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All-Trans Retinoic Acid–Triggered Antimicrobial Activity against Mycobacterium tuberculosis Is Dependent on NPC2

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A role for vitamin A in host defense against Mycobacterium tuberculosis has been suggested through epidemiological and in vitro studies; however, the mechanism is unclear. In this study, we demonstrate that vitamin A–triggered antimicrobial activity against M. tuberculosis requires expression of NPC2. Comparison of monocytes stimulated with all-trans retinoic acid (ATRA) or 1,25-dihydroxyvitamin D3 (1,25D3), the biologically active forms of vitamin A and vitamin D, respectively, indicates that ATRA and 1,25D3 induce mechanistically distinct antimicrobial activities. Stimulation of primary human monocytes with ATRA did not result in expression of the antimicrobial peptide cathelicidin, which is required for 1,25D3 antimicrobial activity. In contrast, ATRA triggered a reduction in the total cellular cholesterol concentration, whereas 1,25D3 did not. Blocking ATRA-induced cellular cholesterol reduction inhibits antimicrobial activity as well. Bioinformatic analysis of ATRA- and 1,25D3-induced gene profiles suggests that NPC2 is a key gene in ATRA-induced cholesterol regulation. Knockdown experiments demonstrate that ATRA-mediated decrease in total cellular cholesterol content and increase in lysosomal acidification are both dependent upon expression of NPC2. Expression of NPC2 was lower in caseous tuberculosis granulomas and M. tuberculosis–infected monocytes compared with normal lung and uninfected cells, respectively. Loss of NPC2 expression ablated ATRA-induced antimicrobial activity. Taken together, these results suggest that the vitamin A–mediated antimicrobial mechanism against M. tuberculosis requires NPC2-dependent expression and function, indicating a key role for cellular cholesterol regulation in the innate immune response. The Journal of Immunology, 2014, 192: 2280–2290.

One key function of the innate immune system is the rapid recognition and destruction of invading pathogens via the activation of antimicrobial pathways. In the case of Mycobacterium tuberculosis, the causative agent of tuberculosis, micronutrients have proven to be critical as part of a successful antimicrobial response. Epidemiological evidence demonstrates an association between vitamin A and tuberculosis: serum vitamin A levels are significantly higher in healthy household contacts compared with tuberculosis patients (1, 2). In the laboratory, the biologically active form of vitamin A, all-trans retinoic acid (ATRA), was shown to inhibit the growth of virulent M. tuberculosis in macrophages (3, 4). However, the molecular mechanisms and cellular processes induced by ATRA that lead to this antimicrobial activity are unclear.

Vitamin D and vitamin A share similar molecular and biochemical characteristics: both are fat-soluble secosteroids that are recognized by and effect changes in cells by binding to the vitamin D receptor and the retinoic acid receptor (RAR), respectively (5). RAR and vitamin D receptor are members of the nuclear hormone receptor family and heterodimerize with the retinoid X receptor (5). In relation to tuberculosis, deficient serum vitamin D levels are associated with tuberculosis (6, 7), and treatment of M. tuberculosis–infected cells in vitro with the active 1,25-dihydroxyvitamin D3 (1,25D3) form of vitamin D triggers antimicrobial activity (8, 9), which is comparable to the epidemiological and biochemical properties of vitamin A. Based on these similarities, we compared the 1,25D3-triggered antimicrobial response, which is dependent on production of the antimicrobial peptide cathelicidin (10, 11), with the ATRA-triggered response to elucidate the vitamin A–mediated antimicrobial mechanism.

Materials and Methods

Statistical analysis

Comparisons between two different conditions were analyzed using the Student t test. All experiments with three or more measurements were analyzed using one-way ANOVA or the Kruskal–Wallis one-way ANOVA on Ranks, as appropriate, with Student–Newman–Keuls method for pairwise analyses. Error bars represent the SEM.

Reagents

ATRA was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in DMSO, and stored at −80°C in small aliquots protected from light. 1,25D3 was purchased from Enzo Life Sciences (Farmington, NY), dissolved in ethanol, and stored at −80°C in small aliquots protected from light. Both ATRA and 1,25D3 were used at 10−8 M. LysoSensor Green DND 189 (Life Technologies) was used at 1:2000 dilution (0.5 nM), as recommended by the manufacturer. Oregon Green 488–dextran m.w. 10,000 and
Alexa Fluor 647–dextran m.w. 10,000 were purchased from Life Technologies and used at 250 and 30 μg/ml, respectively. Nelfinavir (Nel) and ritonavir (Rit) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases.

