Retinoic Acid Decreases Adherence of Murine Myeloid Dendritic Cells and Increases Production of Matrix Metalloproteinase-91,2,3

Denise E. Lackey4, Shanna L. Ashley4, Alvin L. Davis5, and Kathleen A. Hoag4,5,*
4 Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824
5 Biomedical Laboratory Diagnostics Program, Michigan State University, East Lansing, MI 48824

Abstract
Myeloid dendritic cells (DC) are professional antigen presenting cells (APC) that migrate to secondary lymphoid tissues upon antigen stimulation, where they activate naïve T cells. Vitamin A is 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. Male BALB/cJ mouse bone marrow cells cultured with granulocyte-macrophage colony-stimulating factor in the presence of retinoic acid receptor (RAR) α-specific antagonist showed an increase in the percentage of developing DC that remained adherent compared with cells rescued with atRA treatment from d 8 to 10 of culture (P<0.05). Replacement of the RARα antagonist with atRA on d 8 of the culture period decreased DC surface expression of the adhesion molecule CD11a (P<0.0001) but not the gene expression. Rescue with atRA also dramatically increased gene and protein expression of pro-matrix metalloproteinase (MMP)-9 (P<0.05). However, gene expression and protein production of tissue inhibitor of metalloproteinase (TIMP)-1 was unaffected by atRA rescue, altering the molar ratio of secreted pro-MMP-9:TIMP-1, resulting in a fold excess of pro-MMP-9 to its primary inhibitor (P<0.05). These data suggest that atRA is essential to augment MMP-9 expression in myeloid DC and can alter their surface expression of adhesion molecules.

Introduction
Vitamin A has long been known for its role in immunity. It is currently estimated that ~127 million preschool-aged children and 20 million pregnant women in developing countries are vitamin A deficient (1), leading to increased risk of night blindness and mortality (2). Vitamin A deficiency compromises the mucosal barriers of the eye and the respiratory and gastrointestinal tracts, the first defenses against infection (3). Deficiency also compromises the ability of macrophages and neutrophils to migrate to sites of infection, phagocytose, and kill bacteria (3,4), while increasing neutrophil precursor cell expansion (5,6). Marginal vitamin A status has been shown to decrease natural killer cell number and function (7). Vitamin A is also important for the 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)6 1 function] in vitamin A-deficient mice and also increases the serum antibody production in children after diphtheria and tetanus toxoid vaccination (3). In mice, vitamin A deficiency enhances the Th1 response to Trichinella spiralis infection through increased secretion of interferon γ, while suppressing...
the Th2 humoral response through decreased production of interleukin (IL)-5 and IL-10 (8).
All-trans retinoic acid (atRA), a bioactive metabolite of vitamin A, has been shown to inhibit
the synthesis of the Th1 cytokine interferon γ in Th1 cell culture (9) and enhance the
development of CD4+ T cells into Th2 cells through an antigen presenting cell (APC)
intermediate (10). Evidence exists that myeloid dendritic cells (DC) are a relevant APC whose
function is modified by vitamin A (11).

Myeloid DC are professional APC derived from a common myeloid progenitor cell and in mice
are characterized as CD11b+ CD11c+ CD8α− (12). There is evidence that a lineage marker
negative CX3CR1+ CD117+ common progenitor cell in the mouse gives rise to both myeloid
DC (CD11bhi CD11c+/hi) and macrophages (CD11b+/mid CD11cvariable or CD11b−
CD11c+/hi for alveolar macrophages) but not other mature myeloid cell types (13,14). 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 (15). 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 (16,17).

AtRA signals through the retinoic acid receptor (RAR) family, composed of α, β, and γ isotypes
in the class II family of nuclear receptors (18). This family also includes the thyroid hormone
receptor, vitamin D receptor, and peroxisome proliferator-activated receptor (19,20). Upon
atRA ligand binding, RAR heterodimerizes with a retinoid X receptor family member, also
consisting of α, β, and γ isotypes. The RAR/retinoid X receptor dimer then binds to retinoic
acid response elements within the promoter regions of retinoid responsive genes and associates
with coregulating proteins, eventually leading to the promotion or inhibition of transcription
and target gene expression (21,22).

