrophil development (Hengesbach and Hoag 2004). However, supplementation of medium with physiological levels of atRA restored DC development (Hengesbach and Hoag 2004). We cultured mouse bone marrow cells in cIMDM supplemented with GM-CSF and CH-FBS in the presence or absence of various dosages of the RARα-specific receptor antagonist AGN 194301, or CD-FBS (vitamin A depleted) in the presence or absence of atRA for 10 d. Floating cells were harvested alone, or floating and enzyme-detached adherent cells were pooled together for each sample. Cells were then dual

labeled for cell surface expression of Gr-1 and CD11c to identify neutrophils and DC, respectively. We found that the percentage of $CD11c^+$ DC, when adherent and floating cells were combined, did not differ significantly between the treatment groups. However, when only floating cells were harvested, the percentage of $CD11c^+$ DC was significantly reduced from 80% with the CH-FBS positive control to 45% with the addition of 10 nmol/L RARα antagonist treatment ($P$ <0.05). At the same time, the percentage of $CD11c^+$ DC was significantly increased from 10% with the CD-FBS negative control to 60% with the addition of 1 nmol/L atRA ($P$ <0.05, **Figure 2.1***A*). When floating and adherent cells were pooled together, the percentage of $Gr\text{-}1^+$ neutrophils increased slightly with 10 nmol/L receptor antagonist treatment while the neutrophil population was reduced from 50% with CD-FBS supplementation to 20% with the 1 nmol/L atRA treatment in the presence of CD-FBS supplemented IMDM, back to the levels of the CH-FBS control. However, when only floating cells were harvested, the percentage of $Gr\text{-}1^+$ neutrophils increased significantly from 10% in the CH-FBS control to 50% with the 10 nmol/L receptor antagonist treatment ($P$ <0.05), while the percentage decreased significantly from 70% with the CD-FBS control treatment to 20% with the 1 nmol/L atRA treatment in CD-FBS supplemented IMDM ($P$ <0.05, Figure 2.1*B*).

***Rescue of cultures with atRA increases the percentage of floating DC***

Based on the results indicating that RARα signaling is necessary for $CD11c^+$ DC to become non-adherent cells, we next designed experiments to test

whether atRA can rescue DC development after culture with receptor antagonist. Primary mouse bone marrow cells were cultured in the presence of GM-CSF and the RARα-specific receptor antagonist AGN 194301 for eight days. The cells either remained in the presence of receptor antagonist or were rescued by replacement of receptor antagonist with 10 nmol/L atRA on d 8. When cells were grown with the receptor antagonist, large cell clusters that were firmly adherent developed on the surface of the plastic dish. When the cells were rescued with atRA on d 8 of the culture period, the cells within the clusters began to release within 24-48 h (data not shown). On d 10, 48 h post-atRA rescue, the resultant adherent cell yield did not differ between the receptor antagonist treatment and the atRA rescue treatment. However, there was a significant increase ($P$ <0.0001) in floating cell yield in atRA-rescued cultures compared to those that remained with the receptor antagonist (**Figure 2.2***A*). The percentage of cells that remained adherent decreased by 10% with atRA treatment and the number of floating cells increased significantly ($P$ <0.01) compared to cultures that received fresh medium and GM-CSF but remained in the receptor antagonist treatment (Figure 2.2*B*).

Upon harvest on d 10, cells were stained with Gr-1 to identify neutrophils and CD11c to identify myeloid DC. The $CD11c^+$ DC predominated over $Gr\text{-}1^+$ neutrophils in the adherent cells in both treatment groups (**Figure 2.3***A*,*B*). However, atRA rescued cell cultures showed a significantly greater percentage ($P$ <0.05) and yield ($P$ <0.001) of $CD11c^+$ floating DC compared to cells retained in culture with receptor antagonist (Figure 2.3*A*,*C*). Conversely, $Gr\text{-}1^+$ neutrophils

predominated in the floating cell population for both treatment groups, and the percentage of Gr-1$^+$ floating cells was not significantly affected by atRA rescue (Figure 2.3*B*). However, total Gr-1$^+$ neutrophils yield was significantly enhanced in the floating cell population when cells were cultured with atRA for the final 2 days of culture as opposed to remaining with receptor antagonist (Figure 2.3*D*).

***CD11a cell surface expression is decreased by atRA rescue***

Based on the results showing that atRA rescue decreased adherence in DC cultures, we also examined the cell surface for expression of relevant cell adhesion molecules. On d 10 of culture, both adherent and floating CD11c$^+$ DC treated with atRA showed significantly decreased ($P$ <0.0001) levels of the cell surface marker CD11a (integrin-$\alpha_L$) compared to cells that remained in receptor antagonist treatment for the full 10 d culture period, measured by mean fluorescence intensity (MFI, **Figure 2.4***A*,*B*). Surface expression of the adhesion molecule and general myeloid cell marker CD11b was unaffected by atRA treatment (data not shown).

***Matrix metalloproteinase-9 mRNA expression and pro-MMP-9 secretion increases in adherent cells with atRA rescue***

A microarray study for mouse extracellular matrix and adhesion molecule gene expression using a GEArray Q Series array by SuperArray (Frederick, MD) was performed to further explore DC adhesion. We found that 48 h atRA rescue increases *Mmp-9* expression 12-fold in adherent DC and 15-fold in CD11c$^+$ floating cells compared to receptor antagonist treated adherent cells, indicating

that these differences in gene expression were not due solely to the adherent or floating phenotype (**Table 2.1**). *Mmp-7* was the only other metalloproteinase that appeared to be retinoic acid-regulated. However, actual levels of expression were not above background (up to 0.5 arbitrary units (AU), a unit of signal intensity after interquartile normalization) for this gene, whereas atRA up-regulated *Mmp-9* expression to 6 AU (**Appendix A**). *Mmp-3*, *-8*, *-12*, *-13*, and *-19* were also highly expressed in these samples (from 8-20 AU, Appendix A), but their expression was unchanged by atRA treatment (Table 2.1). These results suggest that up-regulation of matrix proteases may play a role in the decrease in DC adhesion mediated by atRA. Complete normalized results of the gene array can be found in Appendix A.

**Table 2.1.** Induction of mouse matrix metalloproteinase mRNA relative to expression of receptor antagonist adherent DC in atRA treated adherent and $CD11c^+$ floating mouse DC cultures.[1]

| mRNA | Retinoic Acid Adherent[2] | Retinoic Acid Floating DC[2] |
|---|---|---|
| *Mmp-1a* | 2.47 | 1.59 |
| *Mmp-2* | 1.12 | 0.79 |
| *Mmp-3* | 1.15 | 0.38 |
| *Mmp-7*[3] | 4.44 | 3.58 |
| *Mmp-8* | 0.78 | 2.06 |
| *Mmp-9* | 12.65 | 15.49 |
| *Mmp-10* | 1.15 | 1.36 |
| *Mmp-11* | 0.63 | 0.67 |
| *Mmp-12* | 0.69 | 2.76 |
| *Mmp-13* | 0.76 | 0.59 |
| *Mmp-14* | 1.27 | 0.94 |
| *Mmp-15* | 1.63 | 1.16 |
| *Mmp-16* | 0.95 | 0.38 |
| *Mmp-17* | 0.72 | 1.94 |
| *Mmp-19* | 0.51 | 0.32 |
| *Mmp-20* | 0.79 | 1.53 |
| *Mmp-23* | 1.95 | 1.37 |
| *Mmp-24* | 1.30 | 0.43 |

[1]RNA extracted from adherent atRA-treated, adherent receptor antagonist-treated, or $CD11c^+$ isolated floating DC. RNA was hybridized to SuperArray GEArray Q Series Mouse Extracellular Matrix and Adhesion Molecules Gene Array (SuperArray Biosciences). Signal intensity between treatments was normalized to the interquartile median value.

[2]Relative expression calculated as retinoic acid expression/receptor antagonist expression.

[3]*Mmp-7* signal intensity is similar to blank sample intensity.

Quantitative real-time PCR was used to confirm the level of gene expression in two independent time course experiments. It was found that *Itgal* transcript abundance decreases over time in both the atRA and receptor antagonist treatments. Both treatments had the same level of *Itgal* transcript expression 24 h post-atRA rescue (data not shown). However, we found that atRA rescue treatment increased the expression of *Mmp-9* transcript three fold within 4 h and continued to increase up to 14 fold within 24 h compared to the 0 h receptor antagonist treatment (**Figure 2.5***A*). Levels of tissue inhibitor of metalloproteinase (*Timp*)-1 transcript, an endogenous inhibitor of MMP-9 activity, remained constant for both treatments throughout the time course (data not shown).

We next used ELISA to determine whether MMP-9 and TIMP-1 protein production increased in conjunction with mRNA expression. Treatment with atRA was found to significantly increase the level of secreted pro-MMP-9 in conditioned medium on both d 9 and 10 of culture (24 and 48 h, respectively after the addition of fresh medium and rescue with atRA or continued treatment with receptor antagonist). Pro-MMP-9 protein increased significantly ($P < 0.05$) to 26 μg/L compared to 4 μg/L in the receptor antagonist group on day 10 (Figure 2.5*B*). At the same time, levels of secreted TIMP-1 increased to 4 μg/L in both receptor antagonist and atRA rescued treatment groups compared to the receptor antagonist treated group on d 8 (data not shown). The molar ratio of pro-MMP-9:TIMP-1 indicates that there was a 2:1 molar excess of pro-MMP-9 on day 10 of culture in the atRA treated group (48 hours after rescue), while those

cultures that retained treatment with the receptor antagonist showed a 4:1 molar excess of TIMP-1 (Figure 2.5*C*).

## Discussion

Vitamin A has been demonstrated to be important in the development of humoral immunity by polarizing naïve $CD4^+$ T cells to a Th2 response through an APC intermediate (Hoag et al. 2002). One of the vitamin A dependent APC has been identified as myeloid DC (Hengesbach and Hoag 2004). However, the mechanisms by which atRA modulates myeloid DC physiology have not yet been fully described. Therefore, we employed the RARα-specific receptor antagonist, AGN 194301, to investigate the effects of atRA on the development of cultured DC via signaling through the nuclear receptor RARα. We explored the ability of atRA to rescue DC development after culture initiation in the presence of the receptor antagonist. A number of new findings resulted from this approach.

First, when primary bone marrow cells are cultured in medium depleted of retinol or in the presence of receptor antagonist in medium containing sufficient concentrations of retinol, a decreased percentage of DC and an increased percentage of neutrophils in the floating cell population results compared to cells cultured in cIMDM supplemented with CH-FBS. In addition, atRA supplementation of CD-FBS containing medium increased the percentage of DC and decreased the percentage of neutrophils to the levels of the positive control treatment in the floating cell population. However, the percentage of DC and neutrophils in the total cell population does not differ between cultures receiving

medium supplemented with CH-FBS in the presence or absence of receptor antagonist or cultures receiving medium supplemented with CD-FBS in the presence of atRA. These data indicate that inhibition of signaling through RARα blocks normal DC development and loss of adherence.

Myeloid DC developed an adhesive phenotype when primary bone marrow cells were cultured in the presence of RARα-specific receptor antagonist, while rescuing these cultures with atRA reduced this phenotype and instead the DC showed a floating phenotype, as found when they are cultured exclusively in vitamin A sufficient medium. These results indicate that atRA acts to increase the percentage of DC that lose adherence from the plastic culture dish surface. However, the proportion of floating neutrophils did not decrease significantly upon atRA rescue compared to cells treated with the receptor antagonist and the actual neutrophil cell yield increased upon atRA rescue. It is possible that neutrophils are still able to develop from precursor cells in the presence of RARα-specific receptor antagonist while DC are not, thus atRA rescue over a limited 48 h period begins the process of DC development while neutrophils have an earlier advantage.

We also found the level of the adhesion molecule CD11a was decreased on the cell surface of both adherent and floating cells in cultures where atRA rescue treatment was provided. CD11a dimerizes with CD18, integrin-$\beta_2$, to form the cell-surface molecule lymphocyte function-associated antigen (LFA)-1 (Elangbam et al. 1997). We chose to focus on CD11a because it was the only cell surface molecule in a panel of adhesion molecules including CD24 (heat

stable antigen), CD31 (platelet/endothelial cell adhesion molecule-1), CD43, CD80 (B7-1), F4/80, and 33D1 that showed differential cell surface expression between receptor antagonist and atRA treated DC cultures (data not shown). Despite the strong reduction in CD11a protein detected on the cell surface of DC rescued with atRA, there is no difference in *Itgal*, the gene for CD11a, transcript expression between adherent cells treated with receptor antagonist and cells rescued with atRA over time.

Pyszniak *et al.* (1994) have shown that soluble intercellular adhesion molecule (ICAM) can bind LFA-1 and compete for binding with anti-LFA-1 antibodies. There is evidence that MMP-9 can act to produce sICAM-1 from cell surface ICAM-1. However, experiments in our lab show there is little variability in *Icam-1* mRNA expression over a 24 h period independent of RARα antagonist or atRA treatment. Likewise, we have not found a difference in sICAM-1 production between the treatments at the different time points over a 48 h period (**Appendix B**). The maximal presence of sICAM-1 in culture supernatant reached only 2.5 μg/L for both culture conditions on d 10 of the culture period. Since sICAM-1 was produced equally regardless of atRA treatment, it is unlikely that sICAM-1 blocking LFA-1 could explain the differences in CD11a cell surface expression.

Overall, our data indicate that atRA may regulate transcription of another molecule through RARα that affects CD11a surface expression. The protein MMP-9, which was up-regulated by atRA rescue, is a possible candidate, and has been shown to cleave a number of other cell surface proteins. These include dystroglycan, allowing for leukocyte entrance through the parenchymal basement

membrane of the blood-brain barrier (Agrawal et al. 2006), and galectin-3, which interacts with the extracellular matrix (Ochieng et al. 1998). MMP-9 has also been shown to proteolytically activate a number of cytokines that are secreted in latent forms, including IL-1β (Schonbeck et al. 1998) and transforming growth factor-β (Yu and Stamenkovic 2000).

AtRA rescue increases expression of the *Mmp-9* transcript and the pro-MMP-9 protein, while leaving expression of TIMP-1 mRNA and protein, the primary endogenous inhibitor of MMP-9 (Vu and Werb 2000) unchanged. This results in a molecular excess of secreted pro-MMP-9 in atRA rescued cell cultures. Conversely, an excess of TIMP-1 is maintained in receptor antagonist treated cell cultures. MMP-9, also known as gelatinase-B, acts to degrade the extracellular matrix and is secreted as a pro-enzyme that must be cleaved for full activity to be realized (Opdenakker et al. 2001). It has been shown that use of monoclonal antibody against MMP-9 inhibits the release of hematopoietic progenitor stem cells from bone marrow in response to IL-8 injection in rhesus monkeys (Pruijt et al. 1999). MMP-9 has also been shown to be critical for DC migration (Randolph et al. 2005). In MMP-$9^{-/-}$ mice, recruitment of bronchial associated lymphoid tissue DC into the airway lumen in response to allergen (Vermaelen et al. 2003) and migration of cultured bone marrow DC through tracheal epithelial tight junctions is impaired (Ichiyasu et al. 2004). Prostaglandin $E_2$-induced TIMP-1 has previously been shown to inhibit the migration of human monocyte-derived DC, while incubation with monoclonal anti-TIMP-1 antibody restores the ability of DC to migrate (Baratelli et al. 2004).

Retinoic acid has been previously linked with negative regulation of MMP-9 levels in diabetic skin cultures (Varani et al. 2002; Lateef et al. 2004), tumor cell invasion (Tsang and Crowe 2001; Andela and Rosier 2004), bronchoalveolar lavage cells (Frankenberger et al. 2001), and emphysema (Mao et al. 2003). Our data directly contradict these findings because we show that atRA rescue up-regulates the expression of the MMP-9 gene and secreted pro-enzyme in myeloid DC cultures compared to cultures treated with receptor antagonist. However, these previous studies all used pharmacological doses of atRA (≥1 μmol/L for cell cultures), while our studies here used more relevant physiological concentrations. It is also possible that the various cell populations studied in the literature differ in their response to atRA treatment. Other cell and tissue types show MMP-9 responses to atRA similar to our results when treated with physiological concentrations. For example, a study completed by Montesano and Soulié (2002) shows that a low dose (100 pmol/L) of atRA induces mammary epithelial cells cultured on collagen to form colonies containing lumen through a mechanism involving MMP-9 production. In rat mammary tissue, Zaragozá *et al.* (2007) showed through chromatin immunoprecipitation studies that atRA acts through RARα on the *Mmp-9* promoter to up-regulate gene expression during involution, despite the lack of a RARE site. The SKNBE neuroblastoma cell line also responds to atRA by increasing production of MMP-9, allowing for production of outgrowing neurites and cell migration to produce a neuronal phenotype (Chambaut-Guerin et al. 2000).

