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Vitamin A Enhances in Vitro Th2 Development Via Retinoid X Receptor Pathway

Charles B. Stephensen,²* Reuven Rasooly,* Xiaowen Jiang,* Michael A. Ceddia,³* Casey T. Weaver,‡ Roshantha A. S. Chandraratna,§ and R. Patterson Bucy†

Vitamin A deficiency increases mortality from common childhood infections (1, 2). The specific mechanisms underlying this increased risk of death have not been clearly defined but presumably involve impairment of specific and nonspecific host defense mechanisms (3). Although data from humans are limited, rodent studies indicate that vitamin A deficiency alters the Ab response to T cell-dependent Ags (4, 5). Vitamin A deficiency decreases the IgA, IgG1, and IgE responses but increases the IgG2a response to viral infection (6–8). The underlying pattern of cytokine production by APCs and T cells is consistent with these effects on Ab production: vitamin A deficiency increases constitutive IL-12 production by macrophages (9), and during secondary in vitro stimulation of lymphocytes with Ag, vitamin A deficiency increases IFN-γ production, but decreases IL-4 and IL-5 production (10). Conversely, supplemental treatment with vitamin A or retinoic acid (RA)¹ decreases IFN-γ and increases IL-5, IL-10, and IL-4 production (11–13). Thus, vitamin A deficiency biases the immune response in a Th1 direction, whereas high-level dietary vitamins may bias the response in a Th2 direction. RA appears to be the metabolite of vitamin A that is most potent in restoring impaired Ab responses (14). Although it is known that exogenous RA can down-regulate IFN-γ transcription (15), little else is known about how RA modulates Th1/Th2 balance.

Vitamin A and other fat-soluble nutrients are precursors for compounds that act as ligands for nuclear receptors that regulate gene transcription in response to changes in nutritional status (16). These receptors regulate key metabolic processes, such as energy metabolism, but are also found in cells of the immune system where they appear to modulate immune function based on environmental (in this case, nutritional) signals. The vitamin A derivatives all-trans and 9-cis RA regulate gene transcription by binding to the RA receptors (RARs) α, β, or γ or to the retinoid X receptors (RXRs) α, β, or γ (17, 18). Both all-trans and 9-cis RA bind to the RXRs, whereas 9-cis RA also acts via the RARs. Docosahexaenoic acid also binds to RXRs and may be a physiologically important ligand in tissues in which concentrations are sufficient (≥10 μmol/L) to trigger transcriptional regulation (19).

RAR and RXR belong to a family of nuclear receptors that also includes the vitamin D receptor (VDR), thyroid hormone receptor, and the peroxisome proliferation/activation receptor (PPAR), which binds specific fatty acids. In brief, the structure of these receptors includes a 5′ “A/B” domain, a DNA-binding “C” domain, a hinge region (“D”) domain, the ligand-binding “E” domain, and a 3′ “F” domain of uncertain function. Receptors bind to specific DNA response elements in the regulatory regions of genes.

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*Abbreviations used in this paper: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; VDR, vitamin D receptor; PPAR, peroxisome proliferation/activation receptor; TTNPB, 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid.

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for which transcription is regulated by these receptors. Transcriptional activity is regulated via specific receptor sequences that interact with coactivator proteins to affect transcription by RNA polymerase II. Sequences in the ligand binding domain are also responsible for formation of heterodimers among these receptors, with RXR being one of the heterodimer partners. Unlike other receptors, RXR can also form homodimers that can positively regulate transcription. In these experiments, we used all-trans RA (which binds to RAR), 9-cis RA (which binds to both RAR and RXR), the RXR-selective retinoid 4-((E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzonic acid (TT-NPB), and the RXR-selective retinoid AGN194204 (20, 21) to demonstrate that stimulation of the RXR pathway enhances Th2 development. Use of these latter compounds is particularly important because they are stable, whereas RA isolomers can interconvert under physiologic conditions (18).

The DO11.10 αβ-TCR transgenic mouse (22) is a useful model for examining early events in the development of Th1 and Th2 memory cells from Ag-naive Th0 cells. The DO11.10 TCR recognizes amino acids 323–339 of the protein OVA (OVA323–339), and the PCR can be identified using the clonotypic mAb KJ1-26 (23). The OVA323–339 synthetic peptide stimulates proliferation and cytokine production when it is presented in the MHC class II context I-Aκ. We have used this system to examine the impact of retinoid treatment during primary in vitro antigenic stimulation on the subsequent development of a Th1 or Th2 pattern of cytokine production.

Materials and Methods
Mice
DO11.10 mice were bred in our facility. BALB/c mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Peripheral blood from DO11.10 mice was used to screen lymphocytes by two-color flow cytometric analysis using anti-CD4 Ab and the TCR clonotypic-specific mAb KJ1-26 (23) to identify transgenic (double-positive) animals when heterozygous breeders were used and when mice were back-crossed onto the BALB/c background.

