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Vitamin D Is Required for IFN- γ -Mediated Antimicrobial Activity of Human Macrophages

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SUPPLEMENTARY MATERIAL

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Materials and Methods

Fig. S1. Correlation between T cell-secreted IL-10 and IL-17 and induction of antimicrobial peptides.

Fig. S2. IFN- γ induces genomic targets in human monocytes.

Fig. S3. IFN- γ induces IL-15 mRNA and cell surface protein expression.

Fig. S4. IFN- γ induces antimicrobial response in *M. tuberculosis*-infected monocytes.

Fig. S5. Effect of serum concentration, heat inactivation, and type of serum on IFN- γ -induced antimicrobial response.

Fig. S6. Effect of 25D supplementation of vitamin D-deficient serum on autophagy and autophagosome maturation in *M. tuberculosis*-infected macrophages.

Fig. S7. Effect of 25D supplementation of vitamin D-deficient serum on antimicrobial activity of *M. tuberculosis*-infected macrophages.

References

Author contributions: M.F. designed and performed the experiments, analyzed the data, and wrote the paper. D.-M.S., J.-M.Y., S.R., H.-M.L., M.S., D.M., S.S.I., and R.T. designed and performed the experiments and analyzed the data. P.T.L. and J.S.A. designed the experiments, analyzed the data, and provided supervisory support. S.R.K. designed and performed the experiments and provided supervisory support. P.A.S. provided T cell clones and supervisory support. H.B. designed the experiments. D.M.L. provided T cell clones. B.W.H. measured the serum vitamin D levels. M.H. and G.C. designed the experiments and provided supervisory support. A.S. and U.Z. provided VAZ. E.-K.J. designed the experiments, analyzed the data, and provided supervisory support. B.R.B. provided supervisory support and wrote the paper. S.S. and R.L.M. designed the experiments, analyzed the data, provided supervisory support, and wrote the paper.

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Abstract

Control of tuberculosis worldwide depends on our understanding of human immune mechanisms, which combat the infection. Acquired T cell responses are critical for host defense against microbial pathogens, yet the mechanisms by which they act in humans remain unclear. We report that T cells, by the release of interferon- γ (IFN- γ), induce autophagy, phagosomal maturation, the production of antimicrobial peptides such as cathelicidin, and antimicrobial activity against *Mycobacterium tuberculosis* in human macrophages via a vitamin D-dependent pathway. IFN- γ induced the antimicrobial pathway in human macrophages cultured in vitamin D-sufficient sera, but not in sera from African-Americans that have lower amounts of vitamin D and who are more susceptible to tuberculosis. In vitro supplementation of vitamin D-deficient serum with 25-hydroxyvitamin D3 restored IFN- γ -induced antimicrobial peptide expression, autophagy, phagosome-lysosome fusion, and antimicrobial activity. These results suggest a mechanism in which vitamin D is required for acquired immunity to overcome the ability of intracellular pathogens to evade macrophage-mediated antimicrobial responses. The present findings underscore the importance of adequate amounts of vitamin D in all human populations for sustaining both innate and acquired immunity against infection.

INTRODUCTION

The acquired T cell-mediated immune response is essential for host defense against intracellular pathogens, including *Mycobacterium tuberculosis*, which is estimated by the World Health Organization to cause 9.3 million new infections and 1.8 million deaths annually (1). HIV-infected individuals with reduced numbers of CD4⁺ T cells have markedly increased susceptibility to tuberculosis, and those individuals with the lowest CD4⁺ T cell counts have the highest frequency of disseminated disease (2). Furthermore, a key role for interferon- γ (IFN- γ) in the immune response to *M. tuberculosis* infection is corroborated by studies indicating that humans with genetic disorders leading to the decreased production of, or response to, IFN- γ are highly susceptible to tuberculosis and other mycobacterial diseases (3–5). Yet, it has been a matter of faith that IFN- γ can activate human monocytes or macrophages to kill intracellular *M. tuberculosis*.

Indeed, multiple studies of IFN- γ treatment of human macrophages have consistently failed to demonstrate antimicrobial activity against intracellular *M. tuberculosis* (6–16). In vitro, IFN- γ is unable to activate human macrophages to restrict or kill virulent *M. tuberculosis* (17), and human macrophages infected with *M. tuberculosis* are desensitized to this cytokine (17). However, the discovery that the innate immune response, activated by Toll-like receptors (TLRs) on human monocytes and macrophages, triggers an antimicrobial response against *M. tuberculosis* that is vitamin D-dependent (18, 19) provided a new opportunity to investigate this phenomenon. We found that the acquired T cell response could activate antimicrobial activity in human cells of the monocyte/macrophage lineage.

RESULTS

T cells activate a vitamin D antimicrobial pathway via secretion of IFN- γ

Within the acquired immune response, the balance of T helper 1 (T_H1) and T_H2 T cell cytokine patterns is pivotal to the ability of humans to defend against mycobacterial infection (20, 21). We therefore derived a panel of *M. tuberculosis*-reactive human T_H1 and T_H2 T cell clones (21) that were activated via their T cell receptor (TCR) by immobilized antibodies to CD3 and collected the supernatants. These supernatants were tested for their ability to induce in monocytes, primary cells of the monocyte/macrophage lineage, expression of the mRNAs encoding the antimicrobial peptides cathelicidin and β -defensin 2 (DEFB4). Previously, both cathelicidin and DEFB4 have been demonstrated to be part of the vitamin D-dependent antimicrobial pathway of the innate immune response against intracellular *M. tuberculosis* (18, 19). The T_H1 cell supernatants, containing IFN- γ , were able to induce cathelicidin and DEFB4 mRNAs in monocytes (Fig. 1A). In contrast, the T_H2 cell supernatants, containing interleukin-4 (IL-4), did not induce cathelicidin or DEFB4. The capacity of supernatants from T cell clones to induce monocyte production of cathelicidin and DEFB4 correlated with the amounts of IFN- γ (Fig. 1B, $P = 0.0004$ and $P = 0.0002$, respectively) and inversely correlated with the amount of IL-4, reaching statistical significance for cathelicidin (Fig. 1B, $P = 0.03$). The T cell clones also secreted GM-CSF (granulocyte-macrophage colony-stimulating factor) (Fig. 1B), IL-10, and IL-17 (fig. S1); however, their production failed to show significant correlation with the induction of antimicrobial peptide gene expression.

To determine whether IFN- γ was responsible for antimicrobial peptide expression, we cultured human monocytes with T cell supernatants in the presence of IFN- γ -neutralizing antibodies or isotype controls. The addition of monoclonal antibodies (mAbs) to IFN- γ reduced the ability of the T cell supernatants to trigger the expression of cathelicidin and DEFB4 by about 65 and 70%, respectively, compared with cultures without antibody (Fig. 1C, $P < 0.05$). However, because of variability in the isotype controls, the relative blocking effect of inhibitory antibodies to IFN- γ failed to reach statistical significance. These data suggest that the acquired T cell response, through the production of IFN- γ , can induce antimicrobial peptide gene expression in human cells of the monocyte/macrophage lineage.