Cell culture
This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Institutional Review Board of the University of California, Los Angeles (UCLA). All donors provided written informed consent for the collection of peripheral blood and subsequent analysis. We obtained whole blood from healthy donors through the UCLA Center for AIDS Research Virology Core with informed consent. Mononuclear cells (PBMCs) were isolated from peripheral blood of healthy donors using Ficoll-Paque, as previously described (12,13). Monocytes were purified using two methods: plastic adherence and negative selection. For plastic adherence, PBMCs were cultured for 2 h in RPMI 1640 medium (Life Technologies) supplemented with 1% FCS (Omega Scientific, Tarzana, CA). The cultures were washed vigorously, and the remaining adherent cells were cultured in RPMI 1640 with 10% FCS. For negatively selected monocytes, we used an EasySep Human Monocyte Enrichment Kit (STEMCELL Technologies, Vancouver, BC, Canada), according to the manufacturer’s recommended protocol. Monocyte-derived macrophages (MDMs) were produced as previously described with M-CSF (14).

M. tuberculosis

M. tuberculosis H37Ra and H37Rv were plated on 7H11 agar plates from frozen stocks. All experiments involving H37Rv were conducted in Biosafety Level 3. Following 3–4 wk of incubation at 37°C in a water-jacketed incubator with 5% CO2, the solid colonies were scraped off the agar plate and placed in 1× PBS. The bacterial suspension was gently separated with a sonicated water bath (Branton 2510) for 30 s and then centrifuged at 735 × g for 4 min to create a single-cell suspension. To enumerate the bacteria, the supernatant was separated from the pellet, and the absorbance at 600 nm was measured using spectrophotometry. Normal monocytes and MDMs were infected at a multiplicity of infection (MOI) of 1 and transfected monocytes were infected at an MOI of 0.5 overnight, and the cells were vigorously washed three times with fresh RPMI 1640 media to remove extracellular bacteria.

Antimicrobial assay
To assess M. tuberculosis viability from infected monocytes, we used the real-time PCR–based method, as previously described (15, 16), which compares 16S RNA levels with genomic DNA (IS6110) levels as an indicator of bacterial viability. Monocytes were purified and infected with M. tuberculosis and stimulated as indicated for 3 d. For H37Ra-infected monocytes, the cells were harvested and divided following the incubation. Half of the cells were lysed by boiling at 100°C for 5 min and then snap-frozen at −80°C. Total RNA was isolated from the remaining half using TRIzol reagent (Life Technologies) via phenol-chloroform extraction, followed by RNA cleanup and on-column DNase digestion using an RNeasy Miniprep Kit (QIAGEN, Valencia, CA). cDNA was synthesized from the total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA), according to the manufacturer’s recommended protocol. The bacterial 16S rRNA and genomic element DNA levels were assessed from the cDNA and cellular lysate, respectively, using real-time PCR using iQ SYBR Green (Bio-Rad). Comparison of the bacterial DNA with the monocyte genomic levels was used to monitor infectivity between all of the conditions in the assay, as well as PCR quality. The relative 16S values were calculated using ΔΔCT analysis, with the IS6110 value serving as the “housekeeping gene.” The following IS6110 genomic element and 16S primer sequences were used: 16S Forward 5′-GGT GCG AGC GTT GTC GGC AA-3′, 16S Reverse 5′-CGG CAC CAC GCT CAC AGT TA-3′ and IS6110 Forward 5′-GGG AAC TTC TAT GAC AAT GCA CTA CGG-3′, IS6110 Reverse 5′-GGT GCG AGC GTT GTC GGC AA-3′, CYP27A1 Forward 5′-GCT ATG CCC TAC GGC ACC A-3′, CYP27A1 Reverse 5′-TCC TTC CGT GGT GAA CGG CCC ATA G-3′, NPC2 Forward 5′-TAT CCC TCT ATA AAA CTG GTG GTG-3′, NPC2 Reverse 5′-CCA GTG CAC CGG AAC TCA AT-3′, and IL6 Forward 5′-GCC CAC CGG GAA CGA CGA AAG AGA-3′, IL6 Reverse 5′-GAC CGA AGG CTC TGG TGC AGA AG-3′.

Cellular cholesterol measurement
Monocytes were cultured and stimulated as indicated for 18 h, collected, and enumerated. The lipid fraction was extracted using 3:2 hexane/isopropanol at room temperature for 30 min. Following 10 min of centrifugation, the supernatant was collected and dried in glass test tubes using nitrogen gas. Cholesterol levels were assessed with the Amplex Red Cholesterol Assay Kit (Life Technologies), using the recommended protocol, and expressed as total cholesterol/cell.