Retinoids
All-trans RA, 9-cis RA, and the RAR agonist TTNPB were purchased from Sigma-Aldrich (St. Louis, MO). The RAR agonist AGN194204 was provided by Allergan (Irvine, CA). Retinoids were diluted aseptically in cell culture grade DMSO (Sigma-Aldrich). The concentration of 9-cis and all-trans RAs was confirmed by HPLC analysis as described (24). Stock solutions at 1 or 10 mM were frozen at −80°C and were discarded after three freeze-thaw cycles. The retinol concentration in FBS was measured using a System 9000 Cell Counter (Baker Instruments, Fort Collins, CO). Concentration was then adjusted to 2 ng/ml OVA323–339 (purchased from the Peptide Synthesis Core Facility of the Comprehensive Cancer Center, University of Alabama, Birmingham, AL) was placed in wells of 24-well plate (1.0 ml) or 48-well plate (0.5 ml) plates (Nunc, Rochester, NY). For preliminary, or retinoid treatment were added, followed immediately by lymph node cells (4 × 10⁶/ml) or CD4⁺ cells (2 × 10⁶/ml) (1.0 ml for 24-well plate, 0.5 ml for 48-well plate). Cultures were placed at 37°C in 5% CO₂. Retinoids were diluted in DMSO. The final concentration of DMSO in the cell culture medium was 0.1% (v/v), which did not affect cytokine production by these cultures (data not shown). Three days after primary stimulation, cultures were expanded into six-well plates by adding 2 or 3 × 10⁶ cells/ml. Secondary stimulation on day 7, cells were collected by centrifugation at 800 × g at 4°C for 10 min, washed twice in Rus, and counted using a System 9000 Cell Counter (Baker Instruments, Fort Collins, CO). Concentration was then adjusted to 2 × 10⁶/ml and cells were restimulated as just described. For tertiary stimulation, on day 14, cells restimulated on day 7 were collected and restimulated in an identical fashion. Supernatants were collected 24 or 48 h after secondary or tertiary stimulation by centrifuging the plates at 4°C for 10 min at 4°C and aspirating supernatants with a micropipette.

In experiments to examine the effect of retinoid treatment on mature Th1 and Th2 populations, DO11.10 lymph node cells were isolated and stimulated with OVA323–339 and splenic APCs (primary stimulation) under Th1-differentiating conditions (IL-12 plus anti-IL-4), Th2-differentiating conditions (IL-4), or without exogenous treatment. One week after primary stimulation, cells were washed and equal numbers of cells were restimulated (secondary stimulation) without any exogenous treatment. One week after secondary stimulation, cells were again washed and equal numbers were restimulated with OVA323–339 (tertiary stimulation) in the presence of 10⁻⁷ M all-trans or 9-cis RA.

In some experiments, naive Th0 cells were stimulated with plate-bound anti-CD3 (1 µg/ml, clone 145-2C11; BD Pharmingen) and soluble anti-CD28 (5 µg/ml, clone 37.51; BD Pharmingen) in the presence of IL-4 (10 ng/ml). Similar results were found with anti-CD3 concentrations of 1 and 10 ng/ml and anti-CD28 concentrations of 5 and 10 ng/ml. Cells were expanded after 3 or 4 days. After 6 or 7 days, cells were washed twice, counted, and treated with PMA (5 ng/ml final concentration; Sigma-Aldrich) to stimulate cytokine production. Supernatants were collected after overnight incubation for measurement of cytokines by ELISA.

Cytokine ELISAs
IL-4 and IFN-γ were measured in cell culture supernatants by capture ELISA using Abs, purified standards, and protocols suggested by the manufacturer (BD Pharmingen). The limit of detection of the IL-4 and IFN-γ ELISAs were 0.03 and 0.06 ng/ml, respectively.

FACS
At various times after stimulation, KJ1-26 and CD4⁺ cells were isolated using a MoFlo (Cyromatics, Fort Collins, CO) high-speed cell sorter. Cells in the lymphocyte gate by forward and side scatter were also gated for
viability using propidium iodide staining. Target cells were from 4 to 10% of total cells, depending on the time point. We routinely obtained purities from 96 to 99%.

mRNA analysis

Total RNA was prepared using the Trizol reagent as described by the manufacturer (Life Technologies). RNA was prepared from CD4+ cells (prepared using Dynabeads, as described in Stimulation protocol) 0, 4, 24, and 48 h after primary antigenic stimulation in two experiments and from CD4+KJ1-26+ cells (purified by FACs, as described above) 0, 24, 48, and 72 h after stimulation in two additional experiments. Analysis of gene expression revealed essentially identical patterns using both cell isolation methods, and results from the four experiments were analyzed together (Table I).