IFN- γ is sufficient to activate a vitamin D-dependent antimicrobial pathway

In monocytes and macrophages, IFN- γ can induce CYP27B1-hydroxylase (22), which converts 25-hydroxyvitamin D (25D) to the bioactive 1,25-dihydroxyvitamin D (1,25D). Furthermore, IFN- γ up-regulates TLR2/1 ligand (TLR2/1L)-induced antimicrobial peptide expression by enhancing CYP27B1 activity (23). To examine the role of IFN- γ in triggering the vitamin D-dependent induction of antimicrobial peptides, we treated primary human monocytes with recombinant human IFN- γ (rIFN- γ) or a TLR2/1L, the *M. tuberculosis*-derived 19-kD triacylated lipopeptide (24), as a control. Both rIFN- γ and TLR2/1L induced cathelicidin and DEFB4 (25) mRNAs to a similar degree (Fig. 2A, $P < 0.05$). In addition, IFN- γ and TLR2/1L equally induced CYP27B1-hydroxylase, as well as vitamin D receptor (VDR), mRNA (Fig. 2B, $P \leq 0.05$) (18). Notably, although nitric oxide is a key effector molecule in activated mouse macrophages (26–28), neither inducible nitric oxide synthase mRNA as measured by quantitative polymerase chain reaction (qPCR) nor nitric oxide as measured by the Griess reaction (28) was detected in IFN- γ -treated human monocytes/macrophages.

Next, we investigated the functional roles of CYP27b1 and the VDR in this system. IFN- γ treatment of human monocytes resulted in enhanced conversion of 25D to 1,25D, indicating increased CYP27b1 enzymatic activity (Fig. 2C, $P < 0.05$). To determine whether CYP27B1

and VDR induction was required for IFN- γ -mediated expression of antimicrobial peptides, we performed small interfering RNA (siRNA) knockdown of each gene on primary monocytes. Both siVDR and siCYP27B1 knocked down the IFN- γ -induced expression of their respective targets by about 50% as measured by qPCR. Transfection of siRNA oligos targeting CYP27B1 and VDR, but not the non-specific control oligo, significantly reduced IFN- γ -induced expression of both cathelicidin and DEFB4 (Fig. 2D, $P < 0.05$). In contrast, knockdown of CYP27B1 and VDR did not inhibit IFN- γ -induced CD64, but instead may have increased CD64 expression, consistent with the ability of 1,25D to inhibit CD64 expression (29). Furthermore, the VDR antagonist VAZ, shown to inhibit 1,25D induction of cathelicidin (30), blocked induction of antimicrobial peptides (Fig. 2E, $P < 0.05$), but not CD64, which was again slightly increased (Fig. 2E). Finally, IFN- γ also induced the VDR downstream gene *CYP24*, providing evidence for activation of the VDR (fig. S2). These data indicate that IFN- γ induction of cathelicidin and DEFB4 mRNAs is dependent on both CYP27b1 and the VDR.

A key early event in the TLR2/1L induction of the vitamin D antimicrobial pathway is the induction of IL-15, which initiates a macrophage differentiation program (31, 32). Because IFN- γ has been reported to induce IL-15 in monocytes (33), we measured IL-15 expression after stimulation with either rIFN- γ or TLR2/1L and found that IL-15 surface expression and mRNA were similarly up-regulated in both cases (Fig. 3A, $P < 0.05$, and fig. S3, A and B). The mechanism of IL-15 induction was investigated in the context of the known signaling pathways for TLR2/1 and IFN- γ . IFN- γ induction of IL-15 was STAT1 (signal transducer and activator of transcription 1)-dependent, whereas TLR2/1L induction of IL-15 was STAT1-independent (Fig. 3B). Conversely, TLR-induced, but not IFN- γ -induced, IL-15 was MyD88-dependent. The addition of anti-IL-15-neutralizing antibodies significantly reduced IFN- γ -induced expression of CYP27B1 by ~60%, cathelicidin by ~90%, and DEFB4 by ~50% (Fig. 3, C and D, $P < 0.05$), demonstrating that IL-15 is a critical cytokine for activating the antimicrobial program in human macrophages. Together, these data show that both the innate and the acquired immune responses converge on a common antimicrobial pathway by inducing IL-15, which is required for up-regulation of CYP27b1 and the subsequent induction of cathelicidin and DEFB4. These data also indicate that the proximal pathways for activation of the antimicrobial response in innate and acquired responses are distinct.

IFN- γ -induced autophagy and autophagolysosomal fusion are vitamin D-dependent

One well-established mechanism by which *M. tuberculosis* evades the macrophage antimicrobial response is by blocking phagosome maturation and phagolysosomal fusion, preventing lysosomal acidification and delivery of lysosomal products into the compartment in which it resides (34–36). A possible host defense mechanism to overcome this blockade is autophagy, which results in the creation of autophagosomes and their subsequent fusion with lysosomes (37–40). Autophagosomes were detected using an anti-microtubule-associated protein 1 light chain 3 (LC3) mAb with confocal laser microscopy. The induction of autophagy is associated with a shift from a homogeneous distribution of LC3 throughout the cytoplasm to aggregation into characteristic puncta (41). Treatment of primary human monocytes with rIFN- γ induced autophagy, as evidenced by an increase in the percentage of cells with LC3-positive vesicles (41) (Fig. 4, A and B, $P < 0.01$). The ability of IFN- γ to induce autophagy in monocytes was inhibited by 3-methyladenine (3-MA) and wortmannin (WM), both of which block the phosphatidylinositol 3-kinase (PI3K) activity known to be required for autophagy (Fig. 4C, $P < 0.05$ and $P < 0.01$). IFN- γ -induced autophagy was also suppressed by lentiviral short hairpin RNA (shRNA) transduction for two key genes of the autophagy pathway, *Beclin-1* and *Atg5* (42) (Fig. 4D, $P < 0.01$), providing further evidence that IFN- γ induced a classical autophagy pathway. Markedly, the ability of IFN- γ to induce

autophagy in human monocytes was also dependent on the vitamin D pathway, because autophagy was blocked in a dose-dependent manner by the VDR inhibitor VAZ (Fig. 4, E and F, $P < 0.05$). In contrast, starvation- or rapamycin-induced autophagy was not vitamin D-dependent (42), thus indicating at least two distinct pathways for inducing autophagy.

IFN- γ treatment of primary human monocytes resulted in the colocalization of vesicles containing the autophagosome marker LC3 with lysosomes identified by LysoTracker (Fig. 4G). In *M. tuberculosis*-infected macrophages, the pathogen accumulates in phagocytic vesicles or early endosomes, which fail to colocalize with lysosomes as identified with the lysosomal marker (Fig. 4H). However, IFN- γ -induced phagosome maturation in macrophages, as evidenced by colocalization of vesicles containing *M. tuberculosis* and the lysosomal marker, was blocked by treatment with the VDR inhibitor VAZ in a dose-dependent manner (Fig. 4, H and I, $P < 0.01$). As previously demonstrated, direct treatment of infected macrophages with 1,25D leads to the vesicular colocalization of cathelicidin peptide with mycobacteria (18). Together, these data indicate that IFN- γ triggers vitamin D-dependent autophagy in human macrophages, facilitating autophagolysosomal fusion and phagosomal maturation and allowing for the delivery of antimicrobial peptides to pathogen-containing compartments.

IFN- γ induces an antimicrobial pathway that requires vitamin D sufficiency

Ultimately, for the host immune response to eliminate an intracellular pathogen, the activation of macrophages ideally should result in a direct antimicrobial activity. Recognizing that *M. tuberculosis* infection has been shown to inhibit several IFN- γ response genes (17, 43), we first infected primary human monocytes with *M. tuberculosis*, then subsequently treated with rIFN- γ , and antimicrobial peptide induction was measured. IFN- γ induced both cathelicidin and DEF4 in *M. tuberculosis*-preinfected monocytes in the presence of vitamin D-sufficient (25D = 98 nM) serum (fig. S4), although quantitative analysis indicated that *M. tuberculosis* infection partially inhibited IFN- γ -induced antimicrobial peptide expression to a greater extent for DEF4 than for cathelicidin.