Recently, Darmanin *et al.* (2007) have shown that immature DC cultured with pharmacological levels of supplemental atRA (1μmol/L) have increased migratory ability *in vitro* and when injected into tumors, compared to that of DC cultured in medium containing physiological concentrations of atRA, and that this migration is greatly diminished by inhibiting MMPs. Our findings that atRA rescue treatment up-regulated expression of MMP-9 and correspondingly decreased myeloid DC adhesion are in agreement with these findings. However, TIMP-1 did not decrease after atRA treatment in our studies, as was shown by Darmanin *et al.*

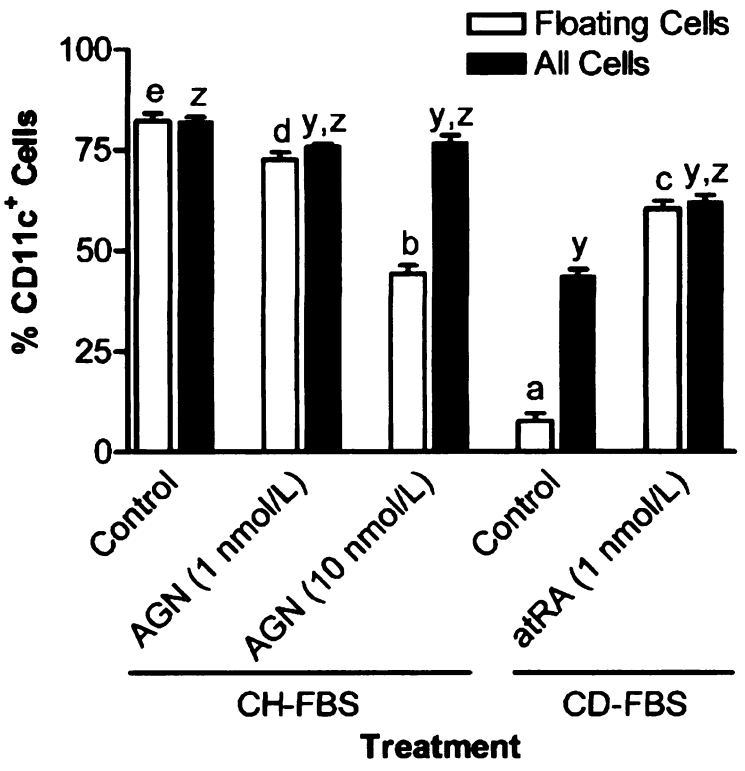
We have shown the progression from adherent DC to floating DC that is inhibited when RARα signaling is blocked can be rescued by atRA treatment, and this progression corresponds with up-regulation of MMP-9 and a decrease in cell surface expression of CD11a. Further studies are needed to verify whether MMP-9, which is up-regulated upon atRA rescue, is proteolytically active and the main effector for loss of DC adherence. These studies will involve the use of chemical inhibitors of MMP-9 and other relevant MMPs as well as functional studies that examine the amount of MMP-9 mediated proteolysis between the different DC culture treatments. DC need to migrate from infected and stressed peripheral sites to relevant lymphoid tissues to activate the adaptive immune response. This migration requires movement through extracellular matrix and changes in adhesion molecule expression (Randolph et al. 2005). Our results indicate that physiological concentrations of atRA may be necessary for optimal DC development and release from or migration through the extracellular matrix.

**Acknowledgements**

We thank Dr. Louis King of the Michigan State University flow cytometry facility in the Research and Technology Support Facilities.

**Figure 2.1.** Mouse bone marrow cells [$CD11c^+$ (A), $Gr\text{-}1^+$ (B)] cultured with RARα receptor antagonist develop an adherent phenotype. Bone marrow cell cultures were generated in the presence of GM-CSF in medium supplemented with CH-FBS with or without receptor antagonist (AGN) or CD-FBS with or without atRA for 10 d. Floating cells only or all cells (floating plus adherent) were analyzed separately by flow cytometry. Values are means + SD, n=5 (floating cells) or 3 (all cells). Within a cell type, means without a common letter differ, $P<0.05$.

A


Floating Cells
All Cells
% CD11c+ Cells
100
75
50
25
0
e
z
d
y,z
b
y,z
a
y
c
y,z
Control
AGN (1 nmol/L)
AGN (10 nmol/L)
Control
atRA (1 nmol/L)
CH-FBS
CD-FBS
Treatment

B

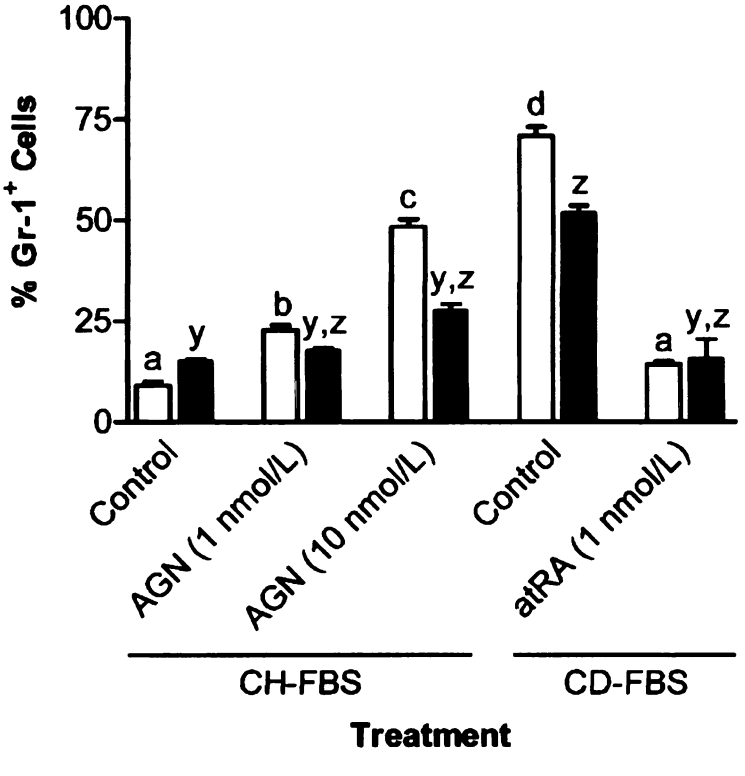

% Gr-1+ Cells
100
75
50
25
0
a
y
b
y,z
c
y,z
d
z
a
y,z
Control
AGN (1 nmol/L)
AGN (10 nmol/L)
Control
atRA (1 nmol/L)
CH-FBS
CD-FBS
Treatment

Figure 2.1

**Figure 2**

with RAR

B) yields

presence

culture w

Floating c

and coun

presented

correspon

**Figure 2.2.** AtRA rescue of mouse bone marrow-derived myeloid DC cultured with RARα receptor antagonist reduces cell adherence [Absolute (*A*) and percent (*B*) yields of adherent and floating cells]. Bone marrow cultures were grown in the presence of GM-CSF and receptor antagonist for 8 d then either remained in culture with 10 nmol/L receptor antagonist or were rescued with 10 nmol/L atRA. Floating cells and adherent cells were harvested separately from each treatment and counted on d 10. Results are from 2 independent experiments and presented as the mean + SD, n=10. Asterisks indicate different from the corresponding receptor antagonist treatment: $^{**}P<0.01$, $^{***}P<0.0001$.

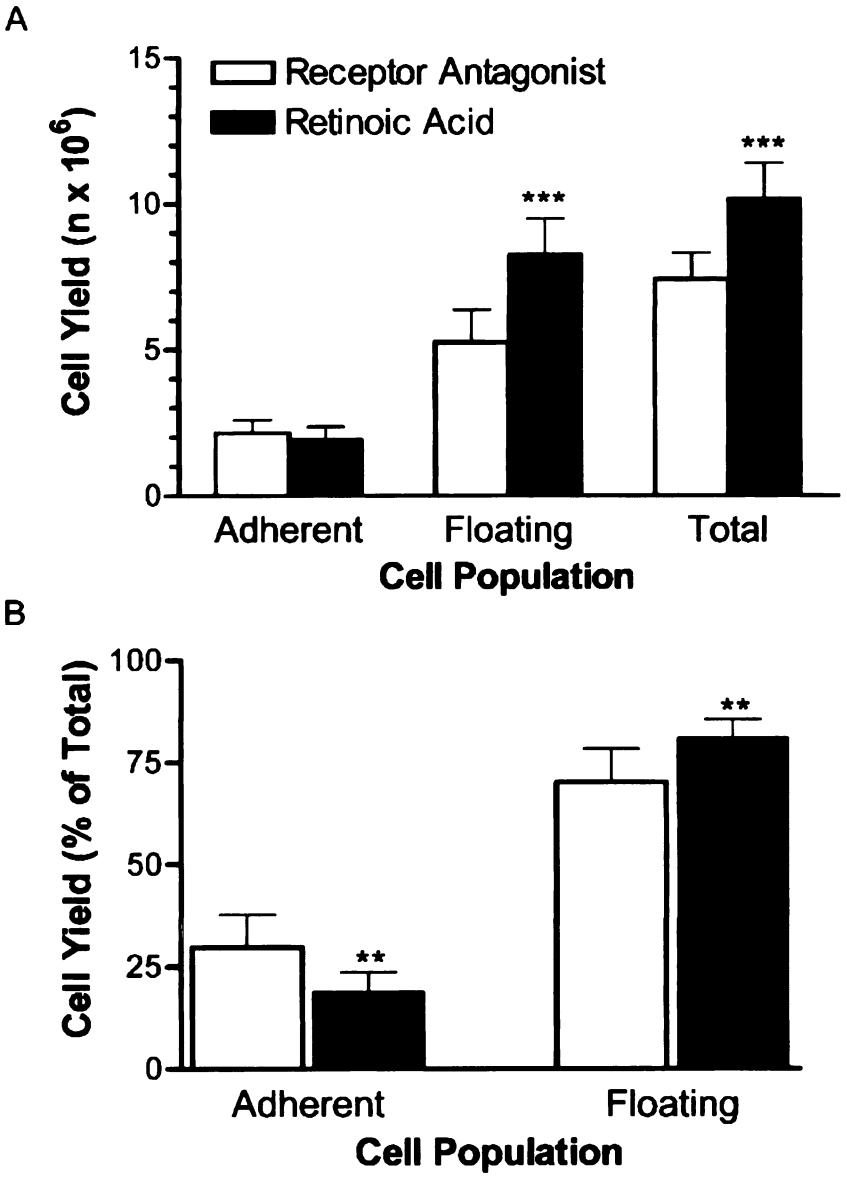

A
15
10
5
0
Receptor Antagonist
Retinoic Acid
Cell Yield (n x 10^6)
***
***
Adherent
Floating
Total
Cell Population
B
100
75
50
25
0
Cell Yield (% of Total)
**
**
Adherent
Floating
Cell Population

Figure 2.2

**Figure 2.3.** The mouse bone marrow-derived floating myeloid DC population is enhanced by atRA rescue [Percent (*A,B*) and absolute (*C,D*) yields of $CD11c^+$ (*A,C*) and $Gr\text{-}1^+$ (*B,D*) adherent and floating cells]. Bone marrow cultures were grown in the presence of GM-CSF and receptor antagonist for 8 d, then either remained in culture with 10 nmol/L receptor antagonist or were rescued with 10 nmol/L atRA. Cells were analyzed by flow cytometry on d 10. Results are representative of 2 independent experiments and presented as means + SD, n=6. Asterisks indicate different from the corresponding receptor antagonist treatment: $*P<0.05$, $**P<0.01$, or $***P<0.0001$.

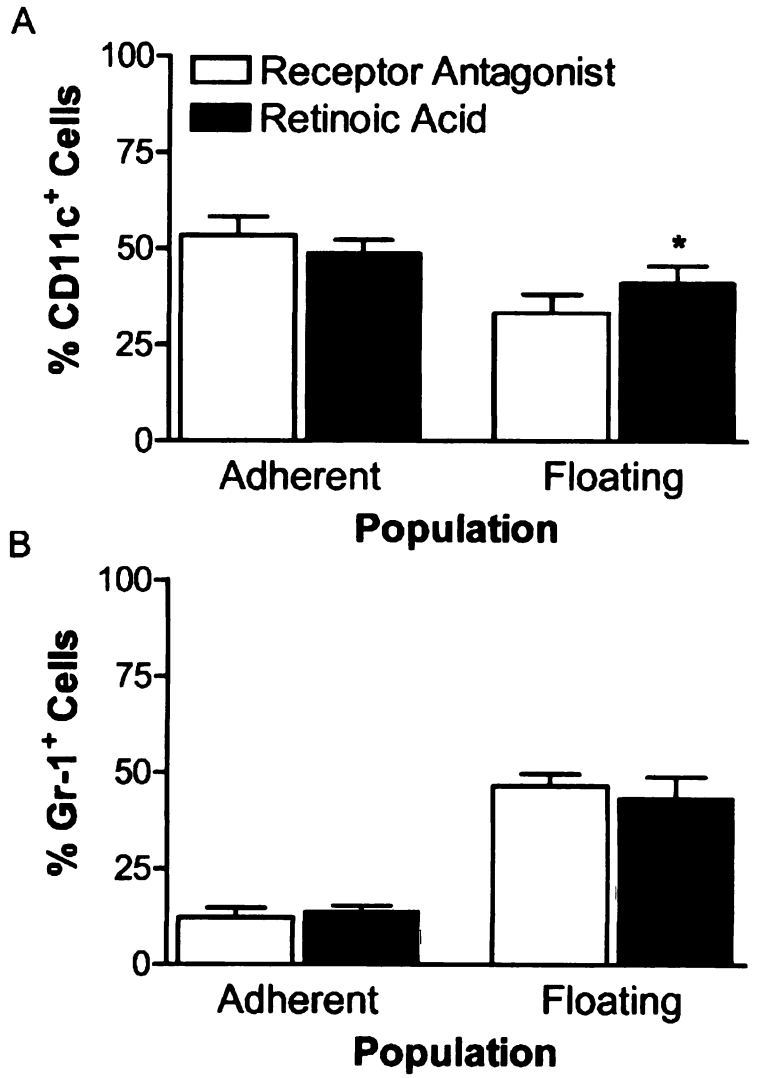
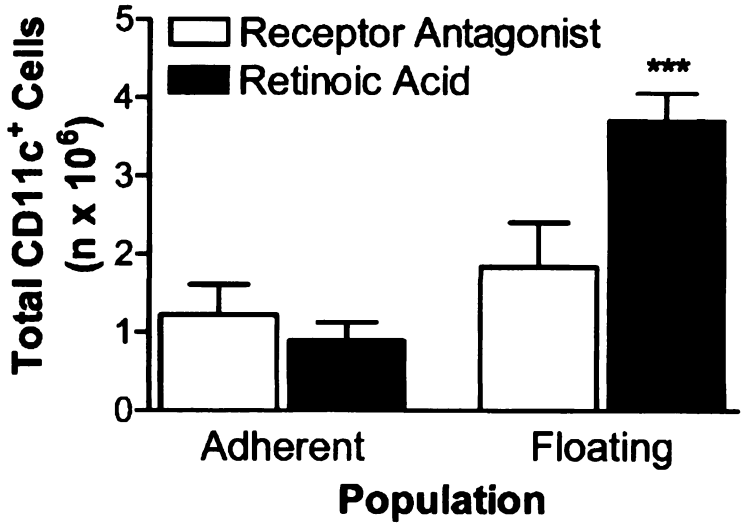

A
100
75
50
25
0
% CD11c+ Cells
Receptor Antagonist
Retinoic Acid
*
Adherent
Floating
Population
B
100
75
50
25
0
% Gr-1+ Cells
Adherent
Floating
Population

Figure 2.3

C




D

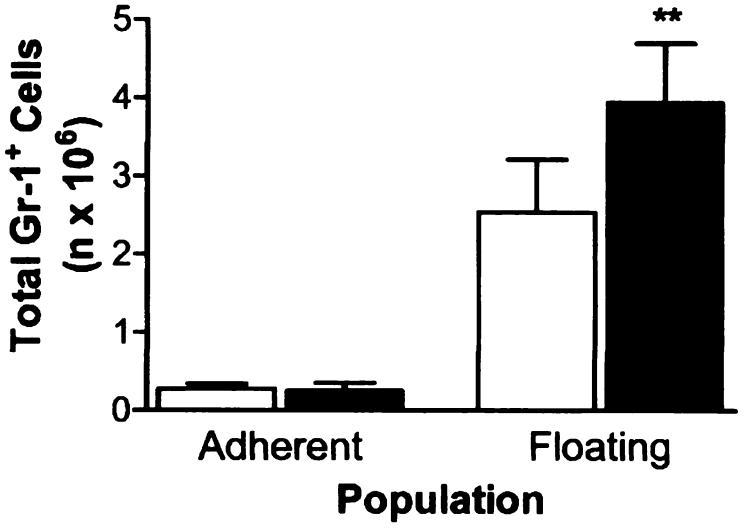



Figure 2.3 continued

**Figure 2.4.** CD11a cell surface expression is reduced by atRA rescue treatment in mouse bone marrow-derived myeloid DC cultures [counts (*A*) and mean fluorescence intensity (*B*) in adherent and floating cells]. Cells were treated with receptor antagonist alone or rescued with atRA on d 8 of the 10-d culture. Cell surface expression for CD11c and CD11a (Itgal) was analyzed by flow cytometry after 10 d. In *B*, results are representative of 2 independent experiments and presented as means + SD, n=6. ***Different from receptor antagonist treatment, $P<0.0001$.

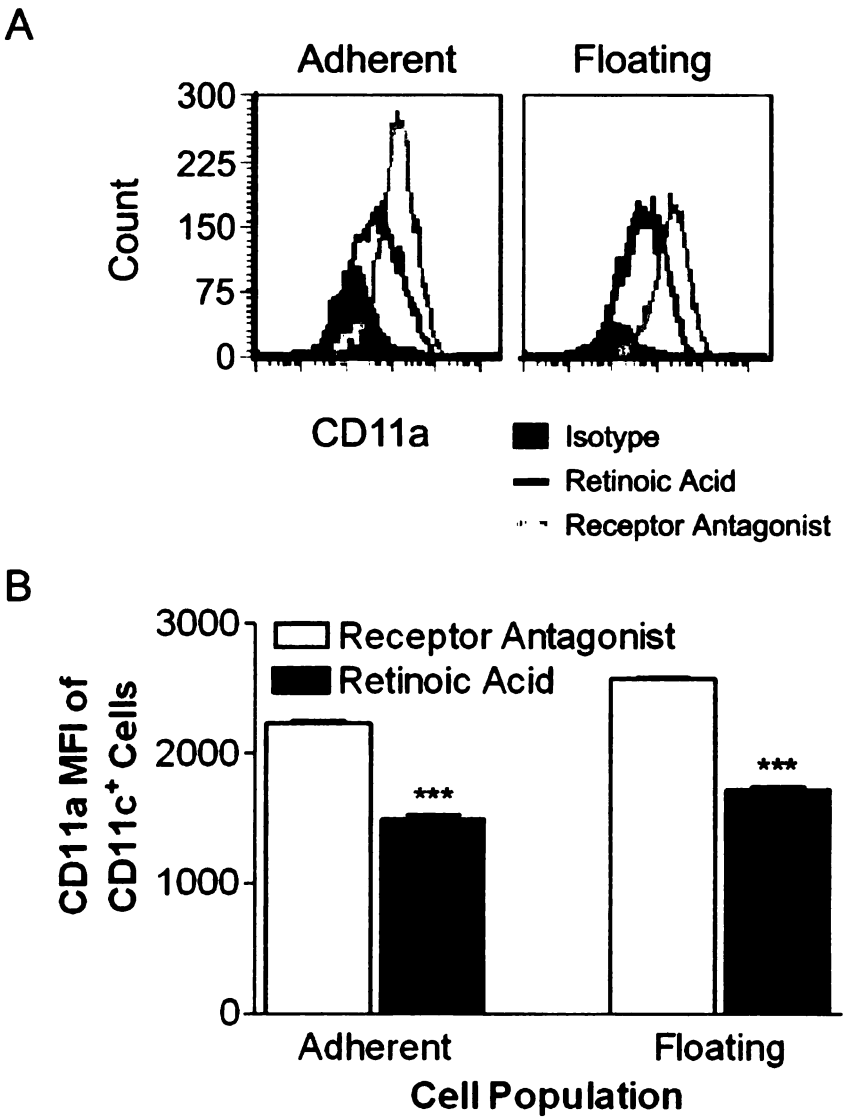

A
Adherent
Floating
300
225
150
75
0
Count
CD11a
Isotype
Retinoic Acid
Receptor Antagonist
B
3000
2000
1000
0
CD11a MFI of CD11c+ Cells
Receptor Antagonist
Retinoic Acid
***
***
Adherent
Floating
Cell Population

Figure 2.4

**Figure 2.5.** AtRA rescue changes the gene expression and secretion of *MMP-9* in mouse bone marrow-derived myeloid DC cultures. *A*, total RNA extracts made from pooled adherent cells on d 8 of culture at 0, 4, 8, 12, and 24 h after receptor antagonist treatment or atRA rescue were reverse transcribed to cDNA and *Mmp-9* gene expression was assayed by quantitative real-time PCR in technical triplicates. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase expression and are shown as fold of the 0 h receptor antagonist treatment. The results are representative of 2 independent experiments. *B*, pro-MMP-9 in cell culture medium supernatants measured by ELISA. *C*, the pro-MMP-9:TIMP-1 molar ratios calculated from the concentration of each protein for each sample measured. The results are representative of 2 independent experiments and are means + SD, n=4. *Different from the receptor antagonist treatment on that day, $P<0.05$.

