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Regulation of vitamin D metabolism during the immune response to mycobacterial infection

A dissertation submitted in partial satisfaction
of the requirements for the degree Doctor of Philosophy
in Molecular, Cell, and Developmental Biology

by

Kathryn Genevieve Zavala

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Molecular, Cell, and Developmental Biology

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Professor John S. Adams, Co-Chair

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Classically, the endocrine vitamin D system is essential for maintaining calcium and phosphate homeostasis. Sufficient vitamin D levels promote bone health and prevent osteopathies, such as rickets and osteomalacia. However, the discovery of an intracrine vitamin D pathway alluded to other roles for vitamin D in regulating the immune system. Specifically, research on the innate immune response to *Mycobacterium tuberculosis* infection demonstrated a vitamin D-mediated antimicrobial response. Here, we continued to investigate the role of vitamin D in the immunobiology of mycobacterial infection.

In Chapter 1, we studied mycobacterial infection using leprosy as a disease model. We demonstrated that induction of the type I IFN gene program during *Mycobacterium leprae* infection is an active immune evasion mechanism used to subvert the vitamin D-dependent antimicrobial response through the suppression of the vitamin D activating enzyme, CYP27B1.

Moreover, our studies comparing macrophages associated with each of the two poles of the leprosy disease spectrum—lepromatous leprosy (L-lep) and tuberculoid leprosy (T-lep)—provided additional insight into susceptibility versus resistance to infection. T-lep-associated macrophages with elevated CYP27B1 expression and activity at baseline were resistant to type I IFN-mediated suppression and capable of inducing an antimicrobial response, whereas L-lep-associated macrophages were not. Taken together, these results demonstrate the importance of control of the vitamin D-dependent antimicrobial response in the host-pathogen interaction.

In Chapter 2, we focused on markers of host defense to identify factors which confer protection against mycobacterial infection. These studies revealed that IL-32 is associated with IL-15- and IFN-γ-induced host defense networks. IL-32 is also necessary and sufficient to induce CYP27B1 activity and the downstream antimicrobial response. These studies pinpoint IL-32 as an important regulator of vitamin D-dependent host defense.

Finally, in Chapter 3, we studied the effects of 1,25D on the iron regulatory protein, hepcidin, which regulates iron homeostasis by binding ferroportin and preventing the export of iron. Our results showed that 1,25D suppresses hepcidin expression in monocytes. These findings are important in the context of mycobacteria which are intracellular pathogens that rely on scavenging iron from the host to survive. Therefore, regulation of hepcidin may be another key battleground in the host-pathogen interaction.

The dissertation of Kathryn Genevieve Zavala is approved.

Atsushi Nakano

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DEDICATION

To my parents, siblings, and fiancé, who encouraged me to keep pounding the rock.

"When nothing seems to help, I go and look at a stonecutter hammering away at his rock perhaps a hundred times without as much as a crack showing in it. Yet at the hundred and first blow it will split in two, and I know it was not that blow that did it, but all that had gone before."

-- Jacob Riis

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PRESENTATIONS/POSTERS

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INTRODUCTION

Vitamin D and the innate immune response

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ABSTRACT

Active vitamin D metabolites play a major role in key functions of the innate immune response. Triggering of the vitamin D receptor (VDR) by those metabolites has been demonstrated to mediate the expression of antimicrobial peptides, induction of autophagy, as well as the production of reactive oxygen species and reactive nitrogen species as mechanisms of host defense. The role of vitamin D has been well characterized in the context of tuberculosis and leprosy from a molecular standpoint. Activation of Toll-like receptors, a family of innate immune pattern recognition receptors, on human macrophages with *M. tuberculosis*-derived ligands, results in activation of the vitamin D pathway, including i) the conversion of 25-hydroxyvitamin D (25(OH)D) to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), ii) activation of the VDR, and iii) antimicrobial activity against intracellular *M. tuberculosis* infection. This provides a potential explanation for the association between the host vitamin D status with susceptibility to tuberculosis infection and disease.

INTRODUCTION

This chapter will examine the role of vitamin D in the innate immune system and host defense against microbial disease, focusing on human infectious diseases, such as tuberculosis and leprosy. The first section will examine the innate immune receptor families that mediate pathogen recognition and the role of vitamin D in their response and regulation. Next, the effects of $1,25(OH)_2D$ on innate immune effectors and processes, such as antimicrobial peptides, oxidative stress, and autophagy will be reviewed. Then, the known associations, genetic and mechanistic, between the vitamin D pathway and tuberculosis susceptibility will be examined. This will be followed by a discussion of the role of vitamin D in barrier function and defense, as well as its association with infectious diseases. Finally, the chapter will conclude with a discussion on the history and potential for adjuvant treatment of tuberculosis with vitamin D.

MYCOBACTERIA

The genus *Mycobacterium* encompasses over fifty species, most of which are non-pathogenic (1). However, a few species have been known to cause disease in humans including *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the etiological agents of tuberculosis and leprosy, respectively. While important to study from a global health perspective, understanding the pathogenesis of tuberculosis and leprosy has also provided valuable insight into the human immune response to intracellular pathogens.

Tuberculosis

Tuberculosis has plagued humans throughout history with prehistoric fossil evidence as well as written recordings of the disease in ancient Egyptian and Chinese manuscripts (2). In 1882, Robert Koch first described *Mycobacterium tuberculosis* as the etiological agent of tuberculosis, which primarily infects lung macrophages leading to pathogenesis of the disease. More than a century later, tuberculosis remains a leading cause of morbidity and mortality worldwide, with one third of the world's population infected and eight million new cases of tuberculosis appearing each year (3). Even developed countries are not spared by this pandemic; an estimated 10-15 million people residing in the United States are infected with *M. tuberculosis* (4,5). The World Health Organization recently reported that the incidence of multidrug-resistant (MDR) *M. tuberculosis* is at the highest in recorded history. More alarmingly, extensively drug-resistant (XDR) as well as totally drug-resistant (TDR) strains, which have no effective treatment, are also rapidly emerging (6).

Studies on tuberculosis have helped delineate several basic immunological paradigms, such as the role of Toll-like receptor 2 (TLR2) in recognition of microbial lipoproteins (7), leading to i) instruction of the adaptive immune response (7-9), ii) macrophage differentiation (8), iii) a nitric oxide-dependent antimicrobial pathway in mice (10) and iv) a vitamin D-dependent antimicrobial pathway in humans (11). TLR2 was shown to be important for

resistance to *M. tuberculosis* in mouse models (12-14), and polymorphisms in both the vitamin D receptor (VDR) and TLR2 are associated with human susceptibility to tuberculosis (15-22). This association of tuberculosis with both the innate immune system (via infection of macrophages) and vitamin D led to both an understanding of vitamin D's immunomodulatory role as well as delineation of the molecular mechanisms by which an individual's vitamin D status could alter their ability to combat pathogens. The vitamin D-mediated innate immune pathway could have implications beyond tuberculosis as other pathogens such as *Escherichia coli* (23), *Helicobacter pylori* (24), and *Aggregatibacter actinomycetemcomitans* (25), have been associated with vitamin D as well.

Leprosy

According to the World Health Organization, across one hundred countries and territories, over two hundred thousand new cases of leprosy were reported during 2012. Additionally, leprosy continues to be the leading cause of disability among communicable diseases (26). Thus, often considered an ancient disease, leprosy presents a significant global disease burden to this day.

Since Gerhard Armauer Hansen first discovered *M. leprae* as the causative agent of leprosy, studies in leprosy have become a powerful tool for studying pathogenic infection and host defense. Particularly, immunologists have benefitted from the correlation of clinical manifestations with specific underlying immunological profiles (27). Patients diagnosed with leprosy fall on a spectrum of disease, which is divided into five main classes (28). The three intermediate groups are separated between two poles. At one end, patients with tuberculoid leprosy (T-lep) have sparse skin granulomas accompanied by few bacilli found in these sites (paucibacillary). At the other end, patients with lepromatous leprosy have much more disseminated skin lesions. Biopsies reveal hundreds to thousands of bacilli residing in these sites (multibacillary). From an immunological perspective, T-lep is characterized by a robust

cell-mediated immune response. Th1 cells dominate and release cytokines: IFN-γ, TNF, IL-2, IL-6, and IL-12 (28-30). IL-15 has also been shown to participate in this immune response (31). Conversely, L-lep is defined by a predominant humoral immune response and the presence of Th2 cells. IL-4 and IL-10 are the main secretion products (28-30) . Some borderline patients experience a natural shift in immunological status known as a reversal reaction (RR). During this acute phase of inflammation, the patient's status is upgraded towards the tuberculoid pole. Immunologically, these patients resemble T-lep with an infiltration of CD4+ T cells and increased release of IFN-γ. Reversal reactions suggest that a patient's disease state may be dynamic.

Furthermore, studies comparing T-lep versus L-lep phenotypes have elucidated many fundamental concepts about host-pathogen interactions including differences in phagocytic properties among macrophage subtypes (32); the use of transcriptional regulation as a mechanism for immune evasion (33); and the role of dendritic cells in antigen presentation (34,35). In the context of vitamin D, various studies have linked vitamin D-related genetic and biological factors to susceptibility to leprosy (36-38). However, the mechanism by which vitamin D mediates leprosy disease outcomes is not well understood.

PATHOGEN DETECTION

Rapid detection and antimicrobial activity against microbes are considered to be key functions of innate immune cells in relation to infection control. However, the mechanisms used by innate immune cells to detect invading pathogens had remained a mystery for many years. Charles Janeway proposed the existence of evolutionarily primitive receptors, termed pattern recognition receptors, which bind conserved microbial constituents (39). In 1996, Lemaitre *et al.* reported that Toll-deficient adult Drosophila were more susceptible to fungal infections (40). Activation of Toll resulted in production of antimicrobial peptides (41), thus implicating Toll as a player in a primitive immune system. One year later, Medzhitov *et al.*

demonstrated that a human Toll homologue, or a Toll-like receptor (TLR), modulated the adaptive immune response by inducing cytokine secretion and co-stimulatory molecule expression (42). Together, these reports first established the importance of Toll and TLRs in host defense.

M. tuberculosis is known to activate at least two different families of pattern recognition receptors: TLRs and the nucleotide oligomerization domain (NOD)-like receptors (NLR). The TLR2 and TLR1 heterodimer (TLR2/1) recognizes a triacylated lipoprotein derived from *M. tuberculosis*, leading to activation of NF-κB, which then results in the production of inflammatory cytokines and direct antimicrobial activity (7,10,11). NOD2 recognizes muramyl dipeptide (MDP), which is a peptidoglycan present on *M. tuberculosis* (43,44). Triggering NOD2 similarly leads to a NF-κB-mediated inflammatory response; however, in contrast to TLRs, NOD2 also results in activation of the inflammasome (45), a protein complex whose function is to cleave and activate the pro-IL-1β protein into the active IL-1β cytokine through the enzymatic actions of caspase-1.

Moreover, studies in leprosy have shown that activation of TLR and NOD receptors triggers distinct differentiation pathways directing the human immune response. Whereas activation of TLR2/1 induces macrophage differentiation thereby orchestrating the direct effector function of the innate response, activation of NOD2 induces dendritic cell differentiation thereby mediating the instructor function of the innate response that modulates the T cell adaptive response (46). Importantly, studies in tuberculosis and leprosy demonstrate synergistic and distinct roles for PRRs in mediating the host immune response during mycobacterial infection.

Toll-like receptors

TLRs have been shown to recognize microbial ligands and trigger functions of the innate immune system. To date, eleven mammalian TLRs have been identified in both the human and murine genomes. Although, TLR1 through TLR9 are conserved between humans and mice, the

murine *Tlr10* gene is nonfunctional, and the human *TLR11* gene harbors a premature stop codon preventing its expression (47). All the mammalian TLRs share a highly similar cytosolic Toll/IL-1 receptor (TIR) domain, which triggers several signaling pathways including the transcription factor, NF-κB (48). The extracellular domains of TLRs include multiple leucine-rich repeat motifs and are responsible for recognition of conserved pathogen-associated molecular patterns (PAMPs). TLR2 is known to heterodimerize with either TLR1 or TLR6, and the dimers mediate recognition of triacylated or diacylated bacterial lipoproteins, respectively (49). The remainder of the known TLR ligands are as follows: viral dsRNA (TLR3), lipopolysaccharide (LPS) (TLR4), bacterial flagellin (TLR5), single-stranded RNA (ssRNA) (TLR7 and TLR8), bacterial unmethylated CpG DNA (TLR9), and protozoan profilin-like molecule (TLR11) (47). The ligand for TLR10 is still unclear. Thus, TLRs provide a rapid first line of defense against a variety of microbial pathogens through the recognition of a milieu of PAMPs.

Activation of TLRs induces a variety of effects, including enhancement of macrophage phagocytosis (50), endosomal/lysosomal fusion (51), production of antimicrobial peptides (52,53), as well as induction of direct antibacterial (10,52) and antiviral activity (54-56). *M. tuberculosis*-infected macrophages can induce a direct antimicrobial activity upon TLR2/1 activation. This was demonstrated in a murine macrophage cell line, where the activity was dependent on the generation of nitric oxide (NO) through inducible nitric oxide synthase (iNOS) activity. Addition of the iNOS inhibitors L-NIL and L-NAME ablated the murine TLR2/1-mediated antimicrobial activity; however, neither had an effect on human monocytes, suggesting fundamentally different mechanisms in human and murine TLR2/1-induced antimicrobial activity (10). This correlated with the finding that upon TLR2/1 activation, human monocytes did not generate detectable levels of NO (57). Accordingly, the mechanism by which human macrophages kill intracellular *M. tuberculosis* intrigued immunologists for many years;

the surprising role of the vitamin D synthetic/metabolic pathway in this mechanism is detailed below.

Nucleotide oligomerization domain

There is building evidence for the participation of the NLR family of pattern recognition receptors in the host defense response against *M. tuberculosis* infection. Studies have demonstrated that the resultant gene programs elaborated by TLR and NOD2 are distinct, despite both receptor families activating the transcription factor NF-κB (58,59). Of the NLRs, NOD2 has been the most extensively studied, and recognizes muramyl dipeptide (MDP), which is a peptidoglycan present on *M. tuberculosis* (43,44). In 2005, Ferwerda *et al.* published a study providing evidence for the role of NOD2 in the recognition of *M. tuberculosis* infection (59). However, conflicting results have emerged regarding the role of NOD2 in the control of *M. tuberculosis* infection.

One study by Gandortra *et al.* demonstrated that NOD2 knockout mice were able to control *M. tuberculosis* infection (32), whereas Divangahi *et al.* showed impaired resistance by NOD2-deficient mice (60). An epidemiological study in humans identified NOD2 polymorphisms associated with susceptibility to tuberculosis in an African American cohort (61); but a separate study conducted in Gambia was unable to identify any NOD2 polymorphisms associated with tuberculosis (62). One potential reason for these conflicting results in humans is that neither study accounted for the vitamin D levels of the patients, an important factor when considering disease linkage in relation to the vitamin D pathway (22). This is highlighted by the fact that $1,25(OH)_2D_3$ strongly induced the expression of NOD2, which then resulted in induction of the antimicrobial peptide hBD2 (DEFB4), as demonstrated by Wang *et al.* in 2010 (63). Therefore, one can infer that an individual's vitamin D status could alter his or her ability to detect and combat an invading pathogen. Taken together, these studies provide sufficient *in vitro* evidence for the role of NOD2 in the host response against

M. tuberculosis infection; however the epidemiological data indicate that there are additional layers of complexity yet to be clarified.

IMMUNE ACTIVITY OF 1,25-DIHYDROXYVITAMIN D

There have been many studies on the role of $1,25(OH)_2D$ on innate and adaptive immune responses (64-66). Insight into vitamin D-induced antimicrobial activity by human monocytes and macrophages against *M. tuberculosis* was first suggested by experiments in the labs of Rook in 1986 (67) and Crowle in 1987 (68). These experiments were performed by adding $1,25(OH)_2D_3$ to the extracellular medium of *M. tuberculosis*-infected human monocytes and macrophages *in vitro,* which resulted in reduction of the intracellular bacterial load. However, the authors noted that "concentrations of $1,25(OH)_2D$ near 4 μg/ml were needed for good protection. These levels seemed unphysiologically high compared with 26 to 70 pg/ml being in the normal circulating range." Nevertheless, these studies opened new questions regarding the role of vitamin D in the physiological response to *M. tuberculosis* and the identity of the vitamin D-dependent antimicrobial effectors.

Nearly a decade later, the vitamin D-induced antimicrobial activity of the macrophage began to be elucidated. One study by Sly *et al.* reported that $1,25(OH)_2D_3$-induced antimicrobial activity was regulated by phosphatidylinositol 3-kinase and mediated through the generation of oxygen intermediates via NADPH-dependent phagocyte oxidase (69). Interestingly, these authors observed that the $1,25(OH)_2D_3$-induced oxidative burst occurred earlier than the resultant antimicrobial activity, thus leading the authors to postulate that there was another key factor (69). Anand *et al.* proposed an alternative mechanism in their study demonstrating that $1,25(OH)_2D_3$-induced antimicrobial activity was associated with downregulated transcription of the host protein, tryptophan aspartate-containing coat protein (TACO) (70). They also demonstrated that TACO plays an important role in *M. tuberculosis* entry and survival in human macrophages (71).

In 2005, using a genome wide scan for vitamin D response elements (VDREs), Wang *et al.* reported that the genes encoding antimicrobial peptides, cathelicidin (CAMP) and hBD2 (DEFB4), were regulated by the VDR (72). Prior to this study, human macrophages were not thought to utilize antimicrobial peptides as a defense mechanism. In the same year, human monocytes were demonstrated to express cathelicidin at both the mRNA and protein levels when stimulated with $1,25(OH)_2D_3$ (11,72,73). Two years later, a critical role for cathelicidin in the $1,25(OH)_2D_3$-induced antimicrobial activity against intracellular *M. tuberculosis* was demonstrated in human monocytic cells using siRNA knockdowns (74).

In contrast to its effects on macrophages, many studies have reported that $1,25(OH)_2D_3$ induces immunosuppressive effects on the adaptive immune response, including but not limited to i) inhibition of IL-12 secretion, ii) inhibition of lymphocyte proliferation and immunoglobulin synthesis and iii) impairment of dendritic cell maturation, leading to the generation of tolerogenic dendritic cells and T-cell anergy (75-78). In particular, it was suggested that $1,25(OH)_2D_3$ produced by macrophages in granuloma-forming diseases, like tuberculosis and sarcoidosis, exerts a paracrine immune inhibitory effect on neighboring, activated lymphocytes expressing the VDR, which slows an otherwise "overzealous" adaptive immune response (79). The physiological significance of this has been highlighted by the development of $1,25(OH)_2D_3$-deficient mouse models where CYP27B1 had been knocked out (80,81). A notable feature of these animals was that they presented with enhanced adaptive immunity signified by multiple enlarged lymph nodes. However, whether this enhancement was due to a loss of $1,25(OH)_2D_3$-mediated suppression of the adaptive immune response, remains to be tested.

Antimicrobial peptides

Antimicrobial peptides consist of a highly diverse family of small peptides, which can function as chemoattractants (82,83), dendritic cell activators (84), and importantly, direct antimicrobial effectors (85,86). They exert antimicrobicidal activity by disrupting the pathogen

membrane through electrostatic interactions with the polar head groups of membrane lipids (87), or through the creation of membrane pores (85). Given this mechanism of activity, antimicrobial peptides exhibit a wide range of microbial targets including bacteria (88), fungi (89,90), parasites (91,92) and enveloped virii (93). Specifically, the antimicrobial peptide, cathelicidin, has been shown to have broad-spectrum activity against Gram-positive and Gram-negative microorganisms (94).

Aside from macrophages, epithelial cells, located at the interface of the outside and inside environments of the host, can express antimicrobial peptides (95). This has been demonstrated in urinary bladder epithelial cells (23), gingival epithelial cells (25), and gastric epithelial cells (24). However, it is the population of innate immune cells buttressing this barrier, such as neutrophils (96), mast cells (97) and monocytes/macrophages (73,98) that is recognized to be the major producer of antimicrobial peptides.

Several antimicrobial peptides produced by macrophages have been demonstrated to have direct antimicrobial activity against *M. tuberculosis*, including but most likely not limited to LL-37 (cathelicidin) (11,99), hBD2 (DEFB4) (100), and hepcidin (101). In humans, the promoter regions of the genes encoding cathelicidin (CAMP) and hBD2 (DEFB4) were found to contain activating VDR response elements (72). Activation of the VDR in monocytes/macrophages resulted in the expression of cathelicidin at both the mRNA and protein levels (11,72,99). siRNA knockdown of cathelicidin in human monocytes resulted in complete loss of $1,25(OH)_2D_3$-induced antimicrobial activity (74), suggesting that generation of antimicrobial peptides by the active vitamin D metabolite represents a major human macrophage host defense mechanism.

Oxidative Stress: Reactive Oxygen Species

Generation of reactive oxygen species (ROS) is dependent upon the enzymatic activity of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and occurs

predominately in neutrophils, and to a lesser degree, in macrophages (102). NADPH oxidase is a complex of both membrane and cytosolic components assembled on the cell membrane. The membrane bound components of the complex include $gp91^{phox}$ and $p22^{phox}$, which are the α/β subunits of flavocytochrome $b_{558,}$ respectively. In addition, the cytosolic proteins include $p47^{phox}$, $p67^{phox}$ and Rac (103,104). Another molecule, $p40^{phox}$, which negatively regulates NADPH-oxidase activity, also associates with the complex (17). The NADPH oxidase complex functions through the transfer of electrons, converting molecular oxygen (O_2) into the superoxide anion ($O_2^{-\cdot}$), which can then be converted to hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$) or other ROS (105). Functionally, ROS have been shown to cause microbial DNA damage, oxidative damage to proteins, and disruption of membrane lipids as possible direct and indirect antimicrobial mechanisms (105,106). All the products of this reaction exhibit varying degrees of antimicrobial ability. The earliest products, O_2^- and H_2O_2,exhibit the weakest antimicrobial activity, whereas downstream products, hypochlorites (OCl^-) and chloramines (RNH_2Cl), are the most potent (106).

The assembly of NADPH oxidase is regulated by the phosphatidylinositol 3-kinase (PI3K), which can be induced by certain proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and granulocyte-macrophage colony stimulating factor (GM-CSF) (107). Induction of PI3K in neutrophils as part of a priming response results in a stronger oxidative burst upon either phagocytosis or encounter with microbial stimuli (107). The induction of NADPH oxidase activity by $1,25(OH)_2D_3$ on human monocytes is through increased expression of the p47 subunit (108) and mediated through PI3K pathway (69). Other immune stimuli, such as interferon gamma (IFN-γ) and microbial products, can directly induce transcription of the NADPH oxidase complex components (109).

Oxidative Stress: Reactive Nitrogen Species

Generation of reactive nitrogen species (RNS) is achieved through a family of nitric oxide synthases (NOS): inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS) (110). The iNOS isoform is predominately responsible for generation of nitric oxide radicals ($NO^{\cdot}$) by cells of the innate immune system, which functions as a potent antimicrobial effector (111). These enzymes are multi-domain proteins with an amino-terminal oxidase domain containing a heme center, a calmodulin-binding domain, and a carboxy-terminal reductase domain. In addition, they contain binding sites for L-arginine, and tetrahydrobiopterin, while the reductase domain contains binding sites for NADPH, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). The transfer of electrons from NADPH to FAD, then FMN, and finally to the heme iron, results in generation of nitric oxide ($NO^{\cdot}$) and citrulline from L-arginine (112). In the presence of oxygen, nitric oxide can be future catalyzed into nitrogen dioxide ($NO_2^{\cdot}$), nitrogen trioxide (N_2O_3), nitrate (NO_3^-), and other RNS (105). NO can also interact with super oxide to form peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH) (102).

The RNS-mediated antimicrobial mechanisms are more complex than those of ROS. NO can inhibit both microbial DNA replication and cellular respiration (113-115). Through interactions with ribonuclease reductase, RNS can limit the precursors required for DNA replication and repair (116). Nitrogen dioxide ($NO_2^{\cdot}$), nitrogen trioxide (N_2O_3), and nitrate (NO_3^-) can potentiate oxidative damage similar to damage by ROS (117-119). Together with H_2O_2 and myeloperoxidase, $ONOO^-$ can nitrate tyrosine residues on proteins (120).