African-Americans have significantly decreased amounts of 25D because their skin melanin content diminishes ultraviolet (UV)-dependent cutaneous vitamin D3 synthesis (44). In addition, African-Americans have increased susceptibility to tuberculosis (45). To determine whether this difference in vitamin D concentration could affect the ability to generate antimicrobial responses, we collected sera from healthy African-American and white donors. The concentrations of 25D reflected the lower amounts of vitamin D in the African-American versus white donors (mean 25D = 56 ± 2 and 113 ± 11 nM, respectively; Fig. 5A, $P < 0.01$). IFN- γ -mediated antimicrobial peptide expression in primary human monocytes was significantly lower when cultured with sera from African-American donors than white donors (Fig. 5A, $P < 0.05$). In addition, IFN- γ -mediated antimicrobial peptide expression correlated with the concentration of 25D (correlation coefficient: cathelicidin versus 25D: $r = 0.68$, $P < 0.05$; DEF4 versus 25D: $r = 0.73$, $P < 0.05$) but not 1,25D (correlation coefficient: cathelicidin versus 1,25D: $r = 0.20$; DEF4 versus 1,25D: $r = 0.11$).

On the basis of these studies, we hypothesized that previous studies failed to detect an antimycobacterial activity by IFN- γ -treated human macrophages because of the different amounts of 25D and/or its availability in the sera used for the in vitro killing experiments. Because IFN- γ was able to up-regulate antimicrobial peptide mRNA expression, we compared the types of sera used in the previous reports in which IFN- γ treatment did not induce an antimycobacterial activity in human macrophages (6, 7, 9–12, 14, 15). We found that IFN- γ -induced antimicrobial peptide expression was optimal in cultures containing 10% or greater vitamin D-sufficient human serum (25D = 98 nM), but could not be induced in 2 or 10% heat-inactivated human serum or 10% vitamin D-deficient fetal calf serum

(FCS) (25D = 16 nM) (fig. S5, A to C, $P < 0.05$), conditions used in one or more of the previously mentioned studies. Markedly, 10% vitamin D–deficient human serum (25D = 45 nM) also failed to support antimicrobial peptide expression in monocytes (Fig. 5B, $P < 0.05$), but this could be restored by supplementation of vitamin D–deficient serum to sufficient concentrations by the addition of 25D in vitro (Fig. 5C, $P < 0.05$).

Because macrophages are the natural hosts of mycobacteria during the course of infection (46), we tested whether IFN- γ treatment would induce the vitamin D antimicrobial pathway in monocyte-derived macrophages (MDMs). As observed in human monocytes, IFN- γ induced cathelicidin and DEFB4 gene expression in MDMs in 10% vitamin D–sufficient serum (Fig. 5D, $P < 0.05$), but not in 10% vitamin D–deficient serum. Likewise, the induction of cathelicidin and DEFB4 gene expression in MDMs could be restored by supplementation of vitamin D–deficient serum by addition of 25D in vitro (Fig. 5D, $P < 0.05$). Moreover, vitamin D–deficient serum did not support autophagolysosomal fusion and phagosome maturation in *M. tuberculosis*–infected macrophages, but this could be rescued by in vitro supplementation with 25D (fig. S6).

Because IFN- γ –induced antimicrobial peptide expression and autophagosome maturation could only be detected in the presence of vitamin D–sufficient human serum, we consequently tested whether vitamin D was required for IFN- γ –induced antimicrobial activity against *M. tuberculosis*. Human monocytes were infected with virulent *M. tuberculosis* and then treated with rIFN- γ in 10% vitamin D–sufficient human serum or 10% vitamin D–deficient human serum in the same experiments. Treatment of infected human monocytes with IFN- γ in the presence of vitamin D–sufficient serum resulted in significant growth inhibition of virulent *M. tuberculosis*, as evidenced by the decreased number of viable *M. tuberculosis* bacilli recovered compared to untreated cells (Fig. 5E, $P < 0.001$). In contrast, IFN- γ , in the presence of vitamin D–deficient serum, had little or no effect on the bacterial viability.

Next, we infected MDMs with virulent *M. tuberculosis* and stimulated the infected cells with IFN- γ in 10% vitamin D–sufficient human serum or 10% vitamin D–deficient human serum in the same experiments. IFN- γ treatment of *M. tuberculosis*–infected MDMs using vitamin D–sufficient human serum resulted in a 90% reduction of *M. tuberculosis* growth at 3 days, relative to untreated cells (Fig. 5F, $P < 0.01$). Markedly, IFN- γ treatment resulted in an 85% reduction in *M. tuberculosis* compared to the initial inoculum (Fig. 5F, $P < 0.001$), formally demonstrating that IFN- γ induced killing of the bacteria. In contrast, IFN- γ treatment of MDM using vitamin D–deficient serum had little effect on the bacterial viability (Fig. 5F); however, antimicrobial activity could be restored by in vitro supplementation of vitamin D–deficient serum with 25D3 (fig. S7). These data reveal a marked difference in IFN- γ –induced antimicrobial activity against intracellular *M. tuberculosis* depending on the serum 25D concentration. Furthermore, these in vitro data are consistent with abundant clinical evidence that links low amounts of serum 25D to both tuberculosis disease susceptibility and progression (47–50).

DISCUSSION

Our data define one mechanism of IFN- γ –triggered antimicrobial killing in human cells of the monocyte/macrophage lineage: Activation of vitamin D–dependent effector pathways results in the production of antimicrobial peptides and induction of autophagy. The innate (TLR) and acquired (IFN- γ –dependent) mechanisms activate different receptors and different signaling pathways, MyD88 and STAT1, respectively (Fig. 6); yet, they converge on IL-15 production and the downstream 25D–dependent antimicrobial peptide pathway. These results emphasize the general importance of the vitamin D–dependent antimicrobial

mechanism, which appears to have evolved in both innate and acquired immune responses in humans (18, 19, 23) through different signaling pathways, to converge on a common effector mechanism. Additional mechanisms may also contribute to IFN- γ and/or other T cell induction of antimicrobial activity, such as granulysin (51) and ubiquitin- or autophagy-derived peptides (39).

We propose a model (Fig. 6) for IFN- γ -induced antimicrobial activity in human cells of the monocyte/macrophage lineage as follows: IFN- γ induction of IL-15 is required for up-regulation of CYP27b1, which results in intracellular conversion of 25D to the bioactive 1,25D, which triggers activation of the VDR, which is required for downstream induction of antimicrobial peptides, autophagy, and phagosome maturation, contributing to the antimicrobial response. Previously, IFN- γ has been shown to up-regulate CYP27B1 activity, but the requirement for IL-15 in mediating this induction was not known (22). Evidence for the key role of the IFN- γ -induced autocrine conversion of 25D to 1,25D in the induction of an antimicrobial pathway is demonstrated by two experiments: (i) siRNA knockdown of CYP27B1 inhibited IFN- γ -induced cathelicidin and DEF4, and (ii) in vitro supplementation of vitamin D-deficient serum with 25D was able to restore IFN- γ -induced antimicrobial peptide expression. Up-regulation and/or expression of the VDR were also required for IFN- γ induction of antimicrobial peptides because induction was not observed when blocking the VDR by either siRNA knockdown or addition of a pharmacologic antagonist.

