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Cutting Edge: Vitamin D Regulates Lipid Metabolism in *Mycobacterium tuberculosis* Infection

Hugh Salamon,^{*,1} Natalie Bruiners,^{†,1} Karim Lakehal,^{†,1,2} Lanbo Shi,^{†,‡} Janani Ravi,[†] Ken D. Yamaguchi,^{*} Richard Pine,^{†,‡} and Maria Laura Gennaro^{†,‡}

Vitamin D has long been linked to resistance to tuberculosis, an infectious respiratory disease that is increasingly hard to treat because of multidrug resistance. Previous work established that vitamin D induces macrophage antimicrobial functions against *Mycobacterium tuberculosis*. In this article, we report a novel, metabolic role for vitamin D in tuberculosis identified through integrated transcriptome and mechanistic studies. Transcriptome analysis revealed an association between vitamin D receptor (VDR) and lipid metabolism in human tuberculosis and infected macrophages. Vitamin D treatment of infected macrophages abrogated infection-induced accumulation of lipid droplets, which are required for intracellular *M. tuberculosis* growth. Additional transcriptomics results showed that vitamin D downregulates the proadipogenic peroxisome proliferator-activated receptor γ (PPAR γ) in infected macrophages. PPAR γ agonists reversed the antiadipogenic and the antimicrobial effects of VDR, indicating a link between VDR and PPAR γ signaling in regulating both vitamin D functions. These findings suggest the potential for host-based, adjunct antituberculosis therapy targeting lipid metabolism. *The Journal of Immunology*, 2014, 193: 30–34.

Effective treatment of tuberculosis, a global infectious disease that kills almost two million people a year, can only be achieved with a protracted, multiantibiotic regimen; the rapid emergence of antibiotic resistance has been eroding even these less-than-optimal treatment options (1). A hallmark of infection with *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is the differentiation of infected macrophages into lipid-rich foam cells (2). These cells accumulate lipid droplets, lipid storage organelles (3) that are re-

quired for intracellular bacillary growth (4), presumably because they provide nutrients (2). Foam cells are located in the tuberculous granuloma at the interface between the caseous center and the lymphocytic cuff; there they contribute to an environment permissive for mycobacterial dormancy (5). Thus, the remodeling of macrophage lipid metabolism that occurs during *M. tuberculosis* infection is likely associated with infection outcome. Finding ways to prevent infection-induced dysregulation of macrophage lipid metabolism might help control infection. In the present study, we asked whether the protective role of vitamin D during infection with *M. tuberculosis* (6–8) involves an effect on macrophage lipid metabolism, in addition to its well-known induction of macrophage antimicrobial functions (9), because vitamin D can have lipid-altering effects (10), and the vitamin D receptor (VDR) interacts with lipid-sensing regulatory molecules affecting macrophage functions (11).

Materials and Methods

Cell growth and infection

In vitro infections were performed with *M. tuberculosis* H₃₇Rv and with the human monocytic cell line THP-1, essentially as previously described (12). Briefly, PMA-differentiated cells were infected with mid-log bacterial cultures at the indicated multiplicity of infection (MOI). Wells were washed three times to remove extracellular bacteria at 4 h postinfection, at which time any compound(s) was added with fresh culture medium, as required, and maintained for the duration of the experiment. All manipulations with viable *M. tuberculosis* were performed under approved biosafety level 3–containment protocols.

Analysis of bacterial growth and lipid droplets

Bacterial CFU were enumerated on 7H10 agar plates. For detection of lipid droplets using flow cytometry, cells were fixed with 4% paraformaldehyde and stained with LipidTOX Deep Red (Molecular Probes, Life Technologies, Grand Island, NY). Data were collected with an Accuri 6 flow cytometer, and data analysis was performed using CFlow Plus software.

RNA extraction for transcriptomics and quantitative RT-PCR

Gene expression profiles for THP-1 cells were determined using Affymetrix Human Gene 2.0 ST, following the vendor's protocols. For measurement of

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H.S., R.P., and M.L.G. conceived the research and wrote the manuscript; H.S. analyzed the bioinformatics data; H.S. and K.D.Y. designed the bioinformatics; N.B., K.L., and L.S. performed experiments; and N.B., K.L., J.R., R.P., and M.L.G. designed experiments and analyzed data.

