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Retinoids inhibit measles virus in vitro via nuclear retinoid receptor signaling pathways

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ABSTRACT

Measles virus (MV) infects 30 million children every year, resulting in more than half a million deaths. Vitamin A (retinol) treatment of acute measles can reduce measles-associated mortality by 50–80%. We sought to determine whether or not retinoids can act directly to limit MV output from infected cells. Physiologic concentrations of retinol were found to inhibit MV output in PBMC and a range of cell lines of epithelial and endothelial origin (40–50%). Near complete inhibition of viral output was achieved in some cells/lines treated with all-*trans* retinoic acid (ATRA) and 9-*cis* RA (9cRA). Important attenuation of the anti-MV effect of retinoids in R4 cells, a subclone of a retinoid-responsive cell line (NB4) deficient in RAR signaling, demonstrates that this effect is mediated at least in part by nuclear retinoid receptor signaling pathways. Inhibition of MV replication could not be fully explained as a result of retinoid effects on cell differentiation, proliferation or viability, particularly at low retinoid concentrations (1–10 nM). These data provide the first evidence that retinoids can directly inhibit MV in vitro, and raise the possibility that retinoids may have similar actions in vivo.

1. Introduction

Measles remains a major cause of mortality and morbidity in developing world children despite the availability of live attenuated vaccines for more than 30 years. It is estimated that MV infects 30 million individuals a year resulting in 0.5–1 million deaths (WHO, 2006). Measles has recently revisited several industrialized nations following spurious allegations of adverse events and decreased vaccine uptake. High dose retinol (vitamin A) is currently the therapy of choice for severe measles (Chabot and Ward, 2002; Joint WHO/UNICEF statement, 1987). Indirect effects of vitamin A on immune function and epithelial repair with prevention of secondary infections have long been assumed to underlie this beneficial effect. Although vitamin A has pleomorphic effects including actions on immune and epithelial cells (Semba, 1999; Stephensen, 2001), little is known about the molecular mechanisms that underlie the clinical effectiveness of vitamin A in measles infection.

Vitamin A is a fat-soluble vitamin obtained from the diet (meat, fish, eggs) that plays a critical role in vertebrate embryogenesis, cellular differentiation, activation, apoptosis, vision and the maintenance of epithelial integrity (Mark et al., 2006). Vitamin A (retinol or ROH) and its synthetic and natural derivatives such as all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9cRA) are collectively referred to as retinoids. Vitamin A is stored as retinyl esters in the liver and transported to target tissues as ROH where conversion to active metabolites occurs (e.g., ATRA, 9cRA) (Napoli, 1999). Although nuclear receptor-independent effects have been observed in the retina (Wolf, 2004) retinoids exert most of their known biological effects via nuclear retinoid receptor signaling. In this pathway, high affinity retinol metabolites such as ATRA and 9cRA bind directly to two families of nuclear receptors: retinoic acid receptors (RAR) and retinoid X receptors (RXR). Once bound by ligand, RAR/RXR heterodimers act as transcription factors for retinoid-responsive genes. The protein products of retinoid-responsive genes are responsible for exerting the effects of retinoids in the cell.

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Although peripheral blood mononuclear cells (PBMC) and a number of common human cell lines were included in this study, the NB4/R4 model was the principal tool used to investigate the role of retinoid receptors in regulating measles virus (MV) growth. NB4 are acute promyelocytic leukemia (APL) cells that contain reciprocal translocations between the RARα gene on chromosome 17 and the promyelocytic leukemia (PML) gene on chromosome 15, resulting in a PML–RARα fusion protein. The RARα component of the fusion protein retains its ligand and DNA-binding domains and can competently mediate signaling at higher concentrations of ATRA (Melnick and Licht, 1999). NB4-MR4 (R4) cells are a subclone of NB4 with a point mutation in the PML–RARα fusion molecule that confers a high level of resistance to retinoids (Shao et al., 1997). The mechanisms of response and resistance to retinoids in NB4 cells and its resistant subclones have been extensively characterized, making these cells a useful model to study retinoid effects on MV.

The experiments described in this manuscript test the hypotheses that retinoids can act against MV in vitro and that activation of nuclear retinoid receptors by their high affinity ligands contributes to the regulation of MV replication. We demonstrate that retinoids are active against MV in many cell lines and that this effect is mediated at least in part by nuclear retinoid receptor signaling pathways.

2. Materials and methods

2.1. Primary cells and cell lines

PBMC were isolated from healthy donors by discontinuous gradient centrifugation (Ficoll-Paque: GE Healthcare, Buckinghamshire, UK) and were activated with anti-CD3 (1 μg/mL). The large majority of PBMC (>80%) activated in this way and exposed to MV at a multiplicity of infection (MOI) of 1.0 express MV proteins within 48 h of infection (B.J. Ward, unpublished observation). Since wild-type MV targets the immune system and epithelial tissues throughout the body, we also assessed the anti-viral activity of retinoids in cell lines of immune (NB4/R4, U937, Jurkat), gastrointestinal (HCT8, T84, CaCo2) and respiratory origin (A549, BEAS-2B, Calu.1). Primary cells and cell lines were maintained in RPMI 1640 medium (Wisent, St-Bruno, QC) supplemented with 10% heat-inactivated fetal calf serum (FCS: Wisent: 56 °C for 30 min) and 10 mM Hepes buffer and 10 μg/mL gentamicin (cRPMI). All cultures were maintained at 37 °C in a 5% CO_2 humidified incubator.