Regulation of iNOS activity by immune cells is predominately at the transcriptional level. Inflammatory cytokines such as interferons (IFNs), IL-1β, and TNF-α can induce transcription of iNOS through the p38-MAPK, NF-κB, as well as the Janus activated kinase (JAK) and signal transducer and activator of transcription (STAT)-IRF3 signaling pathways (121-123). Interestingly, despite numerous reports of human macrophages inducing the iNOS transcript

and promoter activity (124-127), there have been a sparingly small number of publications that have detected robust levels of NO, comparable to those of other animal models (128).

The ability of $1,25(OH)_2D_3$ to regulate iNOS activity in innate immune cells seems to be evolutionarily divergent in animals. Using avian monocytic cells, $1,25(OH)_2D_3$ synthesis through CYP27B1 activity was demonstrated to be dependent upon and regulated by the level of NO production by the same cells (129,130). The authors of the study hypothesized that the NO produced in these cells acted as a source of electrons for the hydroxylation reaction. For rat and mouse monocytic cells, LPS-induced iNOS expression and function was inhibited by $1,25(OH)_2D_3$ (131,132); however the role of NO in $1,25(OH)_2D$ generation was not explored. In contrast, $1,25(OH)_2D_3$ stimulation of bovine monocytes induced the expression of iNOS (133). For human promyeloid cells, $1,25(OH)_2D_3$ stimulation alone did not induce either iNOS mRNA expression or NO, whereas co-stimulation with both $1,25(OH)_2D_3$ and phorbol 12-myristate 13-acetate (PMA) resulted in a robust induction of both iNOS mRNA and NO. These results suggest a more intricate mechanism of iNOS regulation in humans involving multiple pathways, which may include the vitamin D pathway. Primary human monocytes stimulated through TLR2/1 did not produce appreciable levels of NO (10); whether or not sufficient bioavailability of $25(OH)D_3$ would alter this result remains to be tested.

Autophagy

Recent studies have demonstrated autophagy, the cellular process by which a cell degrades its own intracellular compartments, as a previously unappreciated innate immune defense mechanism (134). Stimulation of mouse macrophages with the key immune cytokine, IFN-γ, induced an autophagy-dependent antimicrobial activity against *M. tuberculosis* (134). A separate study demonstrated that lysosomal-hydrolyzed ubiquitin peptides have direct antimicrobial activity against *M. tuberculosis* (Figure 1A) and are delivered in an autophagy-dependent manner to phagosomes harboring mycobacteria (135). Further studies revealed the

mechanism for delivering bacilli to the autophagosome depends on sensing of extracellular bacterial DNA by the STING-dependent cytosolic pathway (136). Mice with macrophages deficient in this pathway showed increased susceptibility to infection with *M. tuberculosis* (136). Autophagy can be induced via a variety of methods in macrophages; most immunologically relevant includes IFN-γ, TLR activation, and $1,25(OH)_2D_3$ (134,137-139). In human macrophages, induction of autophagy was required for $1,25(OH)_2D_3$-induced antimicrobial activity against *M. tuberculosis* (137).

VITAMIN D PATHWAY AND TUBERCULOSIS

Many studies have identified genes that may confer some degree of susceptibility to tuberculosis, including: HLA-DR alleles (140-142), NRAMP1 (143), interferon-□ pathway genes (144), SP110 (145), complement receptor-1 (146), and notably, the VDR (18-22). However, these studies did not identify a clear-cut host defense mechanism explaining the linkage. Several studies have linked serum levels of 25(OH)D, to both tuberculosis disease progression and susceptibility (22,147). In 1985, a study of Indonesian patients with active tuberculosis reported that out of 40 patients, the 10 patients with the highest 25(OH)D levels at the outset of therapy had "less active pulmonary disease" (147).

Another aspect of the vitamin D pathway, which has been extensively studied, is the VDR itself. There are two major VDR polymorphisms that have been studied in terms of tuberculosis susceptibility, yet with conflicting results. These polymorphisms are TaqI (19-21) and FokI (21,148), located in exons nine and two of the VDR gene, respectively (149). Bellamy *et al.* concluded that the tt allele of the TaqI polymorphism protects against tuberculosis; however, studies by two other groups reported no such association (20,148). Liu *et al.* described that the FokI ff allele is associated with active tuberculosis among the Chinese Han population (21), but there are no other studies concluding an association between FokI ff and tuberculosis. These conflicting results became clarified in a study examining the relationship between vitamin D

deficiency, VDR polymorphisms, and tuberculosis in the Gujarati Asians living in West London in the year 2000 (22). The study reported both the TaqI (Tt/TT) and FokI (ff) alleles were associated with tuberculosis only when the individual exhibited serum 25(OH)D deficiency (22). These studies illustrated one problem with regard to vitamin D and human tuberculosis, when comparing the *in vitro* studies with the *in vivo* observations: the *in vitro* studies used the active, $1,25(OH)_2D$ metabolite to affect antimicrobial activity, while the association to tuberculosis was with serum 25(OH)D levels.

Role of 25-hydroxyvitamin D on the innate immune response

Relatively little is known about the direct effects of 25(OH)D on innate immunity. Hewison *et al.* found that 25(OH)D at physiologic levels (100nM) suppressed CD40L-induced IL-12 production in day-7 GM-CSF/IL-4 derived dendritic cells (DCs) *in vitro* (150). Other *in vitro* studies have shown that intracrine metabolism of 25(OH)D to $1,25(OH)_2D$ via endogenous expression of CYP27B1-hydroxylase is a more efficient mechanism for modulating the phenotype of either DCs or monocytes compared to the exogenous addition of active $1,25(OH)_2D$ itself (151). In relation to the adaptive immune response, Yang *et al.* showed significant blunting of the cell-mediated immune response to cutaneous dinitrofluorobenzene (DNFB) challenge in mice with profound reduction of serum 25(OH)D levels (152). Administration of 25(OH)D to humans with head and neck squamous cell carcinoma increased plasma IL-12 and IFN-γ levels, and improved T-cell blastogenesis (153).

Vitamin D-mediated antimicrobial response

In 2006, a potential mechanism by which a person's circulating 25(OH)D status may alter that individual's innate immune response against *M. tuberculosis* was reported (11). Activation of human TLR2/1 on monocytes resulted in the induction of key genes in the vitamin D pathway (Figure 1B), including the vitamin D receptor (VDR) and CYP27B1. Under conditions

in which the extracellular concentration of 25(OH)D was present at sufficient levels, TLR2/1 activation of monocytes resulted in a CYP27B1- and VDR-dependent expression of the antimicrobial peptide, cathelicidin, as well as direct microbicidal activity against intracellular *M. tuberculosis* (Figure 1C). The induction of CYP27B1 and VDR in monocytes was subsequently demonstrated to be mediated through the actions of TLR2/1-induced IL-15 expression (Figure 1D) (154). Interestingly, the human, but not the murine cathelicidin promoter, contains an activating VDRE (73), perhaps suggesting a point of divergent evolution between mice and humans in the antimicrobial effectors used by the TLR-mediated innate immune response. Since murine species are nocturnal and have limited opportunity for UV-mediated vitamin D synthesis, the importance of vitamin D levels to their immune response is unclear.

In addition to cathelicidin, TLR activation of human monocytes resulted in the vitamin D-dependent expression of DEFB4, an antimicrobial peptide gene also characterized with a VDRE in its promoter (72,155). Convergence of IL-1β and vitamin D transcriptional activation was required for the TLR-induced expression of DEFB4 (Figure 1E). Triggering of TLR2/1 was found to modulate IL-1β activity by increasing the cell's responsiveness to secreted IL-1β through the i) simultaneous secretion of IL-β, ii) upregulation of cell surface IL-1R1, and iii) downregulation of the baseline IL-1 receptor antagonist (IL-1RA) (155). These findings provide a potential molecular mechanism for the previously known associations of IL-β and IL-1RA polymorphisms with tuberculosis (156), as well as the requirement for the IL-1R1 in host defense against *M. tuberculosis* (157). Inhibition of the VDR, as well as knockdown of cathelicidin or DEFB4, resulted in ablation of the TLR2/1-induced antimicrobial activity, implicating VDR activation as a critical step in the innate immune response against *M. tuberculosis* (11,155). This potentially explains the association of low 25(OH)D serum levels with susceptibility to tuberculosis: low 25(OH)D levels in circulation cannot provide sufficient substrate 25(OH)D for CYP27B1-mediated production of $1,25(OH)_2D$ and subsequent activation of the VDR-dependent

antimicrobial activity. Interestingly, TLR activation of bovine macrophages in the presence of $25(OH)D_3$ resulted in CYP27B1 activity and expression of iNOS (133). This demonstrates an evolutionary conservation of the TLR-induced vitamin D-dependent antimicrobial pathway as a mechanism of host defense with the exception of the end result: humans induce antimicrobial peptides while ruminants utilize iNOS (11,133). It remains to be seen if other diurnal species also maintain this conservation.

Through rheostatic regulation of CYP27B1 activity and conversion of substrate 25(OH)D to product $1,25(OH)_2D$, the macrophage directly controls its intracellular level of $1,25(OH)_2D$ (158). These data suggest that it is serum 25(OH)D, and not the serum $1,25(OH)_2D$ concentration, which controls the intracellular $1,25(OH)_2D$ level that is essential for the TLR-induced antimicrobial activity. This explains why in previous experiments *in vitro*, a super-physiologic concentration of $1,25(OH)_2D$ in the conditioning extracellular media was required to generate sufficient intracellular levels of the metabolite to affect the VDR and to achieve an antimicrobial effect in human macrophages (67,68).

Adaptive immune response and vitamin D

The preceding studies have provided evidence for the role of $1,25(OH)_2D$ synthesis in TLR2-triggered host defense against *M. tuberculosis* infection. A recent study demonstrated that the adaptive immune response may play a role in regulation of the vitamin D-dependent innate immune response as well. The presence of the key adaptive immune response T-cell cytokine, IFN-γ enhanced the TLR2/1-induced cathelicidin and DEFB4 expression (159). This is biologically significant, since IFN-γ drives the Th1 type adaptive immune response, which is critical for defense against intracellular pathogens such as *M. tuberculosis*. In contrast, the Th2-driving T-cell cytokine, IL-4, blocked TLR2/1L-induced cathelicidin and DEFB4 expression, and the key Th17 cytokine, IL-17, had no effect. The surprising finding was that the effects of IL-4 on the vitamin D pathway was not at the level of $1,25(OH)_2D$ synthesis, since both the mRNA levels

and enzymatic activity of CYP27B1 remained intact; instead, the IL-4 mediated inhibition of the vitamin D pathway was due to increased conversion of $25(OH)D_3$ into the inactive $24,25(OH)D_3$ metabolite. This IL-4-induced conversion of $25(OH)D_3$ into $24,25(OH)D_3$ was regulated at the level of the enzymatic activity of CYP24A1, the enzyme predominately responsible for vitamin D degradation, and not at the level of transcription or post-transcriptional modification (159). However, the mechanism by which IL-4 influences CYP24A1 activity remains to be elucidated. These results further highlight the vitamin D pathway as a key player in host defense and show that the pathway is subject to control by T-cell cytokines, as well as regulated by the metabolism and catabolism of vitamin D metabolites.

VITAMIN D PATHWAY AND LEPROSY

Leprosy presents on a spectrum of disease with self-limiting tuberculoid leprosy (T-lep) at one end and progressive lepromatous leprosy (L-lep) at the other end (28). Studies comparing the two phenotypes have revealed underlying mechanisms driving host resistance versus susceptibility to mycobacterial infection.

Cytokines, macrophages, and vitamin D

Previous studies have shown the association of various cytokines with each leprosy phenotype. Specifically, IFN-γ, TNF, IL-2, IL-6, IL-12 and IL-15 are found in tuberculoid leprosy lesions whereas IL-4 and IL-10 are the main cytokines found in lepromatous leprosy lesions (28-31). Among many cell types found in leprosy skin lesions, macrophages are present and shown to be infected with *M. leprae* (160). Montoya *et al.* studied the effect of cytokines on macrophage differentiation and subsequent function (161). These studies showed that differentiation of macrophages with L-lep associated IL-10 versus T-lep-associated IL-15 resulted in divergent phagocytic properties. IL-10-derived macrophages were programmed for increased lipid uptake leading to the formation of foamy macrophages. On the other hand, IL-

15-derived macrophages were less phagocytic. In the context of vitamin D, IL-15-derived macrophages were primed for vitamin D-mediated antibacterial activity. They had increased expression of CYP27B1 and increased conversion of 25(OH)D to $1,25(OH)_2D$. IL-10-derived macrophages had low CYP27B1 expression and activity. Furthermore, using immunofluorescence, Montoya *et al.* showed the presence of the IL-10-derived macrophage subtype in L-lep lesions and the IL-15-derived subtype in T-lep lesions.

Moreover, Teles *et al.* analyzed gene expression profiles from leprosy skin lesions revealing contrasting interferon signatures in T-lep versus L-lep lesions (162). Type I interferon-induced genes were enriched in L-lep lesions whereas type II interferon-induced genes were enriched in T-lep lesions. Type II interferon, IFN-γ, has previously been shown to activate the macrophage intracrine vitamin D system and associated antimicrobial activity through vitamin D-mediated upregulation of cathelicidin and defensin B2 (159,163,164). Indeed, increased IFN-γ gene expression was detected in T-lep lesions and correlated with increased CYP27B1 and VDR expression. In contrast, type I IFN, IFN-β, and downstream type I IFN-inducible, IL-10, were detected in L-lep lesions and negatively correlated with CYP27B1 and VDR expression. Additionally, monocytes treated with IFN-γ had increased antimicrobial activity that was blocked by the addition of IFN-β demonstrating that type I interferon suppresses type II IFN induction of the vitamin D-dependent antibacterial response.

Taken together, studies by Montoya *et al.,* Teles *et al.* and others have begun to elucidate the signaling pathways by which cytokines regulate immune cell function and influence resistance or susceptibility to mycobacterial diseases. Additional studies showed that IL-32 mediates the induction of the type II IFN-induced vitamin D-dependent antimicrobial response (165). Knockdown of IL-32 ablated IFN-γ-induced CYP27B1 expression (165). These results add to studies to define one pathway for the triggering of antibacterial response as IFN-γ → IL-15 → IL-32 → CYP27B1 and VDR → CAMP and DEFB4 → bacterial killing. Additional studies

on the repression of the vitamin D-dependent antibacterial response have defined one pathway to be IFNβ → IL-27 → IL-10 --/ IFN-γ (162,166).

Vitamin D pathway and immune evasion

Liu *et al* demonstrated that two factors are necessary for the vitamin D-mediated antibacterial response: 1) activation of the intracrine vitamin D system; and 2) availability of substrate 25(OH)D. Thus, in describing a role of vitamin D, these studies also suggest a target for mechanisms to evade host defense. Repression of CYP27B1 hampers the conversion of circulating 25(OH)D to biologically active $1,25(OH)_2D$ necessary for induction of antimicrobial peptides, CAMP and DEFB4. As described above, Teles *et al.* showed that *M. leprae* induces a type I IFN gene program that is correlated with a repressed vitamin D system (162). By blocking IL-10 signaling, type I IFN suppression of the vitamin D-dependent antimicrobial response was reversed. Furthermore, analysis of microRNA expression in L-lep and T-lep skin lesions provides additional evidence of regulation of the vitamin D pathway during immune evasion. microRNA-21 (mir-21) was shown to be highly expressed in L-lep lesions as well as induced by *M. leprae* infection of monocytes (33). Furthermore, mir-21 inhibited TLR2/1 induction of CAMP and DEFB4 by directly binding to the 3' untranslated region and downregulating expression of both CYP27B1 and IL-1β transcripts.

Clinical relevance

This requirement of adequate 25(OH)D in the extracellular environment of the human macrophage for the induction of host defense mechanisms via TLR2/1 provided a link between two well-documented clinical observations: compared to lightly-pigmented human populations, darkly-pigmented black individuals are i) more susceptible to virulent infections of tuberculosis and ii) have lower circulating, serum 25(OH)D levels owing to their relatively diminished capacity to synthesize vitamin D in their skin during sunlight exposure. The biosynthetic

pathway of 25(OH)D in humans involves the absorption of ultraviolet B (UVB) photons from sunlight by 7-dehydrocholesterol (7HDC) in the basal layer of the epidermis and its non-enzymatic conversion to a pre-vitamin D_3 precursor in the skin; in fact, the melanin in pigmented skin will competitively absorb these UVB rays preventing this photoreaction (167). In human monocytes cultured in sera from pigmented African American subjects and stimulated with a TLR2/1 ligand, there was no upregulation of cathelicidin mRNA, whereas the same human monocytes conditioned in sera from lightly-pigmented subjects did. Moreover, supplementation of the African American sera with exogenous 25(OH)D_3 restored the induction of cathelicidin mRNA (11).

The ability of monocytes from human subjects to mount a cathelicidin response following TLR challenge was recently shown to be directly proportional to circulating levels of 25(OH)D but not 1,25$(OH)_2$D (163). Importantly, this study also demonstrated that TLR-induction of cathelicidin was enhanced in subjects supplemented with vitamin D [500,000 IU vitamin D_2 over 5 weeks], indicating that the immunomodulatory effects of 25(OH)D also occur *in vivo*. Another study demonstrated a single oral dose of vitamin D was able to increase the *in vitro* response to mycobacterium infection in a whole blood assay (168). Furthermore, the TLR2/1L-induced vitamin D-dependent antimicrobial pathway was shown to involve autophagy, mediated through the VDR (169), which highlights another key host defense pathway reliant upon the host's vitamin D status.

BARRIER FUNCTION AND ASSOCIATED INFECTIOUS DISEASES

The first line of immune defense is the responsibility of the barrier cells, including the epithelia of the eyes, the oral mucosa, and the cells of the skin, urinary tract, and respiratory and digestive systems. In addition to the specialized functions each of these cell types perform, they also serve as a barrier to exclude invading pathogens, which is the most effective way of preventing infection. Vitamin D has been demonstrated to play an integral role in maintenance

of this barrier (170); however, there is emerging data implicating barrier cells as an active participant in the innate immune response in relation to the detection and elimination of microbial pathogens. In particular, the vitamin D-mediated innate immune response participates in the host defense by cells of the respiratory lining (171), urinary bladder (23), and gingival (25).

Traditionally, activation of endogenously produced vitamin D_3 requires two hydroxylation steps: vitamin D_3 is converted, by a liver 25-hydroxylase, into $25(OH)D_3$ (172), then metabolized into $1,25(OH)_2D_3$ by the 1α-hydroxylase, CYP27B1, in the kidneys (173). Now, studies have revealed that non-renal tissue can also convert $25(OH)D_3$ to the active $1,25(OH)_2D_3$ form (173,174). This has been demonstrated by 1α-hydroxylase activity in innate immune cells, such as macrophages (11), monocytes (175), and dendritic cells (176), as well as in respiratory epithelial cells (171), urinary bladder epithelial cells (23), gingival epithelial cells (25), colonic epithelial cells (177), keratinocytes (178,179), prostatic cells (180), and mammary epithelial cells (181).

While epithelial cells comprise an important physical barrier to microbes, their importance is augmented by the expression of CYP27B1, required for launching the vitamin D-dependent innate immune response to pathogens. This has been clearly demonstrated by respiratory (171), urinary bladder (23), and gingival (25) epithelial cells.

Respiratory epithelia

Several studies have demonstrated that lung epithelial cells express functional Toll-like receptors capable of being triggered by their cognate ligands (182-184). Notably, TLR2 expression in primary small airway epithelial cells was strongly induced following stimulation with the cytokines, IFN-β and TNF-α, both recognized to be important for resistance to tuberculosis (183). Activation of TLR2 on lung epithelial cells resulted in both direct and indirect immune modulatory responses, including the expression of the antimicrobial peptide,

DEFB4, and induction of chemokines (182), whereas $1,25(OH)_2D_3$ stimulation resulted in the expression of the antimicrobial peptide, cathelicidin (171). Furthermore, other studies have shown that respiratory tracheobronchial epithelial cells constitutively express CYP27B1 and thus can activate vitamin D (171). In contrast to monocytes and macrophages, where TLR2/1 activation is needed for induction of the vitamin D pathway, respiratory epithelial cells activate vitamin D at a baseline level (171). In addition, stimulation of respiratory epithelial cells with TLR2 ligands did not induce the expression of CYP27B1 and cathelicidin ; yet TLR2/1-activation of the epithelial cells resulted in upregulation of cathelicidin as well as the TLR coreceptor CD14 (171). Taken together, these studies indicate lung epithelial cells provide several key innate immune mechanisms for defense against infection through i) barrier function, ii) recognition of pathogens through TLRs and iii) induction of direct antimicrobial effectors, i.e. antimicrobial peptides DEFB4 and cathelicidin.

HISTORY OF VITAMIN D, SUNSHINE AND TUBERCULOSIS TREATMENT

Establishment of vitamin D‘s role in host defense against tuberculosis provides new insights into the historical understanding of tuberculosis treatment prior to the advent of antibiotics. In the late 19th century, two young physicians, who themselves had contracted tuberculosis, were instructed by their physicians to travel to mountainous regions of Europe during the summertime as part of their attempt to recover. Their trek into this high UVB environment led to the “remission” of their disease. As a consequence of this success, Hermann Brehmer built the world’s first high-altitude tuberculosis sanitorium in Germany, designed to allow patients to be exposed to “fresh air and sunlight”. At about the same time in the United States, Edward Livingston Trudeau of New York published his original scientific finding that rabbits infected with tuberculosis had a more severe course of disease if caged in the dark and indoors, as opposed to being kept outdoors on a remote island. These experimental observations led him to build the first sanitorium at Saranac Lake, NY. In fact, it was the

success of treatment facilities like these which paved the way to the 1903 Nobel Prize in Medicine awarded to the Danish physician, Niels Ryberg Finsen, who demonstrated that UV light was beneficial to patients with lupus vulgaris, a form of cutaneous *M. tuberculosis* infection. Despite widespread skepticism about the value of sanitoria at the time, it is likely that the prolonged exposure to sunlight increased cutaneous vitamin D production, increased substrate 25(OH)D levels and enhanced innate immunity to combat tuberculosis.

There is a long history of using vitamin D to treat mycobacterial infections with apparent success. In 1946, Dowling et al. reported the treatment of patients with lupus vulgaris with oral vitamin D (185). Eighteen of 32 patients appeared to be cured; nine improved. Morcos *et al.* treated 24 newly-diagnosed cases of tuberculosis in children using standard chemotherapy with and without vitamin D (186); they noted more profound clinical and radiological improvements in the group treated with vitamin D (186). Nursyam *et al.* administered vitamin D or placebo to 67 tuberculosis patients following the 6th week of standard treatment (187). Out of 60 total patients, the group with vitamin D had a statistically significantly higher sputum conversion rate and radiological improvement (100%) than the placebo group (76.7%). Despite the clear benefits of vitamin D treatment for tuberculosis, the mechanism of action had not been elucidated. The fact that TLR-activated macrophages can convert vitamin D to produce antimicrobial peptides illustrates a possible mechanism by which supplementation of patients with inactive vitamin D leads to a positive therapeutic outcome.

CONCLUSION

In all, these studies demonstrate that a host deficient in 25(OH)D could suffer impaired immune responses mediated by barrier cells and innate immune cells, resulting in increased susceptibility to infection. Returning the circulating 25(OH)D to levels that achieve immunosufficiency could restore their host defense mechanisms; however, the appropriate 25(OH)D levels needed for the optimum immune response remains to be defined. As antibiotic-

resistant pathogens continue to develop, understanding the mechanisms by which we can enhance our body's natural immune response will be paramount to the development of successful therapies. As such, insights into the role of human vitamin D metabolism and action in host defense against infection provide hope that vitamin D supplementation could prove to be a safe, simple, and cost-effective strategy for the treatment of infectious diseases.

FIGURE LEGEND

Figure 1. The role of 25(OH)D in the innate immune response. (A) $1,25(OH)_2D_3$-induction of autophagy leads to antimicrobial activity against intracellular *M. tuberculosis* (*M. tb*) infection. (B) TLR activation results in: i) induction of expression of the CYP27B1-hydroxylase and vitamin D receptor (VDR) genes; ii) intracrine generation of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), from substrate 25-hydroxyvitamin D (25(OH)D) when present in sufficient quantities. (C) $1,25(OH)_2D$ triggering of the VDR leads to i) transactivation of the cathelicidin gene via interaction of the $1,25(OH)_2D$-VDR complex with an VDR enhancer element in its promoter; ii) expression of the cathelicidin gene product and iii) killing of ingested mycobacteria. (D) Activation of TLR2/1 induces both IL-15 and key components of its receptor, leading to downstream expression of CYP27B1 and VDR. (E) TLR-induction of IL-1β activity converges with the VDR pathway, resulting in expression of DEFB4.