After RNA isolation, first-strand cDNA was synthesized from 0.5 or 1.0 µg of total RNA using Moloney murine leukemia virus reverse transcriptase (Superscript II; Life Technologies) in a 25-µl reaction with oligo-dT (Life Technologies) as a primer. RNA was then degraded by treatment with RNaseH (Life Technologies). Quantitative PCR analysis was performed using 1 µl of cDNA with 0.25 µM primers in a 20-µl reaction using a LightCycler rapid thermal cycler system (Roche Diagnostics, Indianapolis, IN) and DNA Master Mix reagents (Roche Diagnostics). Amplification program began with a 30-s denaturation step at 94°C followed by cycles with a 94°C denaturation for 1 s, annealing for 5 s at 6°C, and extension at 72°C for 1 s. Upstream and downstream primers (respectively) for genes of interest were as follows: IL-4, 5’-CATCGCGATTTTGACAGGTC-3’, 5’-TTATCGATAGGACGCATCG-3’; GATA-3, 5’-TACTTGGCCTTGCAGAGAGGAGATTATCCG-3’, 5’-GGGCTTCATTCACTCCGCCCT-3’; STAT-6, 5’-TCTGCTGACAGCCAGTTGCC-3’, 5’-GGGCTTGGTTTCGACCCCTTCT-3’; IL-4R, 5’-GGTTGCCCTTTTATTTACTTTCGGACT-3’, 5’-CTGATCTTTGCTCACCACCTGAGCTCTGCGAAA-3’; IFN-γ, 5’-CATTGAAAGCCTTCAAAGGCTAAAGAATCTGC-3’, 5’-CTCATGAATGCATCCTTTTTCG-3’; STAT-5a, 5’-CACAACCTGAGCTCTGCGAAA-3’, 5’-TCTGACTAGACATGATCCCGGAAA-3’. β-Actin primers were purchased from Promega (Madison, WI).

The cDNA copy number for each gene of interest was determined using a four-point standard curve (of plasmid DNA). Standard curves were run with each set of samples. Correlation coefficients (r2) for standard curves were typically ≥0.98. The precision of target gene-β-actin ratios from the same cDNA sample (within run) were quite good. For example, the average coefficient of variation for the 12 IL-4-β-actin ratios from Table I was 9.2 ± 7.5% (range, 0–26%). Furthermore, the day-to-day reproducibility (precision) was also good. For example, the correlation coefficient for IL-4-β-actin ratios from nine samples (measured in duplicate) from a single experiment was r2 = 0.87 (r = 0.93) (quantitative PCR was performed on different days using two different batches of cDNA, prepared on different days).

Table I. mRNA expression in purified Th0 cells from DO11.10 mice following primary stimulation with in vitro with OVA233–250 peptide and treatment with RXR agonist

<table>
<thead>
<tr>
<th>Gene</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGN</td>
<td>IL-4</td>
<td>DMSO</td>
</tr>
<tr>
<td>Th2 genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>0.037</td>
<td>0.046</td>
<td>0.046</td>
</tr>
<tr>
<td>GATA-3</td>
<td>0.19c</td>
<td>0.34b</td>
<td>0.15a</td>
</tr>
<tr>
<td>c-maf</td>
<td>0.033a</td>
<td>0.05b</td>
<td>0.016e</td>
</tr>
<tr>
<td>STAT-6</td>
<td>0.38</td>
<td>0.48</td>
<td>0.29</td>
</tr>
<tr>
<td>IL-13R</td>
<td>0.64</td>
<td>1.11</td>
<td>0.54</td>
</tr>
<tr>
<td>Th1 genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.042</td>
<td>0.048</td>
<td>0.045</td>
</tr>
<tr>
<td>T-bet</td>
<td>0.081</td>
<td>0.099</td>
<td>0.068</td>
</tr>
<tr>
<td>IL-12R</td>
<td>ND in most cases</td>
<td>0.040b</td>
<td>0.063b</td>
</tr>
<tr>
<td>Fyn</td>
<td>0.36</td>
<td>0.26</td>
<td>0.31c</td>
</tr>
</tbody>
</table>

* AGN, 1 × 10–7 M AGN194204; IL-4, 10 ng/ml; or vehicle (DMSO, 0.1% DMSO). SE, pooled SE. The copy number for each gene is expressed relative to β-actin and was measured in duplicate or triplicate by real-time PCR in two (72 h) to four (24 and 48 h) independent experiments.

To confirm that each primer pair correctly amplified the sequence of interest, initial PCR products from T cell cDNA were run on an agarose gel (3% SeaKem 3:1), stained with 0.5 µg/ml ethidium bromide, and viewed by UV transillumination to confirm that a single product of the predicted size was produced. PCR products were cloned into the PCR-II-TOPO plasmid vector (Invitrogen, San Diego, CA) and used to transform Escherichia coli competent cells (Life Technologies). White colonies were picked and grown, and plasmid DNA was isolated and analyzed for the presence of the receptor sequence by digestion with EcoRI restriction enzyme that flanked the insert. Inserts were sequenced completely in one direction using M13 forward and reverse primers.