2.2. MV strains and infections

Three MV strains were used: Chicago-1 (CHI-1) is a tissue culture-adapted genotype D3 strain originally isolated in 1989 (courtesy of W Bellini, CDC, Atlanta, GA), Bilthoven (BILT) is a low passage, genotype C2 wild-type strain originally isolated in 1991 (courtesy A Osterhaus, Rotterdam, Netherlands) and Connaught (CN) is a Moraten-like, vaccine strain attenuated by growth in chick embryo fibroblasts (courtesy R Wittes, Connaught Laboratories, Willowdale, ON). Unless otherwise indicated, cells were infected with the Chicago-1 strain virus at an MOI of 1.0 for 90 min at 37 °C. Following infection, cells were washed and resuspended at a density of 1×10^6 cells/mL in cRPMI containing a reduced concentration of FCS (2%), in a 24 well plate (1 mL/well). Unless otherwise indicated, infected cells were incubated for a period of 72 h in the presence of retinoids or retinoid agonists/antagonists. Ethanol or DMSO diluted in cRPMI was used as the mock control in all experiments. Supernatant MV at 72 h reflects the production of new virions rather than residual input virus, since unattached virions are removed by washing after infection.

2.3. Retinoids and nuclear receptor agonists/antagonists treatments

ROH, ATRA, 9cRA and arotinoic acid (4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid or TTNPB), a selective pan-RAR agonist, were purchased (Sigma–Aldrich Fine Chemicals, ON). A potent RXRα agonist (rexinoid or LGD1305), a RARα agonist (LGD030593) and RARα antagonist (RO 41-5253) were obtained from Ligand Pharmaceuticals Inc. (San Diego, CA) and Roche Applied Science (Laval, QC) respectively. Retinoid stock solutions of 10^{-2} M were prepared in 100% ethanol or 100% DMSO and further dilutions were performed using Hanks buffered salt solution (HBSS) or RPMI. All retinoids were stored in opaque eppendorf tubes at −80 °C. MV-infected cells were treated with various concentrations of retinoids or agonists/antagonists (0.1–1000 nM).

2.4. Viral output by plaque assay

The principle outcome for most experiments was MV output from infected cultures (i.e., replication efficiency) measured by plaque assay. Briefly, Vero cells were seeded in 24-well plates (BD Biosciences, Mississauga, ON) to obtain 90% confluency. Vero monolayers were infected in duplicate with 100 μL of serial 10-fold dilutions of culture supernatants for 90 min at 37 °C. Viral suspensions were then removed, a 16% methylcellulose overlay in Liebovitz's L-15 media (Gibco/Life Technologies, Grand Island, NY) was applied and plates were incubated at 37 °C in 5% CO_2 for 4 days. Neutral red (4%) was added for an additional 24 h and monolayers were fixed with 3.7% formalin for 10 min. Visible plaques were counted to determine the number of plaque forming units (pfu)/mL.

2.5. Propidium iodide staining

Cells were infected at an MOI of 0.1 for 90 min at 37 °C. Following infection, cells were washed and resuspended at a density of 1×10^5 cells/mL in cRPMI containing a reduced concentration of FCS (2%). The infection was carried out for 48 h, after which the number of apoptotic cells was counted as previously described (Nicoletti et al., 1991; Riccardi and Nicoletti, 2006). Briefly, cells were washed in a PBS/5% FBS/0.01 M NaN_3 solution and resuspended in a 50 μg/mL propidium iodide (Sigma–Aldrich Fine Chemicals, ON)/0.1% sodium citrate/0.1% Triton X-100 solution for 20 min at 4 °C. Cells undergoing apoptosis (i.e., sub-G0 DNA content) were measured by flow cytometry and are expressed as a % of total cells.

2.6. Cell proliferation by thymidine uptake

Both retinoids and MV can also have anti-proliferative effects in many cells/cell lines. To assess the potential anti-proliferative effects of retinoid treatments ±MV infection, cells were infected and/or treated with retinoids and seeded in triplicate in 96-well plates (25,000 cells/well) for 6 h. The wells were then treated with 1 μCi of ^{3}H-thymidine/well (ICN, Costa Mesa, CA) for 18 h. Following one freeze-thaw cycle, DNA was harvested onto glass-fiber filters, and thymidine incorporation was measured by scintillation counter (Wallac, Microbeta, Finland). The results are shown as mean counts per minute (CPM) of triplicate samples (±standard deviation).

2.7. Real-time PCR for RARβ mRNA expression

The induction of RARβ was used as a test of intact RARα signaling. Total RNA was harvested from NB4 cells by phenol–chloroform extraction and 5 μg was transcribed into cDNA. The primer

sequences used for real-time PCR were:

RARβ (sense): AGAGTTTGATGGAGTTGGGTGGAC;
RARβ (antisense): CATTCGGTTTGGGTCAATCCACTG;
β-actin (sense): TCATCACCATTGGCAATGAG;
β-actin (antisense): CACTGTGTTGGCGTACAGGT.

For each PCR reaction, a standard curve was generated by amplifying known amounts of an external standard (linearized plasmid DNA carrying a cloned RARβ sequence) and all reactions were normalized with β-actin. The level of expression of the NB4 untreated sample was used as the calibrator against which all other samples were compared. The PCR reaction mixture contained $MgCl_2$ 2 mM, 0.2 μM of each primer (final concentration) and Light Cycler DNA FastSTART DNA Master SYBR Green I® (Roche Diagnostics, Laval, Canada) (2 μL/20 μL reaction). Total PCR volume was 20 μL which included 2 μL of the reverse transcription reaction.

2.8. Expression of differentiation markers by flow cytometry

Expression of the myeloid differentiation marker CD11b was assessed by flow cytometry. Cells were collected and washed twice with HBSS before incubation at room temperature for 30 min with CD11b-PE (BD Pharmingen, ON). Appropriately labelled-IgG isotype (BD Pharmingen) was used as negative controls. Stained cells were analysed with an argon laser FACScan equipped with Consort 30 and Lysys II software (Becton Dickinson, Mississauga, ON); 15,000 events were collected per condition.