Figure 1

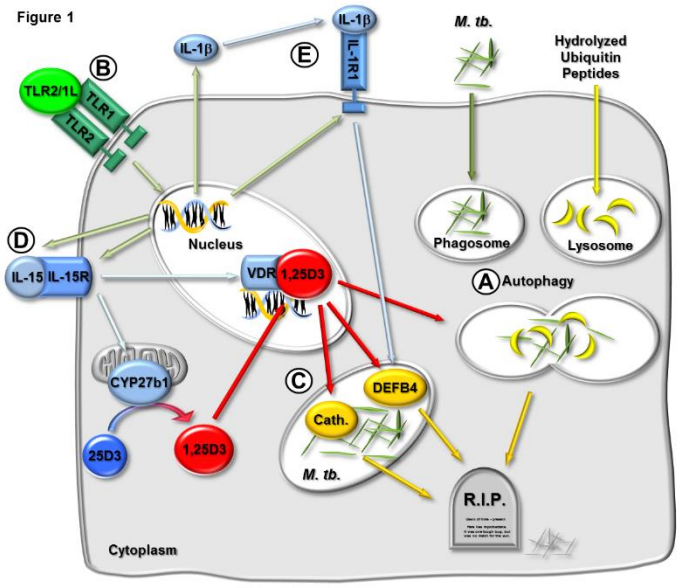
Figure 1
IL-1β
IL-1β
IL-1R1
M. tb.
Hydrolyzed Ubiquitin Peptides
B
TLR2/1L
TLR1
TLR2
E
Nucleus
Phagosome
Lysosome
D
IL-15
IL-15R
VDR
1,25D3
A Autophagy
CYP27b1
C
DEFB4
Cath.
25D3
1,25D3
M. tb.
R.I.P.
Cytoplasm

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CHAPTER 1

Type I interferon induction during *Mycobacterium leprae* infection inhibits vitamin D metabolism

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Abstract

Leprosy is a debilitating disease caused by cutaneous infection of *Mycobacterium leprae (M. leprae)*. Previous studies have demonstrated the role of the vitamin D system in mediating the antimicrobial response to mycobacterial infection; however, it is not understood how dysregulation of the vitamin D system contributes to disease outcomes. The present study investigates the factors that regulate vitamin D metabolism and activation in leprosy. Human monocytes were infected with *M. leprae* and analyzed for vitamin D system gene expression and metabolism. In contrast to stimulation with synthetic toll-like receptor 2 (TLR2) ligand or the type II IFN-γ, *M. leprae* infection did not induce conversion of pro-hormone 25-hydroxyvitamin D to the biologically active 1α,25-dihydroxyvitamin D. Furthermore, gene expression of activating enzyme 1α-hydroxylase (CYP27B1) and vitamin D receptor (VDR) were not induced by infection. However, infection induced the type I IFN gene program demonstrated by increased expression of downstream gene, 2'-5'-oligoadenylate synthetase 1 (OAS1). Blocking of type I IFN signaling decreased expression of OAS1 as well as increased expression of CYP27B1. Moreover, exposure of cells to irradiated *M. leprae* induced expression of CYP27B1 and VDR. This suggests that the pathogen is utilizing the type I IFN pathway to evade the host antimicrobial response through repression of the vitamin D pathway. Interestingly, macrophages already capable of triggering the antimicrobial pathway through vitamin D metabolism, such as those found in tuberculoid leprosy lesions, are resistant to type I IFN-mediated repression. This suggests that type I IFN may play a more pivotal role during the early stages of disease as compared to the chronic state of infection. The major finding of the present study is that successful regulation of the vitamin D system in response to *M. leprae* infection by either the host cell or the pathogen is a critical determinant to the outcome of the infection.

Introduction

For over a century, use of vitamin D as a treatment against pathogenic infections has been investigated [1-7]; however, only in recent years has the mechanisms by which the immune system regulates the vitamin D system been described. Human monocytes and macrophages can synthesize the active vitamin D hormone 1α,25-dihydroxyvitamin D (1,25D) from the inactive prohormone substrate 25-hydroxyvitamin D (25D) upon stimulation via innate or adaptive immune signals as part of an antimicrobial response to infection [8]. Previous *in vitro* studies have demonstrated that the intracrine vitamin D metabolic system plays a major role in macrophage antimicrobial activity against *Mycobacterium tuberculosis* (*M. tb*) infection [8-12]. As the role of vitamin D in the human immune response against mycobacterial infection becomes increasingly established, studies have also emerged linking these mechanistic findings to human diseases.

In particular, studies on leprosy, a dermal granulomatous diseased caused by *Mycobacteria leprae* infection, have indicated a critical role for vitamin D in the outcome of infection. Leprosy presents as a spectrum of disease in which the clinical manifestation indicates the immunological state of the patient, where at one end is the self-limiting tuberculoid form (T-lep) and the other end is the disseminated lepromatous form (L-lep) [13]. Each pole is accompanied by a well-defined immunological profile including cytokines, macrophage subsets and T cell subsets [13]. Gene expression profiling of lesions derived from different forms of leprosy suggested a correlation between the vitamin D pathway components and favorable disease outcomes [12]. However, how the vitamin D system is regulated at the site of infection in either form of leprosy is not well defined. We hypothesized that the host immune response to pathogenic infection is limited by vitamin D availability. Furthermore, disease-associated dysregulation of the vitamin D system contributes to pathology.

Materials and Methods

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained from healthy donors with informed consent. Monocytes were enriched using a plastic adherence protocol in which PBMCs were cultured for two hours in RPMI 1640 media (Invitrogen, Carlsbad, CA) with 1% FBS (Omega Scientific, Tarzana, CA) followed by washing. Monocytes were cultured in RPMI media with 10% FBS and treated with 100ng/mL Pam_3CSK_4, *M. leprae* (MOI 10, gift from J.L. Krahenbuhl), or irradiated *M. leprae* (equivalent to MOI 10, gift from J.L. Krahenbuhl). For macrophage differentiation, monocytes were treated with 10^3 U/mL IL-4 (Peprotech, Rocky Hill, NJ), 10µg/mL IL-10 (R&D Systems, Minneapolis, MN), or 200ng/mL IL-15 (R&D Systems) for 48 hours. For blocking type I IFN signaling, monocytes or macrophages were treated with 10µg/mL anti-IFNAR antibody (PBL, Piscataway, NJ). Vitamin D supplementation experiments were carried out using 25-hydroxyvitamin D_3 (Enzo Life Sciences, Farmingdale, NY).

High-performance liquid chromatography (HPLC): Monocytes or macrophages were incubated with radiolabeled ^{3}[H]-$25D_3$ (PerkinElmer, Waltham, MA) substrate for 5 hours in serum-free RPMI media. [^{3}H]-metabolites were purified using a C18 column and separated using a Zorbax-sil column (Agilent, Santa Clara, CA). Radioactivity was measured in each sample by scintillation counting. The amount of each metabolite present was quantified from counts per minute plotted against elution profiles for $25D_3$, $1,25D_3$, and $24,25D_3$.

Quantitative polymerase chain reaction (qPCR): Total RNA was harvested using TRIzol (Thermo Fisher Scientific, Waltham, MA) and cDNA synthesized using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). mRNA expression levels were assessed for 1α-hydroxylase (CYP27B1), 24-hydroxylase (CYP24A1), vitamin D receptor (VDR), cathelicidin

(CAMP), and interferon beta 1 (IFN-β) using the Taqman system (Applied Biosystems, Foster City, CA). Fold change was quantified by comparing to the housekeeping gene 18S rRNA and using the ΔΔCt method. mRNA expression was also assessed for 2'-5'-oligoadenylate synthetase 1 (OAS1) using the SYBR Green system (Bio-Rad Laboratories, Irvine, CA). Fold change was quantified by comparing to the housekeeping gene h36B4 and using the ΔΔCt method.

Antimicrobial assay: Total RNA was harvested as above and DNA was isolated using a manufacture-provided sodium acetate back-extraction protocol and cDNA was synthesized. Bacterial 16S rRNA and *M. leprae* repetitive genomic element DNA (RLEP) were measured using quantitative PCR. Bacterial viability was calculated by comparing 16S rRNA to RLEP DNA as previously published [14]. The 16S and RLEP primers used were previously described [14].

Results

To determine the effects of *M. leprae* infection on vitamin D metabolism, monocytes were infected with live *M. leprae* at a multiplicity of infection (MOI) of 10 overnight. Following infection, the monocytes were isolated and cultured with radiolabeled 25D for 6 hours, and the levels of 25D, 1,25D and 24,25D were measured using high performance liquid chromatography (HPLC). While monocytes treated with a Toll-like receptor 2/1 ligand (TLR2L), were able to convert 25D into 1,25D, consistent with previous studies [8], *M. leprae*-infected monocytes did not (Figure 1A). The ability of these cells to metabolize 25D was correlated with the gene expression of CYP27B1 which was highly induced following TLR2L treatment but not during *M. leprae* infection (Figure 1A). Conversely, production of the 25D catabolite, 24,25D, was not significantly affected by TLR2/1 activation or infection despite both conditions resulting in a significant reduction of 24-hydroxylase gene expression (Figure 1B). These results suggest that despite having a cell wall-associated lipoprotein capable of activating TLR2/1 [15], *M. leprae* infection does not activate the TLR2/1L-mediated antibacterial response in the same manner as described for purified ligands. Therefore, we hypothesized that expression of CYP27B1 was inhibited during *M. leprae* infection.

To investigate the mechanism by which CYP27B1 expression is inhibited during *M. leprae* infection, we measured IFN-β gene expression, which has previously been shown to inhibit CYP27B1 expression [16, 17] and induced during *M. leprae* infection [18]. As expected, *M. leprae* infection but not TLR2/1 ligand significantly induced expression of IFN-β (Figure S1). To determine if infection induced IFN-β contributes to suppression of CYP27B1, we neutralized IFN-β activity using a monoclonal antibody against the interferon alpha/beta receptor (IFNAR). The IFNAR monoclonal antibody was added to the monocytes prior to *M. leprae* infection, and 24 hours post-infection, IFN-β activity was evaluated by measuring expression of the IFN-β

inducible gene OAS1. Infection with *M. leprae* resulted in significant expression of OAS1 mRNA (Figure 2A). Neutralization of IFN-β signaling by the IFNAR specific monoclonal antibody during *M. leprae* infection resulted in a significant induction of CYP27B1 and VDR gene expression (Figure 2B).

To determine whether inhibition of CYP27B1 gene expression during *M. leprae* infection is an active or passive process, we compared gene expression of CYP27B1, VDR and OAS1 in monocytes treated with live *M. leprae*, irradiated *M. leprae,* and the TLR2/1 ligand. Compared to infection with live *M. leprae*, monocytes treated with irradiated bacteria had significantly higher induction of CYP27B1 and VDR gene expression similar to TLR2/1 activation (Figure 3A). On the other hand, OAS1 gene expression was lower in monocytes treated with irradiated *M. leprae* compared to those infected with live *M. leprae* (Figure 3B). These results indicate that *M. leprae*-mediated suppression of CYP27B1 expression and function was likely through an active bacterial process triggered upon initiation of infection.

Linear regression analysis comparing the expression levels of CYP27B1 and OAS1 in the experiments above indicate a significant inverse correlation between the two genes (Figure 4A). To determine if this correlation extends to the disease state *in vivo*, we examined the co-expression of CYP27B1 and VDR against OAS1 in microarray data derived from skin lesions of T-lep and L-lep patients. Our analysis indicates a dichotomy in which L-lep lesions expressed high OAS1 and low CYP27B1 whereas T-lep lesions expressed low OAS1 and high CYP27B1 (Figure 4B). These result suggest that the counter regulation of IFN-β and the vitamin D machinery maybe an important determinant to disease outcome.

Previous studies have defined macrophage subtypes specific to T-lep and L-lep lesions that also have differential capacity for vitamin D metabolism [12]. To better understand the interplay

between type I IFN and vitamin D metabolism we differentiated L-lep- or T-lep-like macrophages by treating monocytes with IL-10 or IL-15 cytokines, respectively. We conducted HPLC to study vitamin D metabolism as described above in the macrophage subtypes. Consistent with previous findings [12], IL-15-derived-, but not IL-10-derived- macrophages converted 25D to 1,25D. Furthermore, CYP27B1 gene expression was highly induced in IL-15-derived, but not in IL-10-derived, macrophages (Supplemental Figure 2). The effects of *M. leprae* infection on the vitamin D metabolic system in these cells were not addressed; therefore, we infected IL-10- and IL-15-derived macrophages and assayed CYP27B1 gene expression. In IL-10-derived macrophages, there was no change in CYP27B1 expression following *M. leprae* infection whereas TLR2/1 stimulation induced a significant increase (Figure 5A). On the other hand, CYP27B1 gene expression in IL-15-derived macrophages, which have high CYP27B1 gene expression at baseline, was not significantly affected as a result of either infection or stimulation with TLR2/1L (Figure 5A). Similar to monocytes, the IL-10-derived macrophages showed significant induction of OAS1 gene expression with *M. leprae* infection but not TLR2/1 stimulation (Figure 5B). On the other hand, IL-15-derived macrophages did not express OAS1 as a result of infection (Figure 5B). These findings suggested that the T-lep-associated macrophages may be resistant to *M. leprae*-mediated regulation of vitamin D metabolism.

To investigate if *M. leprae* infection has a functional impact on vitamin D metabolism in IL-15-derived macrophages, we used HPLC analysis to assay vitamin D metabolism during infection. Similar to CYP27B1 gene expression findings, IL-15-derived macrophages had high 1,25D conversion at baseline which was not significantly affected by *M. leprae* infection (Figure 6A). Given that CYP27B1 expression and function are not suppressed by *M. leprae* infection in IL-15-derived macrophages, we hypothesized that provisions of the 25D substrate to these infected macrophages would result in activation of the vitamin D-dependent antimicrobial pathways, which is critically dependent on induction of the antimicrobial peptide cathelicidin, encoded by

the gene CAMP [8]. Indeed, when provided with exogenous 25D, IL-15-derived macrophages dose-dependently induced CAMP gene expression (Figure 6B), suggesting that the macrophages retain the ability to engage the vitamin D-dependent antimicrobial pathway during *M. leprae* infection.

In contrast to the IL-15-derived macrophage, our results suggested that the IL-10-derived macrophages are susceptible to type I IFN-mediated regulation of the vitamin D system during *M. leprae* infection. IL-10-derived macrophages were treated with a neutralizing monoclonal antibody specific for IFNAR prior to *M. leprae* infection and assayed for gene expression of CYP27B1 and VDR. Blocking IFN-β signaling during *M. leprae* infection resulted in the significant induction of CYP27B1 and VDR in IL-10-derived macrophages (Figure 7A). As expected, OAS1 gene expression was also reduced when the IFNAR antibody was present, indicating that the type I IFN pathway was active during *M. leprae* infection in these cells as well (Figure 7B). Finally, we addressed the ability of type I IFN signaling to regulate bacterial viability during *M. leprae* infection of IL-10-derived macrophages. When comparing IL-10-macrophages cultured in vitamin D-sufficient serum and treated with isotype control versus the type I IFN neutralizing monoclonal antibody, *M. leprae* viability was significantly lower in the IFNAR neutralized macrophages (Figure 7C). Taken together, these results suggest that *M. leprae* can avoid the macrophage vitamin D-dependent antimicrobial response through type I IFN-mediated inhibition of the vitamin D metabolism, thus contributing to disease pathogenesis.

Discussion

We investigated how the vitamin D system is regulated by the immune response during intracellular infection using leprosy as a model. In contrast to direct activation of TLR2/1 using a purified ligand, monocytes infected with *M. leprae* did not significantly induce CYP27B1 gene expression or 1α-hydroxylase activity. However, both *M. leprae* and TLR2/1L significantly inhibited CYP24A1 gene expression; although, both conditions did not significantly affect 24-hydroxylase activity. Based on previous data showing induction of type I IFN during *M. leprae* infection [18], we showed that blocking type I IFN signaling relieved repression of CYP27B1 gene expression. Additionally, using irradiated *M. leprae,* we determined that CYP27B1 repression was dependent on live bacteria. Analysis of *in vitro* studies showed a negative correlation between CYP27B1 and OAS1 expression that was mirrored in leprosy lesions from L-lep and T-lep subtypes. Moreover, we found that L-lep- and T-lep-associated macrophages had differential susceptibility to type I IFN-mediated repression of CYP27B1. Whereas T-lep-associated macrophages had elevated CYP27B1 expression that was not affected by *M. leprae* infection, the opposite was observed in L-lep-associated macrophages. Under vitamin D-sufficient conditions, *M. leprae* infection triggered the vitamin D-mediated antibacterial response in T-lep-associated macrophages; however, to trigger the same response in L-lep-associated macrophages, type I IFN signaling first had to be neutralized. Nevertheless, our findings suggest that regulation of the vitamin D pathway is a key switch during *M. leprae* infection.

Although the ability of innate immune cells to recognize mycobacterial lipoproteins and subsequently initiate an immune response through Toll-like receptors (TLRs) is well characterized, it is unclear if these same immune mechanisms are engaged when the cells encounter the intact pathogen [19, 20, 21]. For mycobacteria, one of the key immune mechanisms is

the intracrine vitamin D system which initiates an important host defense mechanism upon activation [8]. Currently, there is little evidence that activation of the vitamin D pathway during the immune response has a direct correlation to disease outcome in mycobacterial infections, partly because suitable animal models for studying the utilization of the vitamin D pathway during the innate immune response to mycobacteria have yet to be identified. Therefore, we investigated this defense mechanism in the context of *M. leprae* infection. Our finding of induction of the vitamin D system by irradiated *M. leprae* but not by live *M. leprae* suggested that *M. leprae* utilizes an active immune evasion mechanism to suppress the vitamin D-mediated antibacterial response. Other studies have also suggested potential mechanisms in which *M. leprae* subverts host defenses. For example, several studies point to the role of phenolic glycolipid 1 (PGL-1), a component of the mycobacterial cell wall, in manipulating host defense mechanisms, such as complement activation, phagocytosis, and cytokine release, to inhibit maturation of dendritic cells and modulate T cell responses [22, 23, 24, 25]. Related to vitamin D-mediated antibacterial responses, our previous study showed that microRNA-21 (miR-21) was highly expressed in L-lep lesions versus T-lep lesions and inhibited CYP27B1 gene expression and function [26]. Similar to the results presented here, miR-21 induction was exclusive to live *M. leprae* infection and not induced by purified TLR2/1 ligands or *M. leprae* sonicate. However, the precise mechanism by which live *M. leprae* infection regulates miR-21 expression is unknown. Taken together, these results support the hypothesis that *M. leprae* utilizes an active immune evasion mechanism which directly targets the vitamin D pathway.

Induction of type I interferons (IFN) is a well-studied host defense mechanism against viral infection [27]. However, their role in the immune response against intracellular bacteria is less well-defined [27]. Our recent study has shown the induction of type I IFNs during *M. leprae* infection [18]. Interestingly, there is evidence indicating that although type I IFNs are critical for viral immunity, type I IFN signaling leads to impaired antibacterial responses against the

extracellular pathogen *Streptococcus pneumoniae* [28]. When comparing expression of the type I IFN inducible gene, OAS1, to CYP27B1, we found a consistent inverse correlation in both *in vitro* models of infection as well as the leprosy lesions. Since type I IFN signaling and low CYP27B1 expression was characteristic of lepromatous lesions and vice versa in tuberculoid lesions, it supports a pathogenic role for type I IFNs and a protective role for the vitamin D pathway in mycobacterial diseases. Indeed, there is a direct regulation of the two pathways, as neutralization of type I IFN during *M. leprae* infection relieved inhibition of CYP27B1 expression. These results suggest that *M. leprae* manipulates the host defense system to mount an inappropriate antiviral program to subvert the vitamin D-mediated antimicrobial response.

Naturally, the immune microenvironment within the leprosy lesions is different between the two poles of the spectrum, one key factor being the different macrophage subsets present. The function and response of the macrophage is critical to the outcome of disease since they are the host cell of M. *leprae*. Previous studies have defined macrophage subtypes that are present in each phenotype which can be differentiated *in vitro* using cytokines associated with L-lep or T-lep lesions [9, 12]. Our studies showed that L-lep-associated macrophages were susceptible to type I IFN-mediated inhibition of CYP27B1; on the other hand, T-lep-associated macrophages were resistant. Furthermore, neutralization of type I FN signaling during *M. leprae* infection of L-lep-associated macrophages alleviated CYP27B1 repression leading to decreased bacterial viability. These findings support a potential therapeutic invention that relies on blocking type I IFN signaling in order to mount an effective vitamin D-mediated antibacterial response.

The use of vitamin D to treat mycobacterial disease has been studied in numerous clinical trials showing inconsistent benefits [5-7, 29-32]. Our findings suggest a possible explanation for the varied outcomes. The efficacy of elevation in systemic 25D levels to affect local antimicrobial responses at the site of infection is predicated on the ability of the macrophages to convert the circulating

25D into 1,25D. Thus, if the pathogen bearing macrophages, such as those found in L-lep lesions, were unable to convert 25D, it would not be surprising to see minimal therapeutic benefit following vitamin D supplementation. More broadly, our findings suggest that the clinical management of disease using vitamin D supplementation will require simultaneous management of the vitamin D metabolic system to achieve therapeutic benefit.

Figure Legends

Figure 1. *M. leprae* infection of monocytes does not induce conversion of 25D to 1,25D

Primary human monocytes were treated with 100ng/mL Pam_3CSK_4 or *M. leprae* at a multiplicity of infection of 10:1 for 24 hours followed by incubation with radiolabeled $25(OH)D_3$ for 5 hours. Conversion to (**A**) $1,25(OH)_2D_3$ and (**B**) $24,25(OH)D_3$ was measured by high performance liquid chromatography. Additionally, (**A**) 1α-hydroxylase (CYP27B1) and (**B**) 24-hydroxylase (CYP24A1) gene expression levels were determined by quantitative PCR. Data are shown as mean ± SEM, n >3. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (**p ≤ 0.01, ***p ≤ 0.001).

Figure 2. Blocking type I IFN signal during *M. leprae* infection relieves repression of CYP27B1

(**A**) Primary human monocytes were infected with *M. leprae* and co-treated with either antibody against the type I IFN receptor, IFNAR, or isotype control for 24 hours. Blocking of type I IFN signaling was determined by measuring OAS1 gene expression and represented as percent inhibition. Data are shown as mean ± SEM, n = 8. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). (**B**) CYP27B1 and VDR gene expression levels were measured using quantitative PCR. Data are shown as mean ± SEM, n = 8. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

Figure 3. Irradiated *M. leprae* induces CYP27B1 in primary human monocytes

(**A**) Primary human monocytes were infected with *M. leprae* at a multiplicity of infection of 10:1 or treated with an equivalent amount of irradiated *M. leprae* or 100ng/mL Pam_3CSK_4 for 24 hours. Total RNA was collected from each sample. (**A**) CYP27B1, VDR, and (**B**) OAS1 gene expression levels were measured using quantitative PCR. Data are shown as mean ± SEM, n = 10. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

Figure 4. CYP27B1 gene expression is negatively correlated with OAS1 gene expression in *in vitro* studies and in leprosy lesions

(**A**) Correlation of CYP27B1 and OAS1 gene expression (mean fold change) was calculated by liner regression analysis. (**B**) CYP27B1 and OAS1 expression data from microarray analysis of T-lep (n = 6) and L-lep (n = 5) human skin lesions.

Figure 5. IL-15-and IL-10-derived macrophages differentially respond to *M. leprae* infection

Primary human monocytes were treated with 10μg/mL IL-10 or 200ng/mL IL-15 for 48 hours followed by infection with *M. leprae* or addition of Pam_3CSK_4 for 24 hours. (**A**) CYP27B1 and (**B**) OAS1 gene expression levels were assayed by quantitative PCR. Data are shown as mean ± SEM, n = 3. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (**$p \leq 0.01$, ***$p \leq 0.001$).