Microarray analysis
Total RNA was isolated from monocytes treated as indicated in the Results. The total RNA samples were processed and analyzed by the UCLA Clinical Microarray Core Facility using the Affymetrix U133 GeneChip. Cluster analysis was performed using the Cluster and TreeView software programs from the Eisen Lab (http://rana.lbl.gov/) (17). Biological functions and cholesterol-related functions were identified using Ingenuity Pathways Analysis (http://www.ingenuity.com/). For the caseous tuberculosis granuloma and weighted gene coexpression network analysis (WGCNA) validation microarray analysis, data files were obtained from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/, accession numbers GSE20050, GSE23073, GSE13762, and GSE28995) (18). NPC2 and IL6 levels were normalized to G3PDH. Because there are multiple G3PDH probes represented on the microarray, the NPC2 and IL6 probe values were normalized to every G3PDH probe and averaged.

WGCNA
We performed WGCNA, as previously described, using the R package “WGCNA.” (19) A signed weighted correlation network was constructed using the blockwiseModules() command with a soft thresholding power β = 9. Using an adjacency matrix calculated from pairwise correlations between all pairs of genes across all samples raised to the power β, the topological overlap was calculated as a measure of network interconnectedness. Module eigengenes were calculated for each resulting module and were correlated to ATRA or 1,25D3 treatment using a binary vector representation of treatment status.

WGCNA module preservation
WGCNA can assess whether individual modules are preserved between two data sets (19). To validate our microarray results, we examined the preservation of ATRA- and 1,25D3-specific modules against published and publically available microarray data (20–22). The R function “modulePreservation” in the WGCNA R package was applied to our data and the published data, and the Zsummary value was calculated. Zsummary scores > 10 are interpreted as “strongly preserved,” Zsummary scores between 2 and 10 are interpreted as “weak to moderately preserved,” and Zsummary scores < 2 are “not preserved.” Significance of the Zsummary scores was calculated by permutation analysis (23).

Identification of hub genes
Genes with the highest module membership values, or module eigengene–based connectivity (KME), are referred to as intramodular “hub” genes, which are genes that have the highest number of connections within the module. kME was calculated for each module using the signedKME() command. For each module, genes were sorted by kME, which are genes that have the highest number of connections within the module. kME was calculated for each module using the signedKME() command. For each module, genes were sorted by kME, and VisANT was used to visualize the gene connections among the top 50 hub genes, as ranked by KME (24).

Monocyte viability
Two methods were used to determine monocyte viability: trypan blue exclusion and TUNEL assay. Following infection for 16 h with H37Ra, monocytes were harvested, and viability was assessed. For trypan blue exclusion, the harvested cells were incubated with a final concentration of 0.04% trypan blue (Bio-Rad) and enumerated for the number of blue-labeled cells compared with total cells using an automated cell counter (Bio-Rad TC10). The data are shown as a percentage of viable cells. For

Quantitative real-time RT-PCR for mRNA
Total RNA was extracted from cells using TRIzol reagent (Life Technologies), and mRNA was reverse transcribed using iScript (Bio-Rad). Gene expression of CAMP, CYP27A1, NPC2, and IL6 was analyzed by quantitative real-time RT-PCR (qPCR), as described above, with 36B4 as the housekeeping gene. The following primers were used: CAMP Forward 5′-GGG CCC AGA CAC GCC AAA-3′, CAMP Reverse 5′-GCA CAC TGT CTC CTT CAC TGT GA-3′; CYP27A1 Forward 5′-GCT ATG CCC TAC GGC ACC A-3′, CYP27A1 Reverse 5′-TCC TTC CGT GGT GAA CGG CCC ATA G-3′; NPC2 Forward 5′-TAT CCC TCT ATA AAA CTG GTG GTG-3′, NPC2 Reverse 5′-CCA GTG CAC CGG AAC TCA AT-3′; and IL6 Forward 5′-GCC CAC CGG GAA CGA CGA AAG AGA-3′, IL6 Reverse 5′-GAC CGA AGG CTC TGG TGC AGA AG-3′.

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Transfection of monocytes

Purified monocytes were transfected with pooled small interfering RNA (siRNA) oligonucleotides using the Lonza Nucleofector 4D system with the Human Monocyte Nucleofector kit (Lonza, Allendale, NJ), according to the manufacturer’s recommended protocol. To knockdown the expression of NPC2, the predesigned and validated ON-TARGETplus siRNA targeting NPC2 (siNPC2) oligonucleotide pool was used in conjunction with the ON-TARGETplus Non-targeting Control Pool siRNA oligonucleotide control (siCTRL). The siRNA oligonucleotides were purchased from Thermo Fisher (Rockford, IL) and stored as recommended by the manufacturer.