2.9. Selective index

NB4 cells were plated at 1×10^6 cells/mL and treated with increasing doses of ATRA, 9cRA or ROH for 48 h. The percentage of dead cells was determined by trypan blue exclusion in a hemacytometer (0.1% trypan blue). The 50% cytotoxic dose (CC50) of ATRA, 9cRA and ROH is expressed in M and was determined by non-linear regression using GraphPad Prism 5.0 software. These data represent the average of two separate experiments performed in duplicate. NB4 cells were infected with MV at an MOI of 0.1, treated with increasing doses of ATRA, 9cRA or ROH and titers were determined by plaque assay (described above). The 50% effective concentration (EC50) of retinoids against MV in NB4 cells is expressed in M and was calculated by non-linear regression and was calculated from four separate experiments conducted in duplicate. The selective index of the compounds is defined as SI = CC50/EC50.

2.10. Statistics

Statistical analyses were conducted using a matched one-way ANOVA and Dunnett's post-test.

3. Results

3.1. All cell lines, including NB4/R4 cells, support MV growth

In natural disease, MV has broad tissue tropism, infecting cells of immune origin (e.g., monocytes, follicular dendritic cells, lymphocytes) as well as the respiratory and gastrointestinal epithelia (Chabot and Ward, 2002). We tested a range of cell lines of immune, respiratory and gastrointestinal origin for their ability to support MV replication. MV replication was observed in anti-CD3-activated PBMC and all of the cell lines tested (range of MV output at 72 h $\sim 5 \times 10^2$–10^6 pfu/mL). Because of their well-characterized response to retinoids, the APL cell lines NB4 and R4 were used as the principal model in this study.

3.2. Retinoids have antiviral effects in most cells/cell lines

Vitamin A treatment in measles can reduce both mortality and morbidity (Huiming et al., 2005; D'Souza and D'Souza, 2002). We sought to determine whether vitamin A and other retinoids could exert an anti-viral effect against MV in infected cells in vitro. Reduced MV replication in response to retinol (ROH) was evident in PBMC (∼50% reduction) and in 7/10 of the ATRA-treated cell lines studied (range 5–95%) (there was no significant inhibition in Caco-2, A549 or R4 cells) (Table 1). Significant inhibition of viral output was evident in PBMC and several cell lines at concentration as low as 1 nM. Supplemental retinoids had no impact on the capacity of MV to replicate in the Vero cell monolayers that were used in plaque assays to

Table 1
The effect of retinoids on measles viral output in PBMC and cells lines of immunologic, respiratory and gastrointestinal origin

		N of[a]	PFU/mL[b] of control	ROH			ATRA			9cRA		
				% control	S.D.[c]	p value	% control	S.D.[c]	p value	% control	S.D.[c]	p value
Immune cells	Jurkat	5	209,238	58.9	36.4	NS[d]	14.1	11.9	<0.001	10.5	12.2	<0.001
	U937	5	157,125	39.1	38.4	<0.05	17.8	21.2	<0.001	10.9	26.4	<0.001
	NB4	4	39,603	128.4	111.2	NS[d]	28.1	15.8	<0.01	27.9	12.5	<0.01
	R4	4	3,971	180.6	148.3	NS[d]	114.8	27.3	NS[d]	238.1	231.6	NS[d]
	PBMC	5	11,820	55.2	15.3	<0.01	62.1	26.4	<0.05	61.5	15.4	<0.01
Gastrointestinal cells	T84	3	12,183	23.6	16.9	<0.05	10.7	3.3	<0.001	3.1	0.4	<0.01
	HCT8	3	18,650	35.2	11.9	<0.05	57.3	14.5	<0.05	22.5	11.8	<0.01
	Caco-2	3	29,550	91.2	6.1	NS[d]	78.7	9.3	NS[d]	78.7	9.7	NS[d]
Respiratory cells	Beas 2B	4	82,538	61.3	27.4	NS[d]	28.6	19.5	<0.01	41.3	26.1	<0.05
	Calu-1	6	45,750	53.5	19.0	<0.01	42.7	16.2	<0.01	62.1	57.7	NS[d]
	A549	3	24,033	89.1	14.4	NS[d]	66.0	25.5	NS[d]	68.6	13.4	NS[d]

Immune cells/cell lines included peripheral blood mononuclear cells from 5 healthy volunteers, U937 (Human promonocytic lymphoma cell: ATCC CRL 1593), Jurkat (Human T cell lymphoma cell: ATCC CRL 8163), NB4 (acute promyelocytic leukemia cell: courtesy M. Lanotte) and R4 (acute promyelocytic leukemia cell: courtesy W. Miller). Gastrointestinal cell lines included T84 (Human colon carcinoma cell: ATCC CCL 248), HCT8 (Human ileocecal adenocarcinoma cell: ATCC CCL 244) and Caco-2 (Human colon adenocarcinoma cell: ATCC HTB 37). Respiratory cell lines included Beas2B (Human bronchial epithelial cell: ATCC CRL 9609), Calu.1 (Respiratory epithelial cell: courtesy T. Bradley) and A549 (Human lung carcinoma cell: ATCC CCL 185).

[a] *N* represents the number of independent times each experiment was repeated.
[b] PFU/mL is the number of plaque-forming units per mL at 72 h.
[c] S.D. is the standard deviation obtained from the % control data.
[d] NS indicates data that were not significantly different from control.

Table 2
Selective index of retinoids in NB4 cells

	CC50 (M)	EC50 (M)	SI
ATRA	1.50×10^{-5}	3.56×10^{-10}	4.22×10^{4}
9cRA	1.60×10^{-4}	5.03×10^{-10}	3.18×10^{5}
ROH	5.09×10^{-5}	2.03×10^{-10}	2.51×10^{2}

quantify viral output, thus ensuring that any possible carry-over retinoids did not influence the results of our plaque assay (data not shown).