We also observed that IFN- γ activation of human monocytes led to the vitamin D-dependent induction of autophagy, autophagolysosomal fusion, and phagosome maturation of *M. tuberculosis*-infected macrophages. These processes facilitate delivery of antimicrobial effector molecules including antimicrobial peptides (42) and ubiquitinated peptides (39) to *M. tuberculosis*-containing vesicles, which otherwise block lysosomal fusion. Autophagy also has the capacity to envelop pathogens that escape vacuoles and penetrate into the cytoplasm as has been reported to occur with *M. tuberculosis* (36, 52–55). IFN- γ -induced autophagy was dependent on the expression of Atg5 and Beclin-1. Previously, Yuk *et al.* have shown that cathelicidin is required for 1,25D-induced gene expression of *Atg5* and *Beclin-1*; however, the mechanism is not known (42).

IFN- γ induction of antimicrobial peptides and antimicrobial activity against intracellular *M. tuberculosis* was only detected in the presence of vitamin D-sufficient but not vitamin D-deficient serum. Previously, we demonstrated that the expression of both cathelicidin and DEF4 was required for TLR-induced antimicrobial response against *M. tuberculosis* (56). Our present data indicate that the treatment of *M. tuberculosis*-infected macrophages with IFN- γ resulted in 85% reduction in colony-forming units (CFU) relative to the initial infection, thus establishing killing of the pathogen, but only in the presence of vitamin D-sufficient serum. Together, the proposed model explains the requirement for vitamin D in the IFN- γ -mediated antimicrobial activity of human cells of the monocytes/macrophage lineage.

The present results provide at least one explanation for a fundamental difference in antimicrobial mechanisms seen between mice and humans. The IFN- γ -induced antimicrobial pathway described here is vitamin D-dependent in humans but not in the mouse. The human promoter of cathelicidin contains three vitamin D response elements (VDREs), whereas the promoter of the murine homolog does not contain a single VDRE (57). In addition, the human promoter of DEF4 contains one VDRE (19), whereas there is no mouse homolog for this gene. Therefore, the biological significance of the IFN- γ -induced, vitamin D-dependent antimicrobial pathway must be investigated in human cells and simply cannot be studied in a mouse model. The evolution of distinct antimicrobial

mechanisms makes sense teleologically as well because mice are nocturnal animals and humans are not, and the amount of vitamin D increases with sun exposure.

These findings may contribute to a conceptual framework for understanding the different roles of the innate and acquired immune responses in responding to intracellular pathogens such as *M. tuberculosis*. The innate immune response requires that each infected cell be individually activated via pattern recognition receptors such as TLRs. Although such activation is rapid, it is not optimally efficient, in that protection is likely to be effective mostly early where the number of invading pathogens is low. In humans, the infectious dose of *M. tuberculosis* required to initiate diseases is thought to be only a few bacilli. In contrast, although the acquired immune response requires longer time to develop, it has the great virtue of amplifying the host antimicrobial response by (i) expanding the number of antigen-specific T cells; (ii) providing for the “elaboration of soluble factors,” such as IFN- γ (58), that in a paracrine fashion acts on other cells in the inflammatory microenvironment, such as the granuloma; and (iii) providing long-term memory. Although the innate and acquired immune responses can activate the vitamin D antimicrobial pathway independently, they also may contribute additively or synergistically as shown for the ability of IFN- γ to enhance TLR2/1L responses (23). The fact that only about 10% of immunocompetent individuals infected with *M. tuberculosis* develop active disease is a testimony to the effectiveness of the human innate and acquired immune responses and the common vitamin D-dependent antimicrobicidal mechanism they share. In these individuals, vitamin D deficiency or certain VDR polymorphisms may contribute to the failure of the human innate and acquired immune responses to provide complete protection against tuberculosis.

Because vitamin D production is dependent on exposure to UV light, vitamin D deficiency is increased in dark-skinned populations including African-Americans, who are known to have increased susceptibility to tuberculosis and other infectious diseases (45, 59–64). Our data demonstrate that sera from white individuals with sufficient amounts of vitamin D, but not sera from African-Americans with lower amounts of 25D, could support IFN- γ -induced antimicrobial peptide expression. The identical correlation of ethnicity and 25D amount was previously shown for TLR-induced cathelicidin mRNA (18). Therefore, vitamin D deficiency may compromise both the innate and the acquired antimicrobial host defense pathways against tuberculosis infection and likely other infections known to be greater in blacks (65).

The data indicating that addition of 25D3 to vitamin D-deficient serum restored IFN- γ -induced antimicrobial peptide expression, autophagolysosomal fusion, phagosome maturation, and antimicrobial killing are consistent with the clinical improvement observed in tuberculosis patients supplemented with vitamin D (66, 67). At a time when multidrug-resistant, extensively drug-resistant, and totally drug-resistant forms of tuberculosis are emerging threats, understanding how to enhance both innate and acquired host immunity is of enormous interest. The present findings underscore the importance of maintaining adequate amounts of vitamin D in all human populations, either naturally or by supplementation, for sustaining both innate and acquired immunity against infection. The dependence of the IFN- γ -induced antimicrobial pathway on adequate amounts of vitamin D provides a rationale for translation to larger-scale clinical trials testing the efficacy of vitamin D supplementation to both prevent and treat tuberculosis.

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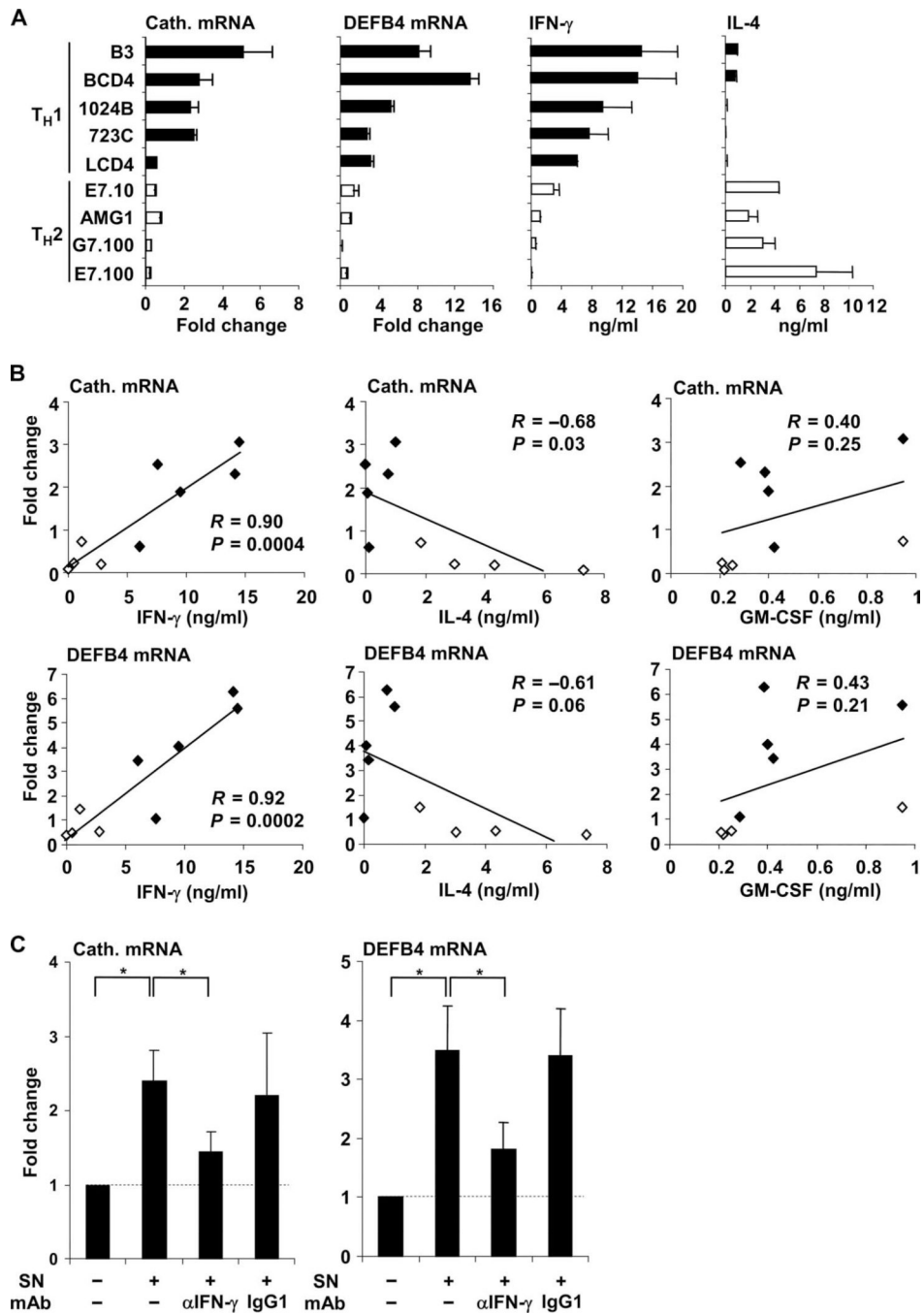