The sequences presented in this article have been submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57028>) under accession number GSE57028.

Address correspondence and reprint requests to Dr. Richard Pine and Dr. Maria Laura Gennaro, Public Health Research Institute, New Jersey Medical School, Rutgers University, 225 Warren Street, Newark, NJ 07103. E-mail addresses: richard.pine@rutgers.edu (R.P.) and marila.gennaro@rutgers.edu (M.L.G.)

The online version of this article contains supplemental material.

Abbreviations used in this article: BCG, bacillus Calmette–Guérin; FDR, false discovery rate; MOI, multiplicity of infection; PPAR γ , peroxisome proliferator-activated receptor γ ; VDR, vitamin D receptor.

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individual gene expression levels, RNA was extracted, oligonucleotide primers and molecular beacon probes were designed, and quantitative RT-PCR was performed as described (13). Gene expression was normalized against that of the reference gene GAPDH.

Gene expression analysis

Transcriptome data were obtained from Gene Expression Omnibus accession no. GSE19491 for clinical samples (14) and from GSE17477 for THP-1 cells (15). Data from the current study have been deposited to the Gene Expression Omnibus under accession no. GSE57028 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57028>). Statistical analysis was performed in R. One-sided and two-sided *t* tests were performed to compare measurements of transcript levels between sample groups, and the resulting *p* values were calculated. To evaluate gene sets defined by Reactome (<http://reactome.org/>), Gene Ontology (<http://www.geneontology.org/>), or by transcriptional modulators (16), each set was tested for extreme ranks of differential expression among all measured genes in each comparison by coincident extreme ranks in numerical observations, and multiple transcript measurements were combined as described (17). The Benjamini–Hochberg method was used to calculate the false discovery rate (FDR).

Results and Discussion

We used transcriptomics to explore associations among VDR signaling, tuberculosis, and lipid metabolism. When we analyzed the transcriptome of *M. tuberculosis*-infected THP-1 cells (15), we found that VDR was highly represented among DNA-binding proteins associated with upregulated genes (Fig. 1A). Reanalysis of human whole-blood transcriptome data from Berry et al. (14) identified altered expression of VDR-bound genes associated with latent *M. tuberculosis* infection and bacillus Calmette–Guérin (BCG) vaccination compared with controls, as well as with pulmonary tuberculosis compared with latent infection or treated disease (Fig. 1B). Thus, mycobacterial

infection leaves a significant fingerprint of VDR-regulated gene expression in leukocytes, and this fingerprint is recapitulated in THP-1 cells. Next, we investigated the link between the VDR signal and the cellular functions associated with infection. Up-regulated gene pathways in THP-1 cell infection included “metabolism of lipids and lipoproteins” (and its subsets “cholesterol biosynthesis” and “sphingolipid metabolism”), in addition to well-studied immune responses, such as IFN- α / β signaling and TLR cascades (Supplemental Fig. 1). Further analysis revealed that VDR was the DNA-binding protein most significantly associated with upregulation of “metabolism of lipids and lipoproteins” (Fig. 1C, Supplemental Fig. 1). VDR was also significant within upregulated lipid metabolism pathways in some of the clinical comparisons in Fig. 1B, including pulmonary tuberculosis versus controls (FDR = 5e–06) and BCG vaccination versus controls (FDR = 2.3e–03). Collectively, the transcriptomics data strongly implicate VDR in the regulation of lipid metabolism in *M. tuberculosis*-infected THP-1 cells and in human tuberculosis.

To assess the mechanistic implications of the transcriptomic results, we experimentally investigated the connection between macrophage lipid metabolism and VDR signaling in the context of *M. tuberculosis* infection. Because lipid droplets form in *M. tuberculosis*-infected macrophages (2), we measured lipid droplet content as a readout for altered lipid metabolism in infected THP-1 cells. We found that addition of vitamin D (1,25 α -dihydroxyvitamin D3) to the infected cells prevented induction of lipid droplets (Fig. 2A) but had no effect on lipid droplets in uninfected cells (Supplemental