3.3. ATRA and 9cRA inhibit MV effectively

Although ROH is used for the treatment of acute measles, many of vitamin A's actions are mediated by metabolites such as ATRA and 9cRA that bind directly to nuclear retinoid receptors. When PBMC and cell lines were treated with ATRA or 9cRA or the synthetic agonist TTNPB (targeting RAR), the suppression of viral output was often greater than that observed with ROH (Table 1 and data not shown). At 1 nM, both ATRA and 9cRA reduced viral titers by 40–50% in PBMCs, indicating that inhibition of MV by retinoids is not restricted to immortalized cells lines and can also be observed in primary cells. Retinoid-induced inhibition of MV output was evident within 24 h of infection and was maintained throughout the 72 h period of infection (data not shown).

3.4. Nuclear receptors mediate anti-viral activity of retinoids

To determine whether or not the anti-viral effect of retinoids requires nuclear receptor signaling, we exploited the well-characterized NB4 and R4 cell lines that differ in their response to retinoids. As noted above, NB4 cells typically respond to ATRA and other RARα agonists at pharmacologic concentrations (1 μM) (Blomhoff et al., 1990; Zelent et al., 2001), while the NB4 subclone R4 is resistant to even pharmacologic doses of ATRA (Shao et al., 1997). In NB4 cells, treatment with ATRA and 9cRA inhibited viral output in a concentration-dependent manner (Fig. 1A) and inhibition was observed at even low physiological concentrations (physiologic concentration of ATRA is 5–10 nM) (Blomhoff et al., 1990). In contrast, treatment of R4 cells with retinoids did not result in any significant change in viral titer (Fig. 1B).

The inhibitory effect of retinoids in NB4 cells was determined to be a selective effect by determining the selective index. The selective indices for ATRA, 9cRA and ROH in NB4 cells were calculated as the ratio between the 50% cytotoxic concentration of the retinoids on the cells (CC50) and the 50% effective concentration against viral replication in NB4 cells (EC50) (SI = CC50/EC50). The SI values are all well above 10 (Table 2) and provide strong evidence that we are observing a selective effect. We also conducted an in vitro virucidal assay to ensure that our results are not due to a virucidal effect of retinoids on the virus. The virus was incubated with 1 μM of ATRA, 9cRA, ROH or the appropriate control for 1 h, and this virus was subsequently titered in Veros using a plaque assay. No difference in viral titer was observed when the virus was directly incubated with retinoids (data not shown), suggesting that the anti-viral effect of retinoids is in fact selective and due to an inhibition of viral replication in infected cells, and not the result of a direct virucidal effect.

TTNPB, a pan-RAR agonist with little RXR activity effectively suppressed MV output from NB4 cells, but had no anti-viral activity in the R4 cells (data not shown). Since NB4 and R4 cells do not express RARβ or RARγ constitutively (Shao et al., 1997), this inhibitory effect presumably acts through RARα signaling. To confirm that the inhibitory effect acts through RARα signaling, we

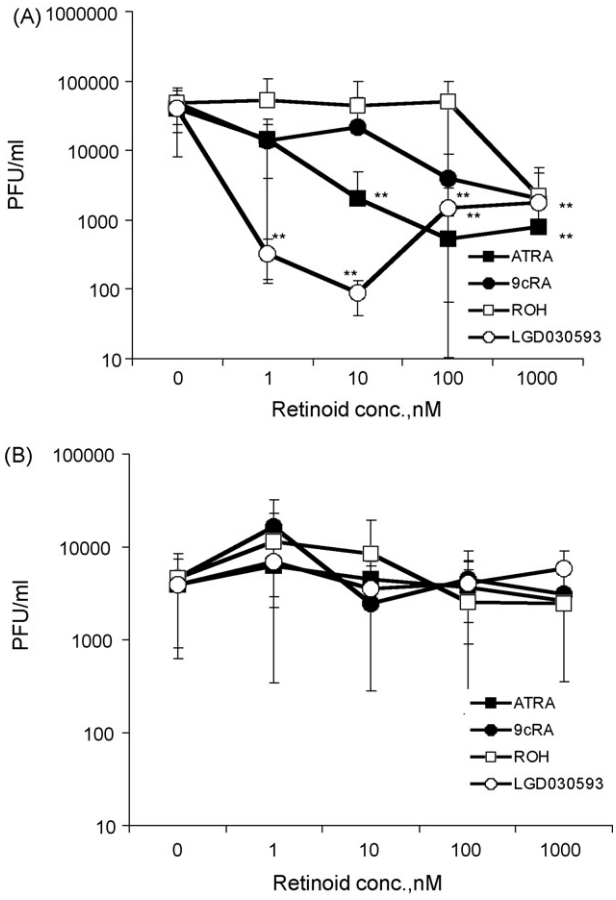

Fig. 1. Effect of retinoids on measles viral output in acute promyelocytic leukemia (APL) cells. NB4 (A) and R4 (B) cells were infected at an MOI of 0.1 for 48 h and treated with ATRA, 9cRA, ROH or an RARα agonist (LGD030593). Values are means ± standard deviation of four experiments. **<0.01.

tested the effect of LGD030593, a specific RARα agonist (Pettersson et al., 2002), in NB4 and R4 cells. We found complete inhibition of MV at all the doses tested in NB4 cells (>95% inhibition for all doses) and no significant inhibition in R4 cells (Fig. 1A and B). These observations suggest that RARα plays a critical role in regulating MV output in this model. The anti-viral role of RARα signaling in the NB4/R4 model was further addressed using RO 41-5253 (RO), a specific RARα antagonist that must be used in excess of ligand (Apfel et al., 1992). The addition of RO to MV-infected NB4 cells significantly reduced the inhibition of viral output mediated by ATRA and 9cRA (Fig. 2A). RO treatment completely reversed the modest inhibition induced by ROH, providing further evidence that RARα signaling is crucial for the anti-viral effect in NB4 cells. As expected, RO had no impact on viral output in either retinoid-treated or control R4 cells (Fig. 2B). Retinoid-induced inhibition of viral output could also be reversed by RO treatment of primary PBMCs (Fig. 2C).