RAR are known for their importance in regulating the process of hematopoiesis (23). RARγ
deletion studies indicate that RARγ is necessary for maintaining the hematopoietic stem cell
population, whereas overexpression of RARα in bone marrow cells indicates RARα activation
favors neutrophil development (24). We originally observed that treating BALB/cJ mouse bone
marrow-derived myeloid DC cultures with the RARα-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 RARz and blocks atRA activity (25). Based on these initial
observations, experiments were designed to assess the ability of atRA to rescue DC
development after culture initiation with the RARα-specific antagonist.

Materials and Methods

Materials

Stock solutions of atRA (Sigma) were prepared in dimethyl sulfoxide (Sigma) and stored at
−70°C under an argon atmosphere in the dark. Recombinant murine granulocyte-macrophage
colonystimulating factor (GM-CSF) was purchased from PeproTech and dissolved in
molecular biology grade water. Iscove’s modified Dulbecco’s medium (IMDM; BioWhittaker)
was supplemented with 10% (v:v) serum [characterized (CH) or charcoal/dextran treated (CD)]

| Abbreviations used: atRA, all-trans retinoic acid; APC, antigen presenting cell; AU, arbitrary unit; CD-FBS, charcoal/dextran treated
fetal bovine serum; CH-FBS, characterized fetal bovine serum; cIMDM, complete Iscove’s modified Dulbecco’s medium; DC, dendritic
cell; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM, intercellular adhesion molecule; IL,
interleukin; Itgal, integrin-αL; LFA, lymphocyte function-associated antigen; MMP, matrix metalloproteinase; RAR, retinoic acid
receptor; Th, T helper; TIMP, tissue inhibitor of metalloproteinase.

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fetal bovine serum (FBS), HyClone], 2 mmol/L L-glutamine, 100 kU/L penicillin, 100 mg/L streptomycin (BioWhittaker), and 10 μmol/L β-mercaptoethanol (Sigma) to make complete IMDM (cIMDM).

Mice

Male BALB/cJ mice (Jackson Laboratories) 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 mice were fed commercial solid pellets (22/5 rodent diet no. 8640, wt:wtt 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) and killed between 6 and 12 wk of age by CO2 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 with a syringe. Cells were filtered and pelleted, then resuspended in ACK lysing buffer (BioWhittaker) to lyse RBC. Bone marrow cells were then pelleted and resuspended in RPMI-1640 medium. A portion of cells was mixed with trypan blue dye solution and counted to determine cell number and viability. We cultured cells for DC development using a method based on that of Inaba et al. (26) and modified by Lutz et al. (27). For the atRA and receptor antagonist full-culture experiments, bone marrow cells were placed 2 × 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), 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 × 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 10 of culture.

Flow cytometric analysis

Floating cells were harvested by rinsing culture dishes with staining buffer [1% FBS, 0.1% (wt:v) sodium azide in PBS, pH 7.4]. Adherent cells were removed with Accutase (Innovative Cell Technologies) according to manufacturer’s instructions. 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-Fcγ RI/II/III monoclonal antibody from the 2.4G2 hybridoma to block nonspecific 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-conjugated hamster anti-mouse CD11c, fluorescein isothiocyanate-conjugated rat anti-mouse Ly-6C and Ly-6G (Gr-1), biotin conjugated rat anti-mouse CD11a, phycoerythrin-conjugated hamster IgG1κ, fluorescein isothiocyanate-conjugated rat IgG2a/κ, biotin-conjugated rat IgG2aκ, and allophycocyanin-conjugated streptavidin (BD Biosciences Pharmingen). Cells were washed and fixed in 2% paraformaldehyde in PBS. Samples were run at the Michigan State University flow cytometry facility using a BD FACS Vantage equipped with DiVA digital system and analyzed using FCS Express v. 3.0 software (DeNovo Software).
Real-time PCR
Total RNA was isolated from adherent cells at 0, 4, 6, 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) for cell lysis. We completed RNA extraction 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). Total RNA was reverse transcribed using the TaqMan Reverse Transcription System with MultiScribe RT and random hexamer primers (Applied Biosystems). Expression of cDNA relative to the ubiquitously expressed glyceralde-hyde-3-phosphate dehydrogenase housekeeping gene was analyzed using Assays-on-Deman Gene Expression primer/probe sets (Applied Biosystems) for integrin-α₁ (Itgal; Mm00801807_m1), matrix metalloproteinase (Mmp-9; Mm00600163_m1), and tissue inhibitor of metalloproteinase (Timp-1; Mm00441818_m1). PCR was performed on an ABI PRISM 7900HT Sequence Detection system (Applied Bio-systems). 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 ΔΔCT 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). We removed medium from the culture dishes and completed RNA extraction as described above. Differential RNA expression was analyzed through an array for mouse extracellular matrix and adhesion molecule gene expression by SuperArray using a GEArray Q Series array by the manufacturer.