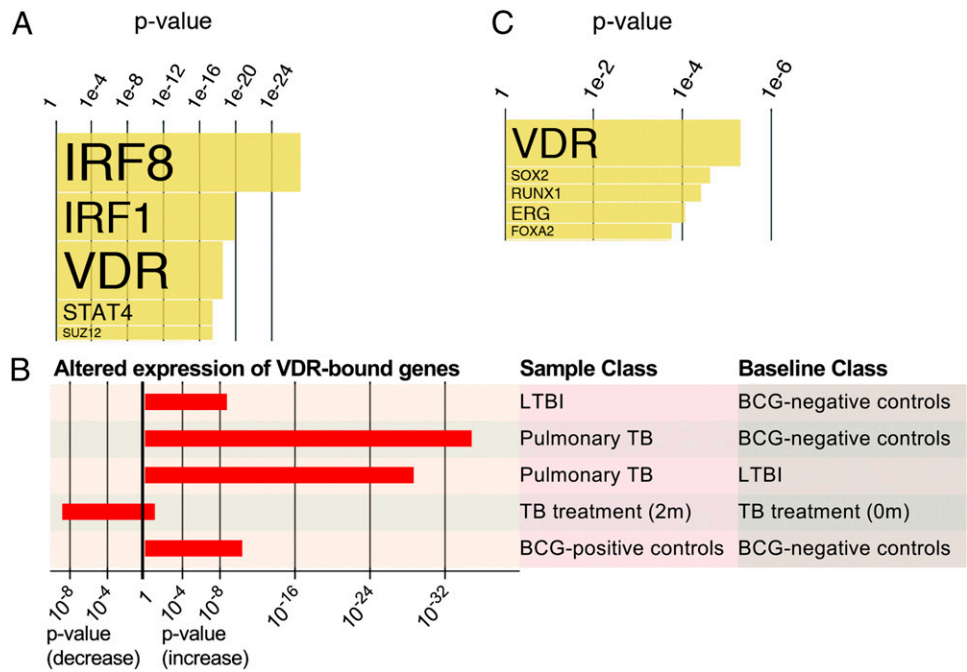


FIGURE 1. Transcriptomic analyses of in vitro THP-1 infection and clinical data. **(A)** DNA-binding proteins implicated by genes upregulated in *M. tuberculosis*-infected THP-1 cells. Gene sets were defined as genes annotated as bound by each transcriptional modulator (16). Differential expression between sample classes for these gene sets was determined by a nonparametric test (coincident extreme ranks in numerical observations) (17). The resulting *p* values were plotted onto the *x*-axis. Of 60 gene sets at FDR < 0.05, the top five are shown (IRF8, *n* = 607; IRF1, *n* = 326; VDR, *n* = 161; STAT4, *n* = 896; SUZ12, *n* = 2812). To represent effect size, sets with fewer genes were given greater bar height than were larger sets that yielded similar *p* values. **(B)** Statistical significance of VDR-bound genes in clinical comparisons, as indicated. Primary data were from a previous study (14). The *p* values were calculated as in (A). **(C)** DNA-binding proteins implicated by analysis of upregulated genes annotated for “metabolism of lipids and lipoproteins” (*n* = 348) in infected THP-1 cells. Gene sets were defined by the overlap between the pathway genes and genes annotated as bound by a transcriptional modulator (Supplemental Fig. 1). Each overlap set was tested for differential expression as in (A). Of 29 gene sets at FDR < 0.05, the top five are shown (VDR, *n* = 23; SOX2, *n* = 165; RUNX1, *n* = 126; ERG, *n* = 64; FOXA2, *n* = 93), plotted as in (A).