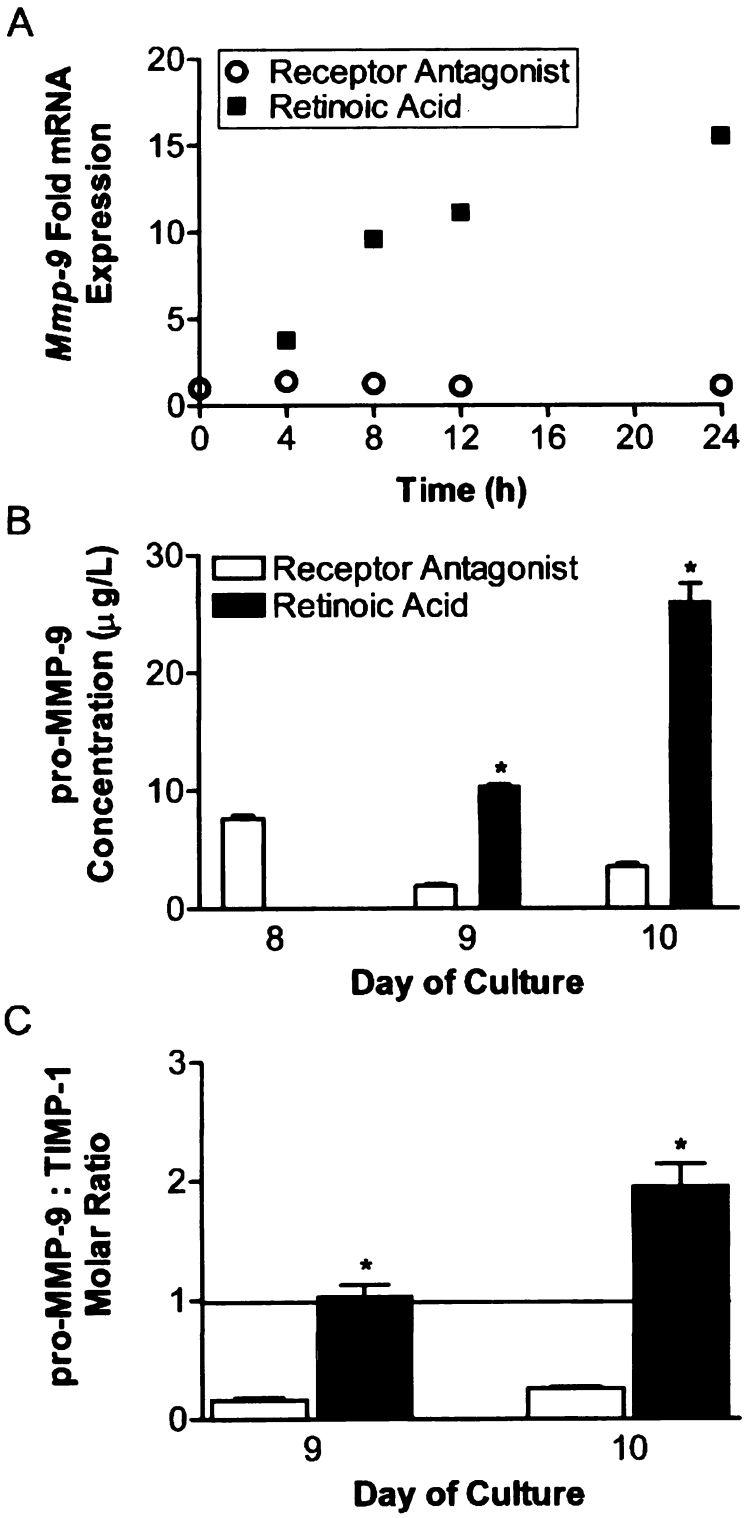

A
Mmp-9 Fold mRNA Expression
Receptor Antagonist
Retinoic Acid
20
15
10
5
0
0
4
8
12
16
20
24
Time (h)
B
pro-MMP-9 Concentration (μg/L)
Receptor Antagonist
Retinoic Acid
30
20
10
0
*
*
*
8
9
10
Day of Culture
C
pro-MMP-9 : TIMP-1 Molar Ratio
3
2
1
0
*
*
9
10
Day of Culture

Figure 2.5

# CHAPTER 3

# VITAMIN A UP-REGULATES MATRIX METALLOPROTEINASE-9 ACTIVITY BY MURINE MYELOID DENDRITIC CELLS THROUGH A NON-CLASSICAL TRANSCRIPTIONAL MECHANISM

**Abstract**

Myeloid dendritic cells (DC) are specialized antigen presenting immune cells. Upon activation in peripheral tissues, DC migrate to lymph nodes to activate T lymphocytes. Matrix metalloproteinase (MMP)-9 is a gelatinase essential for DC migration. We have previously shown that all-*trans* retinoic acid (atRA), a bioactive metabolite of vitamin A, significantly augments DC MMP-9 mRNA and protein production. We investigated the mechanisms by which atRA increases MMP-9 activity *in vitro*. Mouse myeloid DC cultured with atRA demonstrated increased gelatinase activity compared to cells cultured with retinoic acid receptor (RAR)-α antagonist. Adding MMP-9 inhibitor significantly blocked DC gelatinase activity and increased adherence of DC in a dose dependent manner. AtRA-induced *Mmp-9* expression in DC could be blocked by the transcriptional inihibitor actinomycin D. Since the *Mmp-9* promoter contains no classic retinoic acid response element (RARE), we performed additional studies to determine how atRA regulates *Mmp-9* transcription in DC. Electrophoretic mobility shift assays for the consensus Sp1, AP-1 and NF-κB binding sites located in the *Mmp-9* promoter did not increase binding in response to atRA. Chromatin immunoprecipitation assays indicated that atRA increases recruitment of RARα and histone acetyltransferase p300 to, and histone H3 acetylation of, the *Mmp-9* promoter. These data suggest that atRA regulates DC adhesion *in vitro* through MMP-9 gelatinase activity. AtRA increases *Mmp-9* expression through a transcriptional mechanism involving enhancement of RARα promoter binding,

recruitment of p300, and subsequent acetylation of histone H3, despite the absence of a consensus RARE.

KEY WORDS: all-*trans* retinoic acid, dendritic cells, matrix metalloproteinase-9, vitamin A

## Introduction

Worldwide, vitamin A deficiency affects an estimated 190 million pre-school aged children and 19.1 million pregnant women (WHO 2009) and increases morbidity and mortality due to increased susceptibility to infection (Villamor and Fawzi 2005). Vitamin A deficiency (VAD) has multiple effects on innate immunity: 1) mucosal epithelial integrity is impaired (Stephensen 2001); 2) the ability of macrophages and neutrophils to migrate to sites of infection, phagocytose and kill bacteria is abnormal (Twining et al. 1997; Stephensen 2001); 3) there are decreases in natural killer cell number and function in rats (Dawson et al. 1999); and 4) there are increased neutrophil numbers in the spleen, peripheral blood, and bone marrow *in vivo* (Kuwata et al. 2000). Vitamin A is also important in polarizing naïve T helper (Th) cells to a Th2 phenotype. T cells from vitamin A deficient mice do not support $IgG_1$ production by vitamin A sufficient B cells, which requires a Th2 response (Carman et al. 1989). VAD increases Th1 cytokine IFN-$\gamma$ secretion while impairing the Th2 response through diminished IL-5 and IL-10 production during *Trichinella spiralis* infection (Cantorna et al. 1996). *In vitro*, atRA decreases IFN-$\gamma$ production in Th1 cultures while enhancing Th2 development through an antigen presenting cell (APC) intermediate (Hoag et al. 2002). Vitamin A is a crucial nutrient in professional APC myeloid DC development from its precursors, as a vitamin A deficient environment *in vitro* permits granulocyte development instead (Hengesbach and Hoag 2004).

Myeloid DC are professional antigen presenting cells derived from hematopoietic stem cells. All mouse DC express the cell surface protein CD11c. Myeloid DC also possess CD11b but lack CD8α surface expression (Shortman and Liu 2002). Immature DC migrate from the bone marrow and reside in peripheral tissues where they can capture, process, and present antigen on their major histocompatibility complexes (MHC) classes I and II (Banchereau et al. 2000). Upon maturation stimulated by antigen interaction with pattern recognition receptors, DC migrate to T cell areas of the lymph nodes and spleen where they present antigen to naïve T cells (Banchereau and Steinman 1998; Steinman 1999; Hochrein and O'Keeffe 2008). Myeloid DC are the most efficient stimulators of T cells (Hochrein and O'Keeffe 2008).

Matrix metalloproteinase (MMP)-9, a gelatinase secreted as a zymogen, degrades the extracellular matrix through cleavage of gelatin, collagen types I and IV, and laminin (Opdenakker et al. 2001). It is activated by removal of the pro-peptide by MMP-2, -3, -7, or -13, or through chemical cleavage (Opdenakker et al. 2001; Fridman et al. 2003). Experimental impairment of MMP-9 activity inhibits hematopoietic progenitor cell release from the bone marrow (Pruijt et al. 1999), impairs recruitment of DC to the bronchioalveolar associated lymphoid tissue in response to allergen (Vermaelen et al. 2003) and migration through epithelial tight junctions (Ichiyasu et al. 2004), and impairs migration of monocyte-derived DC *in vitro* (Baratelli et al. 2004). *Mmp-9* expression is regulated through AP-1 and NF-κB signaling pathways, including thrombin signaling in human monocytes (Chang et al. 2009), TNF-α in a mouse epidermal

cell line (Hwang et al. 2009), and epidermal growth factor in prostate carcinoma cells (Kuo et al. 2009). *Mmp-9* expression is also often controlled through Sp1 signaling, including in the colon cancer CT26 cell line (Chen et al. 2009).

All-*trans* retinoic acid (atRA), a bioactive metabolite of vitamin A, signals through the retinoic acid receptor (RAR) family consisting of three isoforms, $\alpha$, $\beta$, and $\gamma$ (Chambon 1996). AtRA enters the nucleus and binds RAR which heterodimerizes with one of three retinoid X receptor (RXR) isoforms, creating a holo-RAR-RXR complex. In the classic mechanism of atRA signaling, the complex binds to a retinoic acid response element (RARE) in the gene promoter and associates with coregulatory proteins to affect target gene expression (Bastien and Rochette-Egly 2004; Mark et al. 2006). While this model explains the mechanism approximately 130 vitamin A-regulated genes, at least 400 genes controlled by atRA are not regulated through this mechanism as the promoters lack a consensus RARE (Balmer and Blomhoff 2002). Vitamin A increases MMP-9 expression and activity in the context of cell migration. AtRA induced-MMP-9 activity in mammary epithelial cells results in lumen morphogenesis (Montesano and Soulie 2002) and increases DC migration *in vitro* and *in vivo* (Darmanin et al. 2007). We have previously shown that atRA treatment results in loss of DC adherence in culture and augments expression and secretion of pro-MMP-9 through RAR$\alpha$ signaling (Lackey et al. 2008). However, the promoter region of *Mmp-9* lacks a RARE (Munaut et al. 1999). In the current studies, we sought to determine whether vitamin A-supported MMP-9

activity is essential for decreased dendritic cell adhesion *in vitro* and the mechanism by which atRA regulates *Mmp-9* expression.

## Materials and Methods

### *Materials*

AtRA (Sigma, St. Louis, MO), AGN 194301 (a gift from Vitae Pharmaceuticals), MMP-9 Inhibitor I (Calbiochem, San Diego, CA), and actinomycin D (Sigma) were dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at –80°C under an argon atmosphere in the dark. AGN 194301 was generously provided by Dr. Chandraratna and blocks atRA activity by competing for the ligand binding domain of RARα (Nagpal and Chandraratna 2000). Recombinant GM-CSF was purchased from PeproTech (Rocky Hill, NJ) and dissolved in molecular biology grade water. Iscove's modified Dulbecco's medium (IMDM, Lonza BioWhittaker, Walkersville, MD) was supplemented with 10% (v/v) of characterized (CH) fetal bovine serum (FBS, HyClone, Logan UT), 2 mmol/L GlutaMax (Gibco Invitrogen, Carlsbad, CA), 100 U/L penicillin, and 100 mg/L streptomycin (Lonza BioWhittaker) to prepare complete (c)IMDM.

### *Animals*

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were maintained according to institutional guidelines set by University Laboratory Animal Resources under a protocol approved by Michigan State University's Institutional Animal Care and Use Committee. The animals were fed commercial solid diet pellets (22/5 Rodent Diet #8640, Harlan Teklad, Madison, WI) and killed by carbon dioxide asphyxiation.

***Bone Marrow Harvest***

Femurs and tibiae from mice were collected and kept on ice. Bones were surface-sterilized in 70% ethanol for 2 min and transferred to RPMI-1640 medium (Lonza BioWhittaker) where the bone marrow was flushed by syringe. Bone marrow cells were filtered and centrifuged, then resuspended in ACK lysing buffer (Lonza BioWhittaker) to remove red blood cells. Bone marrow cells were then centrifuged and resuspended in RPMI-1640 medium. A portion of cells were mixed with trypan blue dye solution and counted to determine cell number and viability. Cells were cultured for DC development using a method based on that of Inaba *et al.* (1998) and modified by Lutz *et al.* (1999).

***Cell Culture***

For gelatinase activity assays, bone marrow cells were plated at $2 \times 10^4$ cells per well containing 100 μL cIMDM supplemented with 20 μg/L GM-CSF and 10 nmol/L atRA or AGN 194301. For MMP-9 inhibitor studies, bone marrow cells were cultured at $2 \times 10^6$ cells per 100 mm dish containing 10 mL cIMDM in the presence or absence of 10 nmol/L atRA, 50 nmol/L, 500 nmol/L, or 5 μmol/L MMP-9 Inhibitor I, and equal DMSO concentration. Fresh medium and treatment were given on days 3, 6, and 8 of culture. For gelatinase activity assays, cultures treated with atRA either received 5 μmol/L MMP-9 Inhibitor I or DMSO vehicle control on d 8. For MMP-9 inhibitor studies, cells were harvested on d 10 for flow cytometry staining.

For atRA rescue experiments, bone marrow cells were plated at 2 x $10^6$ cells per 100 mm dish containing 10 mL cIMDM supplemented with 20 μg/L GM-CSF and 10 nmol/L AGN 194301. Cells were given 10 mL fresh cIMDM containing 20 μg/L GM-CSF and 10 nmol/L AGN 194301 on d 3 and 6 of culture. On d 8 of culture, all dishes had a complete medium replacement with cIMDM containing 20 μg/L GM-CSF and either 10 nmol/L of atRA or AGN 194301. Cells were harvested for RNA extraction, nuclear protein extraction, and chromatin immunoprecipitation at various time points on d 8 of culture.

***Gelatinase Assay***

Adherent cell and supernatant gelatinase activities were measured using EnzChek Gelatinase/Collagenase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Specifically, adherent cells or 100 μL supernatant were incubated with 100 μg/mL DQ gelatin and 1 X Reaction Buffer to a total volume of 200 μL for 24 h at room temperature. DQ Gelatin is heavily fluorescein-labeled fluorescently quenched gelatin. Digestion of the gelatin substrate releases fluorescent peptide products. MMP-9 Inhibitor I (5 μmol/L ) was added to adherent cells or supernatant in 1 X Reaction Buffer for 1 h before addition of DQ gelatin substrate. Gelatinase activity was measured in the laboratory of Dr. Michael Orth, Department of Animal Science, Michigan State University using SpectraMax Gemini EM Microplate Spectrofluorometer (Molecular Devices, Sunnyvale, CA) and SoftMax Pro software with excitation wavelength of 485 nm and emission wavelength of 538 nm. Sample fluorescence

was compared to *Clostridium* collagenase standards to measure units of gelatinase activity.

***Flow Cytometric Analysis***

Floating and adherent cells were harvested, counted, washed with staining buffer (1% FBS, 0.1% (w/v) sodium azide, in phosphate buffered saline, pH 7.4), and incubated with anti-$F_{c\gamma}$RII/III monoclonal antibody from the 2.4G2 hybridoma to block non-specific binding. Each sample was then incubated with either an isotype control or a cell surface molecule-specific antibody cocktail, with 1 μg antibody used per 1 x $10^6$ cells. Monoclonal antibodies used were PE-conjugated hamster anti-mouse CD11c, PerCP-Cy5.5-conjugated rat anti-mouse Ly-6G/Ly-6C (Gr-1), PE-conjugated hamster $IgG_{1/\lambda}$, and PerCP-Cy5.5-conjugated rat $IgG_{2b/\kappa}$, (BD Biosciences Pharmingen, San Jose, CA). Samples were run at the MSU flow cytometry facility using a LSR II flow cytometer (BD Biosciences) and data analyzed using FCS Express v.3.0 software (DeNovo Software, Ontario, CA).