ELISA
We purchased quantikine mouse ELISA kits for detection of secreted pro-MMP-9 and TIMP-1 from cell culture supernatants from R & D Systems and followed the manufacturer’s protocol. Briefly, standard dilutions, internal controls, and sample dilutions were incubated in the appropriate precoated 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. We read the well absorbances 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) was used for concentration calculations from the standard curve.

Statistical analysis
Data were analyzed using InStat software version 3.05 (GraphPad Software). Changes in percentages of CD11c⁺ and Gr-1⁺ cells in the atRA and receptor antagonist full culture experiments were determined using 1-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. We made comparisons between 2 groups using Student’s unpaired t test, except for ELISA experiments where we employed the nonparametric Mann-Whitney test. Data are presented as means ± SEM and differences between groups of P < 0.05 were considered significant.

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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 (11). However, supplementation of medium with physiological levels of atRA restored DC development (11). 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 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 increased from 10% with the CD-FBS negative control to 60% with the addition of 1 nmol/L atRA (P < 0.05; Fig. 1A). When floating and adherent cells were pooled together, the percentage of Gr-1+ neutrophils increased slightly with 10 nmol/L receptor antagonist treatment, whereas 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-1+ neutrophils increased from 10% with CD-FBS supplementation to 50% with the 1 nmol/L atRA treatment (P < 0.05; Fig. 1B).

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 nonadherent 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 8 d. 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 an increase (P < 0.0001) in floating cell yield in atRA-rescued cultures compared with those that remained with the receptor antagonist (Fig. 2A). The percentage of cells that remained adherent decreased by 10% with atRA treatment and the number of floating cells increased (P < 0.01) compared with cultures that received fresh medium and GM-CSF but remained in the receptor antagonist treatment (Fig. 2B).

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-1+ neutrophils in the adherent cells in both treatment groups (Fig. 3A,B). However, atRA-rescued cell cultures showed a greater percentage (P < 0.05) and yield (P < 0.001) of CD11c+ floating DC compared with cells retained in culture with receptor antagonist (Fig. 3A,C). Conversely, Gr-1+ neutrophils predominated in the floating cell population for both treatment groups and the percentage of Gr-1+ floating cells was not affected by atRA rescue (Fig. 3B). However, total Gr-1+ neutrophil
yield was significantly enhanced in the floating cell population when cells were cultured with atRA for the final 2 d of culture as opposed to remaining with receptor antagonist (Fig. 3D).

**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 had decreased \( P < 0.0001 \) levels of the cell surface marker CD11a (Itgal), measured by mean fluorescence intensity, compared with cells that remained in receptor antagonist treatment for the full 10-d culture period (Fig. 4A, 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**

Quantitative real-time PCR was used to determine the level of gene expression in 2 independent time course experiments. We found that levels of Itgal transcript decreased over time in both the atRA and receptor antagonist treatments, so that 24 h post-atRA rescue, both treatments had the same level of Itgal transcript (data not shown). However, atRA rescue treatment increased the expression of Mmp-9 transcript compared with the receptor antagonist treatment by 3-fold within 4 h and continued to increase up to 14-fold greater than the 0-h level by 24 h of treatment (Fig. 5A), whereas levels of Timp-1 transcript, an endogenous inhibitor of MMP-9 activity, remained constant for both treatments throughout the time course (data not shown). These results confirm those of a microarray study for mouse extracellular matrix and adhesion molecule gene expression using a GEArray Q Series array by SuperArray. We chose to focus on the effects of atRA on Mmp-9, because the gene expression array showed a 1.1-fold increase in expression over receptor antagonist-treated cells for both adherent and CD11c+ floating cells, indicating that these differences in gene expression were not due solely to the adherent or floating phenotype (Supplemental Table 1). Mmp-7 was the only other metalloproteinase that appeared to be retinoic acid regulated. However, actual levels of expression were barely above background [up to 0.5 arbitrary units (AU)] for this gene, whereas atRA upregulated Mmp-9 expression to 6 AU (data not shown). Mmp-3, -8, -12, -13, and -19 were also highly expressed in these samples (from 8–20 AU; data not shown), but their expression was unchanged by atRA (Supplemental Table 1). These results suggest that upregulation of matrix proteases may play a role in the decrease in DC adhesion mediated by atRA.

