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Vitamin A differentially regulates cytokine expression in respiratory epithelial and macrophage cell lines

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Abstract

Vitamin A is an essential nutrient for the protection of children from respiratory tract disease. Supplementation with vitamin A is frequently prescribed in the clinical setting, in part to combat deficiencies among children in developing countries, and in part to treat respiratory infections in clinical trials. This vitamin influences immune responses via multiple, and sometimes seemingly contradictory mechanisms. For example, in separate reports, vitamin A was shown to decrease Th17 T-cell activity by downregulating IL-6, and to promote B cell production of IgA by upregulating IL-6. To explain these apparent contradictions, we evaluated the effects of retinoic acid (RA), a key metabolite of vitamin A, on cell lines of respiratory tract epithelial cells (LETs) and macrophages (MACs). When triggered with LPS or Sendai virus, a mouse respiratory pathogen, these two cell lines experienced opposing influences of RA on IL-6. Both IL-6 protein production and transcript levels were downregulated by RA in LETs, but upregulated in MACs. RA also increased transcript levels of MCP-1, GM-CSF, and IL-10 in MACs, but not in LETs. Conversely, when LETs, but not MACs, were exposed to RA, there was an increase in transcripts for RAR β , an RA receptor with known inhibitory effects on cell metabolism. Results help explain past discrepancies in the literature by demonstrating that the effects of RA are cell target dependent, and suggest close attention be paid to cell-specific effects in clinical trials involving vitamin A supplements.

Keywords

Vitamin A; Epithelial cell; Macrophage; IL-6; Respiratory tract

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1. Introduction

Invasion of the airway by pathogens engages a variety of cell types. The airway lumen is encased by an epithelial cell layer, creating a physical barrier against bacterial, viral, and fungal pathogens. A pathogen must first permeate a formidable mucus membrane to infect its target. Innate immune cells (e.g. dendritic cells and macrophages) that reside within and below the epithelial layer are rapidly signaled by pathogens in the airway, and in turn trigger pathogen-specific B- and T-cell populations. Once triggered, these adaptive immune cells may durably reside in respiratory tract tissues. Protection against respiratory pathogens is imposed in part by B cell production of antigen-specific IgA antibodies, which are particularly well suited for transcytosis across the airway's epithelial barrier [1, 2].

Vitamin A is essential for a healthy immune response to respiratory tract pathogens. It circulates in the blood in the form of retinol, which upon cellular uptake can be converted to retinoic acid (RA), an active metabolite utilized by the immune system [3]. One mechanism by which RA affects gene expression is through functioning as a ligand for the heterodimeric retinoic acid receptor-retinoid X receptor (RAR-RXR) complex, which binds to promoters and regulates the expression of target genes.

We have previously shown that the enzyme required for RA metabolism is constitutively expressed by epithelial cells in the respiratory tract [4]. We have also shown that an RA precursor (retinol or retinyl palmitate) can be administered intranasally to promote B cell production of antigen-specific IgA toward a respiratory virus pathogen [5]. Additionally, retinol promotes IgA expression *in vitro* when splenocyte B cells are stimulated in the presence of a respiratory epithelial cell line (LETs), in an IL-6 dependent manner [4]. Despite its clear positive influences on IgA induction, vitamin A is commonly reported to have anti-inflammatory properties, and has specifically been shown to negatively regulate IL-6 [6]. In an effort to address these apparent contradictions and to provide insight into RA effects on immune signaling, we designed experiments to test the effects of RA on two different cell types, a respiratory tract epithelial cell line (LETs) and a macrophage cell line (MAC INF429, MACs). Results demonstrate very different consequences of exposing these two cell lines to RA.

2. Materials and Methods

2.1 Cell lines and treatments

MACs were kindly provided by Dr. W.S. Walker. They derived from mouse splenocytes, as previously described [7]. Briefly, spleens were disrupted with a tissue homogenizer to create a single cell suspension. Cells were plated in soft agar in the presence of colony stimulating factor 1-containing medium from a mouse bone marrow cell line, LADMAC. After approximately 10–14 days, colonies were picked and transferred into cultures with liquid medium for expansion in the presence of microcarrier beads. Immortalized cells were identified after several months. For the experiments described here, MACs were cultured in complete tumor medium [CTM; consists of Modified Eagles Medium (Invitrogen, Grand Island, NY) with dextrose (500 µg/mL), glutamine (2 mM), 2-mercaptoethanol (30 µM), essential and non-essential amino acids, sodium pyruvate, sodium bicarbonate and

antibiotics] containing 10% heat-inactivated fetal bovine serum (FBS) and 20% conditioned medium from the cell line LADMAC at 37°C with 10% CO₂.