***Real-Time PCR***

Total RNA was isolated from adherent cells at 8 h post atRA rescue and actinomycin D treatment during the *in vitro* culture period. Medium was removed from the culture dishes and the cells were incubated with TRIzol reagent (InVitrogen, Carlsbad, CA) for cell lysis. RNA extraction was completed according to the manufacturer's instructions. RNA pellets were air-dried, resuspended in molecular biology grade water (Sigma), and stored at –80°C.

RNA concentration was measured at 260 and 280 nm using the CellQuant spectrophotometer (Amersham, Piscataway, NJ). Total RNA was reverse transcribed using the TaqMan Reverse Transcription System with MultiScribe reverse transcriptase and random hexamer primers (Applied Biosystems, Foster City, CA). Expression of cDNA relative to the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) housekeeping gene was analyzed using Assays-on-Demand™ Gene Expression primer/probe sets (Applied Biosystems) for *Mmp-9* (Mm00600163_m1). PCR was performed on an ABI 7300 Real Time PCR System (Applied Biosystems). Monitoring of PCR occurred in real-time by detection of FAM fluorescence on the 5' end of the probe, quenched with a non-fluorescent quencher on the 3' end of the probe. Relative cDNA expression was calculated from $\Delta\Delta C_T$ values and comparison to a standard curve of multiple 10-fold dilutions using ABI 7300 System SDS software v.1.3.1 (Applied Biosystems).

***Nuclear Extracts***

Adherent dendritic cells were harvested in triplicate at 0 h, 30 min, 1, 2, and 4 h post-rescue with atRA treatment. Cells were detached from the culture dish by incubation with Accutase (Innovative Cell Technologies, Inc., San Diego, CA) at 37°C for 10 min. Detached adherent DC were washed twice with ice cold PBS. Nuclear extracts were made using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, ThermoScientific, Rockford, IL) supplemented with Halt Protease and Phosphatase Inhibitor (Pierce Biotechnology) at 1X concentration, according to the manufacturer's instructions.

Protein concentration was determined by BioRad Protein Assay (BioRad, Hercules, CA).

***Electrophoretic Mobility Shift Assays (EMSA)***

The target sequences were synthesized with biotin at the 5' position. Complementary oligonucleotides from transcription factor binding regions of the mouse *Mmp-9* promoter were annealed in a buffer containing 10 mmol/L Tris base, 1 mmol/L EDTA, and 50 mmol/L sodium chloride, pH 8.0 for 5 min at 95°C, then allowed to cool slowly to room temperature. Oligonucleotide sequences used were: Sp1 sequence 1 5'-GGA GGG GAG GGG CGG GGT CA-3', Sp1 sequence 2 5'-GTG TGT CCC CCC GCC CCC CA-3', NF-κB sequence 5'-TTT GCC CCA TGG AAT TCC CCA AAT CCT GC-3', AP-1 sequence 5'-ACA CGC TGA GTC AGC ATA AG-3'. EMSAs were performed using LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology) according to the manufacturer's instructions except that poly(dA-dT) (Sigma) was substituted for poly(dI-dC) to block nonspecific protein-DNA binding when Sp1 target oligonucleotides were used because of the high GC content. Briefly, nuclear extracts and biotin-labeled target DNA were incubated with binding buffer, 2.5% glycerol, 10 mmol/L magnesium chloride, 50 mmol/L potassium chloride, and 50 mg/L poly(dI-dC) or poly(dA-dT), as appropriate, with or without 200-fold excess of unlabeled target DNA competitor for 1 h at room temperature. The reactions were loaded into 5% TBE Ready Gel pre-cast gel (ReadyGel, BioRad) and electrophoresed. DNA and protein were transferred to a Biodyne B nylon membrane (Pierce Biotechnology), UV cross-linked at 120 mJ/cm$^2$, blocked,

incubated with streptavidin-linked horseradish peroxidase, washed, incubated with substrate, and exposed to Amersham Hyperfilm MP (GE Healthcare, Buckinghamshire, UK).

***Chromatin Immunoprecipitation (ChIP)***

Chromatin immunoprecipitation was performed using ChIP-IT Express Enzymatic Magnetic Chromatin Immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturers' instructions. Briefly, protein and DNA in the adherent DC were cross-linked by incubation in 1% formaldehyde in Dulbecco's modified Eagle's medium for 10 minutes, then the reaction was stopped by addition of glycine stop-fix solution for 5 minutes. The adherent DC were scraped in scraping solution (500 μmol/L phenylmethylsulfonyl fluoride (PMSF) in PBS). Cells were centrifuged and resuspended in lysis buffer supplemented with protease inhibitor cocktail and PMSF. Cells were lysed and nuclei released by dounce homogenization on ice. Nuclei were collected by centrifugation. Chromatin was sheared with 10,000 U/L Enzymatic Shearing Cocktail for 10 minutes at 37°C. The shearing was stopped by addition of 10 mmol/L cold EDTA. Sheared chromatin was collected by centrifugation and 50 μL was incubated at 4°C for 4 h with protein G magnetic beads, protease inhibitor cocktail, ChIP buffer, and appropriate antibody for immunoprecipitation. Anti-Acetylated Histone H3 Lys 9/14 (sc-8655-R), anti-RARα (C-20, sc-551X), anti-p300 (N-15, sc-584X) (all from Santa Cruz Biotechnology, Santa Cruz, CA), or purified mouse $IgG_{1\kappa}$ (BD Pharmingen), 3 μg each per immunoprecipitation reaction, was used. Beads were collected on a magnetic stand and washed in supplied ChIP buffers.

Chromatin was eluted and DNA/protein cross-links were reversed by incubation with reverse cross-linking buffer at 94°C, 15 min. The chromatin solution was then treated with 1 μg Proteinase K, 1 h at 37°C followed by treatment with Proteinase K Stop Solution. Chromatin was stored at –80°C for future PCR analysis. For input chromatin samples, chromatin was prepared for PCR analysis by addition of ChIP buffer 2 and a final concentration of 0.1 mol/L sodium chloride and treated for DNA/protein reverse cross-linking and protein digestion.

***PCR analysis of immunoprecipitated chromatin***

Input and immunoprecipitated chromatin were analyzed by PCR with primer pairs corresponding to a 229 bp region of the murine *Mmp-9* promoter, forward: 5'-GGT CTG GGC ACA CAG GAG GC-3', reverse: 5'-GGG GTG ACC TGG ATG GGG CA-3'. *Taq* DNA polymerase, buffer, magnesium chloride (2.5 mmol/L final concentration), and dNTP mixture (0.2 mmol/L final concentration) were from Invitrogen. The thermocycling conditions were 94°C for 3 minutes for 1 cycle followed by 94°C for 45 s for denaturation, 60°C for 30 s for annealing, and 72°C for 90 s for extension for 36-38 cycles. PCR products were electrophoresed through a 3% agarose gel and stained with ethidium bromide. Gel pictures were taken using Gel Doc 1000 (BioRad).

***Statistical Analysis***

Data were analyzed using InStat software version 3.05 (GraphPad Software, Inc., San Diego, CA). One-way ANOVA followed by Bonferroni's multiple comparison post-test was used for gelatinase assays and MMP-9 inhibitor studies. Two-way ANOVA followed by Bonferroni post-test was used for

transcription inhibitor quantitative PCR study. A $P < 0.05$ was considered significant. Each experiment was repeated a minimum of two times.

## Results

### *AtRA rescue treatment increases MMP-9 activity in DC cultures*

DC cultures treated with atRA showed 20% greater adherent cell-associated gelatinase activity than cultures treated with AGN (**Figure 3.1***A*). Conditioned medium from atRA treated cultures also showed significantly greater gelatinase activity compared to cultures only treated with AGN (0.3 U/L compared to 0 U/L, Figure 3.1*B*), but this gelatinase activity was less than that associated with atRA treated adherent cells. Together, atRA treatment resulted in significantly greater total gelatinase activity in DC cultures than in DC cultures treated with AGN 194301 to inhibit RARα signaling (Figure 3.1*C*). Addition of specific inhibitor of MMP-9 activity over the last 48 h of cell culture and during incubation with DQ Gelatin significantly decreased adherent cell-associated gelatinase activity compared to cells treated alone with atRA, but not supernatant-associated activity, resulting in a 30% reduction in total gelatinase activity (Figure 3.1*A-C*). Total DC culture gelatinase activity upon inhibition of MMP-9 activity was comparable to that of DC cultures treated with AGN 194301.

### *MMP-9 inhibitor treatment increases DC adherence*

The percentage of floating and adherent DC ($CD11c^+$ $Gr\text{-}1^-$ cells, analyzed by flow cytometry) cultured from bone marrow cells under DC-generating conditions did not differ greatly between treatments (**Figure 3.2***A*). The total DC yields also did not differ significantly between treatments. However,

the floating cell yield decreased significantly by 35%. The adherent cell yield did not change significantly, as the MMP-9 inhibitor dose increased in culture (Figure 3.2*B*). Because of this, the pattern of the percentage of total DC that were adherent increased significantly, from 32% to 49%, as the dose of MMP-9 inhibitor treatment was increased in culture (Figure 3.2*C*). The results were similar for the total live cell population in the cultures (**Appendix C**). These data indicate that MMP-9, up-regulated by atRA treatment, was important in the release of the adherent cells from the surface of the culture dish. However, MMP-9 appeared to have little effect on the overall proportion of DC within the floating and adherent cell populations.

***AtRA transcriptionally regulates Mmp-9 expression in adherent DC***

Bone marrow-derived DC treated with RARα antagonist were rescued with 10 nmol/L atRA or continued treatment as before. Actinomycin D (5 μg/mL) was added to some DC cultures 1 h before atRA rescue or RARα antagonist treatment and continued following rescue. RNA extracts were made 8 h post-rescue. It was found that atRA rescue of adherent DC significantly increased *Mmp-9* abundance 6-fold compared to DC treated with RARα antagonist ($P<0.0001$). *Mmp-9* expression in DC cultures treated with actinomycin D and atRA was significantly decreased from DC cultures rescued with atRA ($P<0.0001$), while not different from that of DC cultures treated with RARα antagonist only (**Figure 3.3**). These results indicated that atRA treatment of DC up-regulated *Mmp-9* expression through a transcriptional mechanism involving signaling through RARα.

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***AtRA rescue of adherent DC does not increase binding of nuclear protein extracts to common regulation sites in the Mmp-9 promoter***

Because the *Mmp-9* promoter region does not contain a consensus RARE, we hypothesized that the atRA-RARα-RXR complex likely associated with another transcription factor that is known to up-regulate *Mmp-9* expression. Specifically, we hypothesized that atRA treatment would increase nuclear protein extract binding to the Sp1 consensus sequence, as this positive interaction has been previously described in other atRA-induced gene expression in promoters lacking a RARE (Suzuki et al. 1999; Shimada et al. 2001; Xu and Rogers 2007). We used EMSA to determine whether atRA rescue increased binding of nuclear protein extract from adherent DC to consensus binding sequences for AP-1, NF-κB, and Sp1 families of transcription factors. We found that atRA rescue had no reproducible effect on nuclear protein extract binding to Sp1 consensus sequences at any time point tested (Sp1 4 h post-rescue, **Figure 3.4*A***, 0 h, 30 min, 1 h, 2 h, data not shown). There was no nuclear protein extract binding to the AP-1 consensus sequence from any sample, indicating that the binding conditions may not have been the most favorable despite multiple attempts at optimization (data not shown). AtRA rescue resulted in decreased nuclear extract binding to the NF-κB consensus sequence as early as 30 min post-rescue compared to the RARα antagonist treatment (Figure 3.4*B*, 2 and 4 h data not shown). These results indicate that the atRA-RARα-RXR signaling complex does not positively interact with the transcription factors that have been shown to be most responsible for increasing *Mmp-9* expression in the past to increase binding

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to naked DNA. This does not exclude the possibility that the holo-RARα-RXR can interact with one of these transcription factors when binding chromatin.

***AtRA rescue increases association of RARα and p300 with the Mmp-9 promoter region and increases promoter histone acetylation in adherent DC***

Chromatin immunoprecipitation (ChIP) was used to determine the extent of RARα and cofactor association with the *Mmp-9* promoter region after atRA rescue of DC treated with RARα antagonist for 8 d. It was found that RARα association with the promoter increased over time with maximal association 24 h after atRA rescue compared to RARα antagonist treatment. At the same time, p300 histone acetyltransferase association with the promoter and acetylation of histone H3 also increased after atRA rescue to a greater extent than in RARα antagonist treated DC (**Figure 3.5**). These results suggest that RARα associates with the promoter region of *Mmp-9* and recruits p300 to acetylate histone H3, thus activating the promoter and resulting in the increased levels of *Mmp-9* transcript we have previously shown after atRA rescue.

## Discussion

Vitamin A has been demonstrated to induce expression of *Mmp-9* and secretion of pro-MMP-9 by DC in conjunction with the release of DC from the culture dish surface (Lackey et al. 2008). However, it has not been shown previously in this culture system that the pro-MMP-9 released in response to

atRA signaling is active and that the absence of MMP-9 activity inhibits the release of DC from the culture dish surface.

When primary bone marrow cells are cultured under DC generating conditions in the presence of 10 nmol/L RARα antagonist AGN 194301, adherent cell- and conditioned culture medium-associated gelatinase activity is decreased compared to cells cultured in 10 nmol/L atRA. Thus, DC require signaling through RARα to produce active gelatinase *in vitro*. Surprisingly, adherent cell-associated gelatinase activity was greater than that of conditioned culture medium-associated activity in atRA treated DC cultures. Inhibition of MMP-9 activity using 5 μmol/L MMP-9 Inhibitor I results in decreased adherent cell-associated gelatinase activity in atRA treated DC cultures to levels found in RARα antagonist treated cultures. This indicates that MMP-9 is a relevant DC-associated gelatinase whose activity is increased by atRA signaling through RARα. However, there was baseline gelatinase activity in both the RARα antagonist and MMP-9 inhibitor treated cultures. In a previous analysis of adhesion molecule and extracellular matrix (ECM) gene expression, a number of other MMP molecules were expressed at high levels in DC, including the gelatinase MMP-2, though they were not regulated by atRA treatment (data not shown).

While pro-MMP-9 is a secreted zymogen, many studies have shown that it can interact with other proteins on the cell surface. We have previously shown that atRA rescue from RARα antagonist treatment significantly increases production and secretion of pro-MMP-9 into the conditioned medium (Lackey et

al. 2008), but in the current experiments, the greatest gelatinase activity was associated with the cell surface of atRA treated DC. MMP-9 has been shown to associate with the cell surface proteins CD11b, integrin-$\beta_1$ (CD29), and the hyaluronan receptor CD44. The cell association can be useful to direct cell migration through the ECM or to increase activation of MMP-9 (Fridman et al. 2003; Radjabi et al. 2008). The design of the gelatinase assay experiments cannot resolve whether the cell-associated MMP-9 activity is due to MMP-9 association with the cell surface or the ECM. It is possible that active MMP-9 does associate with the DC surface itself as myeloid DC express high levels of CD11b (Shortman and Liu 2002). However, it is also possible that the active MMP-9 is within the DC-produced ECM in the dish where it actively digests that matrix. Pro-MMP-9 has a gelatin binding domain that allows it to associate with collagen $\alpha$2(IV) chain in the ECM or associated with the cell surface with high affinity, inducing gelatinase activity without the proteolytic removal of the pro-peptide (Fridman et al. 2003). Flow cytometric analysis of DC using fluorescently labeled antibody against MMP-9 would resolve whether the MMP-9 is associated with proteins on the DC surface or with the ECM.

Because MMP-9 was determined to be the primary source of gelatinase activity in DC cultures treated with atRA, the role of atRA-induced MMP-9 activity in releasing adherent bone marrow-derived DC was also examined. Inhibition of MMP-9 results in increased total cell and DC adhesion *in vitro*, compared to cultures treated only with atRA. These results indicate that MMP-9, up-regulated by atRA treatment, is important in the release of adherent DC from the culture

dish surface. MMP-9 does not regulate the proportion of bone marrow derived cells that develop into DC (Figure 3.2). This must be a different function of atRA, such as direct up-regulation of differentiation-related transcription factors.

Because we were able to show that atRA increased MMP-9 activity and that this activity resulted in a decreased percentage of cells that were adherent in DC cultures *in vitro*, we next sought to determine the mechanism by which atRA signaling through RARα up-regulates *Mmp-9* expression. While we have shown that atRA up-regulates *Mmp-9* transcript abundance through a mechanism involving RARα (Lackey et al. 2008), the promoter region up-stream of the *Mmp-9* gene does not contain a classic consensus RARE. We aimed to determine the mechanism through which atRA acts to increase *Mmp-9* expression in DC that results in developmental changes (adherent cell to floating cell) in our culture system. We first sought to establish whether the atRA-induced up-regulation of *Mmp-9* expression in adherent DC is transcriptionally activated using the transcription inhibitor actinomycin D. It was found that atRA rescue increased *Mmp-9* expression compared to RARα antagonist treatment, while actinomycin D treatment with atRA rescue did not increase *Mmp-9* abundance. These results indicated that *Mmp-9* expression in adherent DC is controlled by atRA through a transcriptional mechanism involving RARα.

We hypothesized that due to that lack of a consensus RARE in the *Mmp-9* promoter region, the holo-RARα-RXR complex interacts with another transcription factor to positively increase its binding, specifically Sp1. Nuclear protein extracts from primary bone marrow cells cultured under DC generating

conditions in the presence of the RARα antagonist AGN 194301 did not increase association with Sp1, AP-1, or NF-κB oligonucleotide probes upon treatment with 10 nmol/L atRA. In fact, atRA treatment decreased protein binding to the NF-κB consensus sequences. It is likely that fresh GM-CSF supplied during medium change resulted in the initial increased nuclear protein binding to the NF-κB consensus sequence that was inhibited by atRA treatment. GM-CSF, signaling through the GM-CSFRα and common cytokine receptor β chain, phosphorylates Jak2 which in turn activates tyrosine kinase. Tyrosine kinase activates Ras, which further activates Raf (mitogen-activated protein kinase/ERK kinase kinase, MEKK). MEKK phosphorylates and activates inhibitor of kappa B kinase (IKK)-β, which phosphorylates inhibitor of kappa B (IκB) bound to NF-κB subunits to mark it for eventual proteosomal degradation. IκB phosphorylation releases NF-κB subunits to be phosphorylated and translocated to the nucleus. Tyrosine kinase also activates the phosphotidylinositol 3-kinase-Akt pathway, resulting in both IKKβ and Ras activation (Hiraguri et al. 1997; Chang et al. 2003).