We next used ELISA to determine whether MMP-9 and TIMP-1 protein production increased in conjunction with mRNA expression. Treatment with atRA significantly increased 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 \( P < 0.05 \) to 26 μg/L compared with 4 μg/L in the receptor antagonist group on d 10 (Fig. 5B). At the same time, levels of secreted TIMP-1 increased to 4 μg/L in both receptor antagonist and atRA-rescued treatment groups (data not shown). The molar ratio of pro-MMP-9:TIMP-1 indicate that there was a 2:1 molar excess of pro-MMP-9 on d 10 of culture in the atRA-treated group (48 h after rescue), while those cultures that retained treatment with the receptor antagonist showed a 4:1 molar excess of TIMP-1 (Fig. 5C).

**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 (10). One of the vitamin A-dependent APC has been identified as myeloid DC (11). 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 with 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 did 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 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 with 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 whereas 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-β2, to form the cell surface molecule lymphocyte function-associated antigen (LFA)-1 (28). 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 was 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. (29) 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 laboratory 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 (data not shown). 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. Because 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 upregulated 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 (30), and galectin-3, which interacts with the extracellular matrix (31). MMP-9 has also been shown to proteolytically activate a number of cytokines that are secreted in latent forms, including IL-1β (32) and transforming growth factor-β (33).

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 (34) 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 proenzyme that must be cleaved for full activity to be realized (35). 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 (36). MMP-9 has also been shown to be critical for DC migration (37). In MMP-9−/− mice, recruitment of bronchial-associated lymphoid tissue DC into the airway lumen in response to allergen (38) and migration of cultured bone marrow DC through tracheal epithelial tight junctions is impaired (39). Prostaglandin E2-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 (40).

Retinoic acid has been previously linked with negative regulation of MMP-9 levels in diabetic skin cultures (41,42), tumor cell invasion (43,44), bronchoalveolar lavage cells (45), and emphysema (46). Our data directly contradict these findings, because we show that atRA rescue upregulates the expression of the MMP-9 gene and secreted proenzyme in myeloid DC cultures compared with cultures treated with receptor antagonist. However, these previous studies all used pharmacological doses of atRA (≥1 μmol/L for cell cultures), whereas 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 (47) 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. (48) showed through chromatin immunoprecipitation studies that atRA acts through RARα on the Mmp-9 promoter to upregulate gene expression during involution, despite the lack of a retinoic acid response element 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 (49). Recently, Darmanin et al. (50) have shown that immature DC cultured with pharmacological levels of supplemental atRA (1 μmol/L) have increased migratory ability in vitro and when injected into tumors, compared with that of DC cultured in medium containing physiological concentrations of atRA, and that this migration is greatly diminished by inhibiting MMP. Our findings that atRA rescue treatment upregulated 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. (50).

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 upregulation of MMP-9 and a decrease in cell surface expression of CD11a. Further studies are needed to verify whether MMP-9, which is upregulated 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 MMP 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 (37). Our results indicate that physiological concentrations of atRA may be necessary for optimal DC development and release from or migration through the extra-cellular matrix.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Literature Cited


FIGURE 1.
Mouse bone marrow cells [CD11c1 (A), Gr-11 (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 1 SEM, n = 5 (floating cells) or 3 (all cells). Within a cell type, means without a common letter differ, P < 0.05.
FIGURE 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 ± SEM, n = 10. Asterisks indicate different from the corresponding receptor antagonist treatment: **P < 0.01, ***P < 0.0001.
FIGURE 3.
The mouse bone marrow-derived floating myeloid DC population is enhanced by atRA rescue [Percent (A,B) and absolute (C,D) yields of CD11c1 (A,C) and Gr-11 (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 ±SEM, n = 6. Asterisks indicate different from the corresponding receptor antagonist treatment:*P < 0.05, **P < 0.01, or ***P < 0.0001.
FIGURE 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 ± SEM, n = 6. ***Different from receptor antagonist treatment, P < 0.0001.
FIGURE 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 ± SEM, n = 4. *Different from the receptor antagonist treatment on that day, P < 0.05.