Figure 6. *M. leprae* infection induces cathelicidin expression in IL-15-derived macrophages

(**A**) IL-15-derived macrophages were infected with *M. leprae* or treated with Pam_3CSK_4 for 24 hours followed by incubation with radiolabeled $25(OH)D_3$ for 5 hours. Control macrophages were derived by treating primary human monocytes with 10^3 U/mL IL-4 for 48 hours. Conversion to $1,25(OH)_2D_3$ was measured by high performance liquid chromatography. Data are shown as mean ± SEM, n=3. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (*$p \leq 0.05$, **$p \leq 0.01$). (**B**) IL-15-derived macrophages were infected with *M. leprae* and supplemented with 20nM $25(OH)D_3$, 100nM $25(OH)D_3$, or vehicle for 24 hours. Cathelicidin (CAMP) gene expression was assayed by quantitative PCR. Data are shown as mean ± SEM, n = 3. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (**$p \leq 0.01$).

Figure 7. Blocking type I IFN signaling during *M. leprae* infection of IL-10-derived macrophages reduces bacterial viability

IL-10-derived macrophages were infected with *M. leprae* and co-treated with either antibody against the type I IFN receptor, IFNAR, or isotype control for 24 hours. (**A**) CYP27B1, VDR, and (**B**) OAS1 gene expression levels were measured using quantitative PCR. Data are shown as mean ± SEM, n < 5. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (*$p \leq 0.05$, **$p \leq 0.01$). (**C**) IL-10-derived macrophages were infected with *M. leprae* and co-treated with either antibody against the type I IFN receptor, IFNAR, or isotype control for 5 days under vitamin D-sufficient conditions. The ratio of 16S RNA to RLEP DNA was calculated as a measurement of bacterial viability.

Supplementary Figure 1. *M. leprae* induces IFN-β gene expression in primary human monocytes

(**A**) Primary human monocytes were infected with *M. leprae* at a multiplicity of infection of 10:1 or treated with 100ng/mL Pam_3CSK_4 for 24 hours. Total RNA was collected from each sample. Interferon beta 1 (IFN-β) gene expression was measured using quantitative PCR. Data are shown as mean ± SEM, n = 4. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (*$p \leq 0.05$).

Supplementary Figure 2. IL-10- and IL-15-derived macrophages have distinct vitamin D metabolic profiles

(**A**) Primary human monocytes were treated with 10μg/mL IL-10 or 200ng/mL IL-15 for 48 hours. Control macrophages were derived by treating monocytes with 10^3 U/mL IL-4 for 48 hours. Macrophages were incubated with radiolabeled $25(OH)D_3$ for 5 hours and conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ was measured by high performance liquid chromatography. Data are shown as mean ± SEM, n = 3. Statistical significance was determined using one-way ANOVA followed by Bonferroni correction. (***$p \leq 0.001$).

Figure 1

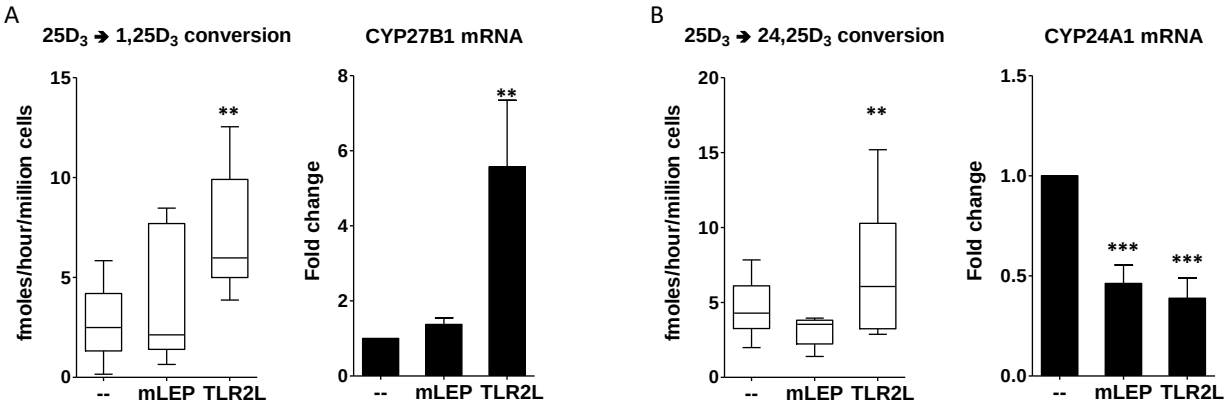

A
25D3 → 1,25D3 conversion
CYP27B1 mRNA
fmoles/hour/million cells
Fold change
**
**
15
10
5
0
8
6
4
2
0
-- mLEP TLR2L
-- mLEP TLR2L
B
25D3 → 24,25D3 conversion
CYP24A1 mRNA
fmoles/hour/million cells
Fold change
**
***
***
20
15
10
5
0
1.5
1.0
0.5
0.0
-- mLEP TLR2L
-- mLEP TLR2L

Figure 2

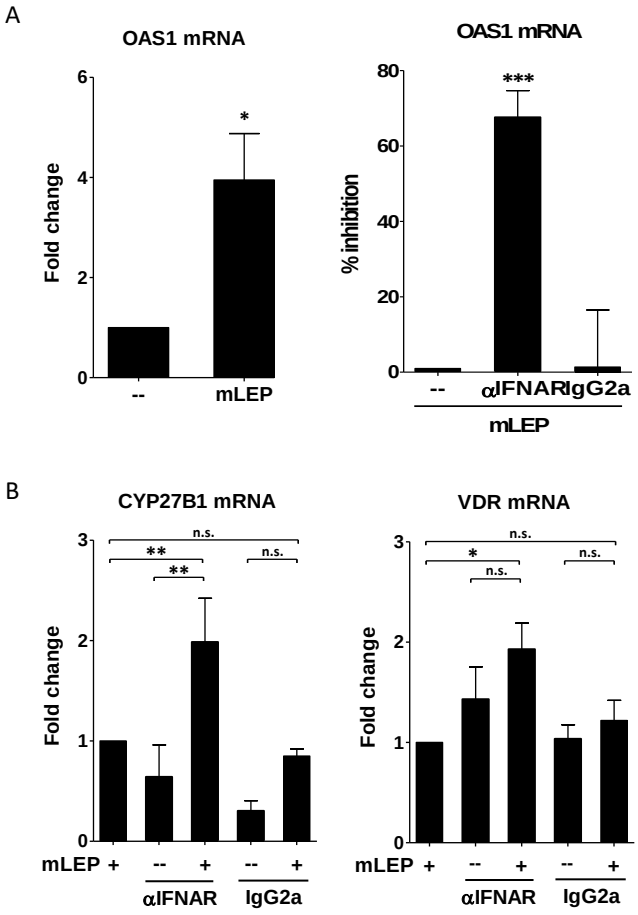

A
OAS1 mRNA
Fold change
6
4
2
0
*
--
mLEP
OAS1 mRNA
% inhibition
80
60
40
20
0
***
--
αIFNAR
IgG2a
mLEP
B
CYP27B1 mRNA
Fold change
3
2
1
0
n.s.
**
**
n.s.
mLEP +
--
+
--
+
αIFNAR
IgG2a
VDR mRNA
Fold change
3
2
1
0
n.s.
*
n.s.
n.s.
mLEP +
--
+
--
+
αIFNAR
IgG2a

Figure 3

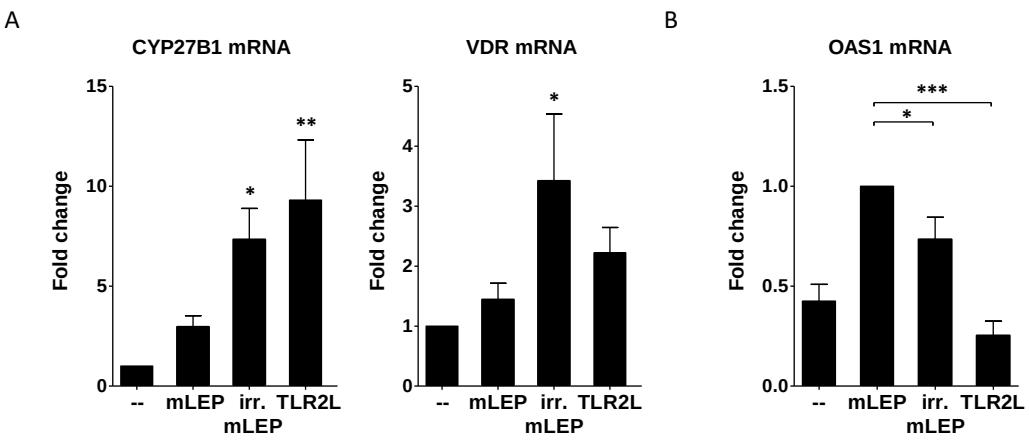

A
CYP27B1 mRNA
Fold change
15
10
5
0
*
**
-- mLEP irr. mLEP TLR2L mLEP
VDR mRNA
Fold change
5
4
3
2
1
0
*
-- mLEP irr. mLEP TLR2L mLEP
B
OAS1 mRNA
Fold change
1.5
1.0
0.5
0.0
***
*
-- mLEP irr. mLEP TLR2L mLEP

Figure 4

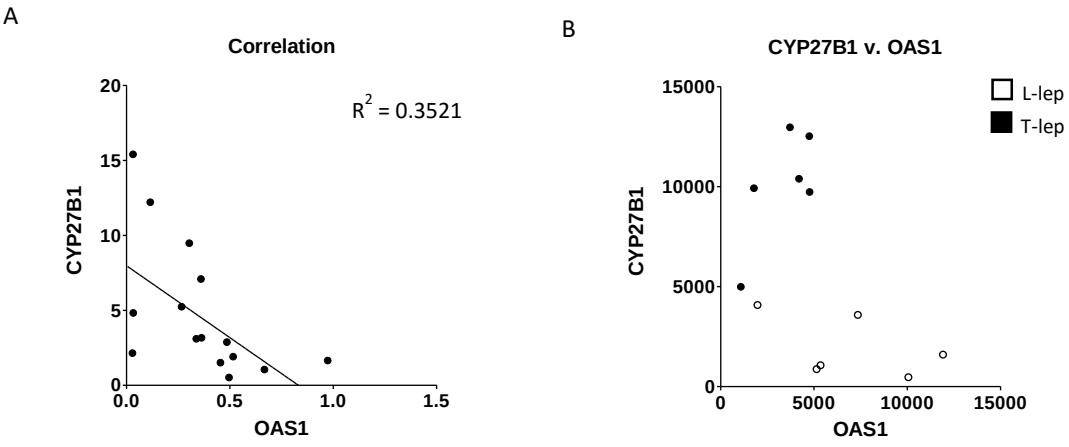

A
Correlation
R^2 = 0.3521
CYP27B1
OAS1
0
5
10
15
20
0.0
0.5
1.0
1.5
B
CYP27B1 v. OAS1
L-lep
T-lep
CYP27B1
OAS1
0
5000
10000
15000

Figure 5

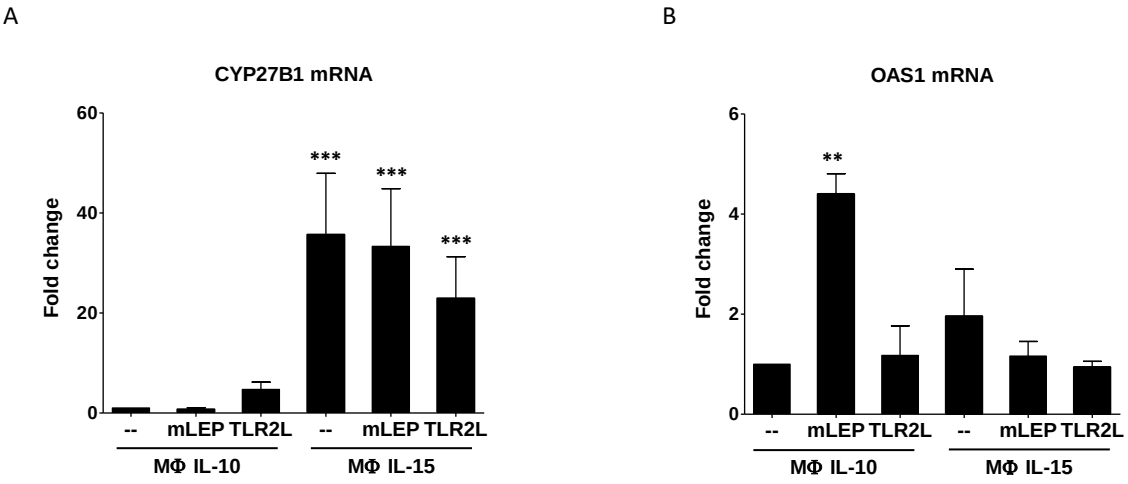

A
CYP27B1 mRNA
Fold change
60
40
20
0
***
***
***
-- mLEP TLR2L -- mLEP TLR2L
MΦ IL-10
MΦ IL-15
B
OAS1 mRNA
Fold change
6
4
2
0
**
-- mLEP TLR2L -- mLEP TLR2L
MΦ IL-10
MΦ IL-15

Figure 6

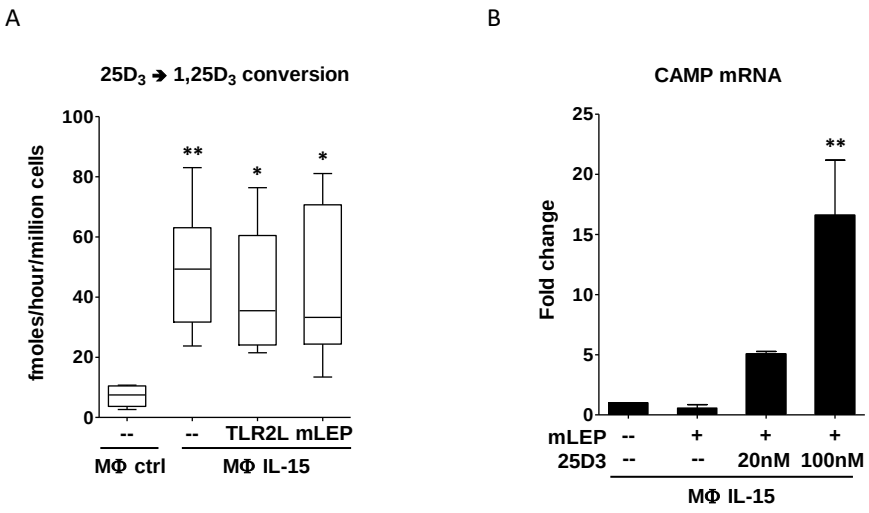
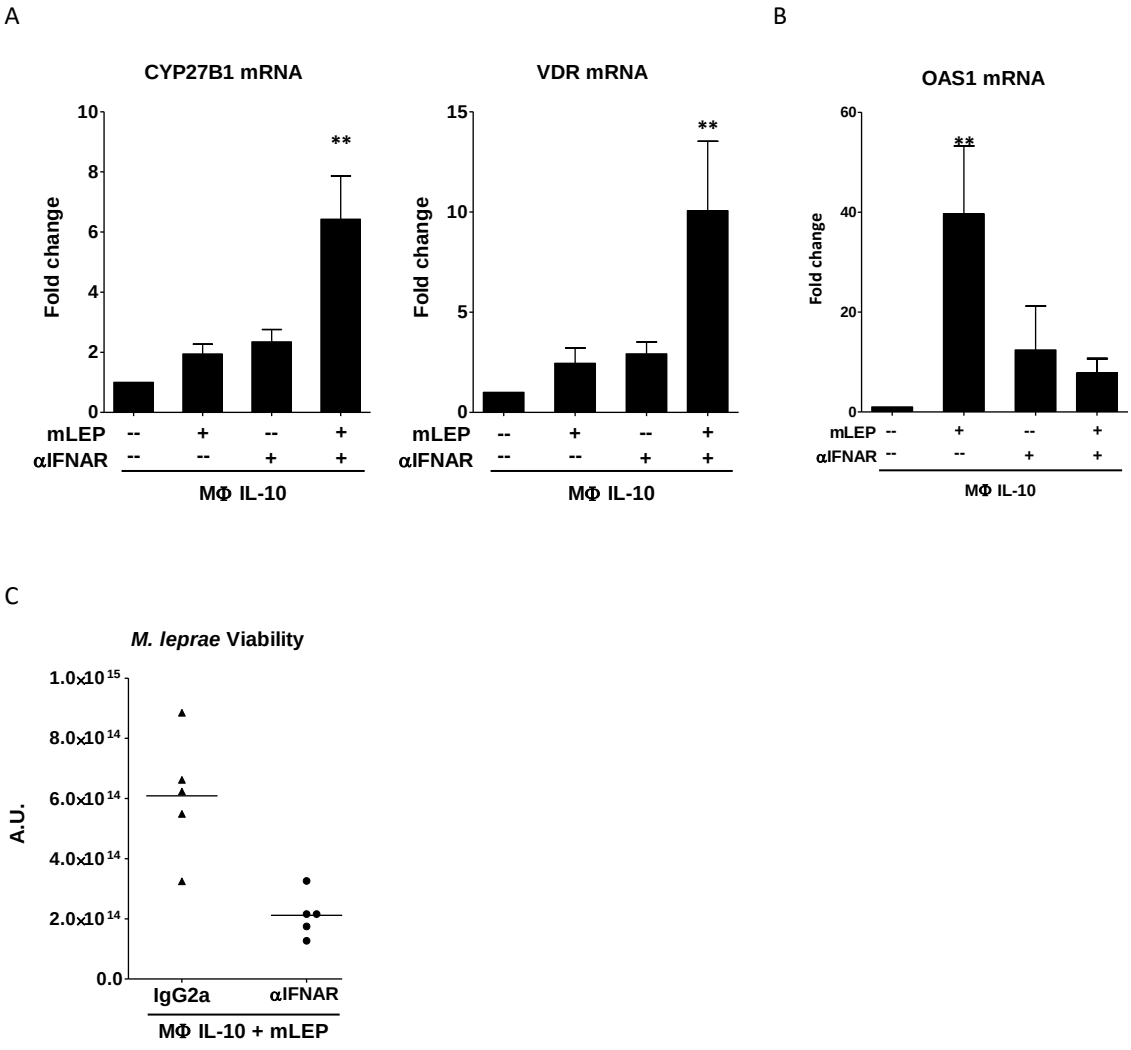
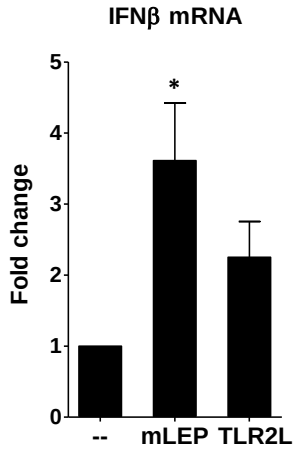

A
25D3 ➔ 1,25D3 conversion
fmoles/hour/million cells
100
80
60
40
20
0
**
*
*
--
--
TLR2L mLEP
MΦ ctrl
MΦ IL-15
B
CAMP mRNA
Fold change
25
20
15
10
5
0
**
mLEP -- + + +
25D3 -- -- 20nM 100nM
MΦ IL-15

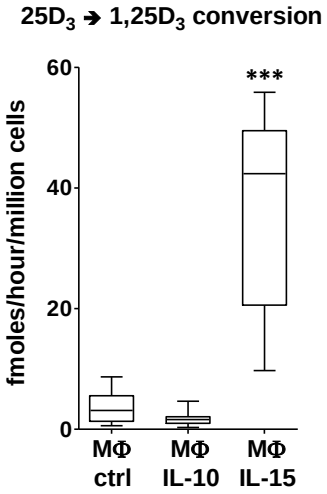
Figure 7


A
CYP27B1 mRNA
VDR mRNA
B
OAS1 mRNA
Fold change
mLEP
αIFNAR
MΦ IL-10
**
C
M. leprae Viability
A.U.
1.0x10^15
8.0x10^14
6.0x10^14
4.0x10^14
2.0x10^14
0.0
IgG2a
αIFNAR
MΦ IL-10 + mLEP

Supplemental Figure 1



Supplemental Figure 2



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CHAPTER 2

IL-32 is a molecular marker of a host defense network in human tuberculosis

TUBERCULOSIS

IL-32 is a molecular marker of a host defense network in human tuberculosis

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Tuberculosis is a leading cause of infectious disease–related death worldwide; however, only 10% of people infected with *Mycobacterium tuberculosis* develop disease. Factors that contribute to protection could prove to be promising targets for *M. tuberculosis* therapies. Analysis of peripheral blood gene expression profiles of active tuberculosis patients has identified correlates of risk for disease or pathogenesis. We sought to identify potential human candidate markers of host defense by studying gene expression profiles of macrophages, cells that, upon infection by *M. tuberculosis*, can mount an antimicrobial response. Weighted gene coexpression network analysis revealed an association between the cytokine interleukin-32 (IL-32) and the vitamin D antimicrobial pathway in a network of interferon-γ– and IL-15–induced "defense response" genes. IL-32 induced the vitamin D–dependent antimicrobial peptides cathelicidin and DEFB4 and to generate antimicrobial activity in vitro, dependent on the presence of adequate 25-hydroxyvitamin D. In addition, the IL-15–induced defense response macrophage gene network was integrated with ranked pairwise comparisons of gene expression from five different clinical data sets of latent compared with active tuberculosis or healthy controls and a coexpression network derived from gene expression in patients with tuberculosis undergoing chemotherapy. Together, these analyses identified eight common genes, including IL-32, as molecular markers of latent tuberculosis and the IL-15–induced gene network. As maintaining *M. tuberculosis* in a latent state and preventing transition to active disease may represent a form of host resistance, these results identify IL-32 as one functional marker and potential correlate of protection against active tuberculosis.

INTRODUCTION

Tuberculosis (TB) is a global disease, with 8.7 million new cases and 1.4 million deaths reported worldwide in 2011 (*1*). In the United States, estimates are that 10 to 15 million people are infected with *Mycobacterium tuberculosis* (*2*, *3*). About one third of the world's population is thought to harbor latent or persistent TB infection (*1*), which refers to those individuals who are infected with *M. tuberculosis* but do not have active disease. The recent emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB in individuals in more than 100 countries is an emerging global threat (*4*). This has underscored the urgency in understanding the immune mechanisms of protection in human TB and developing new strategies for prevention and treatment.

Through the measurement of gene expression profiles in peripheral blood of TB patients, several laboratories have identified sets of genes that distinguish individuals with active TB from those with latent infection (*5*, *6*). Most studies thus far largely identify genes that are differentially expressed in active disease. From these data sets, the focus has been to define biomarkers for disease progression, with the most striking "signature" for active TB being the increase in type I interferon (IFN)–regulated genes (*5*, *7*). The induction of the type I IFN gene program was associated with the extent of disease (*5*) and resolved within months of treatment (*8*). In vitro studies indicated that type I IFN induced interleukin-10 (IL-10), which resulted in the inhibition of human antimycobacterial mechanisms (*9*). The type I IFN gene signature is therefore considered to be one of the "correlates of risk or pathogenesis" for TB.

In contrast, in the absence of a successful vaccine trial in which some individuals are protected and others develop disease, it has been difficult to identify "correlates of protection" for TB. Although some candidate genes have been identified (*5*, *6*), there have not been clear functional data elucidating how the encoded proteins might contribute to human host defense. Our approach was initially to identify genes that might correlate with protection, seeking those related to host antimicrobial pathways against *M. tuberculosis* in human macrophages. Subsequently, these data were integrated with gene expression profiles in blood from individuals with evidence of *M. tuberculosis* infection that do not progress to active disease, that is, individuals with latent TB. We reasoned that because innate and/or adaptive immune killing of intracellular mycobacteria is critical to the outcome of the battle between the host and the pathogen, gene sets regulating the two areas of investigation, microbicidal macrophages, and latent infection compared with active TB might be expressed in common and be informative about mechanisms of protection against TB.