Lysosomal acidification

Lysosomal acidification was measured using two dyes, LysoSensor and Oregon Green (both from Life Technologies), as previously described (25). Monocytes were purified and transfected, as described above, with siCTRL or siNPC2 and then stimulated with ATRA for 18 h. LysoSensor (1:2000) was added to each well, rocked gently for 5 s to mix, and incubated for 30 min. After incubation, the cells were fixed in 1% paraformaldehyde, and fluorescence was acquired using flow cytometry. The mean fluorescent intensity (MFI) was determined on the monocytes in the samples, which were the only cellular population in the samples given that they were magnetically separated as indicated in the “Transfection of monocytes” section above. For Oregon Green labeling, the transfected and stimulated monocytes were harvested, washed, and incubated with Oregon Green 488 (250 μg/ml) and Alexa Fluor 647 (30 μg/ml) for 30 min. The cells were washed and analyzed by flow cytometry. Both Oregon Green 488 and Alexa Fluor 647 were conjugated to 10,000 m.w. dextran for targeting into the lysosome. Data shown are the ratio of the fluorescence detected for Oregon Green 488/Alexa Fluor 647.

Results

ATRA- and 1,25D3-induced monocyte function

Primary human monocytes were infected overnight with M. tuberculosis H37Ra, an avirulent strain used to model mycobacterial infection (10, 15), and then treated with carrier control, ATRA, or 1,25D3 for 3 d. Following treatment, the ratio of bacterial 16S RNA/IS6110 genomic repeat element was determined as an indicator of bacterial viability, as we previously described (15). Both ATRA and 1,25D3 treatment resulted in a decrease in bacterial viability compared with carrier control (representative figure, Fig. 1A). On average, M. tuberculosis viability, measured as \( \log_{10}(16S/IS6110) \) in monocytes treated with ATRA or 1,25D3, was significantly reduced (Fig. 1B) and of comparable magnitude at these concentrations (10–8 M) to previously published studies using macrophages and the CFU assay (3, 8–10).

Given that the 1,25D3-mediated antimicrobial activity is dependent on expression of the antimiicrobial peptide cathelicidin (10,11), we determined whether ATRA could induce cathelicidin (CAMP) mRNA in primary human monocytes. Although 1,25D3 was able to significantly induce expression of CAMP mRNA, ATRA did not (Fig. 1C). In contrast, CYP27A1 mRNA, a known ATRA response gene (26), was induced by ATRA but not 1,25D3 (Fig. 1D), indicating that the cells were responding to ATRA. Because cholesterol is a key factor in the interactions between innate immune cells and M. tuberculosis (27–29), and ATRA is known to induce cholesterol efflux (26, 30, 31), we hypothesized that the ability of ATRA to regulate cellular cholesterol content is linked to antimicrobial activity. The cellular cholesterol concentration (total cholesterol/cell) in monocytes treated with carrier control, ATRA, or 1,25D3 was measured (representative figure, Fig. 1E). ATRA treatment resulted in a significant reduction (∼34 ± 7% versus control, \( p < 0.001 \)) in total cellular cholesterol content, whereas 1,25D3 had no effect (Fig. 1F). These results suggest that ATRA and 1,25D3 induce distinct intracellular pathways and likely use different antimicrobial mechanisms.

Role of cholesterol regulation in ATRA-induced antimicrobial activity

To determine whether an ATRA-induced reduction in cellular cholesterol plays a role in ATRA-mediated antimicrobial activity, we used Nel and Rit, two compounds previously described to inhibit cholesterol efflux from human macrophages and macrophage-derived foam cells (32,33). Monocytes were infected with M. tuberculosis H37Ra for 18 h, washed to remove extracellular bacterium, preincubated for 10 min with Nel at 10 μM and Rit at 30 μM (concentrations previously described to inhibit cholesterol efflux) (32,33), and stimulated with ATRA for 3 d. ATRA induced a decrease in cellular cholesterol compared with carrier control, which was significantly inhibited by the presence of Nel or Rit (Fig. 2A). There were no significant effects of Nel or Rit on baseline total cellular cholesterol levels (Supplemental FIGURE 1. ATRA- and 1,25D3-induced cellular responses. Bacterial viability of M. tuberculosis H37Ra-infected primary monocytes treated with carrier control (CTRL), ATRA, or 1,25D3 for 3 d, displayed as a representative experiment (A) or \( \log_{10}(\text{mean bacterial viability versus CTRL} \pm \text{SEM}) \) (B) \((n = 4)\). mRNA expression levels assessed by qPCR of cathelicidin (CAMP) (C) and CYP27A1 (D) in primary human monocytes stimulated with CTRL, ATRA, or 1,25D3 for 18 h, displayed as mean fold change versus CTRL \( \pm \text{SEM} \) (n = 7). Cellular cholesterol levels/cell of monocytes treated with CTRL, ATRA, or 1,25D3 for 18 h shown as a representative experiment (E) or mean percentage change versus CTRL \( \pm \text{SEM} \) (F) \((n = 5)\).
Fig. 1A). Importantly, ATRA-induced antimicrobial activity was significantly inhibited in *M. tuberculosis* H37Ra–infected monocytes by Nel (from −1.3 to −0.05 ± 0.17, *p* = 0.0002, versus ATRA only) and Rit (from −1.3 to −0.13 ± 0.22, *p* = 0.002, versus ATRA only) (Fig. 2B).