To confirm specificity of the reaction product during each run, the melting profile of each sample was analyzed using the LightCycler. The melting profile was determined by holding the reaction at 55°C for 10 s and then heating slowly to 94°C with a linear rate of 0.2°C/s while the fluorescence emitted was measured. Melting curve analysis demonstrated that each of the primer pairs described amplified a single product. Rare samples that demonstrated a significant second peak in the melting profile were not used in analysis.

Statistical analysis

Statistical analysis was performed with the SigmaStat program (Jandel Scientific, San Rafael, CA). A p value of 0.05 was used to determine statistical significance unless otherwise indicated. All variables were compared among the treatment groups using either one-way or two-way ANOVA. Two-way ANOVA was used when data from experiments done at different times were analyzed together (i.e., data were analyzed by “experiment” and by “treatment group”). Pair-wise multiple comparisons among the treatment groups were routinely made (in conjunction with the ANOVA procedure) by the Student-Newman-Keuls method or Bonferroni’s t test. When data were missing from one cell of a two-way ANOVA, the general linear model was used to perform the ANOVA. This occurred with the 24- and 48-h IL-4 and IFN-γ data in Table I, where one experiment lacked the IL-4 treatment, and with T-bet at 24 h in the IL-4 treatment, where RNA was not available for this assay. Preplanned, two-group comparisons were also made using the Student t test.

Results

DO11.10 T cells have naive phenotype before antigenic stimulation

Before in vitro antigenic stimulation (day 0), CD4+KJ1-26+ cells from DO11.10 mice in our colony had high-level L-selectin expression, consistent with a naive phenotype (data not shown). Seven days after tertiary stimulation, L-selectin expression was low—similar to that of the isotype control Ab (data not shown)—which is consistent with a memory cell phenotype, as has been shown previously for these mice (25).
Because conditions at primary stimulation of Ag-naive Th0 cells influence subsequent Th1/Th2 development, we treated primary cultures with all-trans and 9-cis RA and monitored IL-4 and IFN-γ production at secondary and tertiary stimulation. As shown in Fig. 1, 9-cis, but not all-trans, RA increased IL-4 production at both secondary and tertiary stimulation (p < 0.01), with 10⁻⁷ M 9-cis RA having the greatest effect, stimulating a 10-fold increase over the DMSO control culture. In three additional experiments (one using serum-free medium), 10⁻⁷ 9-cis RA significantly increased IL-4 production at secondary or tertiary stimulation in all cases, whereas 10⁻⁷ all-trans RA increased IL-4 production in just one experiment.

**RXR-selective agonist AGN194204 promotes Th2 development**

Because 9-cis RA can bind to both RARs and RXRs, we used the synthetic RXR agonist AGN194204 to determine whether treatment with an RXR-selective ligand would reproduce the Th2-enhancing effect of 9-cis RA. Parallel cultures were treated with the RAR-selective agonist TTNPB as a control for stimulation via RAR. As shown in Fig. 2, treatment of primary cultures with AGN194204 significantly enhanced Th2 development at concentrations of 10⁻⁶ through 10⁻¹⁰ mol/L. In three of three additional experiments, treatment of primary cultures with 10⁻⁷ M AGN194204 also strongly enhanced IL-4 production at secondary stimulation (p < 0.05; data not shown). On average, the IL-4 concentration was 21-fold greater in the AGN194204 cultures than in the DMSO control cultures (range, 3.6–56).

As expected, treatment of primary cultures with the RAR agonist TTNPB had little effect on IL-4 production at secondary stimulation (Fig. 2). In two of three additional experiments, the IL-4 concentration was significantly higher after treatment with 10⁻⁷ M TTNPB, compared with DMSO control cultures (p < 0.05; data not shown), although the increases were small. On average, IL-4 concentrations were 1.8-fold greater in the 10⁻⁷ M TTNPB cultures than in the DMSO cultures (range, 1.3–2.3). IL-4 concentrations in the AGN194204 cultures were always significantly greater than in the TTNPB cultures (p < 0.05; data not shown).

**IL-4 is required for Th2 enhancement by RXR agonists, but IL-12 and IFN-γ are not**

Because RA treatment can decrease IL-12 (9) and IFN-γ production (26), it is possible that 9-cis RA and the RXR agonist AGN194204 enhance Th2 development by decreasing production of these Th1-enhancing cytokines by APCs or bystander cells (26).