Fig. 1. T cell–secreted IFN- γ induces the vitamin D antimicrobial pathway. (A) T_H1 and T_H2 T cell clones were stimulated with plate-bound mAb to CD3 antibody, and supernatants were collected after 18 hours. T cell supernatants were added to human monocytes for 24 hours in 10% vitamin D–sufficient human serum, and amounts of mRNA encoding the antimicrobial peptides cathelicidin (Cath.) and DEFB4 were measured by qPCR (mean fold change \pm SEM; shown is one representative donor done in triplicate). In parallel, T cell supernatants were characterized according to their secreted concentrations of IFN- γ and IL-4 (protein concentrations in ng/ml \pm SD). (B) The correlation between T cell supernatant cytokine concentrations (ng/ml) and monocyte antimicrobial gene expression (mean fold change) was

determined and represented as the correlation coefficient r . (C) Monocytes were pretreated with mAb to IFN- γ or isotype control antibody for 15 min and then stimulated with the T_H1 T cell clone supernatant for 20 or 24 hours in 10% vitamin D-sufficient human serum. mRNA quantities were measured by qPCR (mean fold change \pm SEM, $n = 7$ to 9). * $P < 0.05$.

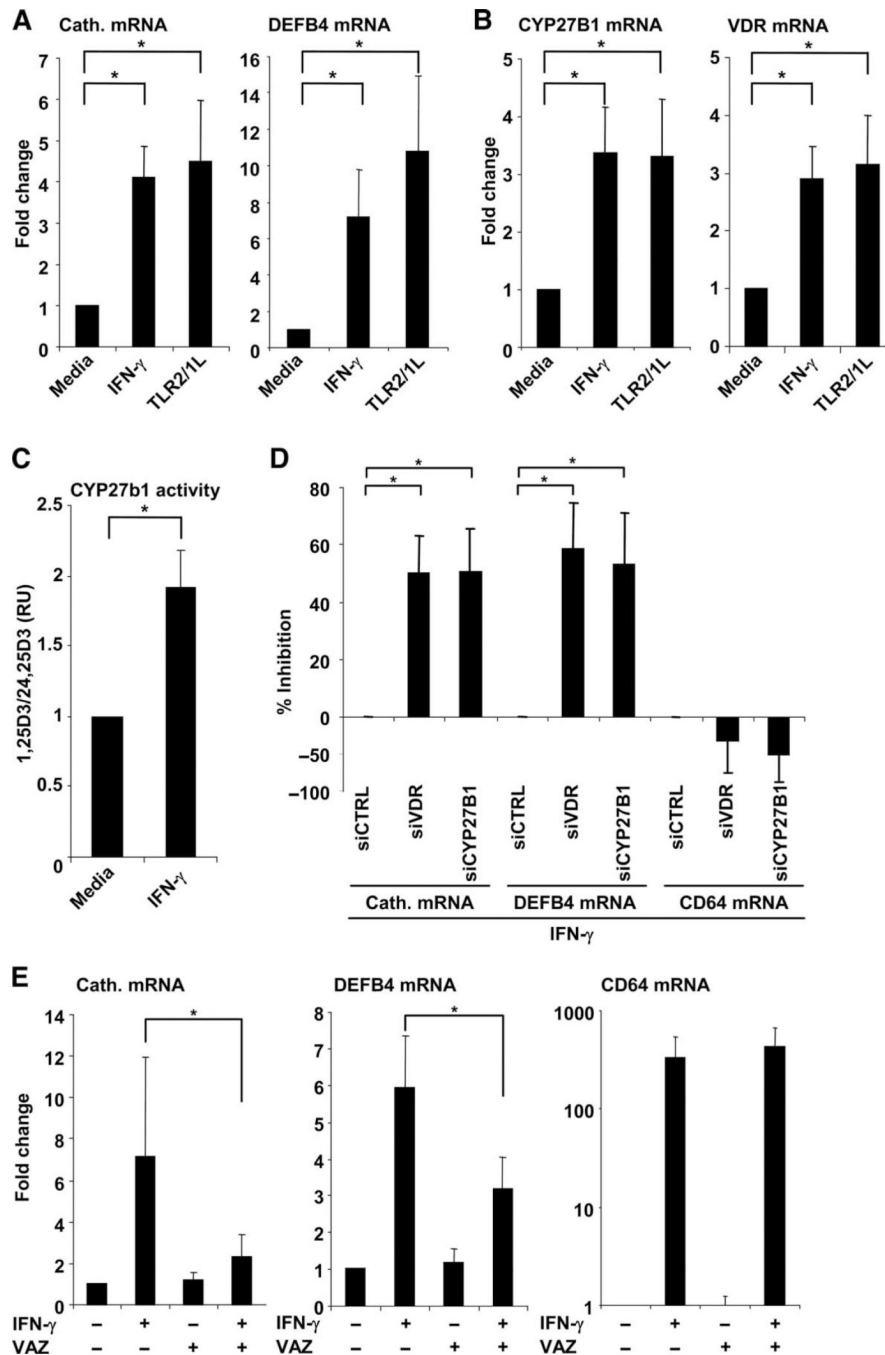


Fig. 2. IFN- γ and TLR2/1L induce a common vitamin D-dependent antimicrobial pathway. Primary human monocytes were stimulated with rIFN- γ or TLR2/1L for 24 hours in 10% vitamin D-sufficient human serum. (A and B) Cathelicidin and DEFB4 (A) and CYP27B1 and VDR (B) gene expression was assessed by qPCR (mean fold change \pm SEM, $n = 3$ to 7). (C) To assess CYP27b1 activity, we cultured human monocytes treated with rIFN- γ in 10% FCS overnight for an additional 5 hours with [3 H]25D3. The amount of conversion to [3 H]1,25D3 and [3 H]24,25D3 was measured by high-performance liquid chromatography, and CYP27b1 activity was calculated as the ratio of 1,25D3/24,25D3 (relative change compared to media \pm SEM, $n = 4$). (D) Human monocytes were transfected with siRNA