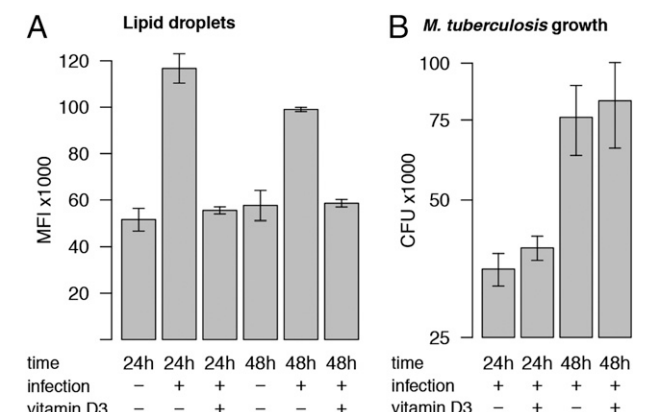


FIGURE 2. Effects of vitamin D on lipid droplet formation and *M. tuberculosis* growth in THP-1 cells. Differentiated THP-1 cells were infected with *M. tuberculosis*, treated with 100 nM vitamin D, as indicated, and harvested at 24 and 48 h postinfection. Results shown are means from triplicate experiments (\pm SEM). **(A)** Cells were fixed and stained with LipidTOX Deep Red for detection of lipid droplets by flow cytometry. Data were calculated as mean fluorescence intensity (MFI). **(B)** Infected cells were lysed, and *M. tuberculosis* CFU were determined. The selected dose of vitamin D caused maximal inhibition of lipid droplets and induced significant expression of VDR target genes *CAMP* and *CYP24A1* in uninfected and infected cells at 24 h postinfection (data not shown).

Fig. 2A, left panel). Moreover, vitamin D did not concurrently reduce bacterial CFU (Fig. 2B), but it did limit bacillary growth at later times postinfection (Supplemental Fig. 2B). Together, the data show that inhibition of lipid droplet induction by vitamin D was dependent on infection and was not merely secondary to reduced bacterial burden.

To explore the underlying mechanisms, we obtained transcriptome data from infected THP-1 cells with and without vitamin D treatment and from corresponding controls. Analysis

of lipid-annotated gene sets showed that, in most cases, vitamin D treatment did not alter the direction of the infection-induced changes (Fig. 3A). However, the gene set “lipid binding” and its subset “phospholipid binding” were significantly upregulated by infection alone but were downregulated by vitamin D treatment of infected cells (Fig. 3A). This expression pattern mirrors the effect of infection and vitamin D treatment of infected cells on the formation of lipid droplets (Fig. 2A). Further analysis of the downregulated “lipid-binding” genes for DNA-binding protein annotations identified a strong signal associated with peroxisome proliferator-activated receptor γ (PPAR γ) in vitamin D-treated, infected cell transcriptomes (Fig. 3B). This result agrees with a role for PPAR γ in VDR inhibition of adipocyte differentiation (18).

The downregulation of PPAR γ -bound genes in the response of infected cells to vitamin D led us to test relationships between PPAR γ and VDR signaling. We found that macrophage infection with *M. tuberculosis* induced PPAR γ expression and activity (the latter assessed as expression of the sentinel PPAR γ target gene CD36) and that vitamin D abrogated this induction (Fig. 4A). We then tested whether PPAR γ agonists reversed the vitamin D inhibition of lipid droplet induction. When *M. tuberculosis*-infected THP-1 cells were treated with vitamin D and with two PPAR γ agonists, rosiglitazone (a thiazolidinedione) and GW1929 (a non-thiazolidinedione), lipid droplets returned to levels comparable to those seen with *M. tuberculosis* infection alone (Fig. 4B) [lipid droplets were unaffected by agonists alone in uninfected (data not shown) or infected cells (Fig. 4B)]. Moreover, the PPAR γ agonists also reversed the antimicrobial effect of vitamin D while having very little, if any, effect on bacterial growth when tested alone (Fig. 4C). Thus, PPAR γ agonism counteracts both antiadipogenic and antimicrobial effects of vitamin D. These results agree with proadipogenic