**FIGURE 1.** 9-cis, but not all-trans, RA treatment at primary stimulation increases IL-4 production at secondary and tertiary stimulation. At primary antigenic stimulation, lymph node cells from DO11.10 mice were treated with the indicated concentrations of all-trans or 9-cis RA or with a vehicle control (0.1% DMSO). Cells were restimulated 7 days later without RA treatment (secondary stimulation). Cells from the secondary stimulation were again restimulated without RA 14 days after primary stimulation (tertiary stimulation). The means ± SD of triplicate cultures are shown.

If this were true, then neutralizing these cytokines during primary stimulation would block the Th2-enhancing effect of 9-cis RA. We conducted three experiments to test this hypothesis and found that when neutralizing Abs and 9-cis RA were added at primary stimulation, significant increases in IL-4 (and reductions in IFN-γ) were still seen in the 9-cis RA cultures at both secondary and tertiary stimulation (Fig. 3) when either IFN-γ (p < 0.001) or IL-12-neutralizing Ab (p < 0.001) were used. However, neutralization of IL-4 blocked the Th2-enhancing activity of 9-cis RA (Fig. 3). In two additional experiments, essentially the same results were reproduced for the RXR agonist AGN194204 (data not shown).

To control for the possible nonspecific effect of Abs on cytokine, isotype-control Abs for the IFN-γ (IgG1), IL-12 (IgG2a), and IL-4 (IgG2b)-neutralizing Abs were included in several experiments. These Abs did not significantly alter the Th2-enhancing effect of either AGN194204 or 9-cis RA. For example, mean IL-4 concentrations at secondary stimulation for IgG1, IgG2a, and IgG2b, and no Ab cultures with 10⁻⁷ M 9-cis RA treatment were 1.88 ± 0.11, 1.99 ± 0.11, 2.14 ± 0.13, and 1.94 ± 0.07 ng/ml, respectively, whereas IL-4 concentrations for the DMSO control cultures that received the same Abs were 0.20 ± 0.01, 0.30 ± 0.00, 0.19 ± 0.03, and 0.15 ± 0.03 ng/ml, respectively.

**FIGURE 2.** The RXR agonist AGN194204 enhances Th2 development of naive DO11.10 T cells, whereas the RAR agonist TTNPB does not. At primary antigenic stimulation, lymph node cells from male DO11.10 mice were treated with the indicated concentrations of the synthetic retinoids AGN194204 and TTNPB. Cells were restimulated 7 days later without retinoid treatment (secondary stimulation). The means ± SD of triplicate cultures are shown. Asterisks indicate significant differences in IL-4 or IFN-γ concentration between the AGN194204 and TTNPB treatments at the indicated concentration (p < 0.05 by Student’s t test).
cultures at 1 ng/ml or 10 ng/ml, both compounds significantly (10 ng/ml IL-4). In six additional experiments, IL-4 levels in 9-cis RA increased IL-4 production under strong Th2-enhancing conditions. The means ± SD of triplicate cultures are shown.

To determine whether Th2 enhancement by RXR agonists was limited to low (endogenous) levels of IL-4 or if further enhancement could be seen under Th2-polarizing conditions, we examined the effect of 9-cis RA and AGN194204 on Th2 development using different levels of IL-4 during primary stimulation. As shown in Fig. 4, blocking IL-4 activity with either 5 or 0.5 μg/ml of neutralizing Ab largely blocked the ability of these compounds to enhance IL-4 production (although small, statistically significant increases were seen). However, both compounds substantially decreased IFN-γ production in the presence of IL-4-neutralizing Ab ($p < 0.001$), with the effect of AGN194204 being greater than that of 9-cis RA ($p < 0.05$). When IL-4 was added to the primary cultures at 1 ng/ml or 10 ng/ml, both compounds significantly increased IL-4 production at secondary stimulation ($p < 0.001$), with the effect of AGN194204 consistently being greater than that of 9-cis RA ($p < 0.05$) (Fig. 4). The 9-cis RA did not consistently increase IL-4 production under strong Th2-enhancing conditions (10 ng/ml IL-4). In six additional experiments, IL-4 levels in 9-cis-RA-treated cultures were from 18% higher to 48% lower than in the DMSO control cultures.

**RXR agonists diminish IL-12-induced Th1 development**

Under Th1-polarizing conditions (i.e., treatment of primary cultures with IL-12 and IL-4-neutralizing Ab), 9-cis RA treatment consistently decreased IFN-γ production. In six experiments, 9-cis RA treatment decreased IFN-γ production from 100 ± 3 (mean ± SE) to 50 ± 0.3 ng/ml, with the percent decrease ranging from 18 to 85%. The mean decrease was 50% ($p < 0.001$ by two-way ANOVA). The RXR agonist AGN194204 had a similar effect (data not shown).