oligos specific for CYP27B1 (siCYP27B1), VDR (siVDR), or nonspecific (siCTRL) and then treated with IFN- γ in 10% vitamin D-sufficient serum for 20 or 24 hours. Cathelicidin, DEFB4, and CD64 gene expression was determined by qPCR (mean fold change \pm SEM, $n = 3$ to 5). (E) Human monocytes were cultured in 10% vitamin D-sufficient human serum, pretreated with the VDR antagonist VAZ (ZK159222) for 15 min, and then exposed to rIFN- γ . Cathelicidin, DEFB4, and CD64 gene expression was determined by qPCR (mean fold change \pm SEM, $n = 4$ to 7). * $P \leq 0.05$.

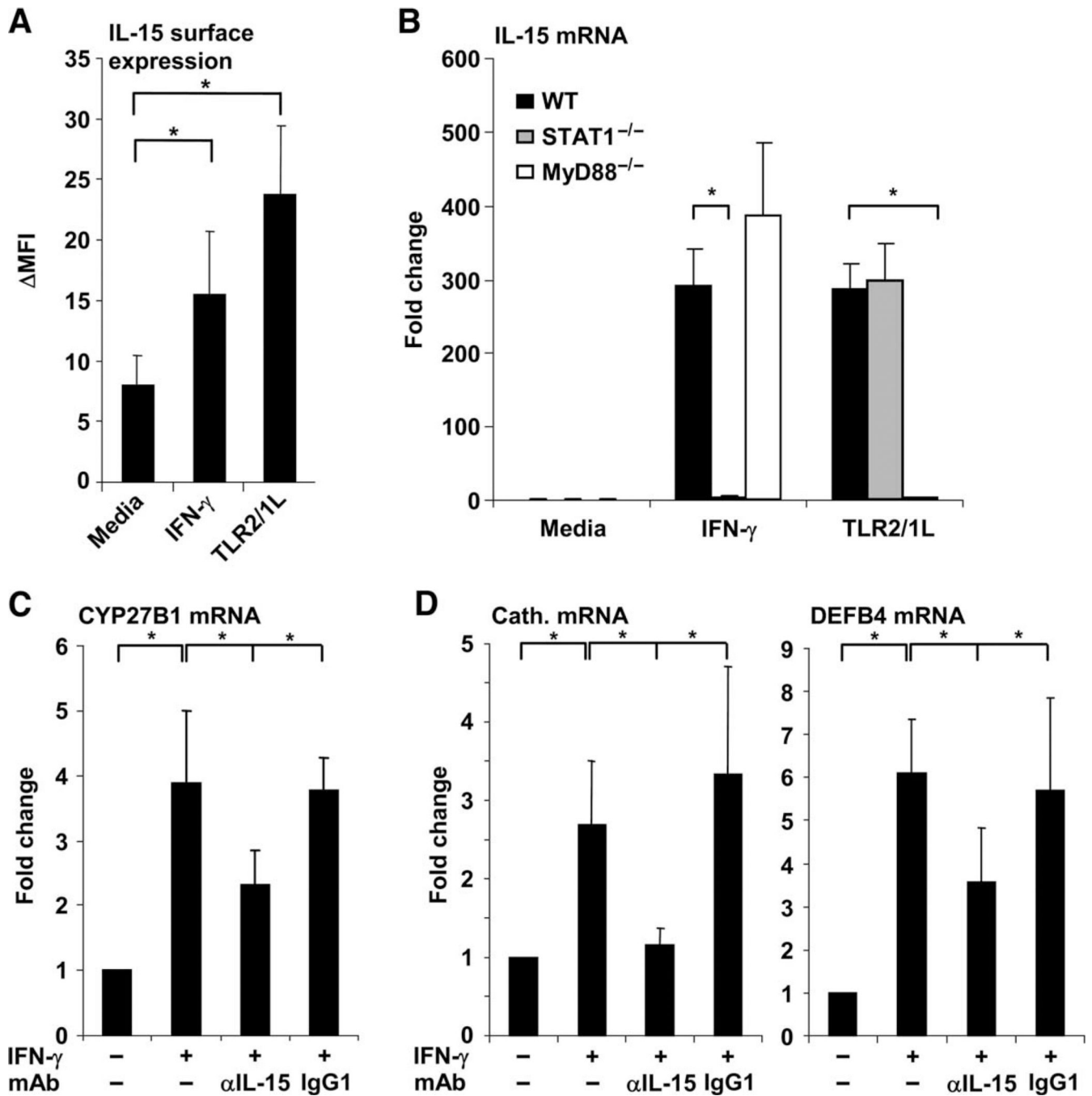


Fig. 3. IFN- γ induction of the vitamin D antimicrobial pathway is IL-15-dependent. (A) IL-15 cell surface expression on monocytes stimulated with rIFN- γ or TLR2/1L as measured by flow cytometry at 24 hours [Δ mean fluorescence intensity (MFI) \pm SEM, $n = 3$]. (B) Wild-type (WT), MyD88^{-/-}, and STAT1^{-/-} bone marrow-derived macrophages (BMDMs) were stimulated with murine rIFN- γ or TLR2/1L for 4 hours. IL-15 mRNA was quantified by qPCR (mean fold change \pm SEM, $n = 4$). (C) Primary human monocytes were incubated with anti-IL-15 mAb, isotype, or media control for 15 min and stimulated with human rIFN- γ in 10% FCS for 24 hours. CYP27B1 gene expression was assessed by qPCR (mean fold change \pm SEM, $n = 5$). (D) Monocytes were cultured as described in (C) in 10% vitamin D-

sufficient human serum for 20 or 24 hours. Cathelicidin and DEFB4 gene expression was assessed by qPCR (mean fold change \pm SEM, $n = 6$ to 8). * $P \leq 0.05$.