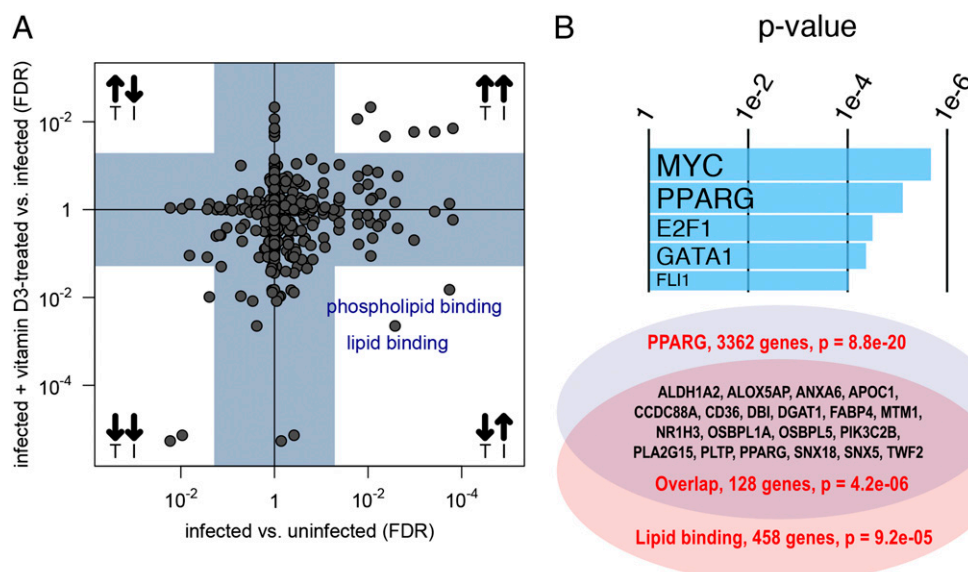


FIGURE 3. Transcriptomic analyses of THP-1 cells infected with *M. tuberculosis* and treated with vitamin D. **(A)** A total of 108 Gene Ontology gene sets containing the terms “lipid” or “cholesterol” was tested for increased and decreased expression. FDR values for these annotations (gray circles) were plotted for the comparison infected versus uninfected cells (infection [I]; x-axis) and for infected cells with/without vitamin D treatment (treatment [T]; y-axis). Test results for increased (up arrows) and decreased (down arrows) gene expression were plotted. The gray area includes nonsignificant results for both comparisons (FDR \geq 0.05), whereas the white area contains significant results (FDR < 0.05). **(B)** DNA-binding proteins implicated by downregulation of genes annotated for “lipid binding” upon vitamin D treatment of *M. tuberculosis*-infected THP-1 (upper panel). Shown are the top five gene sets at FDR < 0.05, plotted as in Fig. 1. Gene set overlap was determined as in Fig. 1C, and the most significantly regulated genes are shown ($p < 0.005$) (lower panel).

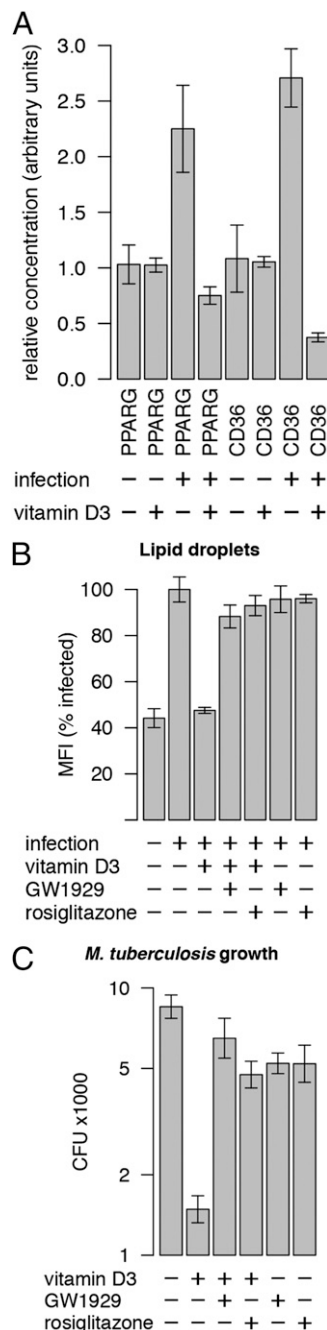


FIGURE 4. Role of PPAR γ . Differentiated THP-1 cells were infected with *M. tuberculosis* and treated with 100 nM vitamin D, 1 μ M PPAR γ agonist GW1929, and 100 nM PPAR γ agonist rosiglitazone, alone or combined, as indicated. Results are means from triplicate experiments (\pm SEM). **(A)** Cells were infected at MOI 1:1, and total RNA was extracted at 24 h postinfection. Transcript measurements were obtained by quantitative RT-PCR using gene-specific primers and molecular beacons. Data are normalized to the reference gene GAPDH. **(B)** Lipid droplets from cells infected as in **(A)** were detected by fluorescence staining and flow cytometry as in Fig. 2B. Mean fluorescence intensity (MFI) is shown. **(C)** *M. tuberculosis* CFU were determined in lysates from cells infected at MOI 1:30 at 4 d postinfection (MOI was reduced in this experiment to prolong infected THP-1 survival). For gene expression **(A)**, lipid droplets **(B)**, and CFU **(C)**, the effect of vitamin D on response to infection and the further effect of PPAR γ agonist were statistically significant ($p < 0.05$).

and bacterial growth-promoting effects of PPAR γ in *M. tuberculosis*-infected macrophages (Supplemental Fig. 2A, 2C) (19). Taken together, our findings show that VDR signaling

decreases lipid droplet formation in *M. tuberculosis*-infected macrophages by limiting the adipogenic function of another nuclear hormone receptor protein, PPAR γ , and the antimicrobial and antiadipogenic effects of VDR both involve reduction in PPAR γ signaling.