LET1 cells (LETs) were kindly provided by Dr. C.M. Rosenberger. They were type I alveolar epithelial cells derived from the lung of a C57BL/6 mouse, as previously described [8]. Briefly, lung cells were isolated using a modified dispase-agarose protocol. Lung epithelial cells were cultured for 5 days and then immortalized by transduction with MSCV-SV40 large T antigen. A majority of cells expressed high levels of the type I epithelial cell lineage marker T1 α . T1 α ⁻ cells were removed by FACS sorting. For the experiments described here, LET cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10 % heat-inactivated FBS, 2 mM L-glutamine and 5 ug/mL gentamycin at 37°C with 5% CO₂ and passaged minimally (<20 passages) before usage. Where indicated, cells were treated with 1 uM RA (Sigma; Cat# RA2625), 1 ug/mL lipopolysaccharide (LPS) from *Salmonella typhosa* (Sigma; L6386), and/or Sendai virus (SeV; Enders strain) at an MOI of 10.

2.2 Cytokine protein analyses

Cells were plated at 2x10⁴ cells/well in a flat-bottomed 96-well plate (overnight, 37°C). Cells were washed, and CTM with test and control variables (RA, LPS and/or SeV) was added for incubation (37°C, 48 hours), after which supernatants were harvested. ELISA plates were coated with 1 ug/ml purified mouse anti-IL-6 antibody (eBiosciences; cat. no. 14-7061; overnight, 4°C), washed 3X with PBS and blocked with 1% BSA in PBS (1 hour). Block was removed and 50 ul/well of culture supernatant (1:10) was added for incubation (overnight, 4°C). Plates were washed 3X with 0.5% Tween in PBS and 50 ul/well of mIL-6 biotin-conjugated antibody (1:1000, eBiosciences; cat. no. 13-7062) was added (3 hours, room temperature). Plates were washed 3X with 0.5% Tween in PBS and 50 ul/well of Streptavidin-Alkaline Phosphatase (1:2000, Southern Biotech; cat. no. 7100-04) was added (1 hour, room temperature). Plates were washed 3X with 0.5% Tween in PBS and 4-Nitrophenyl phosphate disodium salt hexahydrate [pNPP (Sigma; cat. no. 2640)] in 5% Diethanolamine (Sigma; cat. no. D8885) was added (150 ul/well). Plates were developed and read on a Molecular Devices Precision Microplate Reader.

2.3 Real-time PCR

LETs were plated at 1x10⁵ cells/well in a 12-well dish and grown to confluency (~2–3 days, 37° C). MACs were plated at 4x10⁵ cells/well in a 12-well dish (overnight, 37° C). Cells were pretreated with RA (1uM) for 1 hour prior to stimulation with LPS (1ug/mL) or SeV (MOI 10). After 1, 2, and 4 hours for LETs and 2, 4, and 6 hours for MACs, cells were lysed in the plate and RNA was isolated (Qiagen RNeasy Mini Kit, Cat#74104). cDNA was produced using a Superscript III First-Strand Synthesis Kit (Life Technologies; cat. no. 18080-051) with oligo dT. Transcript levels were evaluated by qPCR on an Applied Biosystems 7300 instrument using SYBR green ROX (Qiagen; Cat# 330520). Gene expression was normalized to GAPDH. Primers used included: GAPDH (5'-ccaggtgtctcctcgactt-3', 5'-cctgttgctgtagccgtattca-3'); IL-6 (Qiagen; Cat# PPM03015A); IL-6 pre-transcript (5'-cagaacacgccacaagaaaa-3', 5'-ggaaattgggtaggaagga-3'); IL-10 (5'-ccaggagatcctttgatga-3', 5'-cattccagaggaattgcacat-3'); GMCSF (5'-

ctgtcacgttgaatgaagaggtag-3', 5'-agctggctgtcatgttcaagg-3'); MCP-1 (5'-cccaatgagtaggctggaga-3', 5'-gctgaagaccttagggcaga-3'); C/EBP β (5'-caagctgagcgacgagtaca-3', 5'-cagctgtccaccttctct-3'); I κ B α (5'-ctcacggaggacggagactc-3', 5'-ctcttcgtggatgattgcca-3'); RIG-I (5'-tggttgcccttcttctta-3', 5'-agcactgttccttcctgaa-3'); RAR α (5'-ctcatctgtggagaccgaca-3', 5'-cctgggatctccatcttcaa-3'), RAR β (5'-gaaacaggccttctcagtgc-3', 5'-atgagagtggtgcatgatcc-3') and RAR γ (5'-gggcaagtacaccacgaact-3', 5'-gctgag ccctgtaaaaccag-3'). For mature mRNA analyses, when possible, primers were designed to span an intron. Primers not spanning an intron include: C/EBP β , RIG-I and IL-10.