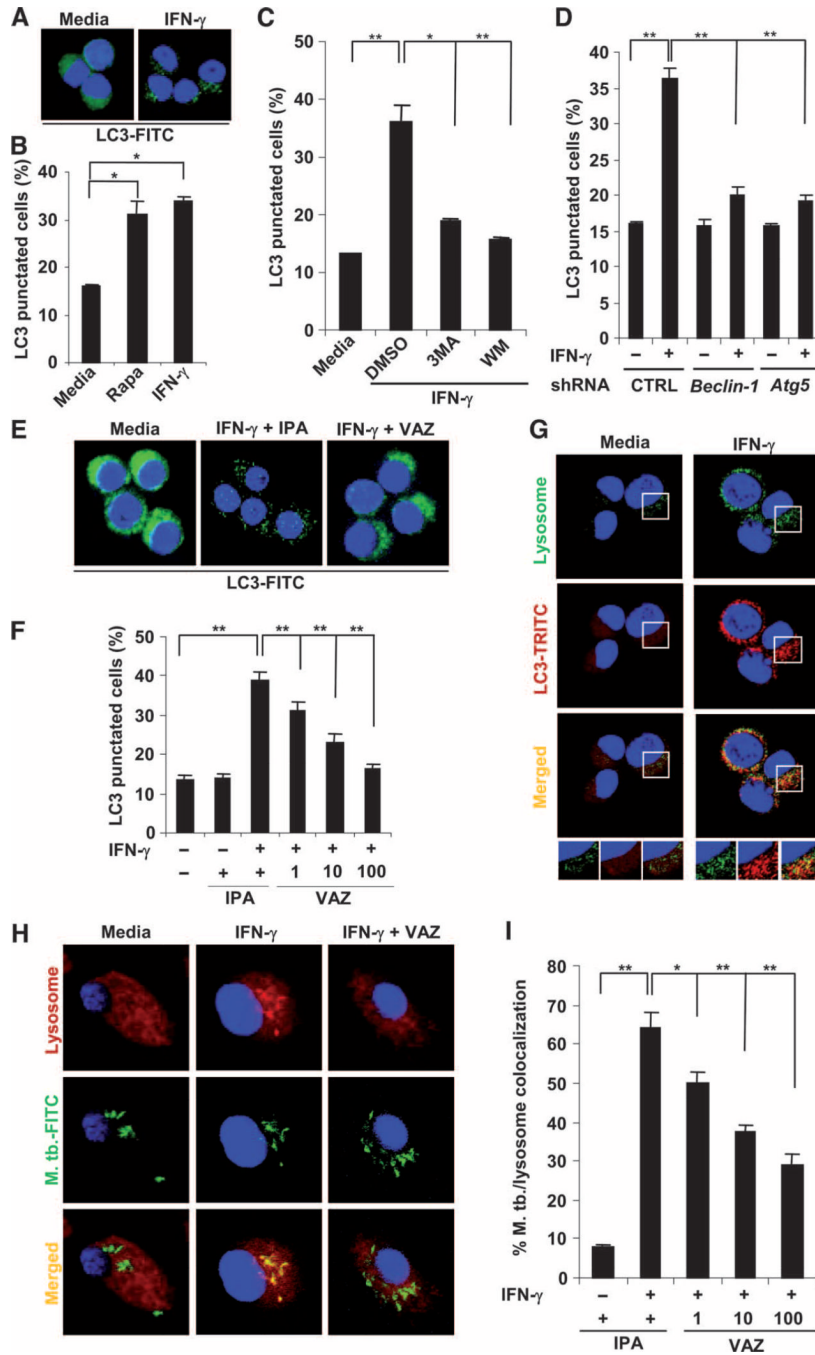


Fig. 4. IFN- γ -induced autophagy and phagosome maturation are VDR-dependent. (A) Human primary monocytes were incubated with rIFN- γ , rapamycin (Rapa), or medium for 24 hours in 10% vitamin D-sufficient human serum, fixed, and immunolabeled with anti-LC3-FITC (fluorescein isothiocyanate) antibody (green). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Representative immunofluorescence images are shown. (B) LC3 punctate cells were quantified (mean of percent positive cells \pm SEM, $n = 5$). (C) Monocytes were incubated with rIFN- γ in the presence or absence of 3-MA or WM for 24 hours, and the percentage of LC3 punctate cells was determined (mean \pm SEM, $n = 4$). (D) Gene expression was knocked down in monocytes with lentiviral shRNA specific for

Beclin-1, *Atg5*, or control shRNA followed by stimulation with rIFN- γ for 24 hours. Percent LC3-positive cells was determined (mean \pm SEM, $n = 4$). **(E)** Monocytes were incubated with rIFN- γ in the presence or absence of VAZ (1, 10, or 100 nM) for 24 hours and labeled as in (A). Representative immunofluorescence pictures of LC3 punctate cells are shown. IPA, isopropyl alcohol. **(F)** LC3 punctate cells were quantified (mean of percent positive cells \pm SEM, $n = 5$). **(G)** Monocytes were stimulated with IFN- γ for 24 hours in 10% vitamin D-sufficient human serum, loaded with LysoTracker (green), and stained for LC3 (red). Representative immunofluorescence images of three independent replicates are shown. TRITC, tetramethylrhodamine isothiocyanate. **(H)** Human MDMs were infected with FITC-*M. tuberculosis* (green) for 4 hours, washed, and stimulated with rIFN- γ in the presence or absence of VAZ (1, 10, or 100 nM) for 30 hours. Lysosomes were stained with LysoTracker (red), and cells were fixed. Representative fluorescence-merged images are shown. **(I)** Quantitative analysis for (H) (mean \pm SEM, $n = 6$). * $P < 0.05$; ** $P < 0.01$.