3.5. Other nuclear receptors may also contribute to the anti-viral action of retinoids

Although both RARβ and RARγ are rapidly expressed in NB4 cells following ATRA stimulation, neither is constitutively expressed in these cell lines (Rosenauer et al., 1996; W.H. Miller, unpublished observation). The potential role of retinoid X receptors in limiting MV output was assessed using the RXR-selective agonist 1305. In both NB4 and R4 cells, viral output was inhibited by 1305 in a concentration-dependent manner (Fig. 3). However,

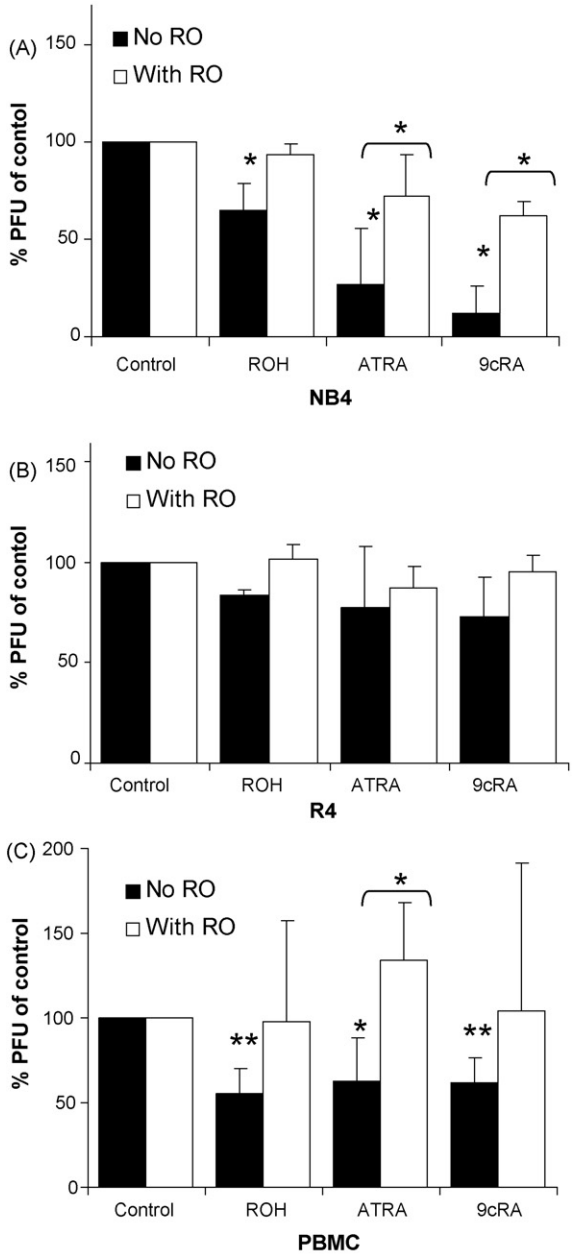

Fig. 2. RARα antagonist (RO 41-5253) blocks the inhibitory effect of RA on MV viral output. NB4 (A), R4 (B), and anti-CD3 activated PBMC (C) were treated with retinoids (ROH, 9cRA or ATRA) at a concentration of 1 nM for a period of 72 h with (with RO) or without (no RO) 1000 nM of the RARα-selective antagonist RO 41-5253. MV output results were standardized to percent of mock-treated controls. Mean PFU/mL values of controls (NB4 = 134,300, R4 = 93,250, PBMC = 11,820). Values are mean ± standard deviation of three separate experiments, except for fresh PBMC ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control values.

the degree of inhibition reached statistical significance only in NB4 cells. Although the RARα pathway is compromised in R4 cells, the RXR pathway is fully functional. Treating R4 cells with another RXR-specific ligand (LGD100153) at 1 μM for 24 and 72 h results in the up-regulation of myeloblastin and CD18 mRNA, two RXR-responsive genes (W.H. Miller Jr., unpublished observation). MV output was inhibited modestly by 1305 in PBMC and six out of eight of the other cell lines tested at 1 nM without obvious cytotoxicity (data not shown). This observation raises the possibility that RXR-dependent nuclear signaling pathways contribute modestly to anti-MV effects.

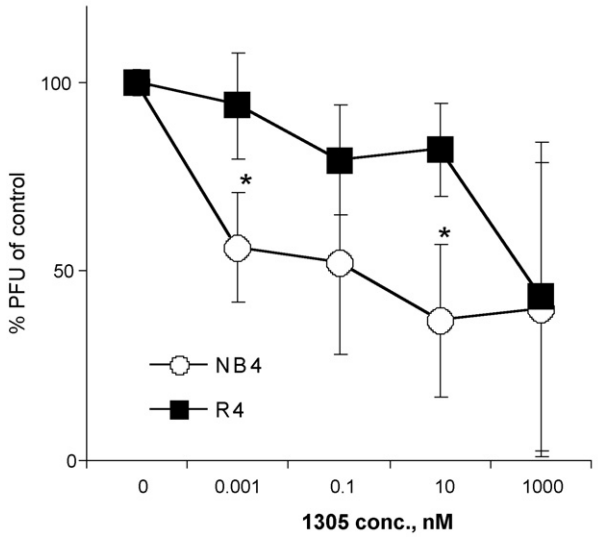

Fig. 3. Effect of RXR-selective agonist, LGD 1305 on MV output from NB4/R4 cells. The effect of LGD 1305, a selective agonist of RXR, on MV output in NB4/R4 cells after 72 h of incubation. PFU/mL of controls were 25,333 for NB4 cells and 33,875 for R4 cells. Values are mean ± standard deviation of three separate experiments ($^{*}p < 0.05$ vs. control values).

