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Short communication

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, GMCSF, 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 RARβ, 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.

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

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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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% CO_2.

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 T1α. T1α$^-$ 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 μg/mL gentamycin at 37 °C with 5% CO_2 and passaged minimally (<20 passages) before usage. Where indicated, cells were treated with 1 μM RA (Sigma; Cat# RA2625), 1 μg/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 2×10^4 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 h), after which supernatants were harvested. ELISA plates were coated with 1 μg/ml purified anti-mouse IL-6 antibody (eBiosciences; cat. no. 14-7061; overnight, 4 °C), washed 3× with PBS and blocked with 1% BSA in PBS (1 h). Block was removed and 50 μl/well of culture supernatant (1:10) was added for incubation (overnight, 4 °C). Plates were washed 3× with 0.5% Tween in PBS and 50 μl/well of anti-mouse IL-6 biotin-conjugated antibody (1:1000, eBiosciences; cat. no. 13-7062) was added (3 h, room temperature). Plates were washed 3× with 0.5% Tween in PBS and 50 μl/well of Streptavidin-Alkaline Phosphatase (1:2000, Southern Biotech; cat. no. 7100-04) was added (1 h, room temperature). Plates were washed 3× 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 μl/well). Plates were developed and read on a Molecular Devices Precision Microplate Reader.

2.3. Real-time PCR

LETs were plated at 1×10^5 cells/well in a 12-well dish and grown to confluency (~2–3 days, 37 °C). MACs were plated at 4×10^5 cells/well in a 12-well dish (overnight, 37 °C). Cells were pretreated with RA (1 μM) for 1 h prior to stimulation with LPS (1 μg/mL) or SeV (MOI 10). After 1, 2, and 4 h for LETs and 2, 4, and 6 h 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′-ccaggttgtctcctgcgactt-3′, 5′-cctgttgctgtagccgtattca-3′); IL-6 (Qiagen; Cat# PPM03015A); IL-6 pre-transcript (5′-cagaacacgccacaagaaaa-3′, 5′-ggaaattggggtaggaagga-3′); IL-10 (5′-ccagggagatcctttgatga-3′, 5′-cattcccagaggaattgcat-3′); GMCSF (5′-ctgtcacgttgaatgaagaggtag-3′, 5′-agctggctgtcatgttcaagg-3′); MCP-1 (5′-cccaatgagtaggctggaga-3′, 5′-gctgaagaccttagggcaga-3′); C/EBPβ (5′-caagctgagcgacgagtaca-3′, 5′-cagctgctccaccttcttct-3′); IκBα (5′-ctcacggaggacggagactc-3′, 5′-ctcttcgtggatgattgcca-3′); RIG-I (5′-tggcttgccctttcttctta-3′, 5′-agcactgttccttccctgaa-3′); RARα (5′-ctcatctgtggagaccgaca-3′, 5′-cctgggatctccatcttcaa-3′), RARβ (5′-gaaacaggccttctcagtgc-3′, 5′-atgagaggtggcattgatcc-3′) and RARγ (5′-gggcaagtacaccacgaact-3′, 5′-gctgagccctgtaaaaccag-3′). For mature mRNA analyses, when possible, primers were designed to span an intron. Primers not spanning an intron include: C/EBPβ, 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 h, 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 (Fig. 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 (Fig. 1B).

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