Because macrophages are the natural cellular host type for *M. tuberculosis* infection, we used MDMs, a cellular model for antimicrobial activity against *M. tuberculosis* in macrophages (14). MDMs were infected with the virulent *M. tuberculosis* H37Rv strain at an MOI of 1 for 18 h, washed to remove extracellular bacteria, preincubated for 10 min with Nel and Rit, and stimulated with ATRA for 3 d. Pretreatment of *M. tuberculosis* H37Rv–infected MDMs with Nel or Rit inhibited ATRA-induced antimicrobial activity (Nel: from −0.9 to −0.05 ± 0.16, *p* = 0.05, versus ATRA only; Rit: from −0.85 to −0.28 ± 0.15, *p* = 0.05, versus ATRA only) (Fig. 2C). These data suggest that regulation of cellular cholesterol may play an important role in ATRA-induced antimicrobial activity in both monocytes and macrophages.

**ATRA- versus 1,25D3-induced gene-expression profiles**

To identify specific genes driving the ATRA-induced cholesterol regulation, we compared the monocyte gene-expression profiles induced by ATRA (10^{-8} M) or 1,25D3 (10^{-8} M) treatment for 18 h from four independent donors using microarrays. Analysis of genes significantly upregulated (1.2-fold versus control, *p* < 0.05) by either ATRA or 1,25D3 revealed three gene groups: induced by ATRA only, induced by 1,25D3 only, and induced by both (Fig. 3A). A total of 868 genes was represented in the ATRA-only group, 2591 genes were induced by both, and 205 genes were induced by either ATRA or 1,25D3. As expected, CYP27A1 was significantly upregulated (2.6 ± 0.3-fold versus control, *p* < 0.001) and was present in the ATRA-only group (Supplemental Fig. 1B).

To confirm the differential gene-expression signatures induced by ATRA or 1,25D3, we applied WGCNA to the microarray data (34). WGCNA identified transcripts that organize into distinct modules of coexpressed genes (Fig. 3B). In particular, the “salmon” module eigengene was significantly correlated with ATRA stimulation (*r* = 0.95, *p* = 2 × 10^{-6}), whereas the “cyan” module eigengene was correlated with 1,25D3 stimulation (*r* = 0.91, *p* = 4 × 10^{-7}) (Fig. 3C). To validate these ATRA- and 1,25D3-induced gene-expression profiles, we determined the preservation of the cyan and salmon modules in published and publicly available microarray studies that examined CD14+ monocytes differentiating into dendritic cells treated with 1,25D3 (10^{-8} M) for 12 h (21), and the THP-1 monocytic cell line treated with ATRA (2 × 10^{-7} M) for 2, 6, or 16 h (22). These studies were chosen for comparison with our current study because they were conducted on myeloid immune cells, used similar doses of ATRA and 1,25D3, and used similar incubation times. Because of the low number of THP-1 samples at 2 h (*n* = 2) and 6 h (*n* = 2) for the ATRA study, the data from these two time points were combined to provide the necessary resolution for the module-preservation test. The cyan module demonstrated a strong preservation in the 1,25D3 studies (Fig. 3D), whereas the salmon module was only preserved in the ATRA study (Fig. 3E). These results suggest that the gene-expression profiles we obtained in ATRA or 1,25D3 stimulated primary human monocytes are representative of the core gene signatures induced by ATRA or 1,25D3.