3.6. MV infection does not inhibit RARα signaling activity

Because our results suggested that signaling through the RARα nuclear receptor was important for the anti-viral effect of retinoids in vitro, we sought to determine whether MV infection itself has an impact on RARα-mediated transcriptional activation in NB4 cells. RARβ is often studied as a classic RARα-induced gene (de The et al., 1990); it is not expressed in resting NB4 cells but is rapidly induced following exposure to ATRA. Using real-time, RT-PCR, we found that ATRA treatment of NB4 cells up-regulated RARβ transcription independent of the presence of MV infection (Fig. 4). ATRA was unable to induce RARβ transcription in R4 cells as previously reported (Shao et al., 1997) (Fig. 4). MV infection by itself had no effect on RARβ transcription in either NB4 or R4 cells. This observation suggests that MV infection does not have a general negative effect on transcriptional activity mediated by RARα signaling.

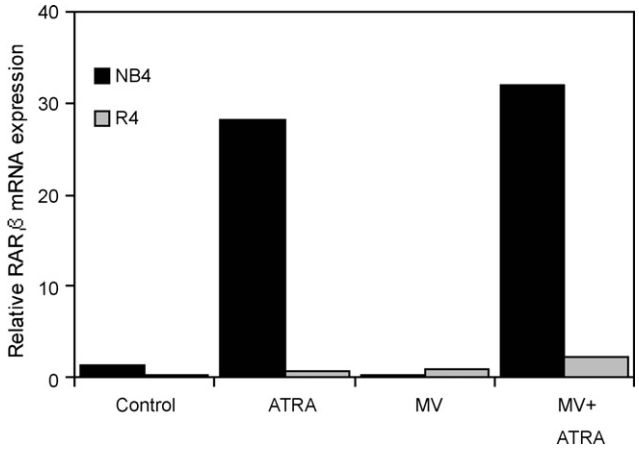

Fig. 4. RARα-dependent transcription is not affected by MV infection. Real time PCR was carried out in triplicate to detect RARβ transcripts at 48 h in NB4 and R4 cells infected with MV with or without supplemental ATRA (10 nM). βactin was used as a control.

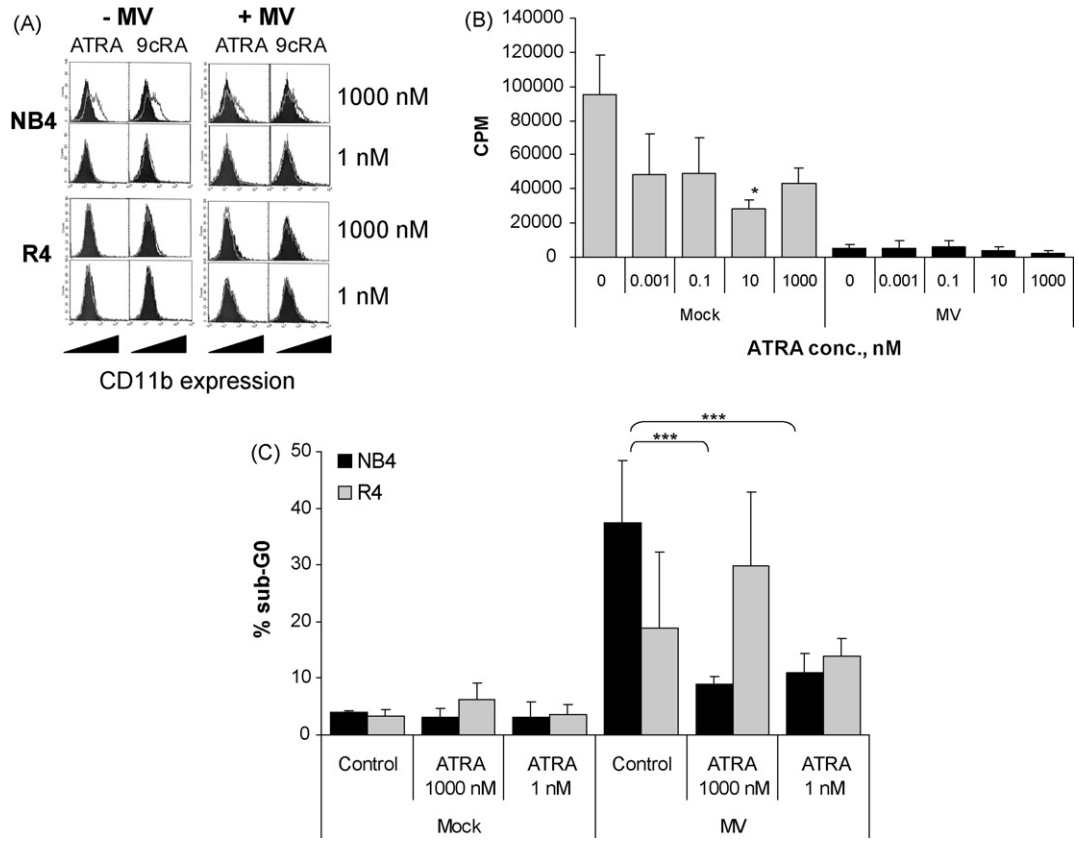

Fig. 5. Inhibition of MV output is not due to changes in cell differentiation, proliferation or viability. (A) CD11b expression on NB4/R4 with (MV+) or without MV infection (MV−) was measured by flow cytometry at 72 h in cultures treated with supplemental retinoids (ATRA, 9cRA) at 1000 and 1 nM. For each experimental group, CD11b staining of retinoid-treated cells (white histogram) was compared to the mock-treated control (black histogram). (B) H^3-thymidine uptake reported as counts per minutes (CPM) in uninfected and MV-infected NB4/R4 cells with and without ATRA treatment. (C) Apoptosis of NB4/R4 cells infected with MV at an MOI of 0.1 measured by PI staining at 48 h with 1000 or 1 nM for each group. Values are expressed as mean ± standard deviation of living cells (as a percentage of total cells) in triplicate experiments ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. mock-treated control).