Because there was no nuclear extract binding to the AP-1 consensus sequence from any sample, it is also possible that the binding conditions used were not optimal. In order to increase nuclear protein extract binding to the AP-1 consensus sequence, we increased the concentrations of glycerol, magnesium chloride, and potassium chloride as well as the time of binding from 20 min to 1 h. Because there was no increase in nuclear protein extract binding to these consensus sequences, it is not likely that atRA signaling through RARα works by positively interacting with one of the common families of transcription factors.

Chromatin was isolated from atRA-rescued DC cultures to determine which proteins associated with the *Mmp-9* promoter following atRA treatment. Using ChIP, we found that both RARα and p300 associated with the *Mmp-9* promoter following 24 h atRA treatment. P300 is a histone acetyltransferase that acetylates histone H3 at lysines 14 and 18 and is a cofactor for RARα-mediated transcriptional regulation. It was also found that atRA rescue increased the acetylation of histone H3 at the lysine 9 and 14 postion as determined by immunoprecipitation. These results indicate that RARα interacted with the *Mmp-9* promoter, despite the lack of a RARE within the promoter, and increased the potential for transcriptional activity due to the acetylation status of histone H3. However, it cannot be determined from these results whether RARα actually makes physical contact with the *Mmp-9* promoter DNA directly or if it interacts with another protein that does. Alternately, the plasmid construct transfected contained 2200 base pairs of the *Mmp-9* 5' untranslated region, but a RARE could further up-stream in the endogenous MMP-9 5' untranslated region.

In an experiment where we transfected DC with *Mmp-9* promoter-firefly luciferase reporter plasmid construct, atRA rescue did not increase *Mmp-9* promoter activity in adherent DC compared to RARα antagonist treated cultures (data not shown), indicating that the holo-RARα-RXR complex acts to increase *Mmp-9* expression through an epigenetic mechanism because plasmid DNA is not histone-associated. The results from the ChIP experiments confirmed those of Zaragoza et al. (2007) who showed increased RARα and p300 associated with the *Mmp-9* promoter region after vitamin A supplementation during mammary

involution in rat dams. We have previously found that atRA treatment increased *Mmp-9* expression within 4 h of treatment (Lackey et al. 2008), but we did not see increased RARα and p300 association with the promoter after 4 h of atRA treatment. This indicates that RARα and p300 may associate with the *Mmp-9* promoter for long-term atRA regulation of *Mmp-9* expression. However, there may be an indirect mechanism for early atRA regulation of *Mmp-9* expression, such as the displacement of NF-κB from the promoter.

It would be interesting to also determine whether there is increased interaction of the histone methyltransferase MLL5 with, and hence increased methylation of, the *Mmp-9* promoter region. It has been previously shown that MLL5, a histone lysine mono- and di-methyl transferase, acts as a co-regulator of RARα-induced transcription by methylating lysine 4 on histone H3 after it has been N-glycosylated (Fujiki et al. 2009). Methylated histone H3 (K4) and acetylated histone H3 also result in more permissive chromatin in a number of promoter regions, including that for *Mmp-9*, as well as increased association with MLL (Yan and Boyd 2006).

We have shown MMP-9 activity, located mainly at the adherent DC surface or in the ECM, is responsible for the loss of DC adherence after atRA rescue through a transcriptional mechanism involving RARα. Further, we have shown that the transcriptional mechanism by which atRA up-regulates *Mmp-9* expression for long-term regulation involves the interaction of RARα with the *Mmp-9* promoter region along with the recruitment of the histone acetyl transferase p300 and increased acetylation of histone H3. AtRA treatment did not

increase transcription factor binding to AP-1, NF-κB, or Sp1 non-chromosomal DNA binding elements. However, these results do not necessarily preclude the possibility that the atRA-RARα-RXR complex could interact with one of these transcription factors to increase binding to the chromosomal DNA associated with histone proteins and specific to the *Mmp-9* promoter. Additional studies are necessary to further elucidate the atRA-induced *Mmp-9* expression mechanism in the mouse DC model. These studies will determine the mechanism of holo-RARα-RXR recognition of the *Mmp-9* promoter region and with what other protein(s) the complex likely interacts. DC migration through the ECM is a critical aspect of their function, from release of immature DC from the bone marrow to the periphery to the migration of mature DC to secondary lymphoid tissues. Our findings suggest that vitamin A may play a large role in bridging innate and adaptive immunity through increased MMP-9 activity at the DC surface, allowing increased cell migration potential.

**Acknowledgements**

We thank Dr. Louis King of the Michigan State University flow cytometry facility in the Research and Technology Support Facilities.

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**Figure 3.1.** DC gelatinase activity after 10 d culture with atRA treatment in the presence or absence of MMP-9 inhibitor or with RARα antagonist, AGN 194301. *A*, Adherent cell and *B*, conditioned cell culture medium gelatinase activity. *C*, Total gelatinase activity, calculated from adherent cell and conditioned cell culture medium activity. Results are presented as the mean + SD, n=5 for each treatment group, and show one representative experiment of two. (N.D., not detectable). Treatment groups designated with different letters are significantly different with $P < 0.05$ as determined by one-way ANOVA.

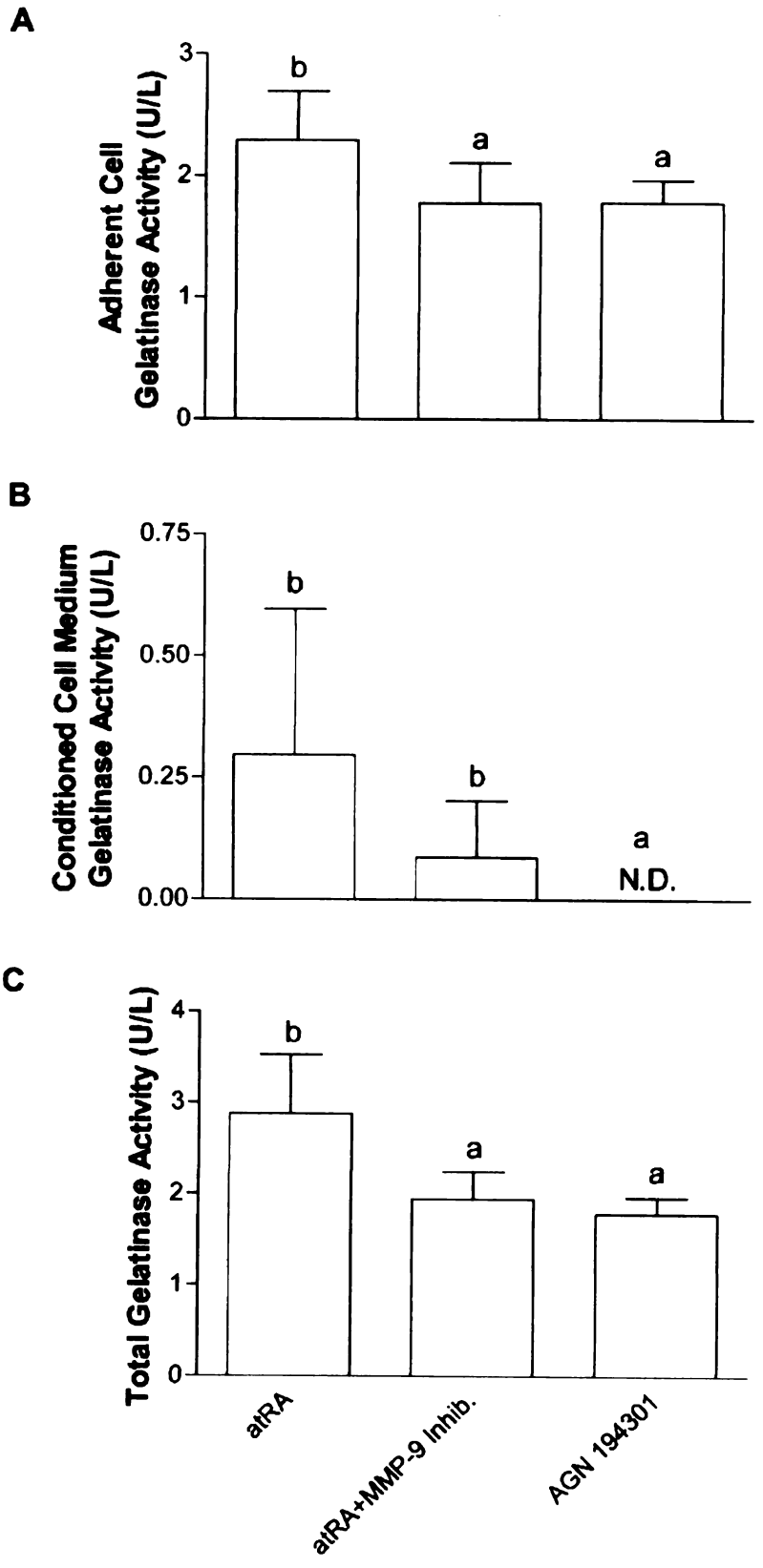
A
Adherent Cell Gelatinase Activity (U/L)
3
2
1
0
b
a
a
B
Conditioned Cell Medium Gelatinase Activity (U/L)
0.75
0.50
0.25
0.00
b
b
a
N.D.
C
Total Gelatinase Activity (U/L)
4
3
2
1
0
b
a
a
atRA
atRA+MMP-9 Inhib.
AGN 194301
Treatment

Figure 3.1

**Figure 3.2.** Dendritic cell proportions and yields after 10 d culture in the presence or absence of MMP-9 inhibitor. *A*, Percent $CD11c^+$ DC floating or adherent to culture dish. *B*, Floating and adherent DC yields, calculated based on cell yield and proportion of cells that are DC. *C*, The percentage of total DC that are adherent, calculated as adherent DC yield divided by total DC yield multiplied by 100%. All cultures were treated with 10 nmol/L atRA. Results are presented as the mean + SD, n=4 for each treatment group, and show one representative experiment of two. Treatment groups designated with different letters are significantly different with $P < 0.05$ as determined by one-way ANOVA.

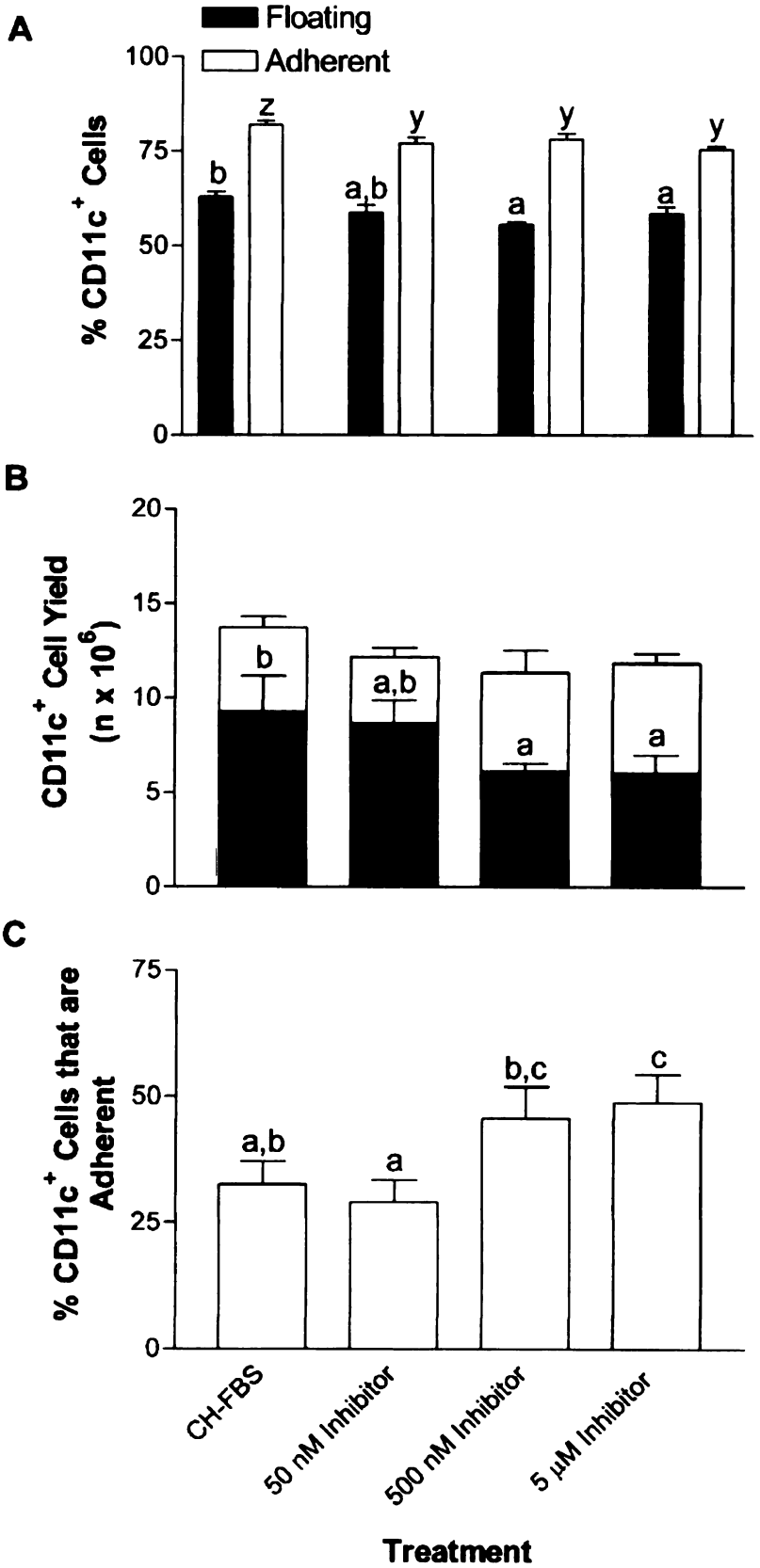
A
Floating
Adherent
100
75
50
25
0
% CD11c+ Cells
b
z
a,b
y
a
y
a
y
B
20
15
10
5
0
CD11c+ Cell Yield (n x 10^6)
b
a,b
a
a
C
75
50
25
0
% CD11c+ Cells that are Adherent
a,b
a
b,c
c
CH-FBS
50 nM Inhibitor
500 nM Inhibitor
5 µM Inhibitor
Treatment

Figure 3.2

**Figure 3.3.** Adherent DC *Mmp-9* expression after d 8 atRA rescue with or without transcriptional inhibitor. Total RNA extracts made from adherent cells on d 8 of culture. Cultures were pre-treated with 5 μg/mL actinomycin D (ActD) 1 h before atRA rescue from RARα receptor treatment. The extracts were reverse transcribed to cDNA and *Mmp-9* gene expression was assayed by quantitative real-time PCR in technical triplicates. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase expression and are shown as fold of the RARα antagonist treatment. The results are combined from 2 independent experiments and are presented as mean + SEM, n=4 for each treatment. Means without a common letter differ, $P<0.05$. Two-way ANOVA indicates significant interaction between atRA treatment and ActD treatment.

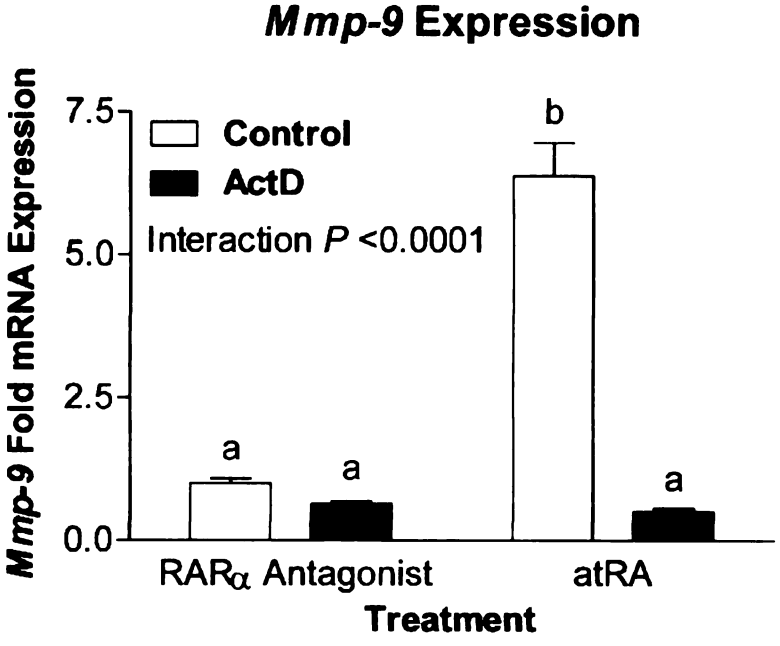
Mmp-9 Expression
Mmp-9 Fold mRNA Expression
7.5
5.0
2.5
0.0
Control
ActD
Interaction P <0.0001
a
a
b
a
RARα Antagonist
atRA
Treatment

Figure 3.3

**Figure 3.4.** AtRA rescue of mouse bone marrow-derived adherent DC does not increase nuclear protein binding to common *Mmp-9* regulatory sequences. Bone marrow cultures were grown in the presence of GM-CSF and RARα antagonist for 8 d, then either remained in culture with 10 nmol/L RARα antagonist (AGN) or were rescued with 10 nmol/L atRA. Nuclear protein was extracted 0 h, 30 min, 1, 2, and 4 h after RARα antagonist treatment or atRA rescue, then subjected to EMSA. Gel shifts using oligonucleotides for *A*, Sp1 (4 h); and *B*, NF-κB (30 min and 1 h) consensus binding sequences matching those found in the mouse *Mmp-9* promoter are shown. The results are representative of 2 independent experiments, n=3 for each treatment, although only 2 of 3 replicates are shown for NF-κB in panel *B*.