**Identification of candidate genes**

To determine the relationship between the gene-expression profiles and cellular function, the upregulated genes were analyzed by Ingenuity Pathways Analysis (IPA), a knowledge-guided bioinformatics tool, to identify biological functions enriched by ATRA or 1,25D3 (analysis scheme displayed in Supplemental Fig. 2). Based on our hypothesis that control of cellular lipids is a key element of the ATRA-induced antimicrobial response, the categories “lipid metabolism,” “molecular transport,” and “small molecule biochemistry” (the second-, third-, and fourth-ranked
functions, respectively) were examined further (Fig. 4A). The same three categories (“lipid metabolism,” “molecular transport,” and “small molecule biochemistry”) were the 27th-, 23rd-, and 2nd-ranked 1,25D3-induced categories, respectively (Fig. 4B). Comparing the ATRA- and 1,25D3-induced genes in the “small molecule biochemistry” category reveals 14 genes in common, which represents 6.2% of the total genes in the category induced by either ATRA or 1,25D3 (Supplemental Fig. 2). Taken together, these analyses suggest that ATRA induces a lipid metabolism and intracellular molecular transport gene profile that is not present in 1,25D3-stimulated monocytes.

The comparison of the ATRA-induced genes in the “lipid metabolism,” “molecular transport,” and “small molecule biochemistry” categories revealed a high degree of similarity among the categories, with 44 genes in common (Fig. 4C). A total of 11 of the 44 common genes also were identified as hub genes by WGCNA (Fig. 4D). Of the 44 common genes, 16 genes were annotated by IPA with functions related to regulation of cellular cholesterol (functions: “accumulation of cholesterol,” “concentration of cholesterol,” and “efflux of cholesterol”), three of which (NPC2, CYP27A1, and LAMP2) were also hub genes. Only one gene, NPC2, was annotated with all three cholesterol-related functions and was induced by ATRA (Fig. 4E) but not by 1,25D3 (Fig. 4F). The kME value for NPC2 was 0.98, which was the highest ranked hub gene identified by WGCNA in the salmon module. These data suggest that NPC2 may play a central role in the ATRA-induced gene signature that mediates the intracellular regulation of cholesterol content.

**Induction of NPC2 by ATRA**

Monocytes were stimulated with ATRA or 1,25D3, and NPC2 mRNA levels were measured by qPCR. Confirming the microarray analysis, the qPCR results demonstrated specific induction of NPC2 mRNA in ATRA-stimulated monocytes compared with 1,25D3 stimulation (Fig. 5A). To better characterize the induction of NPC2, monocytes were stimulated with ATRA at 10^{-8} M for 1, 4, 16, or 24 h. Total RNA was harvested, and NPC2 mRNA levels were measured by qPCR. NPC2 was significantly induced (3.4-fold versus control, \( p = 0.008 \)) at the 16-h time point in monocytes (Fig. 5B). An RAR response element is present 1312 bp upstream of the NPC2 mRNA start site (Supplemental Fig. 3A). These results demonstrate that NPC2, which has an RAR response element, can be induced by ATRA stimulation of monocytes.
Role of NPC2 in ATRA-induced regulation of cellular cholesterol content

Mutations in NPC1 or NPC2 are associated with Niemann–Pick disease, a lysosomal storage disorder that is characterized by abnormally high cholesterol accumulation in cells. NPC1 and NPC2 have common (35) and nonredundant functions related to lysosomal lipid transport (36,37). Stimulation of monocytes with ATRA resulted in expression of NPC2, but not NPC1, detected by qPCR correlating with the microarray results, suggesting a specific role for NPC2 as a lipid transporter in ATRA-stimulated cells (Supplemental Fig. 3B). To ascertain the role of NPC2 in ATRA-mediated regulation of cellular cholesterol concentration, we transfected monocytes with siNPC2 or siCTRL and then stimulated the cells with carrier control or ATRA for 18 h. siNPC2 transfection resulted in a significant decrease in NPC2 mRNA levels in both resting and ATRA-stimulated monocytes (Fig. 5D). Neither siNPC2 nor siCTRL had an effect on the CYP27A1 mRNA levels of resting and ATRA-stimulated monocytes (Fig. 5E), indicating that the siNPC2 knockdown was specific. Correlating with the expression of NPC2, siCTRL-transfected monocytes stimulated with ATRA demonstrated a reduction (±42 ± 15% versus control treated, p<0.05) in total cellular cholesterol content (Fig. 5F). In marked contrast, ATRA stimulation of siNPC2-transfected monocytes resulted in an increase (126 ± 69% versus control treated, p<0.05) in total cellular cholesterol content (Fig. 4F). There was no significant difference in baseline cellular cholesterol concentration.
between untreated siCTRL- and siNPC2-transfected cells (Supplemental Fig. 3C).