**Th2 enhancement by RXR agonist does not require APCs**

Although irradiated cells are unlikely to respond to retinoid treatment, it is possible that APCs, or bystander cells from the splenocyte population, may be required for RXR-mediated enhancement of Th2 development. Thus, we wished to determine whether the RXR agonist acts directly on naive Th0 cells in the absence of APCs. In addition, we wished to determine whether Th2 enhancement is limited to BALB/c mice (the background strain for DO11.10 mice), which are prone to Th2 development, or whether it could be reproduced in C57BL/6 mice, which are not (27). To address these two questions, we purified naive Th0 cells (CD4+CD28-7M9 - L-selectin high) from C57BL/6 mice by flow cytometry and stimulated proliferation using anti-CD3 and anti-CD28 Ab in the presence of IL-4. We conducted two experiments, which gave essentially identical results. As shown in Fig. 5, treatment with AGN194204 enhanced Th2 development compared with the vehicle control, indicating that naive Th0 cells respond directly to stimulation with an RXR agonist.

**All-trans and 9-cis RA decrease IFN-γ production by mature Th1 cells**

In addition to examining the effects of RA treatment at primary stimulation on subsequent Th1 and Th2 development, we also examined the effect of RA treatment on IFN-γ and IL-4 production by Th1 and Th2 populations produced by previous treatment with IL-12 and IL-4, respectively. IFN-γ production by Th1 cultures differed significantly among the three treatment groups ($p < 0.001$) (Fig. 6). IFN-γ production by the all-trans RA-treated cultures was 19% lower than in control cultures ($p < 0.05$) and was...
RXR agonist increases IL-4 and IL-5 and decreases IFN-γ production by naive Th0 cells

IL-4 and IFN-γ are produced by developing Th0 cells and enhance Th2 and Th1 development, respectively. To test the hypothesis that an RXR agonist would enhance production of the former and diminish production of the latter during primary antigenic stimulation of naive Th0 cells, DO11.10 cultures were treated with AGN194204 exactly as described for experiments examining Th1/Th2 phenotype at secondary stimulation. The RXR agonist AGN194204 significantly increased IL-4 concentrations relative to the vehicle control (p = 0.014 by two-way ANOVA); differences were statistically significant 48 and 72 h after stimulation (p < 0.05) (Fig. 6A). The RXR agonist also decreased IFN-γ concentrations relative to the vehicle (p < 0.05) and IL-4 treatments (p < 0.05), which did not differ from one another (Fig. 6B). When individual time points were considered separately, the only significant difference was that the IFN-γ concentration in the IL-4 treatment was greater than in the AGN194204 treatment (p < 0.05). In addition, IL-5 concentrations were increased over both the vehicle control (p < 0.05) and IL-4 treatment groups (p < 0.05) by treatment with the RXR agonist (Fig. 6C). IL-10 concentrations were not affected by treatment with the RXR agonist (Fig. 6D).

RXR agonist increases Th2 and decreases Th1 gene expression by naive Th0 cells

Because RXR is a nuclear receptor that modulates gene expression, we examined mRNA levels by real-time PCR 24, 48, and 72 h after antigenic stimulation and treatment with DMSO (vehicle control), AGN194204, or IL-4. β-Actin mRNA levels before stimulation were 1.5 ± 0.5 × 10^8 copies/50 ng total RNA (mean ± SE, n = 4 experiments). β-Actin mRNA levels increased over time. Levels did not differ by retinoid or IL-4 treatment at 24 and 48 h, but at 72 h, β-actin levels were higher in the IL-4 treatment than in the DMSO treatment (p < 0.05). The β-actin levels 24 h after Ag stimulation in the DMSO, AGN194204, and IL-4 treatments were 2.7 ± 0.3, 2.7 ± 0.3, and 2.9 ± 0.3 × 10^8 copies/50 ng total RNA, respectively. At 48 h, the corresponding values were 4.6 ± 0.4, 5.2 ± 0.4, and 5.3 ± 0.5 × 10^8 copies/50 ng total RNA, respectively. At 72 h, these values had increased to 7.7 ± 0.2, 8.2 ± 0.2, and 9.0 ± 0.2 × 10^8 copies/50 ng total RNA, respectively (n = 2 experiments).

Treatment with the RXR agonist AGN194204 increased IL-4 and decreased IFN-γ mRNA levels 48 and 72 h after primary Ag stimulation (Fig. 8A, C, and E; Table I), consistent with the changes in IL-4 and IFN-γ protein levels seen in supernatants at these same time points (Fig. 7). Because the balance in expression of these genes is important in Th1/Th2 development, we compared the mean IL-4:IFN-γ ratios (Fig. 8A) in the AGN194204, IL-4, and vehicle treatments at 48 h and found that they were 20, 12, and 3.1, respectively (SE = 0.85; n = 4; p < 0.001; all comparisons among means significant at p < 0.05). At 72 h, these ratios were 20, 11, and 2.3 (SE = 0.67; p = 0.007; all means significantly different) (Fig. 8A). This analysis reveals that treatment with the RXR agonist shifted the IL-4:IFN-γ ratio more strongly in favor of Th2 development than did treatment with IL-4.