3.7. Inhibition of MV by retinoids is not due to an effect on differentiation, proliferation or apoptosis

MV replication can be influenced by host cell differentiation, proliferation, and viability (Helin et al., 1999), all of which can be altered by retinoids in some experimental situations. We therefore sought to determine if the observed anti-viral effects of retinoids could be attributed to one of these known actions. In NB4 cells exposed to high concentrations of retinoids, RARα-mediated up-regulation of differentiation markers is observed most strikingly at days 5–7, with no effect on morphology, cell cycle or growth arrest on days 2–3 (W.H. Miller, unpublished observations). Our experiments were carried out for 72 h, making a retinoid-induced effect on differentiation unlikely to fully explain the impact of retinoids on MV infection. High concentrations of ATRA and 9cRA (1000 nM) increased CD11b expression on both infected and uninfected NB4 cells after 72 h, but not on R4 cells (Fig. 5A). However, no CD11b up-regulation on NB4 cells was observed at 1 nM ATRA, a concentration that significantly inhibited MV output (Table 1). NBT reduction, another marker of NB4 cell differentiation, was also unchanged at the retinoid concentrations <100 nM (data not shown). Retinoids also had powerful anti-MV effects in Jurkat cells (Table 1) which are not sensitive to retinoid-induced growth inhibition or differentiation (C. Nervi and W.H. Miller, unpublished observation). These data suggest that the anti-viral effects of retinoids at low concentrations cannot be wholly explained by cell differentiation.

Although one or more retinoids had anti-proliferative effects in several of the cells/cell lines used, there was a clear dissociation of anti-viral and anti-proliferative effects in the infected cells exposed to retinoids at lower doses (Figs. 1 and 5B). Furthermore, MV infection itself has striking anti-proliferative effects in all of the cell lines studied, as measured by thymidine uptake assay (data not shown). In NB4 cells, MV infection reduced cell proliferation by >95% compared to mock-infected cells (Fig. 5B). Treating these infected cells with increasing doses of ATRA had no further effect on proliferation. At the highest dose used (1000 nM), ATRA further decreased proliferation compared to infected, mock-treated controls, but this difference did not reach statistical significance. The additional anti-proliferative effect of ATRA on MV-infected cells is therefore likely to be minimal given the massive effect of MV infection alone.

MV-induced apoptosis has been described and would be expected to occur in both NB4 and R4 cells (Esolen et al., 1995). Using PI staining as a marker of DNA fragmentation, we observed that ATRA treatment of infected NB4 cells significantly reduced the number of dead and dying cells (Fig. 5C). In contrast, the addition of ATRA to infected R4 cell cultures had no effect (Fig. 5C). Therefore, the anti-MV activity of retinoids cannot be explained as result of increased host cell death.

4. Discussion

Since 1988, the WHO has recommended high dose vitamin A treatment for acute measles in many resource-poor settings (Joint WHO/UNICEF statement, 1987) and such treatment has become standard throughout the world. During this same period, high dose vitamin A supplementation in infancy has been recognized as a

pivotal health intervention in many parts of the developing world (Stephensen, 2001). Almost nothing is known about the mechanism of action of vitamin A on either child survival or during MV infection. A full understanding of potential retinoid–MV interactions is important, since high dose vitamin A supplements are often given at the same time as live attenuated measles virus vaccine in developing world children (Semba et al., 1995, 1997; Bhaskaram and Rao, 1997; Benn et al., 1997; Bahl et al., 1999). This manuscript provides evidence, for the first time, that retinoids can have anti-MV effects in vitro in a range of cell lines and in primary cells, and that this effect is mediated primarily by nuclear receptor signaling pathways.

The potential impact of retinoids on viral replication in vitro has been examined by several groups before. To date however, these studies have focused on DNA viruses (e.g., HSV, EBV, CMV and polyoma virus) and HIV (Isaacs et al., 1997; Yamamoto et al., 1979; Russell and Blalock, 1984; Ghazal et al., 1992). Although clinical studies of vitamin A status and supplements are only available for HIV, neither in vitro or in vivo data have shown evidence of consistent benefit (Turpin et al., 1992; Maciaszek et al., 1998; Irlam et al., 2005). Our study was initiated because of the clinical evidence indicating that water-soluble vitamin A supplements can reduce mortality and morbidity due to measles virus infection (Joint WHO/UNICEF statement, 1987; D'Souza and D'Souza, 2002; Huiming et al., 2005). Specifically, two 200,000 IU doses of retinol in a water-based preparation has been shown to be effective in children with acute measles.

One of the central observations of this paper is the ability of retinoids to reduce the level of MV replication in a range of cell lines of immune, respiratory and gastrointestinal origin, as well as in PBMCs (Table 1). The observed inhibition in PBMC is particularly important because these cells are natural targets for MV (Perry and Halsey, 2004) and are not immortalized. Key experiments in NB4 cells were repeated with a low-passage, wild-type strain (BILT) and an attenuated vaccine-strain (CN). Responses to both natural and synthetic retinoids reduced viral output for these viral strains as well (data not shown), suggesting that the anti-viral effect of retinoids on MV replication is not strain-specific. Using the Chicago-1 strain, a significant inhibition of viral replication was detected with 1 nM of ATRA in 7/10 cell lines studied (Table 1). ROH is the form of vitamin A found in the circulation, usually at a concentration range higher than that used in our experiments (2 μM) (Blomhoff et al., 1990). We often observed a greater degree of inhibition using ATRA and 9cRA (Table 1 and Fig. 1), the natural derivatives of ROH that bind directly to nuclear receptors. These compounds are mostly intracellular, but can be found in the serum in the 5–10 nM range (Blomhoff et al., 1990). The surprising observation that physiologic concentrations of retinoids were able to inhibit MV in the normally relatively refractory NB4 cells may be explained by a combined action of the retinoid and viral-derived or virally-induced factors (i.e., innate immune effectors). Natural measles is a monophasic illness characterized by the rapid development of specific and highly effective immunity (Moss et al., 2004). The outcome of infection is therefore a 'race' between the virus and the developing immune response. In this context, it is certainly plausible that even the relatively modest reductions in the rate of measles virus replication that we observed in vitro could contribute to the clinical benefits of vitamin A supplementation.