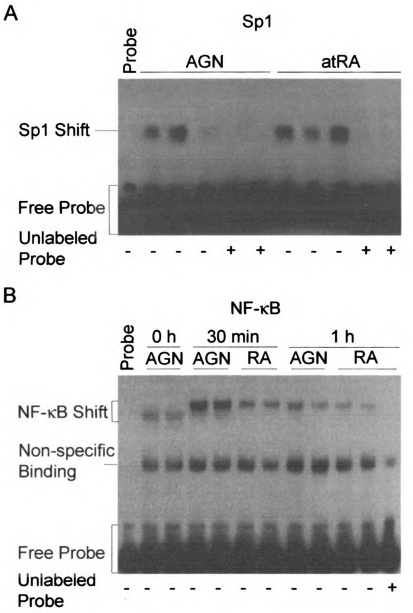
A
Sp1
Probe
AGN
atRA
Sp1 Shift
Free Probe
Unlabeled Probe
- - - - + + - - - + +
B
NF-κB
Probe
0 h
30 min
1 h
AGN
AGN
RA
AGN
RA
NF-κB Shift
Non-specific Binding
Free Probe
Unlabeled Probe
- - - - - - - - - - +

Figure 3.4

**Figure 3.5.** AtRA rescue increases RARα association with and acetylation of histone H3 in the mouse *Mmp-9* up-stream promoter region. Bone marrow cultures were grown in the presence of GM-CSF and RARα antagonist for 8 d, then either remained in culture with 10 nmol/L RARα antagonist or were rescued with 10 nmol/L atRA. Adherent DC were formaldehyde-fixed 0, 4, and 24 h after RARα antagonist treatment or atRA rescue, chromatin sheared, and immunoprecipitated against RARα, p300, acetylated histone H3, or an irrelevant IgG. Immunoprecipitated or diluted input chromatin was then amplified by PCR using primers for a 229 bp region of the murine *Mmp-9* promoter located at -1087 bp to -859 bp from the transcription start site and products were separated by agarose electrophoresis. The results are representative of 2 independent experiments, n=2 for each treatment group.

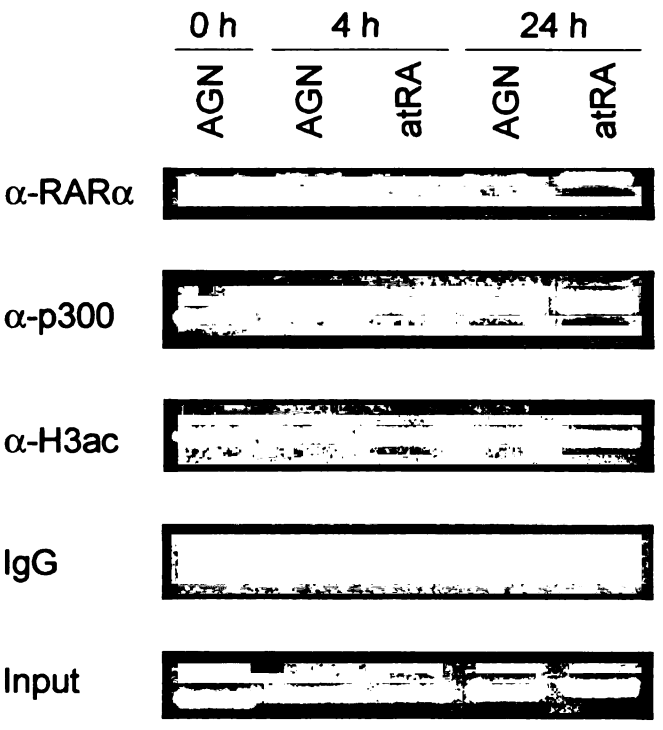
Mmp-9 Promoter
0 h
4 h
24 h
AGN
AGN
atRA
AGN
atRA
α-RARα
α-p300
α-H3ac
IgG
Input

Figure 3.5

# CHAPTER 4

# SUMMARY AND FUTURE DIRECTIONS

**Summary**

Vitamin A deficiency has been known to affect morbidity and mortality since early in its study, including increased infection of the gastrointestinal, urogenital, and respiratory tracts in vitamin A deficient rats (Green and Mellanby 1928). Since the early studies showing an increased prevalence of mucosal site infections, the results have been substantiated by observations of increased respiratory infection (Jason et al. 2002) and intestinal permeability (Quadro et al. 2000) in children with low plasma retinol concentrations. It has been determined that the increased prevalence of mucosal infections is related to impaired production of secretory IgA in response to infection (Stephensen 2001). Vitamin A supplementation increased secretory IgA production in response to *Shigella* infection (Villamor and Fawzi 2000) and hepatitis B vaccine (Newton et al. 2007).

In experimental models of vitamin A deficiency, T cells from vitamin A deficient mice resulted in decreased $IgG_1$ production by B cells from vitamin A sufficient mice, revealing that vitamin A had an effect on the T cells which then led to impaired B cell antibody production (Carman et al. 1989). Production of both $IgG_1$ and IgA isotypes required Th2 help. Vitamin A deficiency in mice has also been linked to decreased symptoms of allergic airway inflammation due to decreased serum IgE and IgG1 and decreased brochoalveolar lavage IL-4 and –5, all responses that require Th2 support for production (Schuster et al. 2008). These observations have led to studies of vitamin A's effect on naïve T cell development.

In vitamin A deficient mice, the Th1 response to *Trichinella spiralis* infection was enhanced through secretion of IFN-γ, which also suppressed the Th2 response by decreasing IL-5 and -10 production leading to an improved cell-mediated immune response and impaired humoral immunity (Cantorna et al. 1996). Addition of atRA to Th1 cell culture inhibited IFN-γ production. AtRA also enhanced the development of naïve T cells to Th2 cells through an antigen presenting cell intermediate (Hoag et al. 2002). Myeloid DC are one type of antigen presenting cell that require atRA to develop from myeloid progenitor cells in culture. Without sufficient atRA, the progenitor cells developed into neutrophils instead (Hengesbach and Hoag 2004). These *in vitro* studies confirmed *in vivo* observations which showed increased neutrophil numbers in the spleen, peripheral blood, and bone marrow of vitamin A deficient mice (Kuwata et al. 2000). However, these expanded neutrophils had impaired migration, phagocytosis, and bacterial killing functions (Twining et al. 1997; Stephensen 2001).

Myeloid DC, characterized as $CD8\alpha^-$, $CD11b^+$, and $CD11c^+$ in mice, are derived from hematopoietic progenitor cells in the bone marrow (Shortman and Liu 2002). DC precursors migrate from the bone marrow, via the blood, into the different lymphoid tissues where they differentiate (Iwasaki 2007). Immature myeloid DC resident in peripheral tissues capture, process, and present antigen in MHC class I and II molecules (Banchereau et al. 2000). DC mature in response to stimulation by antigen and stress signals through the pattern-recognition toll-like receptors (Hochrein and O'Keeffe 2008), leading to reduced

antigen capturing and induced migration to the T cell areas of secondary lymphoid tissues (Banchereau and Steinman 1998; Steinman 1999). There, DC activate naïve T cells through antigen presentation along with the secretion of specific influential cytokines. Myeloid DC are known to polarize naïve $CD4^+$ T cells to Th2 cells through stimulation of the T cells with antigen-bound MHC class II and production of MCP-1 and OX40L (Moser and Murphy 2000; de Jong et al. 2005; Hochrein and O'Keeffe 2008).

MMP-9, a gelatinase that can degrade gelatin, types I and IV collagen, and laminin in the ECM, aids leukocyte migration into blood and lymphatic vessels (Faveeuw et al. 2001; Opdenakker et al. 2001). DC migration has been found to be heavily reliant on MMP-9 activity. Inhibition of MMP-9 activity, through the use of monoclonal neutralizing antibody, the natural inhibitor TIMP-1, and $MMP\text{-}9^{-/-}$ mouse models, impaired the release of hematopoietic progenitor cells from the bone marrow (Pruijt et al. 1999), recruitment of bronchoalveolar associated lymphoid tissue DC to airway lumen in response to allergen (Vermaelen et al. 2003), migration of cultured DC through tight epithelial junctions (Ichiyasu et al. 2004), and migration of cultured monocyte-derived DC (Baratelli et al. 2004). The chemokine CCL5, released under inflammatory conditions, induces a fold increase in pro-MMP-9 production by immature DC within 10 h to aid in migration through basement membranes (Chabot et al. 2006).

MMP-9 production has been reported to be both positively and negatively regulated by atRA signaling. In tumor cell invasion (Tsang and Crowe 2001;

Andela and Rosier 2004) and emphysema (Mao et al. 2003), atRA treatment decreased MMP-9 production. AtRA negatively regulated *Mmp-9* production in squamous cell carcinoma lines through displacement of AP-1's coactivators from the *Mmp-9* promoter (Tsai et al. 2008). However, atRA also positively regulated *Mmp-9* production in mammary epithelial cells (Montesano and Soulie 2002) and rat mammary tissue (Zaragoza et al. 2007) for lumen formation and involution, respectively, through recruitment of p300 to the *Mmp-9* promoter, even though the *Mmp-9* promoter lacks a RARE consensus sequence for the holo-RAR-RXR complex to recognize and bind.

*AtRA decreases DC adhesion*

We found that treatment of bone marrow cell cultures under DC-driving conditions with atRA in combination with CD-FBS supplemented medium led to increased production of $CD11c^+$ DC and decreased yield of $Gr\text{-}1^+$ neutrophils compared to cells in CD-FBS supplemented medium without atRA or in CH-FBS supplemented medium with the RARα-specific receptor antagonist AGN 194301. At the same time, use of the receptor antagonist or CD-FBS supplemented medium without atRA increased the percentage of DC that remain adherent after a 10 d culture period compared to cultures treated with atRA. Rescue of receptor antagonist-treated DC cultures with atRA on culture d 8 resulted in increased overall floating cell yield with no change in adherent cell yield by d 10 of the culture period, resulting in a decreased percentage of cells that remain adherent compared to receptor antagonist treated cultures. The floating $CD11c^+$ DC yield and proportion significantly increased by d 10 after atRA rescue compared to

receptor antagonist treated cultures. The percentage of floating Gr-1$^+$ neutrophils did not differ between the two groups, but the yield increased after atRA rescue due to the overall floating cell yield increase. None of the adherent cell yields or DC percentage differed between the two treatments, though the adherent DC yield decreased insignificantly. These results indicate that atRA signaling through RARα is necessary for normal DC development and loss of adherence. Blocking this signal results in bone marrow cells developing primarily into neutrophils with the majority of DC remaining as adherent cells. Rescue of bone marrow cell cultures with atRA for 48 h after 8 d treatment with RARα antagonist is not long enough to yield a greater proportion of floating DC than floating neutrophils. Bone marrow cells cultured in the presence of GM-CSF, but with blocked signaling through RARα, develop into primary neutrophils, and the atRA rescue allows for their maintenance, while differentiation of myeloid precursors to DC only begins after addition of atRA to the culture medium.

While examining factors that could account for changes in DC adherence after atRA rescue from receptor antagonist treatment with DC, it was found that atRA treatment decreased the magnitude of the cell adhesion molecule CD11a (integrin-$\alpha_L$) expression on both the total cell population and the DC population of both adherent and floating cells. However, there was no difference in *Itgal* transcript expression between the two treatments over 48 h, indicating that atRA does not directly reduce *Itgal* expression at the transcription level. The decrease in CD11a cell-surface expression could not be explained by sICAM-1 binding to CD11a and thus blocking the antibody binding to the relevant epitope, because

there was no difference in sICAM-1 concentrations in conditioned supernatant between the two treatments.

The use of an extracellular matrix and adhesion molecule-specific microarray identified *Mmp-9* as a factor that was increased by atRA rescue of DC. There were a number of other extracellular matrix and adhesion molecule genes that were modulated either by atRA treatment or by developmental stage of the DC (adherent or floating, Appendix A). For example, the proteoglycanase A disintegrin-like and metalloprotease with thrombospondin type 1 motif (*Adamts*)-*8* that, along with ADAMTS-1, -4, -5, -9, -15, and -20, cleaves aggrecan, versican, and brevican (Apte 2009) was down-regulated in both adherent and floating DC. The MMP modulator basigin (CD147) was upregulated in atRA-treated floating DC, but not in atRA-treated adherent DC. It has been reported that the presence of basigin on the DC surface indicates a later developmental stage (Woodhead et al. 1998). N-glycosylated basigin induces the expression of MMP-1, -2, -3, -9, -11. There is evidence that basigin, as a mediator of cell-cell interaction and an adhesion molecule, expression on antigen presenting cells is required for T cell activation (Iacono et al. 2007).

It was confirmed that atRA rescue increased *Mmp-9* production in adherent DC by 4 h with maximal production in 24 h post-rescue. AtRA rescue had no effect on the production of *Timp-1*, the chief natural inhibitor of MMP-9, in adherent DC. Similarly, atRA rescue increased the concentration of secreted pro-MMP-9 to a greater extent than the secretion of TIMP-1, resulting in a molecular excess of pro-MMP-9 and the potential for greater degradation of the ECM upon

atRA rescue compared to cultures treated with receptor antagonist. These results led to the hypothesis that the activity of MMP-9, whose production was increased in adherent DC by atRA rescue, is responsible for the decreased DC adherence due to atRA signaling through RARα. It was also hypothesized that this mechanism was partly due to atRA-induced MMP-9 activity resulting in decreased CD11a cell surface expression in atRA treated cells due to direct cleavage by MMP-9.

*AtRA increases MMP-9 activity*

It was important to determine whether the MMP-9 produced by DC upon atRA signaling through RARα was active. It was found that gelatinase activity was increased on the surface of adherent cells when treated with atRA compared to cells treated with receptor antagonist. When MMP-9 inhibitor was added to atRA treated DC cultures, gelatinase activity was reduced to that of receptor antagonist treated cultures. These results indicate atRA treatment increased gelatinase activity of DC cultures and that MMP-9 was a primary gelatinase responsible for atRA-induced gelatinase activity through RARα signaling. We also found that culturing bone marrow cells under DC-generating conditions in the presence of atRA and MMP-9 inhibitor increased the percent of all cells that remained adherent as well as the percent of $CD11c^+$ DC that remained adherent. These results are summarized in **Figure 4.1**. The inhibitor did not affect the proportion of cells that were DC or the cell yield. The inhibitor also did not affect the cell surface expression of CD11a compared to cultures only treated with atRA. Thus, atRA-induced MMP-9 expression and activity was only responsible

for cell adherence, not progenitor cell differentiation into DC or cell proliferation. Unlike what was hypothesized, MMP-9 activity was not responsible for the atRA-induced decrease in CD11a cell surface expression.

*RARα interacts directly through the MMP-9 promoter*

It was important to determine the mechanism by which atRA regulated *Mmp-9* expression in DC. Through the use of the transcription inhibitor actinomycin D, we found that atRA positively regulated *Mmp-9* transcript abundance through a transcriptional mechanism. Because the *Mmp-9* promoter does not contain a classic RARE, we measured binding of nuclear protein extracts to consensus oligonucleotide sequences in the *Mmp-9* promoter using EMSA and found that neither Sp1, AP-1, nor NF-κB showed increased binding to nuclear protein extracts after atRA treatment. However, the oligonucleotide sequences used in EMSA are not histone associated and the nuclear extract protein binding to the oligonucleotides only indicates global binding to these sequences in response to atRA, not *Mmp-9* promoter specific binding. The binding also does not take place in the context of the cell environment. It is still possible that the holo-RARα-RXR complex interacts with one of these transcription factors in the early period after atRA treatment to increase binding to the chromosomal DNA associated with histone proteins and specific to the *Mmp-9* promoter. While the EMSA results indicate that it is unlikely that the holo-RARα-RXR interacts with transcription factors in the Sp1, AP-1, or NF-κB families to positively increase binding to the *Mmp-9* promoter to induce *Mmp-9* transcription, this conclusion is not definitive. We did not do supershift assays to

determine whether there was increased binding of individual proteins within the nuclear protein extracts in the Sp1 family [Sp1, Sp3, or the transcriptional repressive Sp1-like Krüppel-like factor (KPF) members (Lomberk and Urrutia 2005)] after atRA rescue of adherent DC even without increased overall nuclear protein binding to the Sp1 consensus sequences. It is possible that with RARα antagonist treatment results in increased Sp1-like KPF binding to the Sp1 consensus sequences to block gene transcription, while atRA rescue results in increased Sp1 or Sp3 binding to the same sequences to up-regulate gene transcription. This state could result in a similar band shift as visualized by EMSA despite opposite biological effects *in vivo*.

Through chromatin immunoprecipitation experiments, we found that 24 h atRA rescue increased RARα and histone acetyltransferase p300 association with the *Mmp-9* promoter and increased acetylation of lysine 14 on the tail of histone H3, indicating a state permissive for increased gene expression. We have previously found that atRA treatment increased *Mmp-9* expression within 4 h of treatment (Lackey et al. 2008), but we did not see increased RARα and p300 association with the promoter after 4 h of atRA treatment. These results indicate that the atRA-RARα-RXR signaling complex most likely interacts with an as yet unknown factor that recognizes the *Mmp-9* promoter to increase its interaction with the promoter, recruit p300 to the promoter, and increase histone H3 acetylation and thus open the promoter for interaction with transcription machinery for increased gene expression for long-term expression regulation by atRA (**Figure 4.2**). However, for early atRA regulation, there may be an indirect

mechanism for *Mmp-9* expression, such as the displacement of NF-κB from the promoter or increased binding of Sp1 to the promoter, which was only tested by EMSA and not by ChIP.

**Future directions**

*What other proteins interact with the holo-RARα-RXR complex at the Mmp-9 promoter region?*

Our research indicated that holo-RARα-RXR interacted with the *Mmp-9* promoter to recruit p300 and thus increased the acetylation of the *Mmp-9* promoter and subsequent expression of *Mmp-9*. However, the RARα-RXR signaling complex cannot recognize and bind to the *Mmp-9* promoter through currently recognized mechanisms due to a lack of a consensus RARE (Munaut et al. 1999). It is likely that the holo-RARα-RXR signaling complex interacts with another protein that can recognize and directly bind to the *Mmp-9* promoter. While we could not demonstrate that atRA treatment of DC increased nuclear protein extract binding to the AP-1, NF-κB, or Sp1 consensus oligonucleotide sequences, it is still possible that in the environment of the cell and interaction with the histone associated *Mmp-9* promoter, one of these transcription factors does interact with the holo-RARα-RXR complex. To determine the identity of the potential protein with which the holo-RARα-RXR complex interacts, it will be necessary to make nuclear protein extracts of adherent DC after treatment with atRA or RARα antagonist. The nuclear protein extracts must then be immunoprecipitated against RARα to enrich for proteins that associate with RARα in the nucleus of DC. The immunoprecipitate would be electrophoresed in two dimensions, first by isoelectric focusing, then by size separation. The separated proteins from each treatment of extract could then be compared for

differences in intensity and selected for further identification of the proteins from the atRA treatment that are more intense than those from the RARα antagonist treatment. The selected proteins would then be trypsin digested for preparation for tandem mass spectrometry to identify the possible proteins that interact with RARα in this system.