RXR agonist treatment also enhanced expression of the Th2 transcription factor GATA-3 within 24 h of antigenic stimulation. GATA-3 determines which Th0 cells progress to become Th2 memory cells (28) by enhancing IL-4 and diminishing IFN-γ expression (29). GATA-3 also strongly enhances IL-5 production (30). Treatment with the RXR agonist increased GATA-3 expression at all time points (Fig. 8F; Table I). Treatment with IL-4 had...
and T-bet expression may be important in determining eventual effects, as they were essentially the same (1.55 vs 1.57). Another Th2-specific Th1 and Th2 pathway genes in CD4+/KJ1-26+ Th0 cells purified by FACS after antigenic stimulation with OVA123-339 and irradiated splenic APCs. Cells were treated with 1 × 10^{-7} M AGN194204, 0.1% DMSO, or 10 ng/ml IL-4. Values are means ± pooled SE determined by two-way ANOVA for duplicate measurements. Statistical analysis and mean values for each gene (from three to four independent experiments) are given in Table I.

A similar effect (Table I). Initially, IL-4 treatment caused a greater increase in GATA-3 (relative to the vehicle control) because the promoter region for this gene contains a possible RA response element (our unpublished observation). However, mRNA expression (36). This decrease could enhance Th2 development because Fyn mediates inhibition of Th2 cytokine expression during Th0 development (37). However, treatment with the RXR agonist did not produce a consistent change in Fyn mRNA expression (Table I).

Discussion
We have demonstrated that in vitro treatment of naive DO11.10 Th0 cell cultures with the vitamin A metabolite 9-cis RA during primary antigenic stimulation strongly enhances subsequent development of IL-4-secreting Th2 memory cells. The level of enhancement is comparable to that seen with IL-4 treatment, the classic mechanism of inducing Th2 development (38). This novel activity of 9-cis RA appears to be mediated via the RXR family of nuclear retinoid receptors, because treatment with the RXR-selective agonist AGN194204 has even more potent Th2-enhancing activity than does 9-cis RA (which binds to both RARs and RXRs). In addition, the RXR pathway in T cells seems to be responding directly to this treatment, because enhanced Th2 development occurred in purified, naive Th0 cells stimulated with anti-CD3 and anti-CD28 Ab, as well as in DO11.10 cultures using splenic APCs plus peptide Ag. This effect was also seen in two genetic backgrounds: BALB/c and C57BL/6. These data provide strong evidence that vitamin A-mediated stimulation of the RXR pathway acts directly on naive Th0 cells to enhance Th2 development.

Because Th1 and Th2 development genes can be cross-regulatory (34), we also examined expression of Th1-enhancing genes. Although expression of such genes was not altered at 24 h, we found that expression of both T-bet, a transcription factor that plays a key role in Th1 development (35), and IL-12R were decreased by RXR agonist treatment 48 and 72 h after stimulation (Fig. 8B, 8C, and 8E; Table I). Because the relative levels of GATA-3 and T-bet expression may be important in determining eventual Th1/Th2 development, we compared the mean GATA-3:T-bet ratios at 48 h and found that they were 8.5, 5.7, and 3.2 (SE = 0.14; p < 0.001; all means significantly different) in the AGN194204, IL-4, and vehicle control groups, respectively. Results at 72 h were similar, with the mean ratios being 10, 5.4, and 4.7 (SE = 0.06; p < 0.001; all means significantly different). Treatment with the RXR agonist had an even greater effect than IL-4 in shifting the GATA-3:T-bet ratio in favor of Th2 development.

Finally, we had also hypothesized that an RXR-mediated decrease in mRNA for the protein tyrosine kinase Fyn might occur because RA treatment of embryonic stem cells decreases Fyn mRNA expression (36). This decrease could enhance Th2 development because Fyn mediates inhibition of Th2 cytokine expression. Results at 72 h were similar, with the mean ratios being 10, 5.4, and 4.7 (SE = 0.14; all means significantly different). Our findings suggest that RXR agonists may, in addition, directly enhance Th2 development by acting on naive Th0 cells. Addition of IL-12-neutralizing Ab to cultures during primary stimulation did not diminish the ability of either 9-cis RA or AGN194204 to enhance Th2 development, as would be expected if this Th2-enhancing activity were due to modulation of IL-12 production by APCs. Similarly, 9-cis RA could...
of primary Th0 cultures with AGN194204 did increase IL-4 production (after the 48 h response). These observations do not support a direct role for the presence of 10 ng/ml IL-4 seemed to slightly (but significantly) decrease IFN-γ production by NK or T cells, thus indirectly enhancing Th2 development. Again, addition of IFN-γ-neutralizing Ab did not alter the ability of 9-cis RA and AGN194204 to enhance Th2 development, supporting an IFN-γ-independent mechanism. Thus 9-cis RA and AGN194204 enhance Th2 development by pathways not requiring the presence of extracellular IL-12 or IFN-γ. In addition, we found that the RXR agonist AGN194204 stimulated Th2 development in purified, naive Th0 cells when no APCs were present. This provides strong evidence that the RXR agonist acts directly on Th0 cells.