In conclusion, integrated data from transcriptomic and mechanistic analyses provide evidence for 1) a connection between VDR signaling and lipid metabolism in human tuberculosis, BCG vaccination, and macrophage response to *M. tuberculosis* infection in vitro, 2) an antiadipogenic effect of vitamin D in *M. tuberculosis*-infected macrophages, and 3) cross-talk between PPAR γ and VDR in both *M. tuberculosis*-induced dysregulation of macrophage lipid metabolism and macrophage antimicrobial activity. The observed cross-talk is consistent with the integration of VDR- and PPAR γ -signaling pathways seen in other cell types and contexts (20). Because VDR does not appear to regulate PPAR γ directly (21), the cross-talk might involve effects on interactions with the common heterodimerization partner, retinoid X receptor, which is required for DNA binding and transcriptional activity of either heterodimer (11), and/or on intracellular levels of the cognate lipid ligands required for transcriptional activity (11). The finding that VDR and PPAR γ exert divergent effects on lipid metabolism and bacillary growth in *M. tuberculosis*-infected human macrophages points to opposing roles of the corresponding signaling pathways on macrophage activation state and permissivity for *M. tuberculosis* infection. Although the data clearly indicate that inhibition of lipid droplet formation by vitamin D occurs in the absence of concurrent antimicrobial effects, mechanistic links might exist between antiadipogenic and antimicrobial functions of vitamin D. These remain to be elucidated.

The present findings have clinical relevance. For example, they point to a need for reinterpretation and de novo investigation of epidemiological links among tuberculosis risk, vitamin D levels, and metabolic abnormalities, including diseases such as diabetes. Moreover, identification of drug-targetable host factors involved in lipid metabolism might lead to treatments that enhance host resistance to *M. tuberculosis* infection and serve as adjuncts to antituberculosis chemotherapy.