3. Theory

Vitamin A can exert contrasting and cell-type-specific influences on cytokine expression.

4. Results

4.1 Opposing influences of RA on IL-6 production in two cell lines

To explain the seemingly paradoxical effects of RA on immune cell signaling, we investigated the effects of RA on IL-6 protein production in an epithelial cell line (LETs) and a macrophage cell line (MACs) after stimulation with LPS or SeV (a virus known to infect respiratory epithelial cells), in the presence or absence of RA. After 48 hours, supernatants were tested by ELISA for IL-6. We found that RA had a negative effect on IL-6 production for LPS-treated LETs and little effect on SeV-infected LETs (Figure 1A). Surprisingly, we found the exact opposite effect of RA on MACs. In this case, RA enhanced IL-6 production in response to both stimuli (Figure 1B).

We next asked if IL-6 was regulated by RA at the transcriptional level. LETs were examined over a 4 hour time course following LPS or SeV activation. In both cases, there was a rapid burst of IL-6 transcription that peaked at 1–2 hours and waned by 4 hours post-stimulation (Figure 1C and 1E). Regardless of the stimulant, RA inhibited IL-6 transcription. When MACs were similarly tested, transcript levels increased gradually throughout a 6 hour stimulation period. Unlike our observation in LETs, RA treatment further increased IL-6 transcript levels in LPS (Figure 1D) and SeV (Figure 1F) stimulated MACs throughout the time course.

To determine if the RA-mediated increase in IL-6 transcripts in MACs reflected an enhancement of transcription and/or stabilization of processed mRNA, we examined unspliced, premature IL-6 transcripts using primers spanning an intron/exon splice site. In LPS-stimulated LETs, premature (Figure 1G) and total transcript levels exhibited similar kinetics, and each was reduced by RA. However, in LPS-stimulated MACs, RA differentially increased premature (Figure 1H) and total transcript levels with the latter exceeding the former in upregulation. These results suggest that RA upregulates IL-6 in MACs by increasing transcription and by stabilizing mature transcripts.

4.2 RA effects on transcription of additional cytokines

Cells were also tested for cytokine transcripts including MCP-1, GM-CSF and IL-10. SeV did not reliably stimulate transcription of these cytokine genes in MACs, so we focused on LPS-mediated responses. As observed with IL-6, there was a rapid increase in MCP-1 and GM-CSF transcript levels 1–2 hours following LPS stimulation of LETs, which dropped to near baseline levels within 4 hours (Figure 2A and 2C). No change in IL-10 transcript levels was detected in response to LPS stimulation (Figure 2E). RA had no effect on these transcript levels in LETs. In contrast, RA was a consistent positive regulator of cytokine expression in MACs. LPS stimulated a moderate increase in MCP-1 (Figure 2B), GMCSF (Figure 2D) and IL-10 (Figure 2F) transcript levels in MACs that was consistently enhanced by RA. RA had the most profound effect on IL-10 expression, increasing transcripts approximately 10-fold after 2 hours.

4.3 RA effects on known signaling pathways and RAR

To understand how RA differentially affected cytokine expression, we investigated genes known to regulate IL-6, including CCAAT/Enhancer-Binding Protein Beta (C/EBP β), NF-Kappa-B Inhibitor Alpha (I κ B α), a transcriptional marker of NF- κ B pathway activation, and retinoic Acid-Inducible Gene I (RIG-I), which is known to be induced by LPS. Despite a previous report that C/EBP β expression is regulated by RA [9], we observed only a slight increase in C/EBP β transcripts in response to LPS stimulation in either cell line, which was unaffected by RA (Supplementary Figure 1A–B). LPS increased I κ B α transcript levels within 1–2 hours of stimulation in both LETs (Supplementary Figure 1C) and MACs (Supplementary Figure 1D), but only increased RIG-I transcript levels in MACs (Supplementary Figure 1E–F). Neither I κ B α nor RIG-I transcripts were affected by RA. Finally, we analyzed transcription of nuclear receptors known to be involved in RA signaling, including RAR α , RAR β and RAR γ [10]. Neither LPS stimulation nor RA treatment had a measurable influence on RAR α (Supplemental Figure 2A–B) or RAR γ (Supplemental Figure 2C–D) transcripts in either LETs or MACs. However, LPS did increase RAR β transcript levels in LETs (Figure 2G), but not MACs (Figure 2H). Interestingly, RA substantially increased RAR β transcripts in LETs. This was most notable at the 4 hour time point, when levels remained high in the presence of RA, but dropped below baseline in control cultures (Figure 2G). RA had no influence on RAR β transcript levels in MACs (Figure 2H).