Role of NPC2 in lysosomal acidification

Blocking cholesterol egress from lysosomes prevents acidification (38), which is a key process in antimicrobial activity against M. tuberculosis (39). We sought to address the role of NPC2 in ATRA-induced lysosomal acidification using two dyes: LysoSensor, which accumulates and increases fluorescence intensity in acidic organelles, and Oregon Green 488, a pH-sensitive fluorescent dye (25). siCTRL- and siNPC2-transfected monocytes were treated with ATRA for 18 h and labeled using LysoSensor. Stimulation with ATRA resulted in increased LysoSensor labeling in siCTRL-transfected, but not siNPC2-transfected, monocytes (Fig. 6A).

The change in MFI in siCTRL cells treated with ATRA (11.1%, p = 0.012) was significantly higher (p = 0.009) compared with siNPC2 cells (1.2%) treated with ATRA (Fig. 6B).

Oregon Green 488 is pH sensitive and exhibits a decrease in fluorescence when exposed to acidic environments, whereas Alexa Fluor 647 is pH resistant, and the fluorescence remains constant. Therefore, a decrease in the Oregon Green 488/Alexa Fluor 647 ratio (OG:A647) indicates an increase in acidification. siCTRL- and siNPC2-transfected monocytes were treated with ATRA for 18 h and then colabeled with Oregon Green 488 and Alexa Fluor 647, both conjugated to dextran for lysosomal targeting. Stimulation with ATRA resulted in a decreased OG:A647 in siCTRL-transfected, but not siNPC2-transfected, monocytes (Fig. 6C). The decreased OG:A647 in siCTRL-transfected cells treated with ATRA (16.6%, p < 0.001) was significantly higher (p < 0.001) compared with siNPC2-transfected cells (1.4%) treated with ATRA (Fig. 6D).

These data suggest that ATRA induces lysosomal acidification that is dependent upon the expression of NPC2.

Role of NPC2 during M. tuberculosis infection

To determine whether M. tuberculosis infection regulates NPC2 expression in situ, we analyzed a previously published gene microarray experiment that compared uninvolved lung tissue with caseous tuberculosis granulomas (40). The relative expression of NPC2 and IL6 was compared with G3PDH in the same samples to account for differences between the sample types. Based on this analysis, NPC2 levels trended lower (0.46-fold, p = 0.07), whereas IL6 levels, which is inducible by M. tuberculosis (41), were sig-
significantly higher (2.7-fold, \(p = 0.0004\)) in caseous tuberculosis granulomas compared with uninvolved lung tissue (Fig. 7A). Because lung biopsies contain multiple cell types, including nonimmune cells, the effects of \(M.\) \(tuberculosis\) infection on the NPC2 signal may be confounded. Therefore, we addressed the effects of \(M.\) \(tuberculosis\) infection on NPC2 expression directly in monocytes. In monocytes infected with \(M.\) \(tuberculosis\) H37Ra, we observed a significant decrease (0.4 ± 0.1-fold versus uninfected, \(p = 0.002\)) in NPC2 mRNA after 18 h (Fig. 7B) and an increase in IL6 mRNA (Fig. 7C) correlating with the microarray data. There was no change in monocyte viability following infection, as determined by trypan blue exclusion and TUNEL assay (Fig. 7D).

Treatment of infected monocytes with ATRA for 3 d, which parallels the antimicrobial assay time course, resulted in a significant increase in NPC2 mRNA levels in monocytes (Fig. 7E) and MDMs (Fig. 7F). Knockdown of NPC2 mRNA ablated the ATRA-induced antimicrobial activity (0.29 ± 0.22 versus siNPC2 control stimulated), whereas transfection of siCTRL had no effect (−0.30 ± 0.08 versus siCTRL control stimulated, \(p < 0.05\)) (Fig. 7G). In contrast, knockdown of NPC2 did not affect the ability of 1,25D3 to induce antimicrobial activity (Fig. 7G), which is expected based on the fact that NPC2 was not induced by 1,25D3 and that 1,25D3 uses a cathelicidin-dependent pathway (10). Comparison of siCTRL- and siNPC2-transfected monocytes treated with vehicle control showed no significant difference in bacterial viability during the course of the antimicrobial activity experiment (Supplemental Fig. 3D). Taken together, these findings indicate an important role for NPC2 in the ATRA-triggered antimicrobial response against \(M.\) \(tuberculosis\) infection.