RESULTS

IL-32–associated gene modules were identified during differentiation of microbicidal macrophages

To identify genes that might contribute to macrophage antimicrobial activity against *M. tuberculosis*, we stimulated primary cultures

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of human monocytes from four healthy donors for 6 and 24 hours in vitro with IL-15 to induce M1-like macrophages, which have been associated with host defense against mycobacteria (*10*, *11*), or conversely with IL-10 or IL-4 to induce M2-like macrophages, which have been associated with pathogenesis in mycobacterial infection (*9*, *11*, *12*). As outlined in Fig. 1A, gene expression profiles were obtained from the cytokine-derived macrophages and analyzed using a systems biology approach to identify modules of highly interconnected genes: weighted gene coexpression network analysis (WGCNA). A network was constructed from this microarray data set on the basis of pairwise correlations of gene expression of highly interconnected genes that have significantly correlated coexpression. Subsequently, gene modules of the highly connected genes are derived (Fig. 1B). Two advantages of the WGCNA approach are that it is unbiased by any supervision derived from databases or publications, and it reduces multiple hypothesis testing.

The most significantly correlated module eigenegene (ME) identified with any treatment, represented as MEblack, was associated with IL-15 stimulation at 24 hours ($P = 6 \times 10^{-13}$) and contained 802 probe sets (Fig. 1B), hereto referred to as *IL15black*. Analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) (*13*, *14*) revealed that the black module was enriched for the Gene Ontology term "defense response," a cluster of 48 genes [false discovery rate (FDR) = 3.09×10^{-5}]. The ME of all known genes annotated with defense response, 1706 probe sets, correlated most strongly with IL-15 stimulation at 24 hours as shown in the module-trait relationship diagram ($P = 1 \times 10^{-6}$). We noted that the *IL15black* defense response cluster contained IL-32, which had previously been implicated in host defense in TB. IL-32 is induced by *M. tuberculosis* (*15*) and is reported to stimulate an antimicrobial activity against *M. tuberculosis* in the THP-1 cell line, which was about 20% dependent on caspase-3 (*16*). Although not expressed in resting monocytes, nor yet to be identified in the mouse, IL-32 is induced in human monocytes/macrophages by stimulation with IFN-γ (*15*) or by activation of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) by muramyl dipeptide (MDP) (*17*) or of Toll-like receptor 4 (TLR4) by lipopolysaccharide (*18*).

Analysis of the IL-15–induced defense response cluster by cell type–specific signature scoring was performed because the macrophages were derived from adherent PBMCs. Of the 48 genes in this cluster, 35 were expressed at baseline in the myeloid cell lineage in addition to IL-32 (Fig. 1C).

IL-15 defense response network links IL-32 to the vitamin D antimicrobial pathway

Given that *IL32* was the most highly connected myeloid gene in the module, a "hub gene" (intramodular connectivity, kME = 0.930, table S1), a correlated network of the IL-15–induced defense cluster was displayed in which the relationship of the most highly connected (topological overlap > 0.685) myeloid genes to *IL32* was visualized. Annotation of this cluster showed *IL32* expression correlated with four components of the vitamin D antimicrobial pathway (*19*–*21*): *CYP27B1* (the vitamin D 1-α-hydroxylase), *CD40*, *CYBB*, and *IL15* (Fig. 2). *IL32* was connected to additional genes annotated as antimicrobial, including major histocompatibility complex (MHC) class I presentation, chemotaxis, and lipid metabolism. In the microarray data, *CYP27B1* was induced by 36.4-fold by IL-15 and correlated with *IL32* expression with a topological overlap score of 0.70 (table S2). It is to be noted that although the network indicates correlations, the causal relationships remain to be formally established.

The ability of IL-15 to induce IL-32, as evident in the microarray data (fig. S1 and table S4), was confirmed by polymerase chain reaction (PCR) using additional primary human monocytes and was comparable to induction by IFN-γ (Fig. 3A and table S4). IFN-γ induction of IL-32 was dependent on IL-15 because its knockdown by small interfering RNA (siRNA) significantly reduced IFN-γ induction of IL-32 by 96% (Fig. 3B and table S4) but did not affect a control gene (fig. S2 and table S4).

IL-32 is necessary and sufficient for the induction of the IFN-γ–dependent vitamin D pathway

Although WGCNA of the macrophage subsets revealed a link, defined by topological correlation, between IL-32 and CYP27b1, the causal relationships and the directionality of the relationship remain to be formally established. We found that treatment of adherent monocytes with IL-32 was sufficient to up-regulate CYP27b1 mRNA, at levels comparable with IFN-γ or IL-15 treatment (Fig. 3C and table S4), and that induction was dose-dependent (fig. S3 and table S4). In addition, IL-32 induced conversion of 25D to the bioactive 1,25-dihydroxyvitamin D (1,25D) (Fig. 3D and table S4). The ability of IFN-γ to induce CYP27b1 mRNA in macrophages was dependent on IL-32, as shown by knockdown of *IL32* (Fig. 3E and table S4), but not a control gene (fig. S4 and table S4). The cognate molecular target of 1,25D, vitamin D receptor (VDR), was also up-regulated in monocytes by treatment with IL-32 (Fig. 3F and table S4).

IL-32 triggers a vitamin D–dependent antimicrobial peptide response against *M. tuberculosis*

IL-32 was sufficient by itself to induce mRNA expression of the antimicrobial peptides cathelicidin and DEFB4 in monocytes, at levels comparable with stimulation by IFN-γ or IL-15 (Fig. 4A and table S4), and was similarly dependent on the VDR because addition of the VDR antagonist VAZ (*22*) completely blocked induction (Fig. 4B and table S4). To determine whether IL-32 was sufficient to induce an antimicrobial activity, macrophages were infected with the live virulent *M. tuberculosis* and treated with IL-32. When the viability of the bacilli was assessed 4 days later, IL-32 had induced an antimicrobial activity of 70% when the macrophages were cultured in 25D-sufficient serum (Fig. 4C and table S4). In contrast, when the macrophages were cultured in 25D-insufficient serum, no antimicrobial activity was observed. In addition, when the 25D-insufficient serum was supplemented in vitro with 25D to sufficient levels, the antimicrobial response was restored. In these experiments, a parallel response to IFN-γ was observed, as previously described (*23*). Together, these data indicate that IL-32 induces CYP27b1 and the VDR, as well as the vitamin D–dependent induction of antimicrobial peptides and antimicrobial activity, and is a functional mediator of IFN-γ activation of human macrophage microbicidal activity.

IL-32 is part of an IL-15–induced gene set differentially expressed in latent TB

We reasoned that integration of the IL-15–induced gene set with gene expression profiles in human TB would identify molecular markers of host defense. We sought to integrate the entire IL-15–induced gene module *IL15black* with two distinct analyses of blood gene expression profiles in human TB: ranked pairwise comparisons of gene expression in five different clinical data sets of latent versus active TB or healthy controls, and a coexpression network derived from gene expression in patients with active TB undergoing chemotherapy (Fig. 5A). Latent TB infection was defined by a positive blood interferon-γ release assay (IGRA) test in those studies.

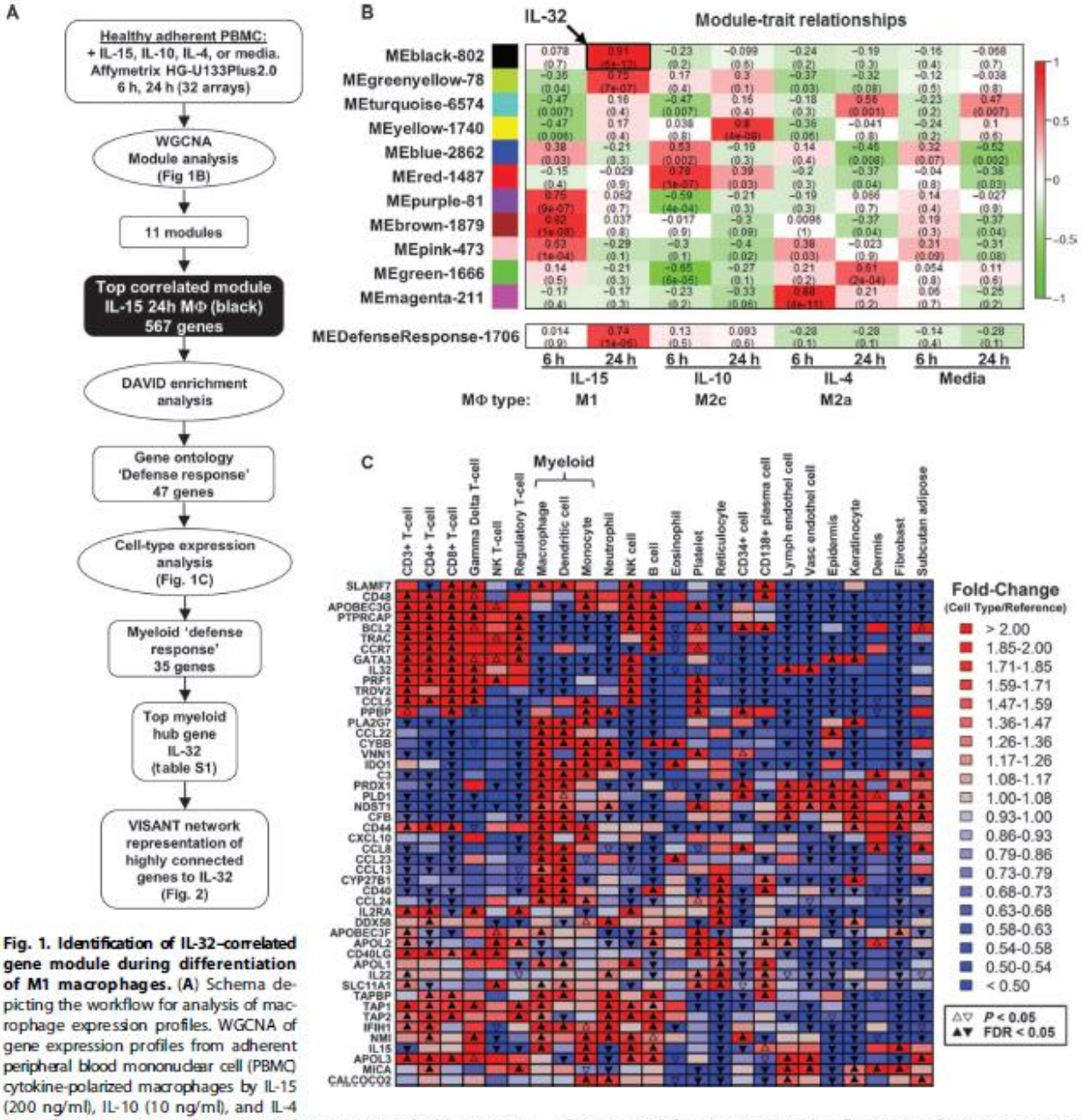

Fig. 1. Identification of IL-32–correlated gene module during differentiation of M1 macrophages. (**A**) Schema depicting the workflow for analysis of macrophage expression profiles. WGCNA of gene expression profiles from adherent peripheral blood mononuclear cell (PBMC) cytokine-polarized macrophages by IL-15 (200 ng/ml), IL-10 (10 ng/ml), and IL-4 (1 U/ml) into M1, M2a, and M2c macrophages, respectively. (**B**) Heat map depicts correlation of each module eigengene (ME) to treatment condition with corresponding *P* values. Number of probe sets per module indicated at beginning of each row; red indicates positive correlation, and green indicates inverse correlation. Lower panel in (B) depicts correlation of treatments to eigengene of all genes annotated by Gene Ontology term "defense response." (**C**) Cell type–specific signature scores of genes from defense response of the black module. Each row represents the expression of each gene across a reference data set from separated cell types. Color depicts fold change expression in a given cell type, relative to its expression among the 23 other cell types; statistical significance is indicated by arrowheads.

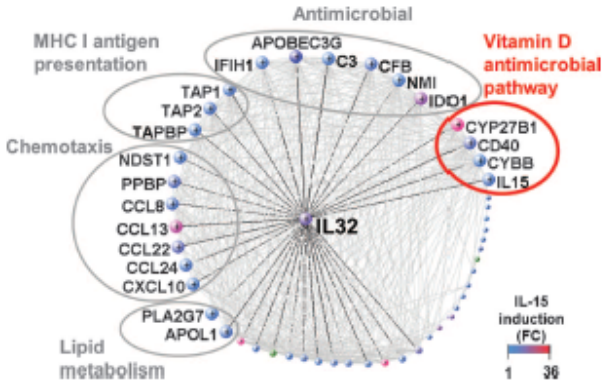

Fig. 2. IL-15 defense response network links IL-32 to the vitamin D antimicrobial pathway. Visualization of IL-15–induced defense response connectivity network by topological overlap (>0.685). Only those genes expressed in myeloid cells (FDR < 0.05) determined by cell type–specific score and connected to IL-32 are labeled. Color of each node depicts fold-change (FC) induction by IL-15 at 24 hours as indicated in the legend.

The first analysis was based on the hypothesis that those genes with elevated expression in latent TB versus active TB and healthy controls were likely to be relevant to host defense against active disease. A pairwise comparison of gene expression profiles derived from five different data sets (table S3) of peripheral blood from individuals with latent versus active TB infection {Berry *et al.* [UK'10 (*5*)], Berry *et al.* [SA'10 (*5*)], Maertzdorf *et al.* [GER'12 (*6*)], Bloom *et al.* [SA'12 (SA) (*8*)], and Kaforou *et al.* [KAF'13 (*24*)]} was computed. Similarly, comparisons of peripheral blood gene expression profiles were calculated for each of two data sets of latent TB versus healthy controls {Berry *et al.* [UK'10 (*5*)] and Maertzdorf *et al.* [GER'12 (*6*)]} (table S3). In total, 366 gene expression profiles were analyzed, from active TB patients ($n = 168$), individuals with latent TB ($n = 173$), and healthy controls ($n = 25$). We used a nonstringent cutoff (fold change ≥1.2) to identify positive genes and ranked these on the basis of the number of data sets in which they passed the threshold (*25*). We then determined which of the genes in the *IL15black* module were differentially expressed in at least three of five latent versus active TB data sets and one of two latent TB versus healthy control data sets. Eighteen genes were identified (hypergeometric $P = 0.002$), of which only *IL32* was up-regulated in latent versus active TB and latent TB versus healthy controls by at least 1.2-fold in seven of seven data sets ($P = 9.6 \times 10^{-7}$, expected value 0.016 genes) (fig. S4A).

In the second analysis, we analyzed a data set of serial blood samples from TB patients undergoing chemotherapy ($n = 29$) (*8*). We performed WGCNA of this data set, which revealed four modules that were significantly enriched in the latent TB group (fig. S5B). Of these, the tan module, referred to as *LATENTtan*, contained 88 probe sets corresponding to 64 genes, including *IL32*. The tan ME (MEtan) was significantly associated with latent TB ($P = 0.03$) and inversely correlated with active TB ($P = 0.002$) but became significantly elevated in active TB after 6 months of chemotherapy ($P = 0.0005$). Overlap of the *IL15black* module with the *LATENTtan* treatment module revealed 14 common genes (hypergeometric $P = 1.5 \times 10^{-7}$), including *IL32* (fig. S5C). The intersection of the comparisons of the *IL15black* module with the pairwise analysis of the five clinical data sets and the latent TB gene modules identified eight common genes, including *IL32* (Fig. 5, B and C). At least three of the identified genes may derive from $CD8^+$ T cells or natural killer cells, such as *CD8A*, *GZMH*, and *PRF1*, because *IL15black* was obtained by studying adherent PBMC, and the clinical data sets involve gene expression profiles from blood.

IL-32 mRNA levels were greatest in peripheral blood of patients with latent TB, lower in healthy controls, and lowest in active TB patients (Fig. 5D). The GER'12 latent TB versus active TB pairwise comparison of IL-32 mRNA was significantly different. Although IL-32 mRNA GER'12 data were 1.3-fold greater in latent TB versus healthy controls, similar to the UK'10 data, the GER'12 data did not achieve significance because of the small number of individuals with latent TB. In addition, IL-32 mRNA expression was lowest in a group of patients categorized as having "other diseases," including pneumonia, malignancy, and a variety of other infections in which TB was a possible differential diagnosis (Fig. 5E). We note that IL-32 mRNA expression was also comparatively low in peripheral blood gene expression profiles from patients with sarcoidosis (fig. S6), although the gene signatures of sarcoidosis and active TB were reported largely to overlap (*6*).

Gene expression profiles were also obtained from peripheral blood of healthy infants in South Africa vaccinated with attenuated modified vaccinia virus Ankara expressing *M. tuberculosis* antigen 85A (MVA85A) (*26*, *27*). This vaccine induced an IFN-γ response in antigen-activated T cells in vitro but failed to engender protection against infection or disease (*28*). We note that there was no induction of IFN-γ or IL-32 mRNAs in the unstimulated blood at 2 or 7 days after vaccination (fig. S7), in contrast to the elevated IL-32 mRNA in unstimulated blood of latent TB versus both active TB and healthy controls.

In the data set from TB patients undergoing chemotherapy ($n = 29$) (*8*), IL-32 mRNA, although lowest in active TB patients, increased during chemotherapy as early as 2 weeks, reaching the levels observed in latent TB infection by 6 months of treatment (Fig. 5F).

DISCUSSION

Most individuals infected with *M. tuberculosis* develop, in addition to innate immunity, an acquired cell-mediated immune response against the pathogen, as determined by tuberculin skin testing or in vitro IFN-γ release assays. In about 90% of immunocompetent individuals, the infection is contained; in an unknown percentage of individuals, infection persists in a latent or persistent state for long periods and can reactivate, resulting in clinical disease. There are a number of markers that have been associated with active TB in patients, including many IFN-β–induced genes (*29*).

Molecular markers for immune responses responsible for containing the pathogen in a latent state would thus represent a valuable measure of immune protection against progression to active disease. Given the difficulty in identifying a set of genes that are biomarkers for protection and have a plausible biologic function in host defense, we initiated studies to identify the pathway activated by IFN-γ leading to antimicrobial activity of human macrophages. We had previously shown that IFN-γ induced IL-15, which rapidly induces the differentiation of monocytes into macrophages with antimycobacterial activity. Our strategy here was initially to identify gene modules in IL-15–treated monocytes that might be critical to antimicrobial activity and then to compare those with gene expression modules in databases characterizing gene expression of latent TB patients, seeking genes that were

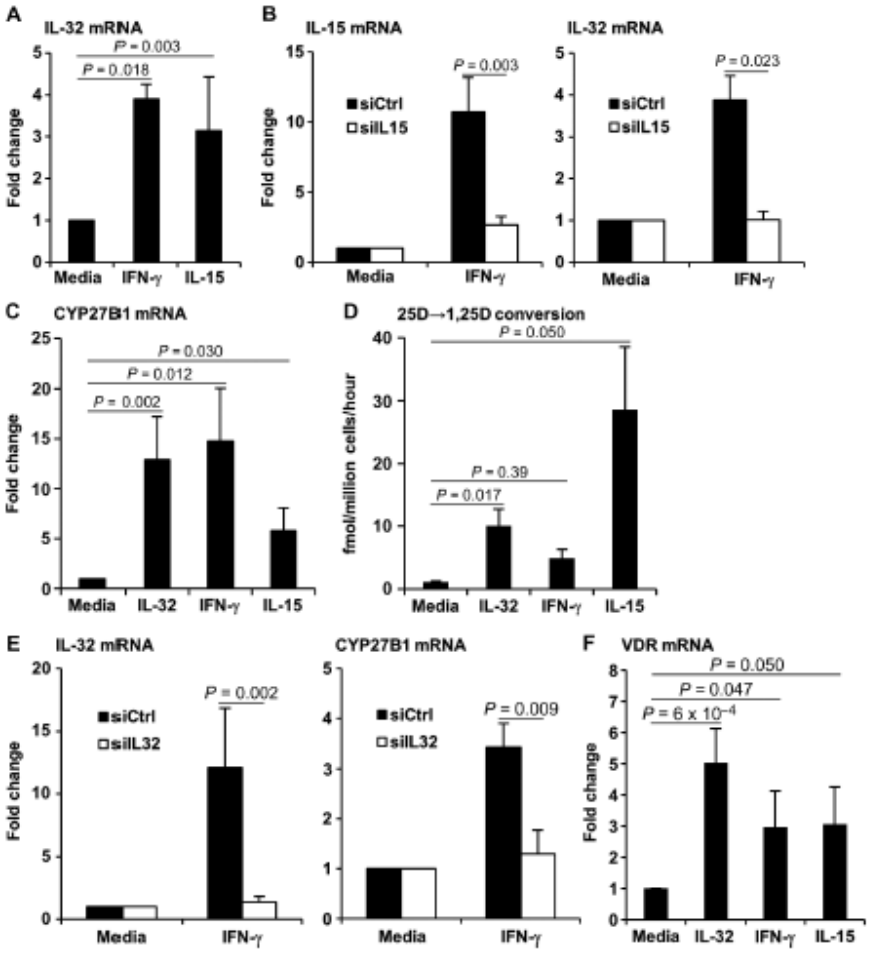

Fig. 3. IL-32 is necessary and sufficient for the induction of the IFN-γ–dependent vitamin D pathway. (**A**) Adherent PBMCs were treated with IFN-γ (1.3 ng/ml) or IL-15 (200 ng/ml) for 24 hours, and IL-32 gene expression was measured by qPCR (mean fold change ± SEM, $n = 4$). (**B**) Monocyte-derived macrophages (MDMs) were transfected with siRNA oligos specific for *IL15* (siIL15) or nonspecific (siCtrl) and then treated with IFN-γ (1.3 ng/ml) for 24 hours, and IL-15 and IL-32 mRNAs were assessed by qPCR (mean fold change ± SEM, $n = 4$). (**C** and **F**) Adherent PBMCs were stimulated with IL-32 (50 ng/ml), IFN-γ (1.3 ng/ml), or IL-15 (200 ng/ml) for 24 hours in 10% fetal calf serum (FCS), and CYP27B1 (C) or VDR (F) expression was assessed by qPCR (mean fold change ± SEM, $n = 5$ to 7). (**D**) CYP27b1 activity measured by treating adherent monocytes with IL-32 (100 ng/ml), IFN-γ (1.3 ng/ml), or IL-15 (200 ng/ml) in 10% FCS for 48 hours and for an additional 5 hours with [^{3}H]25D3. The amount of conversion to [^{3}H]1,25D3 was measured by high-performance liquid chromatography (HPLC). (**E**) MDMs were transfected with siIL-32 or siCtrl and treated with IFN-γ (1.3 ng/ml) for 24 hours. IL-32 and CYP27B1 gene expression was determined by qPCR (mean fold change ± SEM, $n = 7$). *P* value by Student's *t* test.

shared in common between microbicidal macrophages in vitro and peripheral blood of latent TB patients.

Activation of human monocytes/macrophages either by the innate immune system [TLRs (*19*, *30*–*32*)] or the acquired immune response [IFN-γ (*23*)] converges on a common pathway through the induction of IL-15 (*33*) and up-regulation of CYP27b1 and the VDR. Our analysis revealing a linkage between IL-32 and the vitamin D antimicrobial pathway was an unexpected finding, demonstrating the power of WGCNA to reveal previously unknown functional associations. This analysis identified an IL-15–induced host defense network in macrophages, which included both IL-32 and CYP27b1. We uncovered the directionality of this predicted connection by demonstrating in vitro that (i) the ability of IFN-γ to induce IL-32 was dependent on IL-15; (ii) IFN-γ induction of CYP27b1 was IL-32–dependent; (iii) IL-32 triggered the up-regulation of CYP27b1 and IL-32 induced the conversion of 25D to 1,25D; (iv) IL-32 induced the expression of the vitamin D–dependent antimicrobial peptides cathelicidin and DEFB4; and (v) IL-32 induced antimicrobial activity in vitro against *M. tuberculosis* in macrophages. IL-32 is a human secreted protein, not yet identified in mouse, that is reported to trigger monocyte activation including cytokine release and differentiation (*34*) and thought to contribute to the pathogenesis of infectious (*35*, *36*) and autoimmune (*37*) diseases, as well as inflammatory bowel disease (*38*) and cancer (*39*). Infection of monocytes by *M. tuberculosis* (*15*), as well as activation by MDP via NOD2 (*17*), induces IL-32. In the THP-1 cell line, IL-32 induced an antimicrobial activity against *M. tuberculosis* that was about 20% dependent on caspase-3 (*16*). We found that the ability of IL-32 to trigger an antimicrobial response in the presence of 25D-insufficient serum required the addition of sufficient levels of 25D in vitro, as has been found for other in vitro stimuli or after vitamin D supplementation in vivo (*19*, *23*, *40*–*42*). Our data demonstrate that in primary human macrophages, the IL-32–induced antimicrobial response was completely dependent on the level of 25D, confirming the importance of sufficient extracellular levels of 25D to support an optimal human antimicrobial response against *M. tuberculosis*.