5. Discussion

Here we show that RA can have opposing effects on cytokine expression in different cell types. Specifically, we found that RA increased transcript levels for IL-6 (by upregulation of transcription and stabilization of mature transcripts), MCP-1, GMCSF, and IL-10 in MACs, but not in LETs. In contrast, transcript levels for RAR β were increased in LETs, but not in MACs. RAR β is known to have suppressor properties and to promote apoptosis [11], possibly explaining the negative regulation of IL-6 by RA in epithelial cells [12]. Overall, the fact that RA enhanced cytokine expression in MACs argues against the simple dogma commonly presented in the literature that vitamin A mitigates inflammation [6]. Data demonstrate that the outcomes of RA signaling are diverse and contextual.

Cytokine transcript and protein expression patterns by the two cell lines, one macrophage cell line and one epithelial cell line, were differentially influenced by RA in this study. These cell-specific results were reminiscent of a previous study of two human cell lines, one myeloid (NB4), and one alveolar epithelial (A549) [13]. Each cell was examined for MCP-1 expression in the presence of RA. RA substantially increased (100-fold) MCP-1 expression in the myeloid line, but not in the epithelial cells. Based on combined findings, we now propose a working hypothesis. We propose that RA may assist pathogen clearance in the respiratory tract in at least two ways: (i) by upregulating cytokine production by antigen presenting cells (e.g. macrophages) to promote virus-specific IgA, and (ii) by instructing a conservative transcriptional program and senescence in epithelial cells, thereby preventing amplification of pathogen.

As mentioned previously, vitamin A supplementation is administered to infants in developing countries, often with routine vaccinations. It is generally accepted that supplementations are beneficial, but this is not always the case [14, 15]. Perhaps conflicting clinical results can be explained, at least in part, by the differential effects of RA on target cells, as the composition of cell populations engaged by an invading pathogen can be variable and context-specific.

6. Conclusion

In conclusion, we have described contrasting and cell-line-specific influences of RA on cytokine expression. Results encourage (i) further analyses of mechanisms by which RA may up- or down-regulate cytokines, and (ii) attention to the potential opposing effects of vitamin A in the design of future clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

LETs	Respiratory tract epithelial cell line LET1
MACs	macrophage cell line MAC INF429
SeV	Sendai virus
LPS	lipopolysaccharide
RA	retinoic acid
RAR	retinoic acid receptor

RXR	retinoid X receptor
RARE	retinoic acid response element

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Figure 1. Retinoic acid has opposite effects on IL-6 expression in epithelial cells and macrophages

LET (A) and MAC (B) cells were treated with 1 μ M RA or nothing and then stimulated with 1 μ g/mL LPS or infected with SeV (MOI 10). After 48 hours of incubation with indicated components, supernatants were harvested and the amount of secreted IL-6 was determined by ELISA. LET (C, E, G) and MAC (D, F, H) cells were treated with 1 μ M RA or nothing and then stimulated with 1 μ g/mL LPS (C–D, G–H) or infected with SeV (E–F) at an MOI of 10. RNA was collected at indicated times points, converted to cDNA and evaluated by qPCR for total (C–F) and unprocessed (G–H; “premature”) IL-6 transcripts. All gene expression was normalized to GAPDH and fold-change was calculated relative to untreated cells.

Figure 2. Retinoic acid differentially affects transcripts for additional cytokines and the apoptosis-associated nuclear receptor RARβ in a cell-specific manner

LETs (A, C, E, G) and MACs (B, D, F, H) were treated with 1 μ M RA or nothing and then stimulated with 1 μ g/mL LPS. RNA was collected at indicated time points, converted to cDNA and evaluated by qPCR for MCP-1 (A–B), GMCSF (C–D), IL-10 (E–F) and RARβ (G–H) mRNA levels. All gene expression was normalized to GAPDH and fold-change was calculated relative to untreated cells. Note: GMCSF was undetectable in untreated MAC cells, so absolute mRNA levels relative to GAPDH are shown.