**Discussion**

Although vitamin A has been associated with host protection against \(M.\) \(tuberculosis\) both in vivo (1,2) and in vitro (3,4), the precise vitamin A–induced antimicrobial mechanism remained unclear. In this study, we sought to explore the mechanism(s) driving the vitamin A–triggered antimicrobial response by comparing vitamin A (ATRA)-induced and vitamin D (1,25D3)-induced cellular and genomic responses, given that both are known to induce antimicrobial activity in \(M.\) \(tuberculosis\)–infected monocytes and macrophages (3,8,9). Previously, we demonstrated that 1,25D3-induced antimicrobial activity was dependent on expression of the antimicrobial peptide cathelicidin (10,11); however, our current study found that ATRA did not induce cathelicidin expression. In contrast, ATRA, but not 1,25D3, stimulation resulted in the reduction of cellular cholesterol content. Blocking cholesterol egress inhibited ATRA-mediated antimicrobial activity in monocytes infected with \(M.\) \(tuberculosis\) H37Ra, as well as MDMs infected with \(M.\) \(tuberculosis\) H37Rv. Bioinformatic analysis combining WGCNA and IPA revealed NPC2, a lysosomal to endoplasmic reticulum lipid transporter, as a potential mediator of ATRA-induced regulation of cellular cholesterol content. NPC2 expression is decreased in caseous tuberculosis granulomas and infected monocytes compared with normal lung tissue and uninfected cells, respectively. Stimulation of \(M.\) \(tuberculosis\) H37Ra–infected monocytes or \(M.\) \(tuberculosis\) H37Rv–infected MDMs with ATRA recovered NPC2 expression levels, and knockdown of NPC2 expression ablated the ATRA-induced anti-
microbial activity, suggesting that NPC2 plays a pivotal role in vitamin A–mediated host defense. These results demonstrate that vitamin A–induced immune defense against *M. tuberculosis* is dependent on the expression and function of NPC2.

Regulation of cholesterol is an important facet of the host–pathogen interaction between immune cells and *M. tuberculosis*. In a caseous tuberculosis granuloma there is an increased expression of genes involved in lipid sequestration and metabolism, as well as an accumulation of cholesterol, cholesteryl esters, triacylglycerols, and lactosylceramide (40). The presence of foamy macrophages in the granuloma (42) and host hypercholesterolemia both correlate with loss of protection against *M. tuberculosis* (43). Studies also demonstrated that phagocytosis of *M. tuberculosis* (27) and bacterial persistence within the macrophages are dependent on cholesterol (28). *M. tuberculosis* can accumulate and use cholesterol as a source of nutrition (29, 44, 45), as well as exploit host-derived lipids to reduce metabolic stress (46), which could be a determinant of pathogen virulence and immunogenicity (47). Accumulation of lipids within lysosomes alters the pH of the vesicle to favor bacterial survival (38), and fusion of lysosomes with phagosomes harboring *M. tuberculosis* is a critical host defense process against the infection (39,48,49).

Our data demonstrate that the ATRA-mediated decrease in cellular cholesterol requires the expression and function of NPC2. Mutations in the NPC2 gene have been well defined to be responsible for Niemann–Pick disease type C2, a lysosomal storage disease characterized by abnormally high cholesterol accumulation in cells. Our experiments show that loss of NPC2 expression ablated the ATRA-mediated reduction in cellular cholesterol content, and it resulted in a significant accumulation of cellular cholesterol, paralleling the cellular etiology of Niemann–Pick disease. Important to macrophage antimicrobial defense against *M. tuberculosis*, knockdown of NPC2 expression also ablated ATRA-induced lysosomal acidification, which is required for antimicrobial activity against the infection (38,39,48,49). Thus, decreasing NPC2 expression levels during infection can inhibit
important macrophage defense mechanisms, as well as increase a nutrient source within the cell, which favors bacterial survival. These data suggest that regulation of cellular cholesterol through proteins, such as NPC2, may be a key part of the innate immune response, and further studies investigating the consequence of cellular cholesterol modulation on bacterial viability are warranted.

Although vitamin A deficiency is associated with tuberculosis, vitamin A supplementation has not proven effective as a treatment (50,51). This is likely because vitamin A status is determined by serum retinol levels, and vitamin A supplementation similarly modulates retinol levels. In contrast, the ability to induce antimicrobial activity through vitamin A metabolites, such as in this study and previous studies (3,4) use the active ATRA form. This suggests that, although activation of infected macrophages with ATRA results in antimicrobial activity against intracellular *M. tuberculosis* infection, the intricate pathways that regulate retinol metabolism, especially in tuberculosis patients, are still unclear. If retinol metabolism were inhibited by infection, then the effects of vitamin A supplementation will be effectively negated. Further studies are needed to understand how vitamin A is metabolized during the immune response to *M. tuberculosis* infection.

In summary, our data demonstrate a novel role for NPC2 in the ATRA-mediated innate immune response against *M. tuberculosis*, which suggests that regulation of intracellular cholesterol may be an important facet of defense against infection. Understanding how vitamin A–mediated functions are regulated during infection in the host will be an important step in determining how this micronutrient can influence the outcome of disease.

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