Although the presence of IFN-γ and IL-12 are not required to mediate the Th2-enhancing effect of 9-cis RA and AGN194204, both compounds require the presence of IL-4 to enhance Th2 development. This point was clearly shown in experiments demonstrating that treatment of primary cultures with IL-4-neutralizing Ab blocks the ability of these compounds to increase IL-4 production by secondary and tertiary cultures. This requirement for IL-4 in primary cultures suggests that these retinoids could enhance production of IL-4. However, treatment of mature Th1, Th2, or uncommitted Th cells with 9-cis RA did not enhance IL-4 production (Fig. 6), and treatment of naive Th0 cells with 9-cis RA in the presence of 10 ng/ml IL-4 seemed to slightly (but significantly) decrease IL-4 concentrations at secondary stimulation (data not shown). These observations do not support a direct role for the RXR pathway in enhancing IL-4 production. However, treatment of primary Th0 cultures with AGN194204 did increase IL-4 production as well as decrease IFN-γ production by these cultures. Both effects were seen at 48 h, in addition to a significant increase in IL-5. These findings could be due to a direct effect of the RXR pathway on increasing IL-4 production and subsequent enhancement of Th2 development, but a direct effect of RXR agonists on other genes in the Th1 or Th2 development pathway could also indirectly enhance IL-4 production in primary cultures.

Results of our mRNA expression studies using purified Th0 cells isolated from primary stimulation cultures support the hypothesis that the RXR pathway indirectly increases IL-4 production during primary stimulation by enhancing GATA-3 and c-maf transcription. Treatment with AGN194204 first increases mRNA levels for the GATA-3 and c-maf genes (24 h), followed by increased IL-4 mRNA and protein (48 h), increased IL-5 protein (48 h), decreased IFN-γ mRNA and protein (48 h), and decreased mRNA levels for the Th1-enhancing genes T-bet and IL-12R (48 h). This scenario is plausible because GATA-3 and c-maf both enhance IL-4 transcription, whereas GATA-3 also enhances IL-5 and diminishes IFN-γ transcription. The decreased mRNA levels for T-bet and IL-12R could be a direct effect of the RXR agonist or an indirect effect of IL-4 on the expression of these Th1 pathway genes. Because the promoter regions of GATA-3 and c-maf are not well characterized, further work is clearly needed to determine how the RXR pathway acts to enhance Th2 development. Interestingly, a recent study found that stimulation of naive Th0 cells with anti-CD3 and anti-CD28 plus vitamin D also enhanced GATA-3 and c-maf RNA levels (and subsequent Th2 development) compared with a vehicle control (40). Because vitamin D also binds to a nuclear receptor (VDR) and can form heterodimers with RXR, it is possible that the same pathway may be stimulated by two nutrients: vitamins A and D.

The observations that treatment of T cells with agonists for RAR, RXR, VDR (discussed above), and PPAR-γ (41) modulate T cell activity emphasize the important role of nutrients in regulating immune function via nuclear receptors. Nuclear receptors represent an important second level of regulation of the immune response (after the first-level regulation by direct exposure to pathogens and the regulatory molecules that they induce) by a variety of important physiological stimuli. Although not yet widely studied in immunology, nuclear receptors are a family of molecules that modulate immune function, as well as other physiologic processes, in response to a variety of signals, including diet (e.g., vitamins act via RAR, RXR, and VDR; fatty acids act via PPAR; energy and iodine modulate thyroid hormone receptor activity; and cholesterol-derived oxysterols bind to liver X receptor), gender and reproduction (sex hormones act via the estrogen, androgen, and progesterone receptors), stress (cortisol acts via the glucocorticoid receptor), and exposure to xenobiotics (some of which bind to the pregnane X receptor) (16). Their role in the regulation of immune function deserves further study.

In summary, we have found that RXR agonists strongly enhance Th2 development by acting directly on naive Th0 cells. This activity appears to be independent of the previously demonstrated ability of RA to decrease the production of the Th1-enhancing cytokine IL-12 by APCs and IFN-γ by T cells. Our data suggest that RXR agonists may enhance production of the Th2 transcription factors GATA-3 and c-maf, although this hypothesis requires confirmation. Thus, RXR agonists may play a significant role in both diminishing Th1 development and enhancing Th2 development by distinct pathways. This work also suggests that the use of RXR-selective compounds might provide a means of modulating the Th cell response in treating autoimmune or chronic inflammatory diseases, which may be Th1-mediated, or in promoting Th2 responses, which are beneficial in protecting against infectious diseases caused by extracellular pathogens.

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