Further experiments could test whether candidate proteins identified by mass spectrometry interact with the *Mmp-9* promoter through the use of sequential chromatin immunoprecipitation (ChIP-ReChIP). First, fixed and sheared chromatin-protein complexes would be immunoprecipitated against RARα, then immunoprecipitated again using an antibody specific for the candidate protein. The complexes would then be reverse cross-linked followed by treatment with proteinase K, and the resulting chromatin would be amplified by PCR using primers designed to amplify the *Mmp-9* promoter region.

Our research has inferred an interaction between RARα and p300 in DC because they both show increased association with the assayed *Mmp-9* promoter region after atRA treatment at 24 h. However, we have not shown that RARα and p300 interact in the same complex on the *Mmp-9* promoter. ChIP-ReChIP could be used to determine whether these proteins interact in the same complex on the *Mmp-9* promoter. DC cultures would be treated as before with 10 nmol/L RARα antagonist for 8 d followed by 24 h treatment with atRA or RARα antagonist. DC would be fixed and lysed, followed by chromatin shearing. Because p300 is more abundant 24 h after atRA treatment, it would be best to first immunoprecipitate the fixed chromatin-protein complexes using the antibody

against p300 followed by immunoprecipitation against RARα. The immunoprecipitated chromatin would then be amplified by PCR using the *Mmp-9* promoter primers to visualize whether atRA rescue treatment increases binding of RARα and p300 in the same complex to the *Mmp-9* promoter.

Histone acetylation is not the only epigenetic change leading to increased chromatin accessibility to transcription machinery and increased gene expression. Even though methylation, such as at the K9 position on histone H3, is most frequently associated with gene silencing, methylation of histone H3 (K4) is associated with transcription elongation (Jenuwein and Allis 2001). It has been previously shown that the histone methyltransferase MLL5, a histone lysine mono- and di-methyl transferase, after it has been N-glycosylated, acts as a co-regulator of RARα-induced transcription by methylating lysine 4 on histone H3 (Fujiki et al. 2009). Methylation of histone H3 (K4) and acetylation of histone H3 also result in chromatin more permissive to transcriptional activity in a number of promoter regions, including that for *Mmp-9*, as well as increased association with MLL (Yan and Boyd 2006). Experiments using 24 h 10 nmol/L atRA rescue of DC after RARα antagonist followed by ChIP against MLL5 and lysine 4-methylated histone H3 followed by PCR amplification of the immunoprecipitated chromatin using *Mmp-9* promoter primers would be useful in further determining the mechanism of atRA-induced *Mmp-9* expression in DC.

*Does vitamin A deficiency impair DC migration and MMP-9 production in vivo?*

We have shown that MMP-9 produced by DC in response to atRA treatment is active in culture and contributes to a change in adherence. The next

step is to determine whether vitamin A status is linked to MMP-9 production and DC migration *in vivo*. To determine whether vitamin A deficiency has a systemic effect on MMP-9 production, the serum from vitamin A deficient mice could be tested for a significant decrease in pro-MMP-9 production by ELISA compared to the serum of vitamin A sufficient mice. If there is no difference in serum pro-MMP-9 between vitamin A deficient and sufficient groups, immature DC from the spleen could be purified and RNA extracted and reverse transcribed for quantitative PCR analysis of differences in *Mmp-9* expression between the two diet groups.

Based on the results determining whether MMP-9 production is systemically reduced during vitamin A deficiency in the mouse, either bone marrow-derived DC would be injected into vitamin A deficient or sufficient mice, or, if vitamin A deficiency does not result in systemically reduced MMP-9 production, bone marrow-derived DC treated either with atRA or RARα antagonist would be injected into vitamin A deficient mice. Mice would be sensitized to ovalbumin by intraperitoneal injection along with alum adjuvant, followed by exposure to 1% ovalbumin aerosol for 7 consecutive days two weeks later. Fluorescently-labeled bone marrow-derived DC would be injected into vitamin A deficient or sufficient mice with allergic airway inflammation. After the last day of aerosol exposure, bronchoalveolar lavage cells would be collected along with the lung and lymph nodes for processing into single cell suspensions for analysis by flow cytometry to determine the level of DC infiltration. It is expected that vitamin A deficiency, either of the animal or of the cultured DC,

would result in impaired DC migration to the site of ovalbumin-induced inflammation and to the lymph nodes.

*How are other extracellular matrix and adhesion molecules on DC affected by atRA treatment?*

Many other genes for extracellular matrix and adhesion molecules were found to be regulated either by atRA or by floating versus adherent phenotype or developmental state. Two of these genes are *Adamts8* and *basigin*. *Adamts8* expression is likely down-regulated by atRA, while *basigin* expression seems to be related to the later developmental state of the floating DC. As basigin expression is more closely related to the DC developmental state, it is likely that it may be up-regulated by atRA, but through an indirect mechanism related to its role in DC development. For both of these genes, it would be important to confirm gene expression level using reverse transcription and real-time PCR techniques over a time course to determine gene expression kinetics. It would next be important to confirm gene expression with protein expression. For ADAMTS8, protein expression could be confirmed by western blot using total protein extracts from adherent DC or ELISA using conditioned medium from DC cultures. For basigin, flow cytometric analysis of both floating and adherent cultured cells stained for CD11c, Gr-1, and CD147 (basigin) would be useful for determining the percentage of DC expressing basigin and the average expression level on the cell surface of both floating and adherent DC.

*How does atRA regulate CD11a surface expression?*

AtRA treatment of DC resulted in decreased cell surface expression of the adhesion molecule CD11a (integrin-$\alpha_L$), but mRNA expression was not affected. We have also shown that inhibition of MMP-9 activity in the presence of atRA had no effect on the CD11a cell surface expression (**Appendix D**). The next step would be to determine how atRA regulates DC CD11a surface expression. The role of atRA treatment on CD11a protein expression could be explored by inhibiting both the rate of CD11a translation and the protein degradation machinery. In experiments where CD11a translation is inhibited, bone marrow cells would be grown in DC generating conditions in the presence of AGN 194301 as described in previous atRA rescue experiments. On day 8 of the culture period, cells would receive 20 mL fresh cIMDM supplemented with 20 μg/L GM-CSF and either 10 nmol/L AGN 194301 or 10 nmol/L atRA. Some culture plates in each treatment group would also be supplemented with the translational inhibitor cycloheximide on day 8. On day 10, the floating and adherent cells would be harvested separately, counted, and stained with fluorescently labeled monoclonal antibodies against Gr-1, CD11c, and CD11a for analysis by flow cytometry. A significant decrease in cell surface CD11a expression in cultures treated with both AGN and cycloheximide compared to cells only treated with AGN to a level of expression as seen in cultures rescued with atRA in the presence or absence of cycloheximide would indicate that atRA acts by decreasing the rate of translation from *Itgal* mRNA to protein.

It would also have to be taken into consideration that atRA signaling through RARα could act to decrease CD11a protein half-life. If there were a

significant decrease in CD11a cell surface expression after atRA rescue and cycloheximide treatment compared to cells only rescued with atRA, the data would indicate that atRA signaling does not affect the translation rate of *Itgal* mRNA to protein but that the rate of degradation is greater in the presence of this signaling. In this case, it would be necessary to follow the experiment with studies using inhibitors of 20S and 26S subunits of the proteasome. Cells would be cultured in the same way as for the protein synthesis inhibitor studies, except the $AdaAhX_3L_3VS$ proteasome inhibitor at 10 μmol/L dose would be used in the place of cycloheximide. If atRA acts to increase the degradation rate of CD11a, blocking degradation would lead to an increase in CD11a cell surface expression comparable to that of AGN treated cells. Overall, the results of these experiments would help to explain how atRA acts to decrease cell surface expression of CD11a on DC cultures *in vitro*.

It is also possible that atRA treatment increased recycling of CD11a from the cell surface. To explore this possibility, bone marrow cells cultured under DC generating conditions and either treated with RARα antagonist or rescued with atRA could be harvested after 10 days and then incubated with fluorescently-labeled antibody against CD11a. Cellular localization could be determined by immunocytochemistry. If there was greater internally located CD11a after atRA rescue compared to RARα antagonist treated cells, these results would indicate that atRA did regulate cell surface CD11a expression through a mechanism based on recycling of the protein from the surface.

**Figure 4.1. Model of retinoic acid's effect on dendritic cell development.** Retinoic acid receptor (RAR)-α antagonist treatment results in decreased DC yield and increased DC adhesion with mostly neutrophil development. Addition of retinoic acid to cultures increases MMP-9 production and activity, especially at the adherent DC surface, resulting in more floating DC.

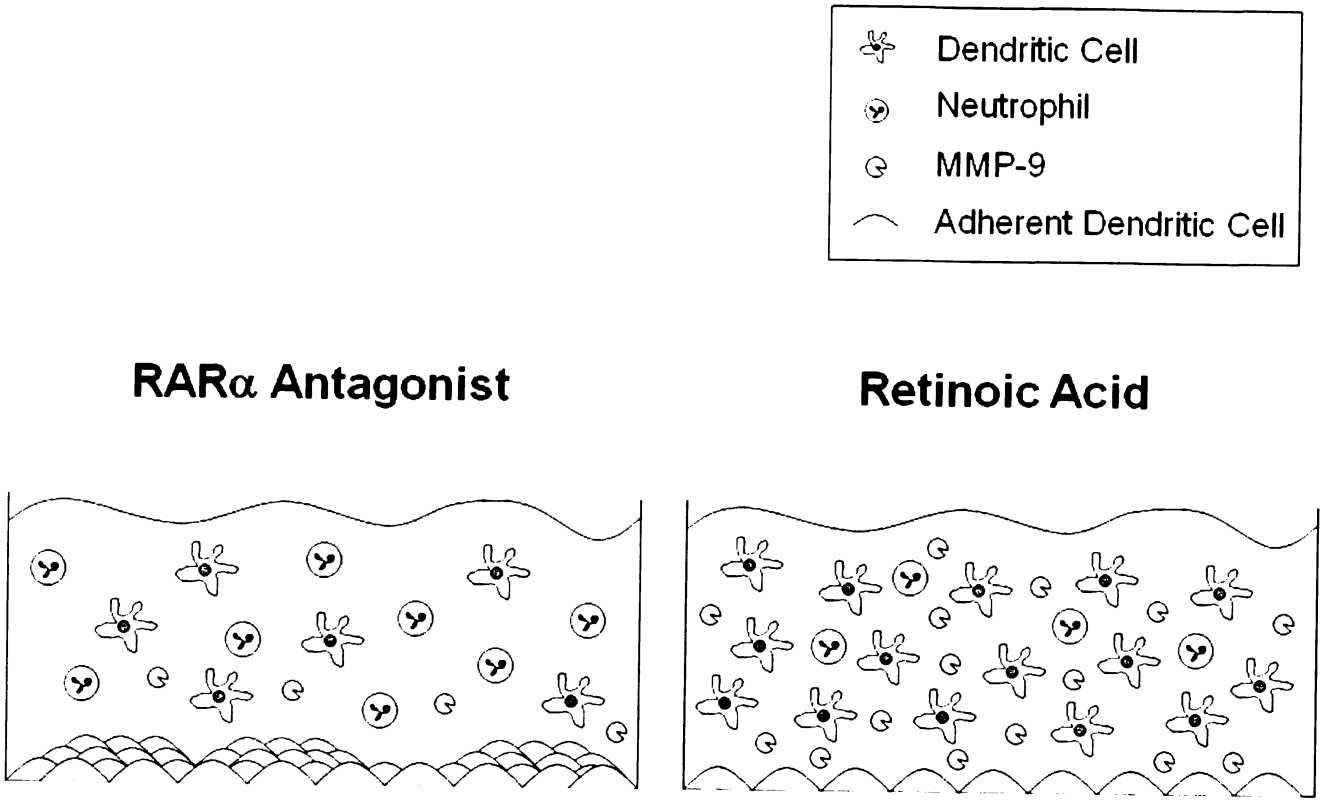
Dendritic Cell
Neutrophil
MMP-9
Adherent Dendritic Cell
RARα Antagonist
Retinoic Acid

Figure 4.1

**Figure 4.2. Model of retinoic acid signaling to increase *Mmp-9* production.**

*A*, The holo-retinoic acid receptor (RAR)-α-retinoid X receptor (RXR) complex interacts with an unknown protein that recognizes a sequence in the *Mmp-9* promoter region. *B*, Binding of the holo-RARα-RXR complex recruits p300 association with the promoter and acetylation of the tail of histone H3 (K14), resulting in transcriptional machinery binding and *Mmp-9* transcription.

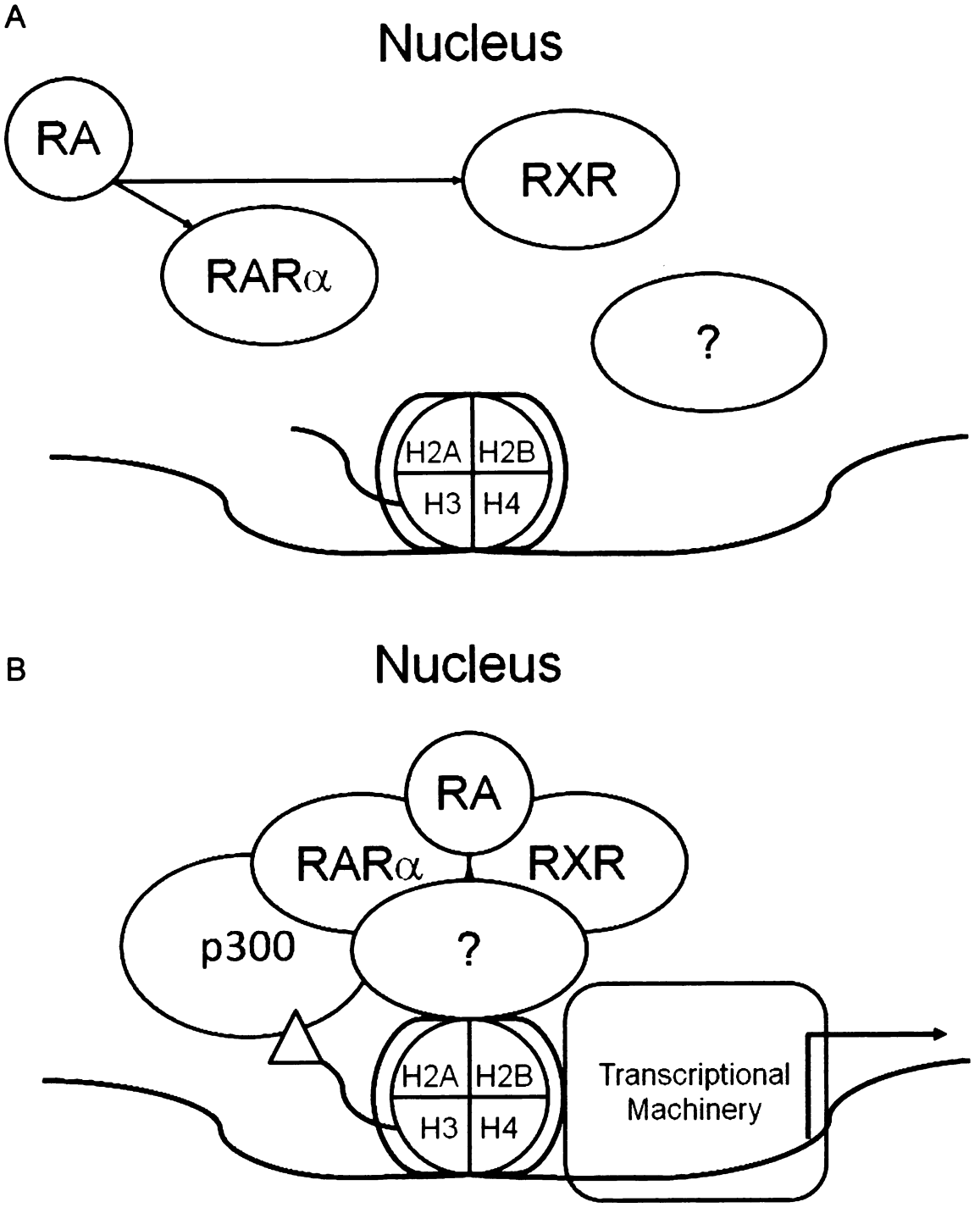
A
Nucleus
RA
RXR
RARα
?
H2A
H2B
H3
H4
B
Nucleus
RA
RARα
RXR
p300
?
H2A
H2B
H3
H4
Transcriptional
Machinery

Figure 4.2

# APPENDICES

## APPENDIX A

**Table A.1.** Expression of all genes assayed on the Extracellular Matrix and Adhesion Molecules Gene Array after 48 h atRA rescue treatment or RARα antagonist treatment.[1,2]

| Gene | AGN Adherent[3,4] | AtRA Adherent | AtRA Floating DC |
|---|---|---|---|
| *Adamts8* | 3.115 | 0 | 0 |
| *Basigin* | 29.281 | 21.371 | 73.481 |
| *Caspase 8* | 9.196 | 9.082 | 13.866 |
| *Catenin alpha 1* | 3.801 | 3.655 | 5.192 |
| *Catenin beta* | 5.868 | 5.505 | 7.786 |
| *CD44 antigen* | 3.287 | 3.156 | 2.739 |
| *Ceacam 1* | 6.889 | 7.921 | 16.061 |
| *Contactin 1* | 0 | 0.637 | 0 |
| *Cystatin C* | 1.611 | 1.539 | 2.332 |
| *Cathepsin B* | 15.893 | 14.914 | 13.369 |
| *Cathepsin D* | 14.120 | 17.797 | 21.055 |
| *Cathepsin E* | 0 | 0.676 | 0.762 |
| *Cathepsin H* | 1.185 | 1.410 | 1.661 |
| *Extracellular matrix protein 1* | 1.378 | 0.975 | 0 |
| *Integrin alpha 4* | 13.080 | 13.957 | 31.277 |
| *Integrin alpha 5* | 0.975 | 0.863 | 1.416 |
| *Integrin alpha 6* | 0 | 0.880 | 1.053 |
| *Integrin alpha L* | 1.227 | 0 | 1.026 |
| *Integrin alpha M* | 29.691 | 20.904 | 71.243 |
| *Integrin alpha V* | 2.504 | 2.955 | 1.795 |
| *Integrin alpha X* | 30.229 | 20.780 | 55.131 |
| *Integrin beta 1* | 21.563 | 17.171 | 33.403 |
| *Integrin beta 2* | 3.826 | 4.895 | 4.536 |
| *Integrin beta 3* | 0.688 | 0 | 0 |
| *Integrin beta 4* | 0.886 | 0 | 0 |
| *Integrin beta 5* | 26.604 | 20.529 | 57.982 |
| *Integrin beta 7* | 0 | 0.607 | 1.434 |
| *F11 receptor* | 0.828 | 0.825 | 0.890 |
| *Mmp-3* | 7.129 | 8.168 | 2.726 |
| *Mmp-8* | 25.570 | 20.067 | 52.662 |
| *Mmp-9* | 0 | 5.289 | 6.479 |
| *Mmp-12* | 30.360 | 21.067 | 83.866 |
| *Mmp-13* | 26.448 | 20.112 | 15.668 |
| *Mmp-14* | 0.695 | 0.884 | 0 |

Table A.1 (cont'd)

| | | | |
|---|---|---|---|
| *Mmp-19* | 19.609 | 10.058 | 6.255 |
| *Meningioma expressed antigen 5* | 2.129 | 2.071 | 3.444 |
| *Plasminogen activator, urokinase* | 29.170 | 20.371 | 33.010 |
| *Plasminogen activator, urokinase receptor* | 1.660 | 1.085 | 0.808 |
| *Selectin, lymphocyte* | 0.728 | 1.935 | 4.054 |
| *Serpine1* | 0.955 | 1.039 | 0 |
| *Serpinb2* | 12.386 | 8.883 | 8.184 |
| *Sparc* | 1.504 | 1.198 | 0 |
| *Thrombospondin 1* | 6.370 | 5.513 | 6.286 |
| *Thrombospondin 2* | 1.631 | 0.941 | 1.005 |
| *Tissue inhibitor of metalloproteinase 1* | 2.616 | 1.202 | 0 |
| *Tissue inhibitor of metalloproteinase 2* | 0.995 | 0 | 0 |
| *Vascular cell adhesion molecule 1* | 2.376 | 1.938 | 0 |

[1]RNA extracted from adherent atRA-treated, adherent receptor antagonist-treated, or $CD11c^+$ isolated floating DC. RNA was hybridized to SuperArray GEArray Q Series Mouse Extracellular Matrix and Adhesion Molecules Gene Array (SuperArray Biosciences).