MV replication in vitro is subject to a number of host cell influences including cell type, cell-cycle progression, apoptosis, activation state and differentiation (Helin et al., 1999; Schnorr et al., 1997; Ryon et al., 2002; Moss et al., 2004). The wide-range of effects of retinoids on human tissues includes several of these actions. We used several approaches to determine whether or not the retinoid-induced changes in MV output we observed could be explained by one or more of these known effects. Many viruses, including MV, disrupt cell cycle progression to avoid mitosis. Cell cycle arrest at G_0 has been described in MV-infected PBMCs (Schnorr et al., 1997), and marked reductions in thymidine incorporation were evident in our models within 24 h following MV infection alone (Fig. 5B). However, the anti-proliferative and anti-viral effects of retinoids on MV-infected cells could be dissociated at 1 nM ATRA, a dose that inhibited MV but had no effect on proliferation (Fig. 5B). Similarly, our results with low dose retinoid supplementation argue against retinoid-induced differentiation as the primary factor in limiting viral replication in our models (Fig. 5A). MV can lead to apoptosis in some cells and cell lines (Esolen et al., 1995). Although retinoid-driven, premature apoptosis could plausibly account for decreased production of virions, we observed precisely the opposite effect. In NB4 cells, ATRA treatment partially rescued infected cells from apoptosis (Fig. 5C). Surprisingly, ATRA was unable to rescue MV-infected R4 cells from apoptosis (Fig. 5C), suggesting the involvement of RARα in this effect. Studies are underway to further explore the role of nuclear retinoid receptor signaling in the modulation of MV-induced apoptosis.

This work demonstrates that nuclear retinoid receptor signaling is central to the anti-viral effect of retinoids against MV. Although more than one nuclear receptor may be involved, a crucial role for RARα appears likely. Intact RAR signaling was required for the anti-viral effect in the NB4/R4 model and the actions of RARα-selective agonist (LGD030593), the pan-RAR agonist (TTNPB) and the RARα antagonist (RO) were consistent with this hypothesis (Figs. 1 and 2). The ability of the RXR-selective agonist 1305 to inhibit viral output in most of the cells and cells lines tested suggests that RXR may also play a significant role. Together, these observations suggest that RAR and/or RXR contribute to the anti-MV effects of retinoids in our in vitro models of infection.

There are many sites in a host cell at which retinoid signaling could plausibly act to mediate the observed anti-viral effects, from initial viral entry through transcription and translation all the way to the budding of new virions. Although direct viral modulation of nuclear receptor-mediated transcriptional activity has been reported in hepatitis C infection (Tsutsumi et al., 2002), we found no evidence that a similar mechanism was operative in our MV infection models (Fig. 4). Our observation that nuclear receptor signaling is necessary for the inhibitory effect of retinoids suggests that retinoid-responsive genes could be involved in mediating this effect. Given that the anti-viral effect was evident in several cell lines of distinct lineages, one of the most attractive hypotheses to explain this observation is retinoid-induction or – enhancement of innate immune effector mechanisms such as the production of type I interferons or anti-microbial peptides. Type I interferons (IFNα/β) are highly effective anti-viral cytokines and both *Paramyxoviridae* and retinoids are known to modulate their expression (Takeuchi et al., 2003; Palosaari et al., 2003; Dimberg et al., 2000; Pelicano et al., 1999; Kolla et al., 1997; Luo and Ross, 2006). A role for apoptosis in mediating this effect is also possible. Experiments are underway to fully characterize the molecular mechanism(s) that link MV and retinoid signaling pathways to induce an anti-viral state at the level of the infected cell.

The central observation of the current work has possible implications for programs that administer MV vaccine and vitamin A supplements simultaneously. Live-attenuated MV vaccines require viral replication in order to elicit a protective immune response. Even low concentrations of maternal antibody can have major impact on vaccine efficacy (Crowe, 2001). In vivo inhibition of vaccine-strain MV replication in vaccines might be expected to have a similar effect. Studies performed to address this issue have yielded conflicting results. Several groups have shown either no effect or improvement in seroconversion rates when measles vaccine and

high dose vitamin A were administered together (Bhaskaram and Rao, 1997; Benn et al., 1997; Bahl et al., 1999). However, other studies have shown lower seroconversion rates, lower geometric mean titers and/or less durable antibody responses in children who received supplemental vitamin A with measles vaccine (Semba et al., 1995, 1997). There is no obvious explanation for these discrepant results, but the studies used different retinol formulations and vaccines and were carried out in different geographic locations in children of varying ages. Our data support further examination of the potential interaction between these two critical health interventions.

Paramyxoviridae such as MV, respiratory syncytial virus (RSV), canine distemper virus (CDV), phocine distemper virus as well as the newly described Nipah and Hendra viruses are important viral pathogens in humans and animals. Commercial vaccines are not yet available for most of these viruses and standard anti-viral drugs have limited or no efficacy (Chabot and Ward, 2002). The anti-MV effects that we have demonstrated raise the possibility that retinoids may have activity against other members of this virus family as well. Our preliminary data suggest that retinol, ATRA and 9cRA have potent anti-viral activity against CDV and Mumps virus in vitro and that retinol supplements can rescue ferrets from otherwise lethal CDV infection (B.J. Ward, unpublished observation; Rodeheffer et al., 2007). On the other hand, high dose vitamin A supplements in children infected with RSV have no benefit and may in some circumstances cause harm (Ni et al., 2005). These clinical and laboratory observations suggest that efforts to better understand the mechanisms of retinoid actions in viral infections may reveal both positive and negative aspects of retinoid supplementation.

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