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Disclosures

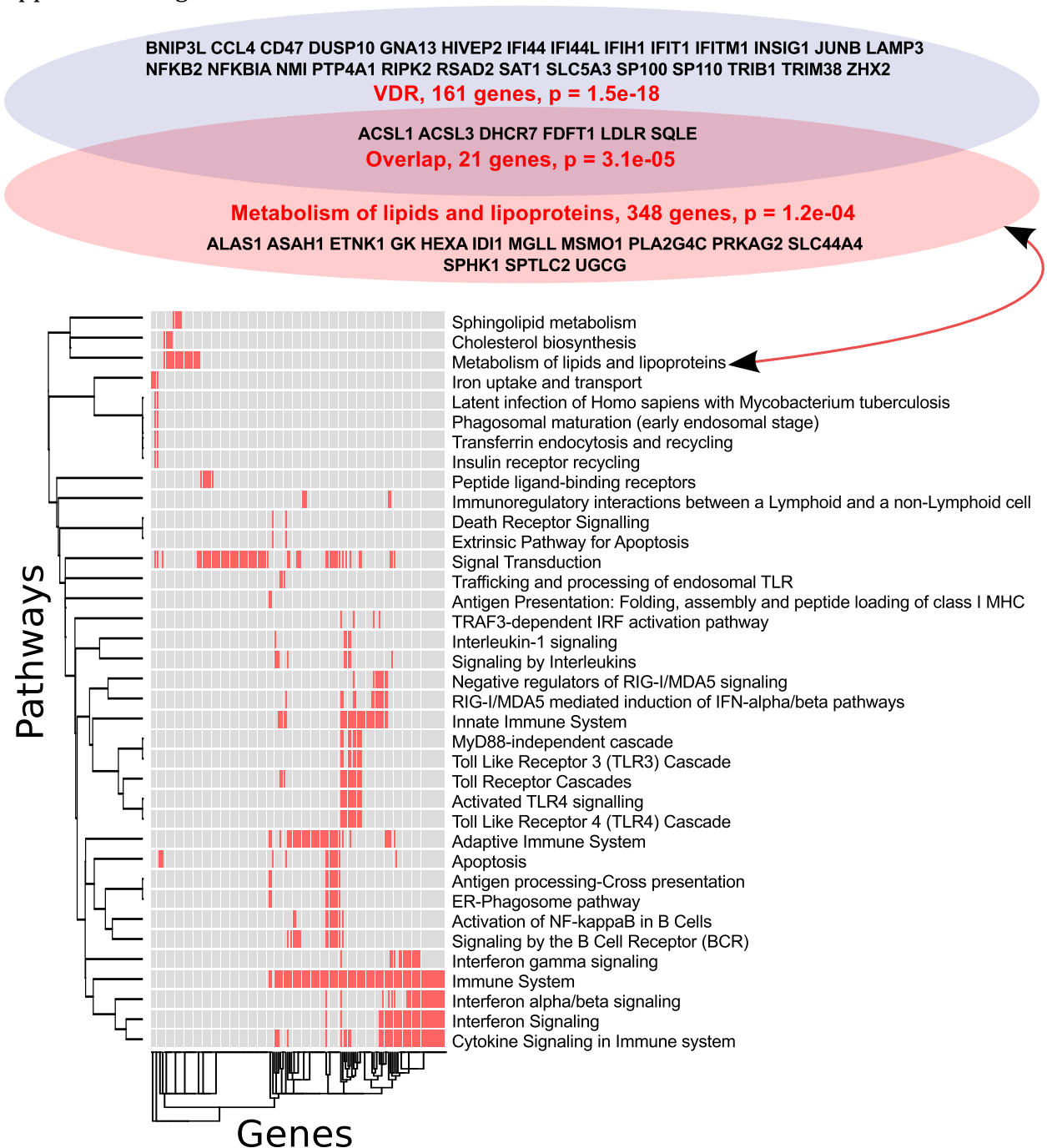
H.S. and K.D.Y. are the beneficial owners of Knowledge Synthesis Inc. There are no patents, consultancies, products, or other interests with respect to this article.

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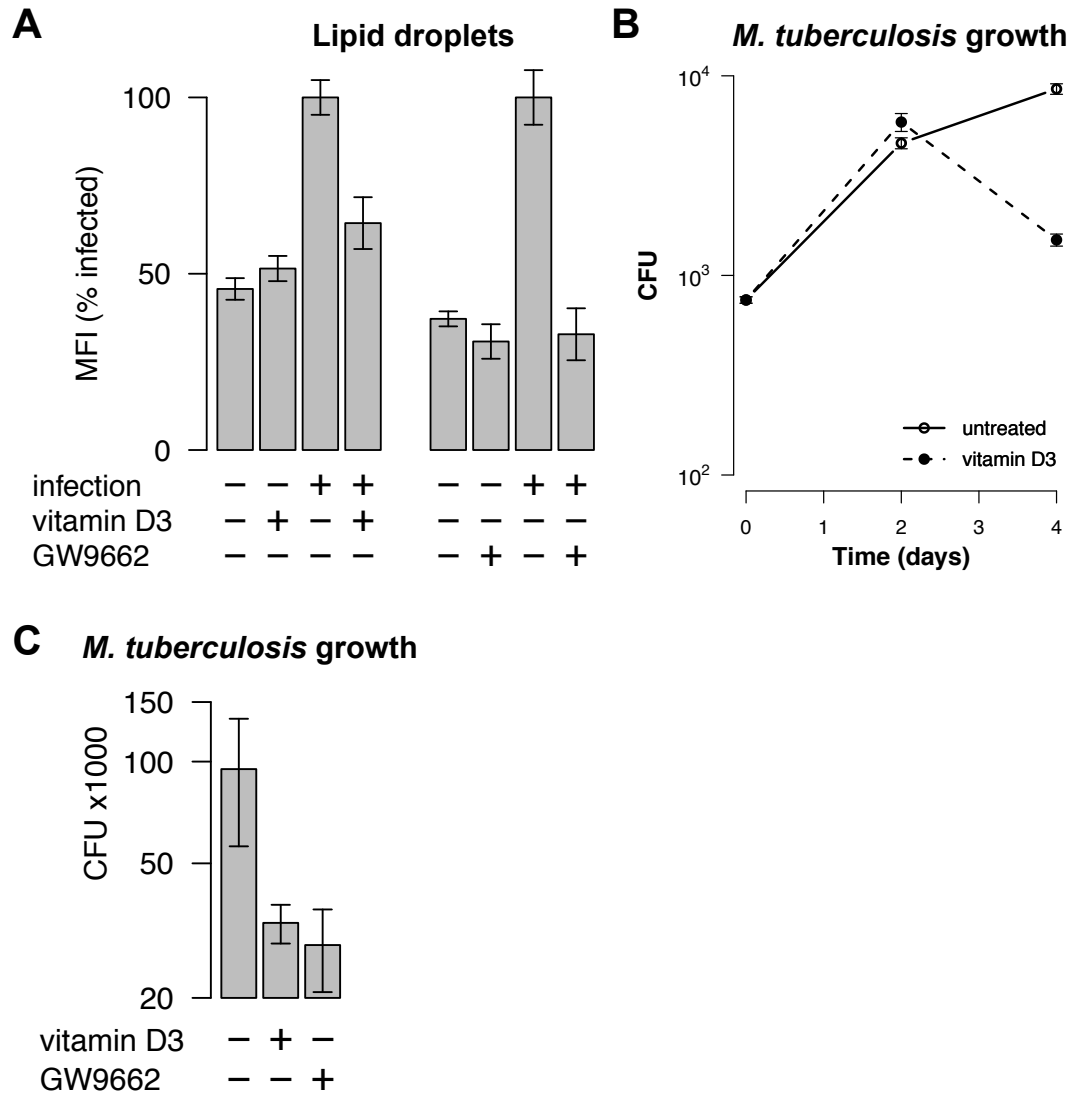
Supplemental Figure 1