In addition to inducing an antimicrobial response, IL-32 triggers the differentiation of monocytes into dendritic cells with enhanced capacity to cross-present antigen via MHC class I to CD8$^+$ T cells (*17*) (fig. S8). A role for CD8$^+$ T cells in protective immunity against TB has been shown in animal studies and inferred from human studies (*43*). Human CD8$^+$ T cells were found to be both cytolytic and able to activate an antimicrobial response against *M. tuberculosis*–infected macrophages (*44*–*46*). These CD8$^+$ T cells express cell surface tumor necrosis factor–α and contain granulysin in cytotoxic granules, an antimicrobial protein

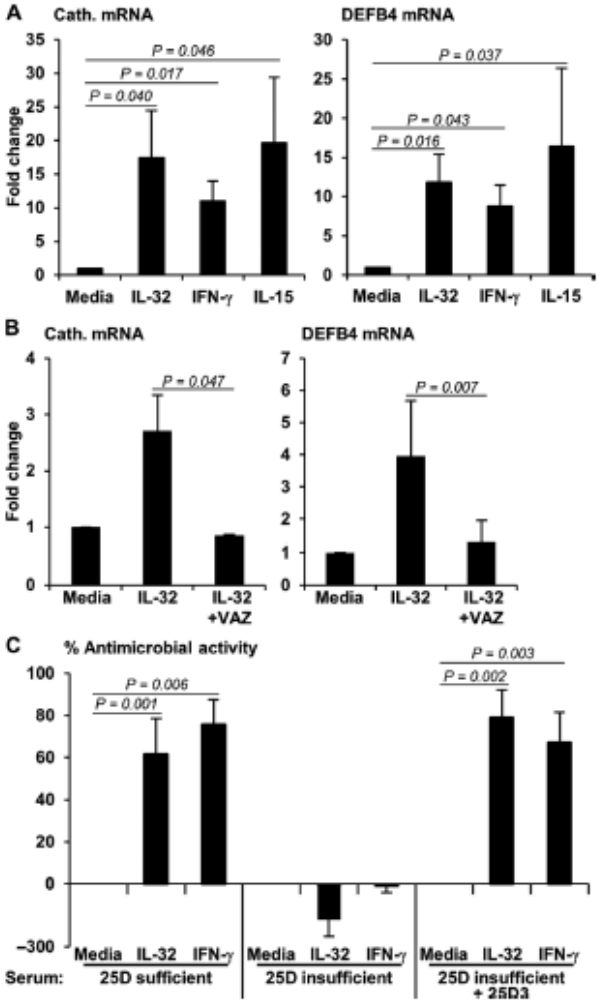

Fig. 4. IL-32 triggers a vitamin D–dependent antimicrobial response against *M. tuberculosis*. (**A**) Human monocytes purified by negative selection were cultured in 10% vitamin D–sufficient human serum and stimulated with IL-32 (50 ng/ml), IFN-γ (1.3 ng/ml), or IL-15 (200 ng/ml) for 24 hours. mRNA expression of the antimicrobial peptides cathelicidin (Cath.) and DEFB4 was determined by qPCR (mean fold change ± SEM, $n = 3$ to 5). (**B**) Purified monocytes were pretreated with the VDR antagonist VAZ (ZK159222) for 15 min and treated with IL-32 (50 ng/ml) for 24 hours. RNA expression of the indicated genes measured by qPCR (mean fold change ± SEM, $n = 3$). (**C**) Human MDMs were infected with *M. tuberculosis* H37Rv overnight. After infection, cells were treated with IL-32 (100 ng/ml) or IFN-γ (1.3 ng/ml) for 4 days. Viability of *M. tuberculosis* was calculated by the ratio of bacterial 16*S* RNA and DNA (IS6110) as measured by qPCR, and percent increase or decrease relative to no treatment (media) was determined (mean fold change ± SEM, $n = 3$). *P* value by Student's *t* test.

with activity against *M. tuberculosis* (*47*, *48*) also not found in mouse macrophages. Three of the eight genes identified in the overlap between the IL-15–induced network and the latent TB gene profile are expressed by $CD8^+$ T cells, consistent with a role for this T cell subset in host defense. It is possible that IL-32 may sequentially induce the killing of the intracellular pathogen within phagolysosomes by a vitamin D–dependent pathway, releasing antigen to the MHC class I pathway to further induce and/or activate $CD8^+$ T cells (fig. S8). Induction of the vitamin D pathway involves autophagy (*23*, *31*, *32*) and phagolysosomal fusion, which contribute to the antimicrobial activity, but these cellular processes may also facilitate antigen presentation to T cells (*49*, *50*).

The IL-15–induced "host defense" network in macrophages as determined by WGCNA identified the connection between *IL32* and genes involved in the vitamin D antimicrobial pathway (*CYP27B1*, *CD40*, *CYBB*, and *IL15*). An additional set of genes implicated in antimicrobial responses was identified (*IFIH1*, *APOBEC3G*, *C3*, *CFB*, *NMI*, and *IDO1*). Of these, *NMI* is noteworthy for its ability to regulate type I IFN responses (*51*), which block type II IFN (IFN-γ) antimicrobial responses against mycobacterial infection (*9*). It is interesting that *IDO1* (indoleanine 2,3-dioxygenase) is part of this network, indicating that the host is responding as if defending against a tryptophan auxotroph, for example, *Leishmania* spp., but because *M. tuberculosis* is a prototroph, it is not effective in killing this pathogen (*52*). In the IL-15–induced network, *IL32* is linked to MHC class I antigen presentation (*TAP1*, *TAP2*, and *TAPBP*), consistent with its role in cross presentation (*17*). There are a number of genes that are involved in chemotaxis required to direct the immune response to the site of infection. Finally, the IL-15–induced host defense network includes genes involved in lipid metabolism (*PLA2G7* and *APOL1*), reflecting the connection between mycobacterial infection and host lipid metabolism (*53*–*55*). The functional role of these genes in the IL-15 network and the role of IL-32 in orchestrating their participation in antimicrobial activity remain to be determined.

Two different approaches were used to determine whether the genes induced by IL-15 treatment of adherent monocytes were relevant to the pathogenesis of clinical TB. First, by a ranked analysis of the gene expression profiles in the blood of individuals with TB spanning five different data sets, a set of genes was identified, which when overlapped with the IL-15 macrophage modules identified 18 genes. Second, informatics analysis of a set of data from individuals with latent TB, and patients with active TB, before and during chemotherapy, identified an IL-32–containing gene module associated with latent TB. When this module was overlapped with the IL-15–induced host defense network, 14 genes were identified. Together, both approaches identified a set of eight common genes. In all of these analyses, IL-32 was the only gene associated with latency in five of five data sets comparing latent versus active TB and two of two data sets comparing latent TB versus healthy controls.

We recognize that there are almost certainly other genes and mechanisms likely to be involved in human macrophage antimicrobial activity yet to be defined. We acknowledge that this type of study has several limitations. Because of the different platforms used in the different studies, most likely IL-32 is one of a set of genes, some of which we identify here, that together could serve as candidates for potential biomarkers for latency. There were two data sets comparing individuals with latent TB versus healthy controls; one data set (GER'12) contained only four samples of latent TB, thus limiting the statistical power of our analysis. However, a reanalysis comparing only data sets from Illumina platforms and omitting the one Agilent platform data set GER'12 did not contribute any additional protein-coding genes that overlapped with *IL15black* and *LATENTtan*. Ideally, it would

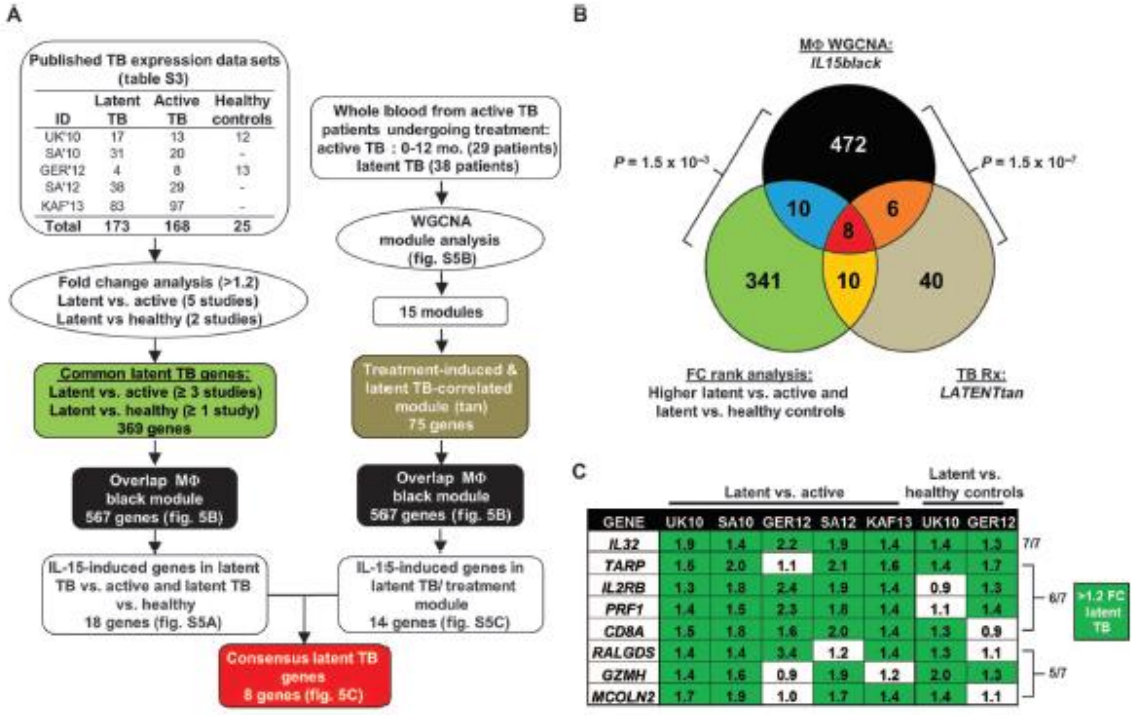

Fig. 5. IL-32 is part of an IL-15–induced gene set differentially expressed in latent TB. (**A**) Schema of workflow for analysis of TB expression data sets from whole blood. (**B**) Overlap of entire *IL15black* module with common latent TB blood genes derived from fold change (FC) rank analysis of latent TB, active TB, or healthy control patients versus WGCNA of active TB patients undergoing chemotherapy treatment (TB Rx). Hypergeometric distribution *P* value for enrichment of genes indicated for each overlap. (**C**) Fold change expression of the eight common latent TB genes (rows) across all TB cohorts (columns) indicated inside each box; green indicates FC >1.2 in latent TB over active or healthy control patients. Genes are ranked in order of consensus across the seven comparisons as indicated on right. (**D**) Raw intensity values for IL-32 mRNA expression in each data set by cohort; red line indicates mean. *P* value by unpaired Student's *t* test. (**E**) Raw data of KAF'13 cohort of latent and active TB patients and other diseases in which TB was a differential diagnosis *P* value by unpaired Student's *t* test. (**F**) IL-32 mRNA expression data from active TB patients undergoing standard chemotherapy treatment. *P* value by paired Student's *t* test.

be important to test candidate biomarkers in longitudinal studies or in vaccine trials that show some protection for their correlation with protection against disease and then to validate them in independent study populations to establish true correlates of protection.

The emergence of MDR and XDR strains of *M. tuberculosis* and the rather small pipeline of new drugs against TB have made the importance of developing immunological approaches to controlling TB compelling. Because of the low prevalence of TB in most populations and the confounding effects of HIV/AIDS and environmental mycobacterial exposure, TB vaccine efficacy trials must inevitably be large, complex, long-term, and very expensive. A 15-year major randomized controlled trial of Bacille Calmette-Guérin in 366,000 people in India in the 1950s showed no protection in any age groups (*56*). In the recent phase 2b clinical trial of the modified vaccinia Ankara expressing *M. tuberculosis* antigen 85A (MVA85A) against TB, an IFN-γ response was detected in recipients, but the vaccine failed to engender any protection against either infection or disease (*28*). Our analysis of the gene expression profile data of vaccinated subjects (*27*) revealed that IL-32 mRNA levels were not increased at day 2 or 7 after vaccination, which is consistent with the immunologic findings that neither an antimicrobial nor a $CD8^+$ T cell response against antigen 85A was detected. Although many studies have established that IFN-γ is a necessary condition for protection against TB, the findings that IFN-γ is produced in most patients with active or latent disease, as well as in vaccine recipients who fail to show protection, clearly mean that it is not itself a sufficient condition or useful correlate of protection. Without some credible molecular biomarkers or correlates of protection derived from small human studies, it is unlikely that there will be the resources to test many of the current and future vaccine candidates (*57*) in large-scale clinical efficacy trials.

In summary, our analysis indicates that IL-32 is linked in an interaction network with a number of genes associated with various aspects of human immune defense, including genes involved in synthesis of the bioactive form of vitamin D, antimicrobial activity, chemotaxis, and MHC class I–restricted antigen presentation. Further, with vaccine trials against TB being enormously costly and requiring years to evaluate, there is an urgent need for molecular correlates of protection. The existing gene expression data on immunologic responses in latent and active TB reveal IL-32 to be at least one gene that correlates with the latent state and not active disease and which has a functionally defined role in host defense, which we believe justifies its consideration as one in a set of potential biomarkers for protective immune responses against TB.

MATERIALS AND METHODS

Study design

The objective of this study was to identify human candidate markers of protection against TB by use of three different computational and experimental approaches. First, to gain insight on the microbicidal activity of macrophages, a total of 32 gene expression profiles of differentially polarized macrophages from four healthy human donors (blood from healthy donors was obtained with informed consent) at two time points were contrasted via gene coexpression analysis to identify an antimicrobial network. Second, mechanistic studies elucidating the antimicrobial network were performed in vitro on cells from healthy blood donors without blinding or randomization. The number of replicates is indicated for each experiment in the respective figure legends. Third, the in vitro antimicrobial network was integrated with five publically available clinical data sets of whole blood gene expression profiles from active TB patients ($n = 168$), individuals with latent TB ($n = 173$), and healthy controls ($n = 25$), and 29 patients with TB undergoing chemotherapy at various time points ($n = 103$) were retrieved from the Gene Expression Omnibus (table S3). In total, 469 gene expression profiles were analyzed, each patient cohort was obtained, and patients were classified according to criteria defined by authors of each study (*5*, *6*, *8*, *24*). Briefly, active TB was defined as culture-positive pulmonary TB patients, and latent TB as asymptomatic $IGRA^+$ patients. The GER'12 cohort originally reported four $IGRA^+$ patients classified as healthy controls (*6*) but were clearly identified as latent TB. Normalized data as processed by author were analyzed unless study was normalized to latent TB (*8*), in which raw data from each patient group were normalized by robust multichip average (RMA).

Reagents

Recombinant human IL-32γ, IL-10, IL-15, and M-CSF (macrophage colony-stimulating factor) were purchased from R&D Systems and used at the indicated concentrations. IFN-γ was purchased from BD Biosciences. 25D3 was purchased from BioMol. VDR antagonist ZK 159 222 (VAZ) was from Bayer Schering AG and used at 10^{-8} M.

Monocyte and macrophage cultures

Adherent PBMCs were isolated from healthy human donors as previously described (*9*). Monocytes were enriched by negative selection by EasySep Human Monocyte Enrichment Kit without CD16 Depletion (STEMCELL Technologies) according to the manufacturer's protocol and confirmed by flow cytometry to have greater than 90% purity for $CD14^+$ monocytes. MDMs were differentiated from CD14 positively selected monocytes stimulated with M-CSF (50 ng/ml) for 4 days as previously described (*23*). Cells were stimulated with the indicated serum: 10% FCS (Omega Scientific) or 10% pooled non–heat-inactivated human serum with 25D3 concentrations determined to be 40 ± 1 ng/ml (25D3-sufficient) or 14 ng/ml (25D3-insufficient as previously described (*23*).

Microarray of macrophage subsets

Adherent PBMCs from four healthy donors were stimulated with IL-10, IL-15 (R&D Systems), or IL-4 in RPMI 1640 supplemented with 10% FCS. Cells were harvested at 6 and 24 hours after stimulation, and monocytes were purified by CD14 MicroBeads (Miltenyi Biotec) for a confirmed monocyte purity of at least 90%. Total RNA was isolated and then processed by the University of California Los Angeles Clinical Microarray Core Facility using Affymetrix Human U133 Plus 2.0 array and normalized as previously described (*9*).

Weighted gene coexpression network analysis

WGCNA was applied to data as previously described (*58*). Probes were filtered according to mean probe set expression across all samples to yield a target number of probe sets between 15,000 and 20,000. The function "blockwiseModules()" was used to construct unsigned, weighted correlation networks with a soft thresholding power of 4 for macrophage data and 9 for TB data, based on the edge distribution of each data set. For each network, modules of coexpressed genes were constructed using a measure of network interconnectedness, topological overlap, which is calculated from an adjacency matrix of pairwise

correlations of all genes raised to the soft thresholding power. MEs, linear combinations of genes that capture a large fraction of the variance in each module, were calculated for each module and correlated to cytokine treatment status (for adherent PBMC data) or TB disease status (for TB data) by taking the correlation of each ME to the expression profiles of each condition. Correlations and corresponding *P* values were displayed in a heat map using the WGCNA command labeled Heatmap(), with ME correlations and a binary matrix representation of sample treatments where a "1" corresponded to the time point and cytokine treatment used for that particular sample and a "0" corresponded to all other time points/treatments not used. Similarly, eigengenes for all human genes annotated with the Gene Ontology term "defense response" or *IL32* were correlated to expression profiles of cytokine treatment status. For each module, hub genes, or genes with high module membership, were identified on the basis of kME. The signedKME() function was used to rank genes within each module. Gene relationships within a module were visualized using the visANT program and the exportNetworkToVisANT() function (*59*).

Cell type–specific signature scores

Cell type–specific signature scores for each gene were based on a database of publically available microarray samples of 24 different cell types, as previously reported (*60*). Briefly, 687 publicly available microarray samples on the Affymetrix HG-U133 Plus 2.0 platform were selected as being representative of specific cell types, with the number of samples per cell type roughly equal. Using this database, we evaluated genes for cell type–specific expression, even though such genes might have detectable expression in multiple cell types. Genes were determined to have expression significantly higher in samples for a particular cell type, compared to the other 23 cell types, by fold changes estimated as the ratio of a gene's expression in a given cell type (numerator) relative to its expression among the 23 other cell types (denominator). *P* values were calculated on the basis of the empirical Bayes approach and moderated *t* statistic. FDR was determined by adjusting *P* values using the Benjamini-Hochberg method.

M. tuberculosis infection

M. tuberculosis H37Rv were cultured and infected into macrophages as previously described (*61*) at a biosafety level 3 facility. Briefly, *M. tuberculosis* was plated on 7H11 agar plates from frozen stocks for 3 to 4 weeks of incubation at 37°C, 5% CO_2, and the solid colonies were picked and placed in 1× phosphate-buffered saline. The bacterial suspension was gently separated with a sonicating water bath (Branson 2510) for 30 s and then centrifuged at 735*g* for 4 min to create a single-cell suspension and enumerated by absorbance at 600 nm using spectrophotometry. MDMs were infected at a multiplicity of infection of one bacterium per cell overnight, and subsequently, the cells were vigorously washed three times with fresh RPMI 1640 medium to remove extracellular bacteria. *M. tuberculosis*–infected MDMs were then stimulated as indicated and incubated for 4 days.

Antimicrobial assay

M. tuberculosis viability from infected MDMs was assessed by the real-time PCR–based method as previously described (*61*–*63*), which compares 16*S* RNA levels with genomic DNA (IS6110) levels as indicator of bacterial viability. Genomic DNA was isolated from the interphase by phenol-chloroform method using the back-extraction protocol, as described by the manufacturer. Total RNA and genomic DNA was isolated using TRIzol reagent (Life Technologies) via phenol-chloroform extraction from the aqueous phase or interphase, respectively. RNA was further purified, and deoxyribonuclease digestion was performed using an RNeasy Miniprep Kit (Qiagen). Complementary DNA (cDNA) was synthesized from the total RNA using the iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's recommended protocol. The bacterial 16*S* ribosomal RNA and genomic element DNA levels were assessed from the cDNA and DNA using quantitative PCR (qPCR), and the relative 16*S* values were calculated using the $\Delta\Delta C_T$ analysis, with the IS6110 value serving as the "housekeeping gene." Each reaction was normalized to the control media, and percent decrease of TB viability after stimulation relative to media alone was reported.

Real-time qPCR

RNA from monocytes/macrophages was isolated, cDNA was synthesized, and qPCR was performed for vitamin D pathway genes [CYP27b1, VDR, cathelicidin (CAMP), and DEFB4] as previously described (*19*) or H37Rv viability elements 16*S* and IS6110. Primer sequences specific for IL-32γ are IL-32γ forward, GTAATGCTCCTCCCTACTTCT, and IL-32γ reverse, AAAATCTTTCTATGGCCTGGT. TLR7: TLR7 forward, TCACCAGACTGTTGCTATGATGC, and TLR7 reverse, CAGCCAAA-ACCCACTCGGT. Reactions were done using SYBR Green PCR Master Mix (Bio-Rad) and normalized to h36B4, and relative arbitrary units were calculated using $\Delta\Delta C_T$ analysis as described (*19*).

Measurement of vitamin D bioconversion

The rate of CYP27b1 activity was assessed in 48-hour IL-32– and IFN-γ–treated adherent monocytes as previously described (*11*). Briefly, [^{3}H]25D3 was added to 10^6 treated cells in 200 μl of serum-free medium and then incubated for 5 hours at 37°C. Vitamin D metabolites were extracted and separated by HPLC, and elution profiles were determined by ultraviolet absorbance at 264 nm.

Primary macrophage siRNA transfection

ON-TARGETplus siRNA pools targeting *IL32* (L-015988-00-0005) and control nontargeting pool (D-001810-10-05) were purchased from Dharmacon. Lipofectamine-siRNA complexes were formed using 1 to 2 μl of Lipofectamine 2000 and 20 to 60 pmol of siRNA according to the manufacturer's instructions and acceptable levels of cell viability (>90%) as determined by trypan blue exclusion. MDMs were seeded in a 24-well plate at 4×10^4 cells, and each well was transfected with Lipofectamine-siRNA complexes for 4 hours at 37°C and 5% CO_2, then washed three times, and placed in fresh RPMI 1640 in 10% FCS for 24 hours to recover. Transfected MDMs were then stimulated as indicated for 24 hours.

Analysis of latent TB signature

For each patient cohort, fold change was calculated by dividing the average latent TB value by the average active TB or healthy control value for all genes annotated with gene symbols common to all microarray platforms (16,727 genes); for genes that have more than one probe, the probe with the highest average intensity across all samples was chosen. The genes were ranked by having a consistent fold change greater than 1.2 across at least three of the five latent versus active data sets and at least one of two latent versus healthy control data sets. *P* values and expected values of top-ranked genes were calculated through MATLAB simulations of randomized data sets with exact number and proportions of genes with fold change greater than 1.2 for each cohort. IL-32 mRNA

expression were displayed as arbitrary units, which are the postnormalization intensity values from each array; if an array platform contained more than one probe for *IL32*, the probe with the highest average intensity across all samples was chosen. Venn diagrams of gene lists were compared using Venny (*64*).

Statistics

Differences in individual gene expression among TB patients were tested on GraphPad Prism software on the log-transformed intensity values. Pairwise comparisons between patient groups within each data set were analyzed using an unpaired two-tailed Student's *t* test. SA'12 data set of individual active TB patients undergoing treatment over time was analyzed using a paired *t* test. Differences in qPCR data were analyzed on log-plus-one transformation of fold changes compared to media using a paired Student's *t* test.

SUPPLEMENTARY MATERIALS

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Fig. S1. Expression of IL-32 in adherent PBMC microarray.
Fig. S2. IFN-γ–induced DDX60 expression is unchanged by siIL15 knockdown.
Fig. S3. IL-32 induction of CYP27b1 is dose-dependent.
Fig. S4. IFN-γ–induced TLR7 expression is unchanged by siIL-32 knockdown.
Fig. S5. Common genes expressed in latent TB.
Fig. S6. IL-32 higher in latent TB patients versus patients with sarcoidosis.
Fig. S7. IL-32 and IFN-γ expression after treatment with MVA85a vaccine.
Fig. S8. Role of IL-32 in host defense.
Table S1. Top hub genes of *IL15black* defense response network.
Table S2. Myeloid genes correlated with IL-32 in the IL-15 defense response functional cluster.
Table S3. TB data sets used in this study.
Table S4. Original data used for graphs (provided as separate Excel file).