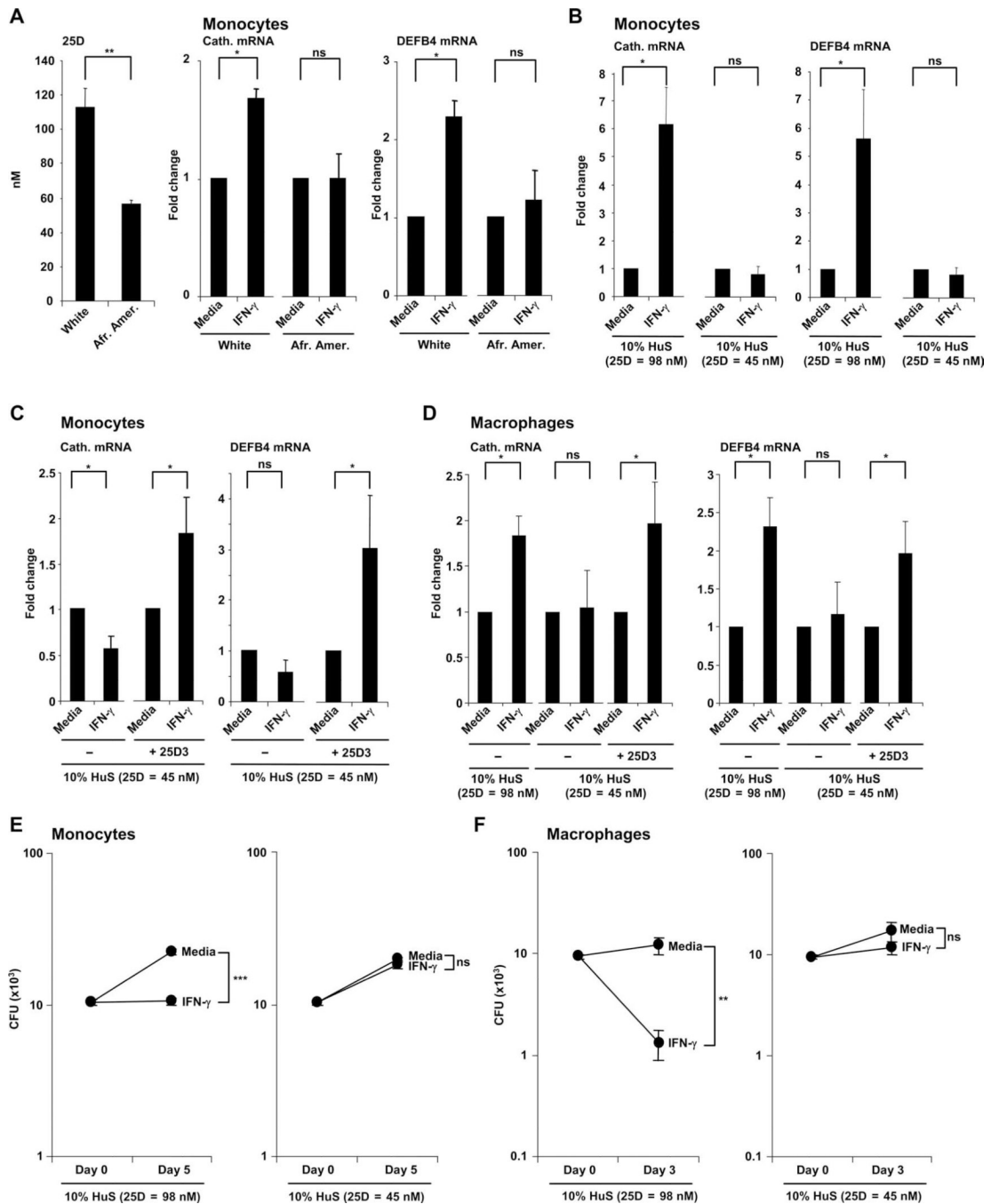


Fig. 5. IFN- γ induction of antimicrobial response is dependent on the concentration of serum vitamin D. **(A)** Concentrations of 25D of individual sera obtained from white ($n = 4$) and African-American ($n = 4$) donors were measured (mean 25D serum amounts \pm SEM). Monocytes were cultured with 10% serum from either white or African-American individuals and stimulated with IFN- γ for 20 hours. Cathelicidin and DEFB4 gene expression was determined by qPCR (mean fold change \pm SEM). **(B)** Monocytes were cultured in vitamin D-sufficient (25D = 98 nM) or vitamin D-deficient (25D = 45 nM) pooled human serum (HuS) and stimulated with rIFN- γ for 20 or 24 hours. Cathelicidin and DEFB4 gene expression was determined by qPCR (mean fold change \pm SEM, $n = 3$ to 4).

(C) Monocytes were cultured in 10% vitamin D–deficient serum (25D = 45 nM), with or without the addition of 25D3 to reach sufficient concentrations, and stimulated with rIFN- γ . Concentrations of cathelicidin and DEFB4 gene expression were measured at 20 or 24 hours (mean fold change \pm SEM, $n = 3$ to 4). (D) MDMs were cultured in 10% vitamin D–sufficient serum (25D = 98 nM) or vitamin D–deficient serum (25D = 45 nM), with or without the addition of 25D3 to reach sufficient concentrations, and stimulated with rIFN- γ . Cathelicidin and DEFB4 gene expression was measured at 20 to 24 hours (mean fold change \pm SEM, $n = 5$ to 6). (E and F) Primary human monocytes (E) and MDMs (F) were infected with *M. tuberculosis* H37Rv and cultured with medium or rIFN- γ in 10% vitamin D–sufficient (25D = 98 nM) or vitamin D–deficient (25D = 45nM) human serum. Viable bacteria were quantified by CFU assay after days 0 and 3 (E) or days 0 and 5 (F) (mean \pm SEM, $n = 9$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns, not significant.

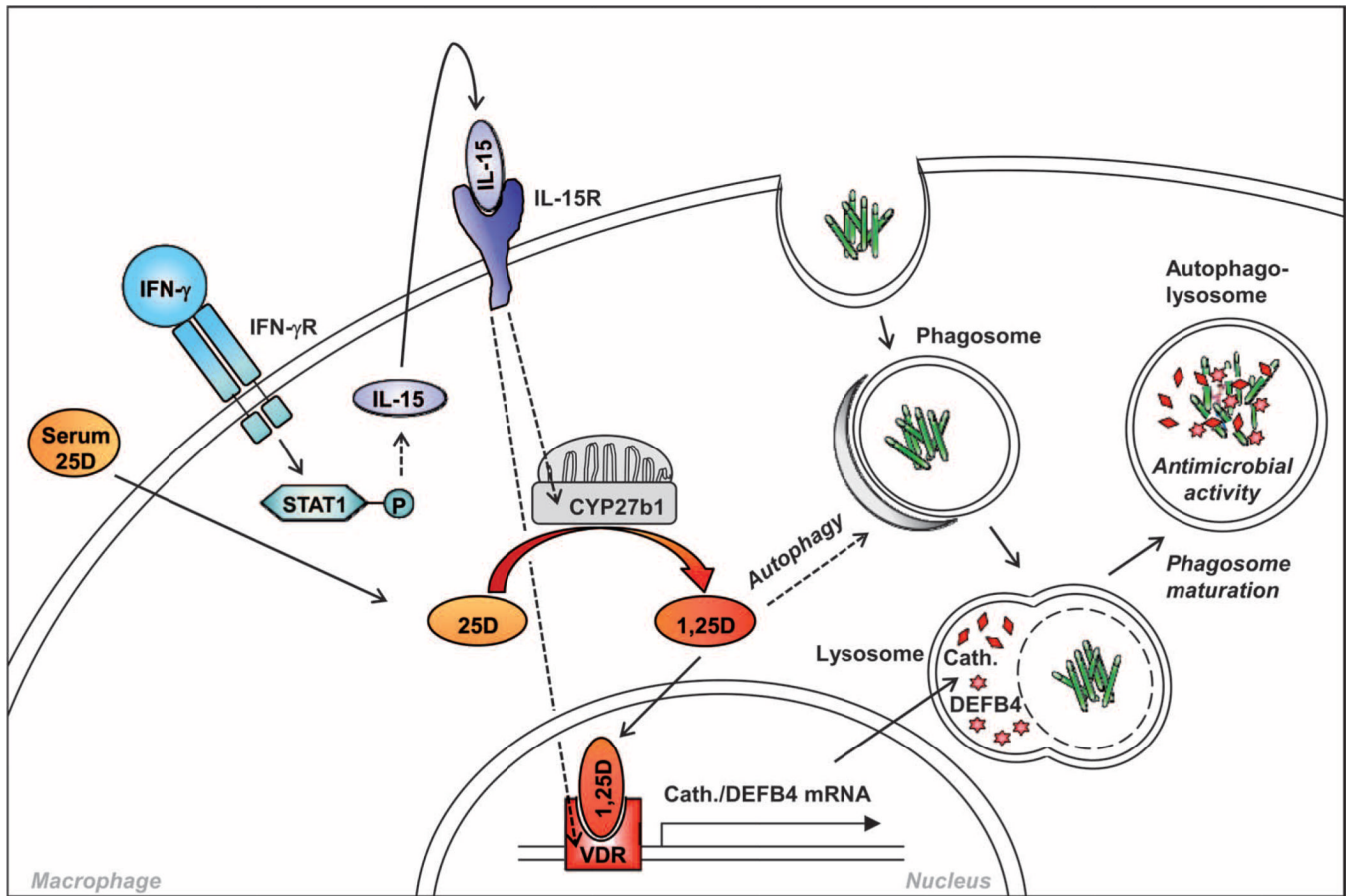


Fig. 6. IFN- γ induces an antimicrobial pathway in human monocytes/macrophages. This model shows that STAT1-dependent induction of IL-15 by IFN- γ leads to the up-regulation of VDR and CYP27b1. CYP27b1 hydroxylates the inactive form of vitamin D (25D) into the active form (1,25D), which mediates the up-regulation of antimicrobial peptides cathelicidin and DEFB4. The intracrine-produced 1,25D also triggers autophagy, which overcomes the *M. tuberculosis*-induced phagosome maturation block, leading to autophagolysosomal fusion and antimicrobial activity against *M. tuberculosis*.