Supplemental Fig 1. Transcriptomics reveals *M. tuberculosis* effects on macrophage lipid metabolism and links to VDR-regulated gene expression.

A. Relationship of lipid metabolism to VDR regulation. The Venn diagram shows the numbers of probed genes annotated as VDR-bound (light blue) or annotated for Metabolism of lipids and lipoproteins (light red), and the number of overlapping genes, along with the p-values calculated for the three sets of genes. Gene symbols list the most significant genes ($P < 0.005$) from each set and the overlap. **B. Upregulated Reactome pathways.** The matrix shows the most significant pathways ($FDR < 0.005$) and the most significant genes ($P < 0.005$) from those pathways. Gene membership in each pathway is indicated by red bars in the matrix of 161 genes (columns) and 37 pathways (rows). The matrix was clustered to bring together similar patterns of membership, as indicated by the dendrograms.

Supplemental Figure 2



Supplemental Fig 2. Effects of vitamin D and PPAR γ antagonist on lipid droplet formation and *M. tuberculosis* growth in THP-1 cells.

Differentiated THP-1 cells were infected with *M. tuberculosis*, treated with 100nM vitamin D and 1 μ M PPAR γ antagonist GW9662, as indicated. Results shown are means from triplicate experiments (+/- standard error of the mean). **A. Effect on lipid droplets.** Cells were infected at MOI 1:1, fixed and stained with LipidTOX Deep Red for detection of lipid droplets by flow cytometry at 24 hrs post-infection. Data were calculated as MFI. Vitamin D (left) and GW9662 (right) significantly inhibited lipid droplets in infected cells ($p < 0.05$). **B. Time course of effect of vitamin D on *M. tuberculosis* growth in macrophages.** *M. tuberculosis* CFU were determined in lysates from cells infected at MOI 1:30 at various times post-infection, as indicated. (Time 0 refers to cells harvested at four hrs post-infection, after removal of extracellular bacteria with three washes). **C. Effect on *M. tuberculosis* growth in macrophages.** *M. tuberculosis* CFU were determined in lysates from cells infected at MOI 1:30 at 4 days post-infection. PPAR γ antagonist (panel C) and Vitamin D (panels B and C) significantly inhibited growth ($p < 0.05$) at 4 days post-infection. Data in panels A and C show that (i) PPAR γ is required for both lipid droplet induction (A) and bacterial growth (C), and (ii) the effect of the PPAR γ antagonist is very similar to that of vitamin D.