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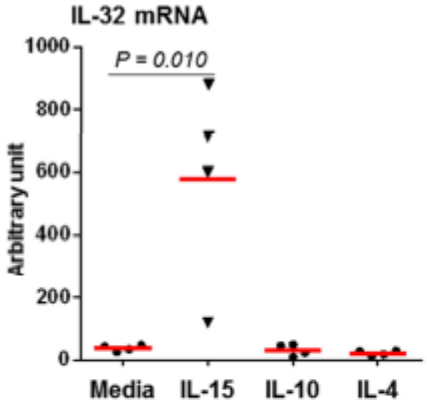

Fig. S1. Expression of IL-32 in adherent PBMC microarray. Adherent peripheral blood mononuclear cells (PBMC) from four healthy donors were stimulated by IL-15, IL-10, IL-4, or media alone for 24h. Expression of IL-32 shown as arbitrary units as measured by Affymetrix Human U133 Plus 2.0 expression array. Red line indicates mean intensity. *P*-value by paired student's t-test.

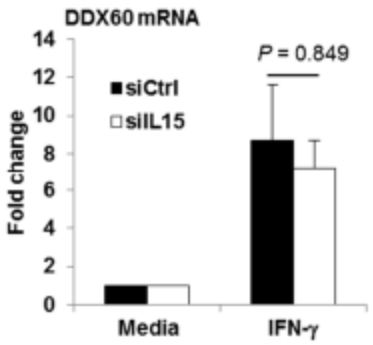

Fig. S2. IFN-γ–induced DDX60 expression is unchanged by siIL15 knockdown. Monocyte-derived macrophages were transfected with siRNA oligos specific for *IL15* (siIL15), or nonspecific (siCtrl) and subsequently treated with IFN-γ for 24 h. DDX60 mRNA assessed by qPCR (mean fold change ± SEM, $n = 4$). *P*-value by paired student's t-test.

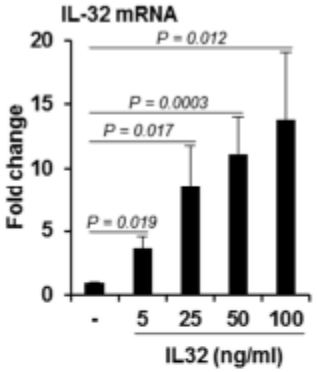

Fig. S3. IL-32 induction of CYP27b1 is dose-dependent. Adherent PBMC were stimulated with indicated concentration of IL-32 for 24h in 10% FCS and CYP27B1 mRNA expression assessed by qPCR (mean fold change ± SEM, n = 4 to 10).

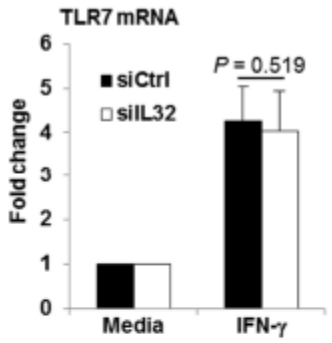

Fig. S4. IFN-γ–induced TLR7 expression is unchanged by siIL-32 knockdown. Monocyte-derived macrophages were transfected with siRNA oligos specific for *IL32* (siIL-32), or nonspecific (siCtrl) and then treated with IFN-γ for 24 h. TLR7 mRNA assessed by qPCR (mean fold change ± SEM, $n = 7$). *P*-value by paired student's t-test.

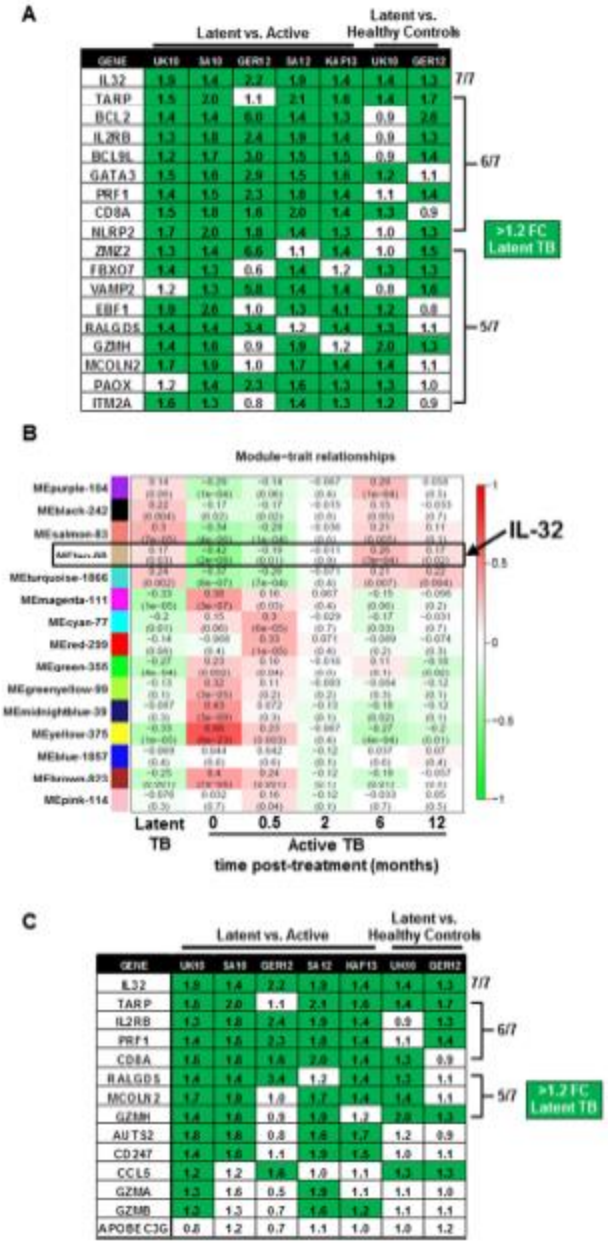

Fig. S5. Common genes expressed in latent TB. (**A**) Top eighteen genes expressed in the blood of latent TB vs. active TB and latent TB vs. healthy controls by fold-change analysis. Average fold-change of genes (rows) across TB cohorts (columns) indicated inside each box, green indicates FC > 1.2 in latent TB over active or healthy controls. Genes are ranked in order of consensus across the seven comparisons as indicated on right. Genes are filtered to have higher expression in latent TB vs. active TB ≥ 3 studies and latent TB vs. healthy controls ≥ 1. (**B**) Heatmap of weighted gene co-expression analysis (WGCNA) of SA'12 cohort with active patients undergoing chemotherapy treatment and latent TB patients. Number of probe sets per each module indicated at beginning of each row, red indicates positive correlation, green inverse correlation. *LATENTtan* module containing IL-32 indicated. (**C**) Average fold-changes depicted across TB cohorts of the 14 gene overlap of *LATENTtan* and *IL15black* from Figure 5B, green indicates FC > 1.2 in latent TB over active or healthy controls.

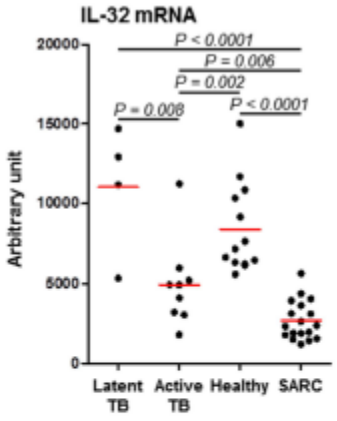

Fig. S6. IL-32 higher in latent TB patients versus patients with sarcoidosis. Raw intensity values of IL-32 from Maertzdorf et al 2012. Red line indicates mean value. *P*-value by unpaired student's t-test.

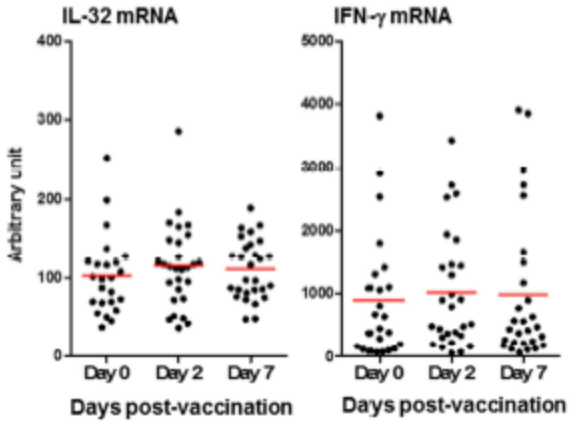

Fig. S7. IL-32 and IFN-γ expression after treatment with MVA85a vaccine. Healthy volunteers were vaccinated with candidate TB vaccine, MVA85a (27). RNA was isolated for microarray expression analysis from unstimulated peripheral blood mononuclear cells at indicated timepoints post-vaccination as described in original study. Raw intensity values of IL-32 or IFN-γ mRNA shown, red line indicates mean value.

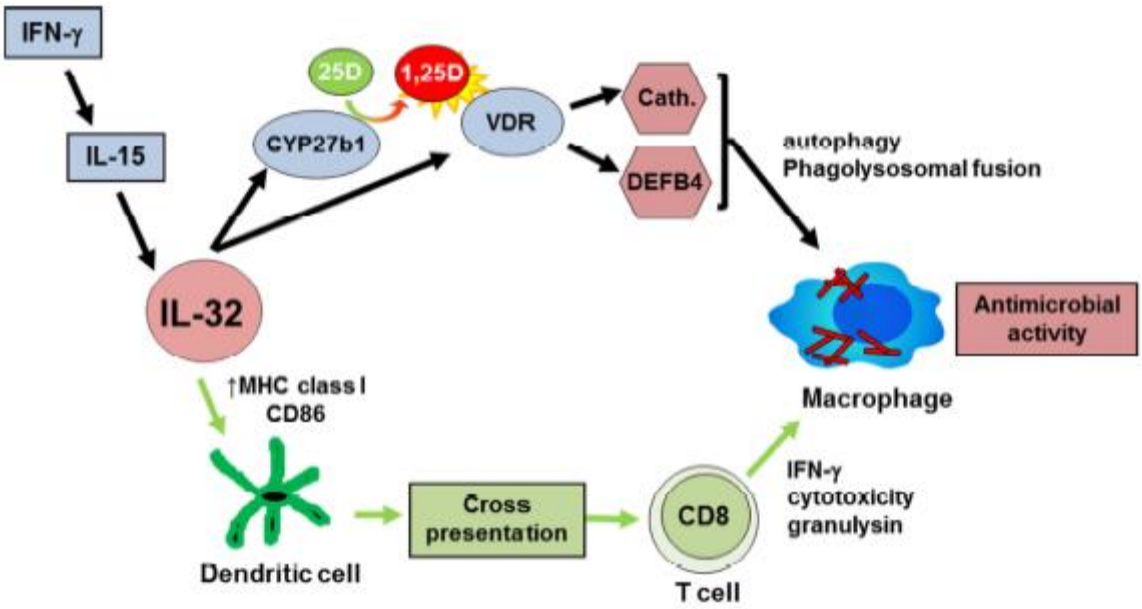

Figure S8. Role of IL-32 in host defense. This model shows the IL-15 dependent induction of IL-32 by IFN-γ leads to dual pathways in which IL-32 can enhance antimicrobial activity of macrophages infected with *M. tuberculosis*. The current study demonstrates IL-32 can directly induce the vitamin D-dependent antimicrobial pathway, while previous studies have shown IL-32 triggers differentiation of dendritic cells that can cross-present to activate $CD8^+$ T cells which can also activate an antimicrobial response against *M. tuberculosis*.

Defense response hub genes	Module eigengene based connectivity (kME)
CCR7	0.936
TRAC	0.932
IL32	**0.930**
IDO1	**0.923**
GATA3	0.902

Table S1. Top hub genes of *IL15black* defense response network. Hub genes as defined by intramodular connectivity (kME > 0.90). *IL32* and *IDO1* are expressed in myeloid cells as depicted in cell-type specific scoring analysis (Fig. 1C).

Gene	Induction by IL-15 (FC)	Topological Overlap to IL-32
CYP27B1	36.4	0.702
CCL13	29.3	0.691
IDO1	21.6	0.717
CCL22	9.8	0.689
CD40	8.3	0.711
PPBP	7.6	0.691
CFB	5.5	0.699
CCL8	5.1	0.713
APOL1	4.4	0.702
TAP2	3.6	0.691
CCL24	2.9	0.684
NDST1	2.9	0.687
TAP1	2.9	0.706
IL15	2.6	0.688
APOBEC3G	2.4	0.726
TAPBP	2.3	0.712
IFIH1	2.3	0.691
CXCL10	2.0	0.695
C3	1.6	0.699
PLA2G7	1.5	0.685
CYBB	1.4	0.717
NMI	1.3	0.732

Table S2. Myeloid genes correlated with IL-32 in the IL-15 defense response functional cluster. Gene induction as measured by Affymetrix Human U133 Plus 2.0 expression array by shown as fold-change (FC) over media at 24 h from IL-15 stimulated adherent peripheral blood mononuclear cells. Topological overlap (connectivity) to *IL32* for each myeloid gene in the IL-15 induced 'defense response' network as determined by weighted gene co-expression analysis

ID	Country of cohort	Latent TB	Active TB	Post-treatment active TB	Healthy controls	Sarcoidosis	Other diseases[1]	Study author	Microarray platform	GEO accession
UK '10	United Kingdom	17	13	-	12	-	-	Berry et al.	Illumina Human HT-12 V3.0	GSE19439
SA '10	South Africa	31	20	-	-	-	-	Berry et al.	Illumina Human HT-12 V3.0	GSE19442
GER '12	Germany	4[2]	9	-	13	18	-	Maertzdorf et al.	Agilent-014850	GSE34608
SA '12	South Africa	38	29	29	-	-	-	Bloom et al.	Illumina Human HT-12 V4.0	GSE40553
KAF '13	Malawi/South Africa	83	97	-	-	-	83	Kaforou et al.	Illumina Human HT-12 V4.0	GSE37250
Total patients:		**173**	**168**	**29**	**25**	**18**	**83**			

[1]Other diseases of which TB is differential diagnosis
[2]IGRA+ patients reclassified as latent TB from healthy control

Table S3. TB data sets used in this study.

CHAPTER 3

Suppression of iron-regulatory hepcidin by vitamin D

BASIC RESEARCH www.jasn.org

Suppression of Iron-Regulatory Hepcidin by Vitamin D

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ABSTRACT

The antibacterial protein hepcidin regulates the absorption, tissue distribution, and extracellular concentration of iron by suppressing ferroportin-mediated export of cellular iron. In CKD, elevated hepcidin and vitamin D deficiency are associated with anemia. Therefore, we explored a possible role for vitamin D in iron homeostasis. Treatment of cultured hepatocytes or monocytes with prohormone 25-hydroxyvitamin D or active 1,25-dihydroxyvitamin D decreased expression of hepcidin mRNA by 0.5-fold, contrasting the stimulatory effect of 25-hydroxyvitamin D or 1,25-dihydroxyvitamin D on related antibacterial proteins such as cathelicidin. Promoter-reporter and chromatin immunoprecipitation analyses indicated that direct transcriptional suppression of hepcidin gene (*HAMP*) expression mediated by 1,25-dihydroxyvitamin D binding to the vitamin D receptor caused the decrease in hepcidin mRNA levels. Suppression of *HAMP* expression was associated with a concomitant increase in expression of the cellular target for hepcidin, ferroportin protein, and decreased expression of the intracellular iron marker ferritin. In a pilot study with healthy volunteers, supplementation with a single oral dose of vitamin D (100,000 IU vitamin D_2) increased serum levels of 25D-hydroxyvitamin D from 27±2 ng/ml before supplementation to 44±3 ng/ml after supplementation ($P<0.001$). This response was associated with a 34% decrease in circulating levels of hepcidin within 24 hours of vitamin D supplementation ($P<0.05$). These data show that vitamin D is a potent regulator of the hepcidin-ferroportin axis in humans and highlight a potential new strategy for the management of anemia in patients with low vitamin D and/or CKD.

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Patients with CKD require iron supplementation and erythropoiesis stimulating agents (ESAs) to correct disease-associated anemia.[1] However, ESA hyporesponsiveness is common, with the iron homeostasis factor hepcidin (encoded by the *HAMP* gene) emerging as a possible culprit.[2] Hepcidin post-translationally suppresses membrane expression of ferroportin, the only known exporter of intracellular iron.[3] Elevated plasma hepcidin, common to patients with CKD[4] or inflammation,[5] causes intracellular sequestration of iron and increases risk of anemia. By contrast, patients with hemochromatosis or iron deficiency exhibit decreased hepcidin.[6]

Studies of patients with CKD suggest that vitamin D status (serum concentrations of the prohormone 25-hydroxyvitamin D [25D]) correlates inversely with the prevalence of anemia[7] and ESA resistance[8] and directly with blood hemoglobin levels.[8] In hemodialysis patients with anemia, vitamin D repletion has been shown to correlate with lower ESA requirements.[9,10] Vitamin D is known to exert physiologic activities beyond its classic skeletal function, notably as a potent

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inducer of antimicrobial proteins such as cathelicidin antibacterial protein (encoded by the cathelicidin [*CAMP*] gene).[11,12] In this respect, it is interesting that hepcidin was initially described as an antimicrobial peptide (encoded by the gene for hepcidin antibacterial protein, *HAMP*),[13] with its role in iron homeostasis being a later observation. We therefore hypothesized that vitamin D can act to regulate expression of hepcidin, in a similar fashion to its effects on other antimicrobial proteins. To test this hypothesis, vitamin D–mediated changes in hepcidin and cathelicidin were compared using *in vitro* and *in vivo* models.

RESULTS

Vitamin D Metabolites Suppress Expression of *HAMP*

Studies *in vitro* using PBMC monocytes, THP1 cells, and HepG2 cells showed that treatment with 25D (100 nM) or 1,25-dihydroxyvitamin D (1,25D) (5 nM) for 6 hours decreased expression of mRNA for *HAMP* (Figure 1A). In PBMC monocytes and THP1 cells, this response contrasted the effect of 25D and 1,25D in stimulating expression of mRNA for antibacterial *CAMP* (Figure 1B) and the vitamin D catabolic enzyme *CYP24A1* (Figure 1C). In HepG2 cells, treatment with 25D or 1,25D appeared to have no effect on expression of *CAMP*, and 1,25D induced only a small increase in mRNA for *CYP24A1*. Additional experiments with PBMC monocytes showed that suppression of mRNA for *HAMP* was also observed after 24-hour treatments with 100 nM 25D (0.57-fold±0.21, $n=3$; $P<0.05$) or 1,25D (0.27-fold±0.36, $n=3$; $P<0.01$).

To determine whether vitamin D–mediated suppression of hepcidin also occurs in nonhuman models, further studies were carried out *in vitro* using mouse monocytes. Peripheral blood-derived monocytes from wild-type C57BL/6 mice showed no change in mouse hepcidin (*Hamp*) gene expression after 24-hour treatment with increasing doses of 1,25D (Supplemental Figure 1A). Similar results were also observed for the mouse monocyte cell line J774 after 6-hour treatment with 25D (100 nM) or 1,25D (10 nM) (Supplemental Figure 1B). To assess possible effects of vitamin D on hepatic expression of *Hamp in vivo*, 12-week-old C57BL/6 male mice were placed on a vitamin D–deficient diet for 6 weeks and then transferred to a 4 ppm iron diet for 1 week. Groups of mice ($n=4$ in each case) were then treated with intraperitoneal injections of either 0.2 μg/g body weight 25D, 1 μg/g 25D, or 0.2 μg/g 1,25D. A similar volume of intraperitoneal saline was used as a control. Analysis of liver mRNA from these mice 24 hours after treatment showed no effect on expression of *Hamp* (Supplemental Figure 1C).

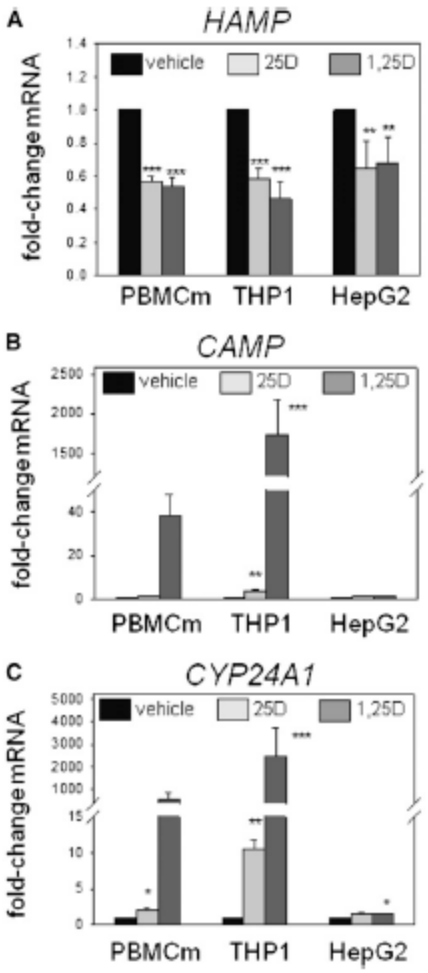

Figure 1. Vitamin D suppresses expression of hepcidin (*HAMP*) in human monocytes and hepatocytes. Effect of *in vitro* treatment of PBMC monocytes (PBMCm), monocytic THP1 cells, and HepG2 hepatocytic cells with vehicle, 25D (100 nM), or 1,25D (5 nM) for 6 hours on expression of mRNA for *HAMP* (A), *CAMP* (B), and *CYP24A1* (C). Data are shown as mean fold-change in gene expression (±SD) relative to vehicle (0.1% ethanol) controls. *$P<0.05$; **$P<0.01$; ***$P<0.001$, statistically different from vehicle-treated cells. For RT-PCR data, $n=8$ separate donors for PBMC monocytes, $n=4$ separate cultures of THP1 cells, and $n=5$ separate cultures of HepG2 cells.

Vitamin D Receptor–Mediated Transcriptional Repression of *HAMP*

In human cells, the suppression of *HAMP* expression by 1,25D or 25D appears to be due to direct inhibition of *HAMP* transcription. *In silico* analyses identified consensus vitamin D response elements (VDREs) within a 1071-bp *HAMP* proximal promoter DNA sequence (Supplemental Table 1). As shown in Figure 2A, chromatin immunoprecipitation (ChIP) assays using PBMC monocyte extracts demonstrated binding of vitamin D receptor (VDR) protein to DNA from a 1-kb fragment of the *HAMP* proximal promoter that includes the VDREs originally identified in Supplemental Table 1. Further ChIP analyses using extracts from the same cell type demonstrated similar VDR binding to promoter fragments for known VDR target genes such as *CAMP*

A

Treatment	Enrichment	*CYP24A1* -1Kb	*CYP24A1* -2Kb	*CAMP* -1Kb	*HAMP* -1Kb	*IGX1A* (-VE)
Vehicle	RNA Pol II	116.6	72.0	229.9	747.9	24.4
1,25D	RNA Pol II	1418.4	407.3	636.9	660.2	23.2
Fold-change		**12.2**	**5.7**	**2.8**	**0.88**	**0.95**
Vehicle	VDR	41.4	33.9	108.4	272.5	7.62
1,25D	VDR	407.3	105.1	161.5	130.7	5.68
Fold-change		**9.8**	**3.1**	**1.5**	**0.48**	**0.75**

B

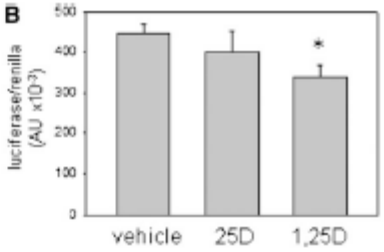

Figure 2. VDR-mediated suppression of hepcidin (*HAMP*) gene expression by 1,25D. (A) ChIP analysis of VDR and RNA Pol II interaction with the *HAMP* gene promoter. PBMC monocytes are treated with 1,25D (5 nM, 24 hours), chromatin extracts are prepared, and ChIP-grade antibodies are used to detect VDR and RNA Pol II interactions. The resulting enriched genomic DNA is qPCR amplified using primers for *CYP24A1* (−1 kb from the transcription start site and −2 kb from the transcription start site), *CAMP* (−1 kb from the transcription start site), *HAMP* (−1 kb from the transcription start site). A negative control sequence for VDR and RNA Pol II (*IGX1A*) is also used. Data are shown first as mean arbitrary units for PCR amplification of DNA associated with RNA Pol II or VDR in cells treated with vehicle or 1,25D (mean of two separate chromatin preparations). In addition, fold-change values are presented showing the change in RNA Pol II or VDR binding to DNA after treatment with 1,25D relative to vehicle-treated cells. (B) Effect of 25D (100 nM) and 1,25D (5 nM) on *HAMP* promoter-reporter activity in VDR-expressing MC3T3 mouse osteoblastic cells. Data are shown as *HAMP* target gene firefly/*Renilla* housekeeping luciferase activity ($\times 10^{-3}$). *$P<0.05$, statistically different from vehicle-treated cells.

and *CYP24A1*. Treatment of PBMCs with 1,25D enhanced VDR enrichment on the *CYP24A1* and *CAMP* promoters (Figure 2A), consistent with the transcriptional induction of these genes by 1,25D (Figure 1, B and C). By contrast, VDR enrichment decreased 0.5-fold for the *HAMP* promoter after treatment with 1,25D. A similar differential promoter response to 1,25D was also observed for ChIP analysis of RNA polymerase II (RNA Pol II), which is essential for gene transcription. Further analysis of the effects of vitamin D on *HAMP* gene expression using a luciferase promoter-reporter construct transfected into VDR-expressing MC3T3 cells showed that treatment with 1,25D produced a 24% decrease in transcription relative to vehicle-treated cells (Figure 2B). However, in the absence of *CYP27B1* expression/1α-hydroxylase activity in MC3T3 cells, treatment with 25D was without effect.