[2]Genes assayed but considered absent in all samples tested were: *Adamts1, Casp9, Catna2, Catnal1, Cav1, Cdh1, Cdh2, Cdh3, Cdh4, Cdh5, Col4alpha2, Col18alpha1, Catnd2, Ctsg, Fn1, Icam1, Itga2, Itga2b, Itga3, Itga7, Itga8, Itgae, Itgb6, Lamc1, Mmp-1a, Mmp-2, Mmp-7, Mmp-10, Mmp-11, Mmp-15, Mmp-16, Mmp-17, Mmp-20, Mmp-23, Mmp-24, Ncam1, Ncam2, Pecam1, Plat, Sele, Selp, Serpinb5, Thbs3, Thbs4, Tnc, Vtn.*

[3]Expression is presented in arbitrary units of signal intensity after interquartile normalization.

[4]A value of 0 indicates expression considered absent.

## APPENDIX B

**Figure B.1.** AtRA rescue does not change sICAM-1 secretion into cell culture medium in bone marrow-derived myeloid DC cultures. Soluble ICAM-1 in conditioned cell culture medium was measured by ELISA. The values for each treatment group are plotted as the mean + SD (n=4). The results are representative of one experiment. There was no significant difference between treatment groups.

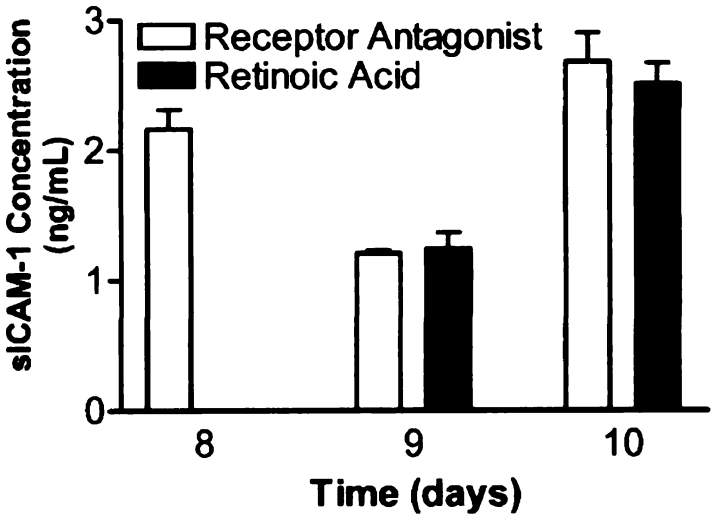
sICAM-1 Concentration (ng/mL)
3
2
1
0
Receptor Antagonist
Retinoic Acid
8
9
10
Time (days)

Figure B.1

## APPENDIX C

**Figure C.1.** Cell culture yields after 10 d culture in the presence or absence of MMP-9 inhibitor. *A*, Floating and adherent cell yields. *B*, The percentage of total cells that are adherent was calculated as adherent cell yield divided by total cell yield multiplied by 100% (n=4 for each treatment group). All cultures were treated with 10 nmol/L atRA. Results are presented as the mean + SD and show one representative experiment of two. Treatment groups designated with different letters are significantly different with $P < 0.05$ as determined by one-way ANOVA. Letter designations in *A* refer to differences in adherent cell yields.

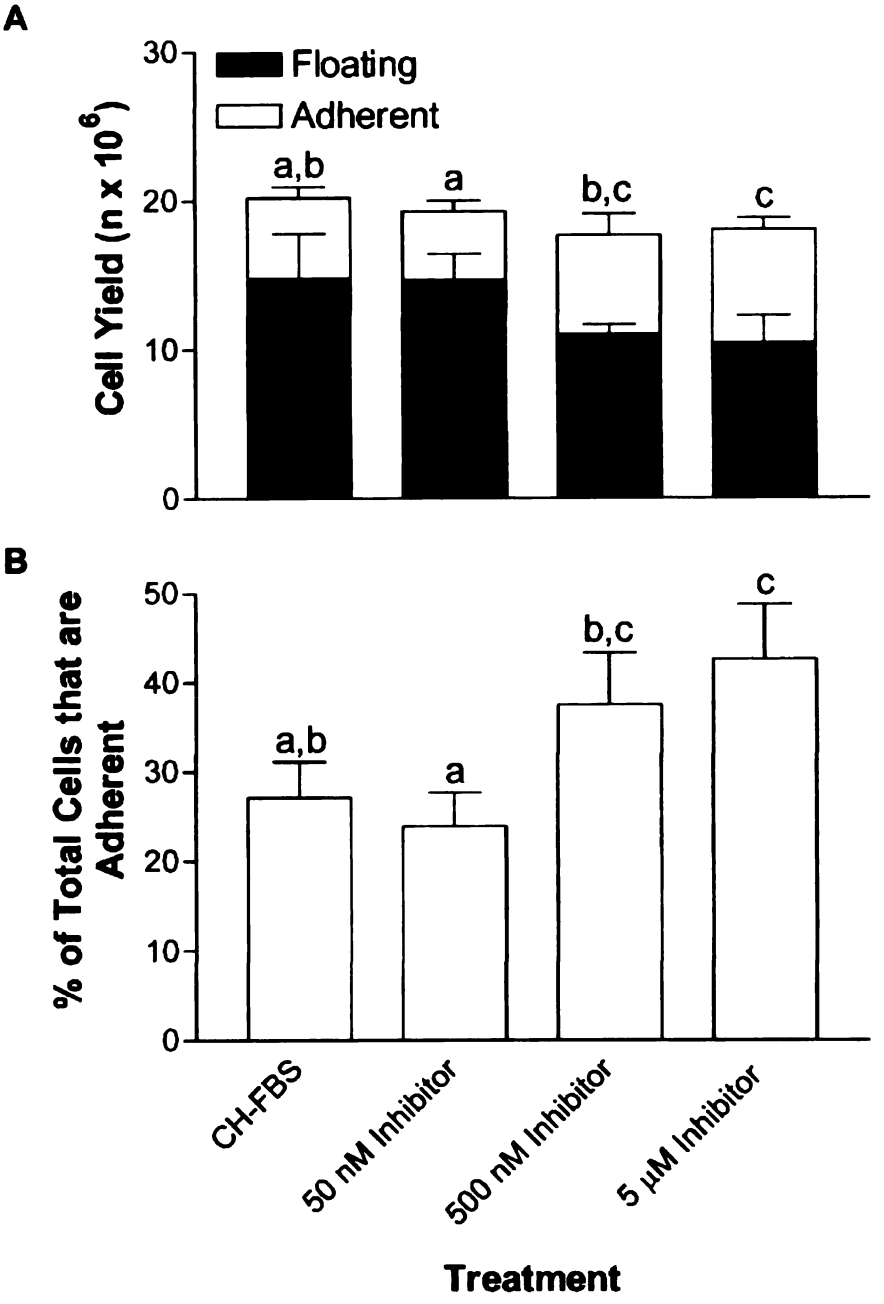
A
Floating
Adherent
Cell Yield (n x 10^6)
30
20
10
0
a,b
a
b,c
c
B
% of Total Cells that are Adherent
50
40
30
20
10
0
a,b
a
b,c
c
CH-FBS
50 nM Inhibitor
500 nM Inhibitor
5 μM Inhibitor
Treatment

Figure C.1

# APPENDIX D

## Introduction

It has not been determined whether MMP-9 activity is responsible for the decrease in cell surface CD11a found in DC cultures in response to atRA signaling through RARα.

## Materials and Methods

For the atRA full-culture experiments, bone marrow cells were plated 2 x $10^6$ per well containing 10 mL cIMDM supplemented with10% characterized (CH)-FBS or charcoal dextran-filtered (CD)-FBS, 2 mmol/L GlutaMax, 100 U/L penicillin, and 100 mg/L streptomycin, 20 ng/mL GM-CSF and 10 nmol/L atRA or AGN 194301 and in the presence or absence of 10 nmol/L atRA, 50 nmol/L, 500 nmol/L, or 5 μmol/L MMP-9 Inhibitor I, and equal DMSO concentrations. Fresh medium and treatment were given on d 3, 6, and 8. Cells were harvested on d 10 for FACS staining. AGN 194301 was generously provided by Dr. Chandraratna and blocks atRA activity by competing for the ligand binding domain of RARα (Nagpal and Chandraratna 2000).

Floating and adherent cells were harvested, counted, washed with staining buffer (1% FBS, 0.1% (w/v) sodium azide, in phosphate buffered saline, pH 7.4), and incubated with monoclonal antibody against $F_{C\gamma}$R-II/III from the 2.4G2 hybridoma to block non-specific binding. Each sample was then incubated with either an isotype control or a cell surface molecule-specific antibody cocktail, with 1 μg antibody used per 1 x $10^6$ cells. Each sample was then incubated with

0.25 μg PE-Cy7-conjugated streptavidin per 1 x $10^6$ cells. Monoclonal antibodies used were PE-conjugated hamster anti-mouse CD11c, PerCP-Cy5.5-conjugated rat anti-mouse Ly-6G (Gr-1), biotin-conjugated rat anti-mouse CD11a, PE-conjugated hamster $IgG_{1/\lambda}$, PerCP-Cy5.5-conjugated rat $IgG_{2b/\kappa}$, and biotin-conjugated rat $IgG_{2a/\kappa}$, along with PE-Cy7-conjugated streptavidin (BD Biosciences Pharmingen, San Jose, CA). Samples were run at the MSU flow cytometry facility using a LSR II flow cytometer (BD Biosciences) and analyzed using FCS Express v.3.0 software (DeNovo Software, Ontario, CA).

**Results**

CD11a cell surface expression is increased on both DC alone and on the total cell population, floating and adherent, when cells are grown in CD-FBS containing medium that is vitamin A deficient. It was hypothesized that MMP-9 can cleave CD11a from the cell surface and inhibition of MMP-9 activity would lead to elevated CD11a cell surface expression in atRA treated cultures similar to that found in AGN treated cultures (Lackey et al. 2008). However, the cell surface expression of CD11a remains unchanged by culture treatment with increasing doses of MMP-9 inhibitor compared to atRA treated cultures (**Figure D.1*A-B***). Thus, it is unlikely that MMP-9 activity is the mode by which atRA down-regulates CD11a surface expression.

**Discussion**

Increased MMP-9 activity through atRA signaling does not regulate the cell surface expression of CD11a in bone marrow-derived DC cultures because

inhibition of MMP-9 activity did not change the cell surface expression. Because MMP-9 inhibition did significantly increase the percentage of cells that develop an adherent phenotype, it is likely that the presence of CD11a on the cell surface is not significantly involved in the change of adherence seen in atRA treated cells compared RAR$\alpha$ antagonist treated cells. It is also likely that atRA must decrease CD11a expression through another mechanism, such as decreased rate of translation or increased rate of degradation. AtRA has been known to increase the rate of protein degradation through increased ubiquitination. In human bronchial epithelial cells, atRA increases the rate of cyclin D1 degradation through polyubiquitination of lysine residues (Feng et al. 2007), while atRA increase degradation of insulin receptor substrate-1 through increased ubiquitination in atRA-sensitive breast cancer cell lines through activation of PKC-$\delta$ (del Rincon et al. 2004).

**Figure D.1.** CD11a cell surface expression after 10 d culture in the presence or absence of MMP-9 inhibitor. *A*. Floating and adherent CD11a cell surface expression on all cells population. *B*. Floating and adherent CD11a cell surface expression on $CD11c^{+}$ $Gr\text{-}1^{-}$ DC. MFI, mean fluorescence intensity, n=4 for each treatment group. All treatment groups contained 10 nmol/L atRA in characterized (CH)-FBS-containing medium except charcoal-dextran filtered (CD)-FBS group. Results are presented as the mean ± SD and show one representative experiment of two. Treatment groups designated with different letters are significantly different with $P < 0.05$ as determined by one-way ANOVA.

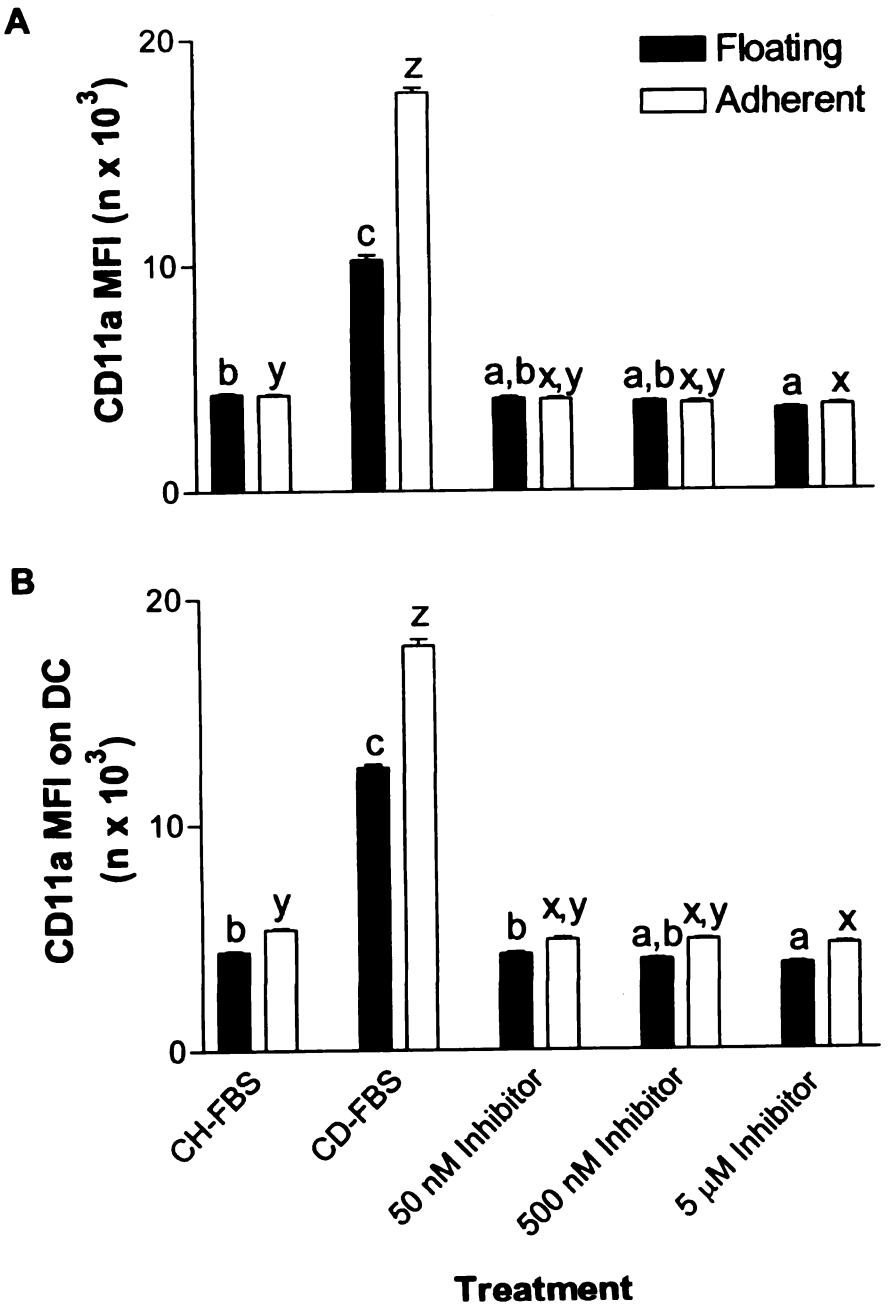
A
20
10
0
CD11a MFI (n x 10^3)
Floating
Adherent
b y
c z
a,b x,y
a,b x,y
a x
B
20
10
0
CD11a MFI on DC (n x 10^3)
b y
c z
b x,y
a,b x,y
a x
CH-FBS
CD-FBS
50 nM Inhibitor
500 nM Inhibitor
5 μM Inhibitor
Treatment

Figure D.1

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