Vitamin D–Induced Suppression of HAMP Is Associated with Changes in Ferroportin and Ferritin Expression

In contrast to the suppression of *HAMP* in human monocytes or hepatocytes, 25D and 1,25D had no effect on levels of mRNA for *ferroportin* in PBMC monocytes, THP1 cells, or HepG2 cells (Figure 3A). However, Western blot and immunohistochemical analyses showed that treatment with 25D or 1,25D increased expression of ferroportin protein in hepatocytes and monocytes (Figure 3, B and C), suggesting a post-transcriptional mode of action for the effects of vitamin D metabolites on ferroportin. To assess the functional effect of vitamin D–mediated induction of ferroportin, further studies were carried out to determine expression of ferritin, an established marker of intracellular iron concentrations. Treatment with 25D or 1,25D decreased expression of mRNA for *ferritin* in PBMC monocytes, THP1 cells, and HepG2 cells (Figure 3D). Immunohistochemical analysis of ferritin protein in HepG2 cells confirmed that treatment with 25D or 1,25D also decreased expression of ferritin protein (Figure 3E).

Effect of Vitamin D Supplementation on Circulating Hepcidin in Healthy Volunteers

To assess the *in vivo* effect of vitamin D on hepcidin, a single-arm supplementation study was performed in seven healthy volunteers receiving a single oral dose of vitamin D_2 (ergocalciferol, 100,000 IU). Analysis of serum samples before and after supplementation showed that circulating levels of 25D increased from 27.0±2 ng/ml (67.5±5 nM) before supplementation to 43.5±3 ng/ml (108.8±7.5 nM, $P<0.001$) after supplementation (Figure 4A). By contrast, supplementation with vitamin D had no effect on circulating levels of active 1,25D (Figure 4B). Analysis of circulating hepcidin levels by ELISA showed no significant variation in serum hepcidin between baseline samples. However, after vitamin D supplementation, circulating hepcidin levels decreased by 34% at 24 hours after supplementation ($P<0.05$) and 33% at 72 hours after supplementation ($P<0.01$) (Figure 4C). First, analysis of serum ferritin concentrations showed that baseline (−24 hours) data for all the healthy volunteers were within normal ranges (18–160 ng/ml for female volunteers and 18–2710 ng/ml for male volunteers). Second, supplementation with vitamin D resulted in a small (10%) but significant ($P=0.04$) decrease in serum ferritin after vitamin D supplementation (138.83±25.03 ng/ml at −24 hours versus 124.50±21.70 ng/ml at 72 hours). No statistically significant changes in serum iron concentration, iron binding capacity, or transferrin saturation were observed 72 hours after vitamin D supplementation.

In parallel with analysis of hepcidin and ferritin, assays were also carried out to assess circulating concentrations of hormones classically associated with vitamin D function.

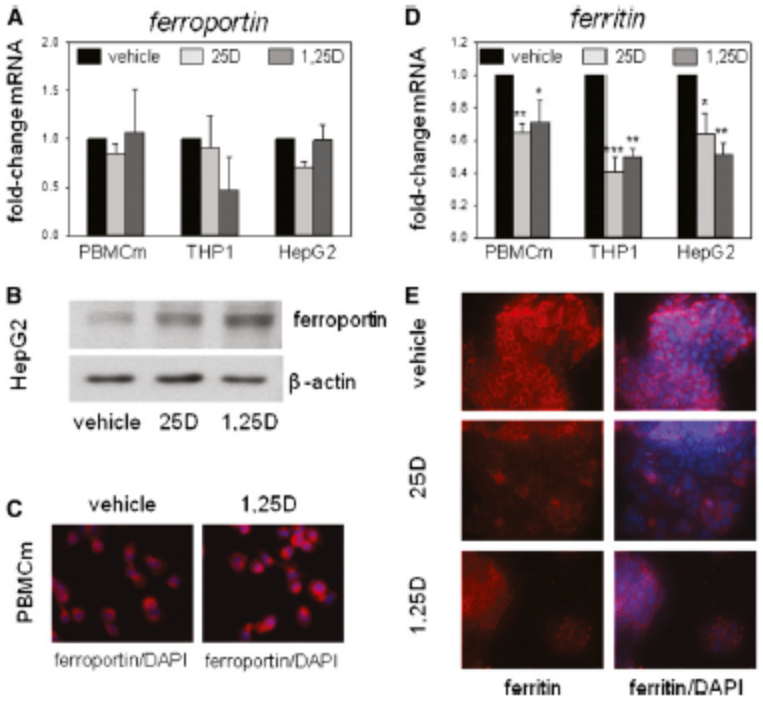

Figure 3. Effect of vitamin D metabolites on ferroportin and ferritin expression in human monocytes and hepatocytes. (A) Effect of *in vitro* treatment of PBMC monocytes (PBMCm), monocytic THP1 cells, and HepG2 hepatocytic cells with vehicle, 25D (100 nM) or 1,25D (5 nM) for 6 hours on ferroportin mRNA expression. Data are shown as mean fold-change in gene expression (±SD relative to vehicle [0.1% ethanol] controls). (B) Western blot analysis of protein for ferroportin protein in HepG2 cells treated with vehicle, 25D (100 nM), or 1,25D (5 nM) for 24 hours. Loading is normalized by analysis of the housekeeping protein β-actin. (C) Immunohistochemical analysis of ferroportin protein in PBMC monocytes after treatment with vehicle or 1,25D (5 nM) for 24 hours. Ferroportin protein is shown in red with 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shown in blue. (D) Effect of *in vitro* treatment of PBMC monocytes, THP1 cells, and HepG2 cells with vehicle, 25D (100 nM), or 1,25D (5 nM) for 6 hours on ferritin mRNA expression. Data are shown as mean fold-change in gene expression (±SD relative to vehicle controls). (E) Immunohistochemical analysis of ferritin protein in HepG2 cells after treatment with vehicle, 25D (100 nM), or 1,25D (5 nM) for 24 hours. Ferritin protein is shown in red with DAPI staining of nuclei in blue. *$P<0.05$; **$P<0.01$; ***$P<0.001$, statistically different from vehicle-treated cells. For RT-PCR data, $n=8$ separate donors for PBMC monocytes, $n=4$ separate cultures of THP1 cells, and $n=5$ separate cultures of HepG2 cells.

Fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH) concentrations were analyzed for six subjects at −24 hours and +72 hours after vitamin D supplementation. Data indicate that supplementation with vitamin D resulted in a significant increase in serum concentrations of FGF23 (62.57±6.70 RU/ml versus 74.71±9.88 RU/ml; $P=0.03$, two-tailed t test), and phosphate (3.27±0.67 mg/dl versus 3.61±0.33 mg/dl; $P<0.001$, two-tailed t test). Vitamin D supplementation resulted in a trend toward decreased serum PTH concentrations (58.86±6.43 pg/ml versus 51.29±7.47 pg/ml; $P=0.06$, two-tailed t test) but no significant change in serum calcium concentrations (9.19±0.12 mg/dl versus 9.07±0.2 mg/dl; $P=0.36$, two-tailed t test).

DISCUSSION

Data presented in this study show for the first time that vitamin D is a potent regulator of the iron-regulatory protein hepcidin in both monocytes and hepatocytes. ChIP and promoter-reporter assays indicate that this occurs as a consequence of direct transcriptional suppression of the *HAMP* gene proximal promoter by 1,25D bound to its cognate nuclear receptor, VDR. This response contrasts the 1,25D-VDR–mediated induction of related antibacterial proteins such as cathelicidin and β-defensin-2.[14] However, in a similar fashion to cathelicidin,[15] 1,25D-mediated regulation of hepcidin expression appears to occur directly, with liganded VDR binding to a specific VDRE within the *HAMP* gene promoter. Previous studies have shown that transcriptional regulation of *CAMP* by 1,25D-VDR is primate specific,[15,16] indicating that antibacterial responses to vitamin D have evolved relatively recently. It was therefore interesting to note that suppression of *HAMP* by 25D or 1,25D was not observed in murine models, suggesting that vitamin D–mediated regulation of hepcidin may be part of the same evolutionary adaptations observed for other antibacterial proteins.

In all three cell types studied, regulation of *HAMP* was observed after treatment with either active 1,25D or inactive 25D, suggesting an intracrine mode of action similar to that previously described for other antibacterial actions of vitamin D.[13] Intracrine regulation of *HAMP* is endorsed by the fact that PBMC monocytes, THP1 cells, and HepG2 cells express mRNA for the enzyme that catalyzes conversion of 25D to 1,25D, 1α-hydroxylase/CYP27B1, as well as the VDR (data not shown). A similar mode of action has been reported for antibacterial effects of vitamin D in monocytes[11,12] and antiviral responses to vitamin D in human hepatocytes.[17] Intracrine responses to vitamin D appear to be exquisitely sensitive to the availability of substrate 25D,[11,12,18] suggesting that the hepcidin-ferroportin homeostasis system may be influenced by serum vitamin D

(25D) status (Figure 5). This hypothesis is supported by supplementation data from healthy volunteers showing that elevated serum concentrations of 25D (but not 1,25D) after a single oral dose of vitamin D_2 produced a 34% decrease in serum hepcidin concentrations that persisted for 72 hours (Figure 4). Moreover, the regulation of serum hepcidin after vitamin D supplementation *in vivo* was at least as sensitive as more established markers of serum 25D status such as PTH, suggesting that hepcidin may be a useful marker of vitamin D function for future studies.

Data for ferroportin and ferritin expression in vitamin D–treated monocytes and hepatocytes are consistent with the actions of hepcidin in promoting post-transcriptional suppression of ferroportin protein.[19] On the basis of data presented in this study, we speculate that 25D and 1,25D can act to oppose this response and maintain membrane expression of ferroportin (Figure 5). Hepcidin-mediated loss of membrane ferroportin is known to be associated with intracellular retention of iron, leading in turn to iron-restrictive anemia.[5] This has immediate implications for the control of systemic iron homeostasis but will also influence host defense during acute infections. Iron is essential for the survival and growth of almost all organisms, and an important strategy for mammalian antimicrobial defense is based on depriving pathogens of this essential nutrient.[20] Thus, another facet of hepcidin physiology is its contribution to host innate immune function.[3,21] By targeting ferroportin and decreasing extracellular iron, hepcidin appears to play a pivotal role in the so-called "hypoferremia" or "anemia" of infection, in which there is restriction of systemic iron to pathogens.[5,22] Conversely, the resulting accumulation of intracellular iron will promote the growth of internalized pathogens such as *Salmonella typhimurium*,[23] *Mycobacterium tuberculosis*,[24–26] and *Chlamydia psittaci*,[27] and innate immune and viral stimuli are known to stimulate the expression of hepcidin.[25,28] In this setting, the effects of vitamin D in suppressing hepcidin and promoting ferroportin are consistent with its established intracellular antibacterial activity.[13] We therefore hypothesize that regulation of the hepcidin-ferroportin axis is another key facet of vitamin D–mediated innate immune function, complementary to its reported effects on antibacterial proteins,[11,13,29] and autophagy.[30,31] In future studies, it will be interesting to determine the extent to which regulation of monocyte/macrophage iron homeostasis contributes to the generalized antimicrobial actions of vitamin D.

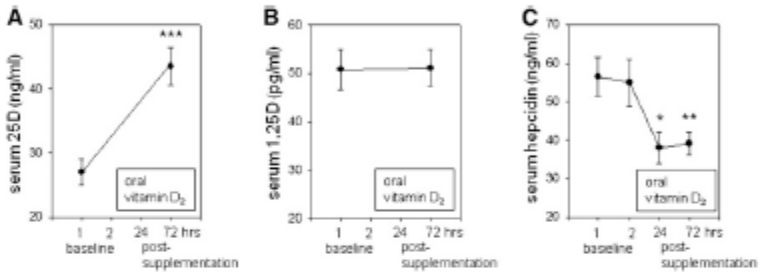

Figure 4. Effects of supplementation with vitamin D_2 on circulating hepcidin levels in healthy humans. A single-arm pharmacokinetic study is performed in seven healthy volunteers (four men; median age 42 years; range, 27–63) to assess changes in serum levels of hepcidin after a single dose of oral vitamin D_2 (100,000 IU). Two blood samples are drawn before supplementation and two are drawn after supplementation. Serum concentrations of 25D (ng/ml) (A), 1,25D (pg/ml) (B), and hepcidin (ng/ml) (C). Data are shown as the mean±SEM. Experimental means were compared statistically using a paired *t* test.*$P<0.05$; **$P<0.01$; ***$P<0.001$, statistically different from baseline values.

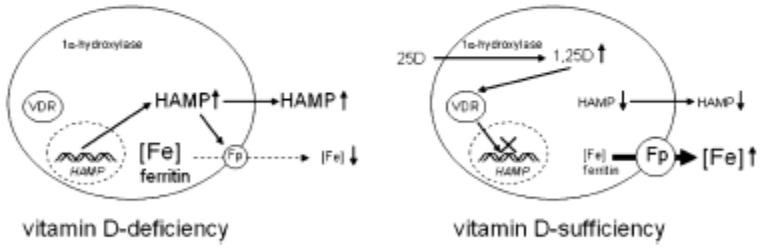

Figure 5. Vitamin D and the hepcidin-ferroportin iron-regulatory axis. Schematic representation of a proposed mechanism for vitamin D regulation of hepcidin/*HAMP*–ferroportin (Fp) interaction in hepatocytes and monocytes. Under conditions of vitamin D deficiency, elevated synthesis of hepcidin by hepatocytes or monocytes may increase intracellular and systemic concentrations of hepcidin and decrease membrane expression of Fp in these cells. The resulting suppression of iron export will, in turn, lead to intracellular accumulation, increased cellular ferritin, and decreased systemic levels of iron. Under conditions of vitamin D sufficiency, decreased transcription of *HAMP* may lead to decreased intracellular and systemic concentrations of hepcidin and concomitant increased membrane expression of Fp. The resulting enhancement of iron export may then lead to decreased intracellular iron and ferritin and increased systemic levels of iron.

Hepcidin was originally identified as an antibacterial protein, but its potential as a clinical target stems primarily from its role in the anemia of inflammation. Unlike dietary iron restriction, anemia of inflammation involves intracellular retention of iron by monocytes and macrophages,[32] and is thus intimately linked to aberrant hepcidin activity and associated dysregulation of membrane ferroportin function.[33] Although this is a problem that is common to many chronic diseases,[34] it is likely to be particularly important in patients with

CKD where elevated hepcidin levels may be a crucial factor in the development of multiple pathologic complications associated with renal impairment.[2] Consequently, targeting of hepcidin has been proposed as an alternative to supplementary iron and/or ESAs as therapy for the anemia associated with CKD.[35] Potential strategies for suppression of hepcidin include direct effects of antibodies to hepcidin,[36] hepcidin-binding oligoribonucleotides,[37] and inhibitors of hepcidin expression,[38] as well as indirect targeting *via* anti-inflammatory responses.[39] However, we believe that this is the first demonstration of rapid direct suppression of hepcidin in humans. High-dose testosterone has also been shown to suppress hepcidin, but effects were only evident after 1 week of therapy.[40] It was also interesting to note that the suppressive effect of vitamin D supplementation *in vivo* on serum hepcidin concentrations was accompanied by decreased levels of serum ferritin. The precise significance of this remains unclear but may reflect a generalized anti-inflammatory response to elevated serum levels of 25D.

The effect of vitamin D on the hepcidin-ferroportin axis also suggests that low vitamin D status may be a contributing factor to the anemia of chronic disease. CKD is characterized by impaired vitamin D status, which is closely associated with adverse CKD health outcomes.[41] In CKD patients, low serum levels of 25D correlate inversely with the prevalence of anemia[7] and ESA resistance,[8] and correlate directly with blood hemoglobin levels.[8] Data presented in this study suggest that these observations are linked by the effects of vitamin D on the hepcidin-ferroportin axis. In hemodialysis patients with anemia, vitamin D repletion has been shown to correlate with lower ESA requirements.[7,9] We therefore propose that by acting to suppress expression of hepcidin in hepatocytes and monocytes, simple vitamin D supplementation may provide a cost-effective and safe adjuvant therapy for managing the anemia associated with this disease.

CONCISE METHODS

Isolation, Treatment, and Culture of Cells

Human hepatocellular carcinoma HepG2 cells (American Type Culture Collection [ATCC], Manassas, VA) were cultured in DMEM with 10% FBS. Human THP1 monocytic cells (ATCC) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) and 10% FBS. Mouse J774A macrophages (ATCC) were cultured in RPMI 1640 medium and 10% FBS. Ficoll-isolated PBMCs derived from anonymous healthy donors (screened in accordance with standard transfusion medicine protocols) were obtained from the Center for AIDS Research Virology Core/BSL3 Facility (supported by National Institutes of Health award AI-28697 and by the University of California Los Angeles [UCLA] AIDS Institute and the UCLA Council of Bioscience Resources). PBMC monocytes were isolated as previously described.[42] PBMC monocytes were seeded in RPMI 1640 (Invitrogen) with 10% human AB serum (Omega Scientific, Tarzana, CA) and GM-CSF (10 IU/ml; PeproTech, Inc., Rocky Hill, NJ). MC3T3-E1 (MC3T3) murine osteoblastic cells (ATCC) were maintained in α-MEM plus 10% FBS before transfection with promoter-reporter constructs. MC3T3 cells were cultured to 50% confluence before transfection with luciferase firefly or *Renilla* constructs. For all cell types, *in vitro* treatments at 37°C and 5% CO_2 included 25D (100 nM) and 1,25D (5 nM), with 0.1% ethanol as vehicle.

Animals

All of the animals used in these studies were subject to recommendations for animal use and welfare outlined by the UCLA Division of Laboratory Animal Medicine, as well as guidelines from the National Institutes of Health. The UCLA Animal Research Committee approved the protocol (no. 2012-024-02A) for the use of mice in our study. Twelve-week-old male C57BL/6 mice were placed on a vitamin D–deficient diet (Research Diets, Inc., New Brunswick, NJ) for 6 weeks then transferred to a 4 ppm iron diet for 1 week. Groups of mice (n=4 in each case) received the following intraperitoneal injections: 0.2 μg/g of 25D3 (Enzo Life Sciences, Farmingdale, NY), 1 μg/g 25D, 0.2 μg/g 1,25D (Enzo Life Sciences), or a similar volume of saline. In each case, mice were euthanized 24 hours after treatment and RNA were extracted from livers as previously described.[43]

Prospective Vitamin D Supplementation Pilot Study

A single-arm pharmacokinetic study was performed in seven healthy volunteers to examine the change in hepcidin serum levels, assessed by competitive ELISA (Intrinsic Life Sciences, La Jolla, CA) after a single dose of oral vitamin D_2 (100,000 IU). For serum 25D, a rapid, direct RIA developed in the laboratory of Dr. Hollis and manufactured by Diasorin Corporation (Stillwater, MN) was used.[44] An RIA manufactured by Diasorin Corporation was used to measure total circulating 1,25D concentrations.[45] Circulating levels of PTH and FGF23 were measured with an RIA kit, a first-generation immunometric assay (normal range, 10–65 pg/ml; Immutopics, San Clemente, CA), and a second-generation C-terminal kit (Immutopics), respectively. Two samples were drawn before supplementation (so that each subject acted as its own control) and two samples were drawn after supplementation (24 and 72 hours). All biologic samples were obtained at 8 AM after an overnight fast. This human study was approved by the UCLA Human Subjects Protection Committee, and consent was obtained from all subjects.

Extraction of RNA and Quantitative RT-PCR

Adherent cell cultures or centrifuged pellets of PBMC monocytes, THP1 cells, or HepG2 cells were initially lysed with 1 ml RNAzol. Each sample was then transferred to Eppendorf tubes and RNA was extracted by adding 0.2 ml chloroform in a fume cabinet, and then vortexing samples for >5 seconds followed by centrifugation at 12,000×g for 15 minutes at 4°C. The aqueous phase was collected for each sample and mixed with 1 μl glycogen and 0.5 ml isopropanol. Samples were then vortexed and centrifuged again as described above. All fluid was then discarded and the resulting RNA pellet dried in the Eppendorf tube. The resulting pellet was then washed with 1 ml 70% ethanol and vortexed and centrifuged again, followed by further air drying of the resulting pellet. Finally, RNA pellets were resuspended

in ≈14 μl of RNase free water and stored at −20°C after RNA quantification and quality assessment (260/280 ratio; Nanodrop).

Aliquots (300 ng) of resuspended RNA were reverse-transcribed using the SuperScript III RT enzyme as recommended by the manufacturer (Invitrogen) and as previously described.[12] Quantitative real-time RT-PCR was performed in a Stratagene cycler (La Jolla, CA), using TaqMan probes and primers (Applied Biosystems, Foster City, CA).[12,46] Expression of mRNA for the VDR (*VDR*) (Hs001721113_m1), the vitamin D–activating enzyme 1α-hydroxylase (*CYP27B1*) (Hs00168017_m1), the vitamin D catabolic enzyme 24-hydroxylase (*CYP24A1*) (Hs00167999_m1), *HAMP* (Hs00221783_m1), ferritin (Hs00830226_gH), ferroportin (Hs00205888_m1), *CAMP* (Hs00189038_m1), and mouse hepcidin (*Hamp*) (Mm00842044_g1) was quantified using TaqMan human gene expression assays, as previously described.[12] All reactions were amplified under the following conditions: 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. Data were obtained as ΔCt values (the difference between Ct of the target gene and Ct of the housekeeping 18S rRNA gene). For visual representation, ΔCt data for each treatment were converted to fold-change relative to ΔCt for control vehicle-treated cells (ΔΔCt) using the equation $2^{-\Delta\Delta Ct}$.

Western Blot and Immunofluorescence Analyses

Expression of ferroportin protein was assessed by Western blot analyses using previously reported protocols,[46] involving overnight incubation with primary ferroportin antibody (1/1000; Amgen, Thousand Oaks, CA). A β-actin antibody (Sigma-Aldrich, St. Louis, MO) was used as a loading control. Immunofluorescence analysis of ferroportin and ferritin was carried out using adaptations of previously described methods.[46] Briefly, monocytes or hepatocytes were seeded on 4-well glass slides, and incubated for 1 hour with antibodies to ferroportin (1/200; Amgen) or ferritin (1/200; Abcam). Secondary antibodies labeled with Alexa 594 were applied for 1 hour, and finalized with 4′,6-diamidino-2-phenylindole (1/10,000, 5 minutes).

ChIP-qPCR Analyses of the Hepcidin Promoter

To predict potential *HAMP* promoter chromatin binding sites for the VDR protein, the NubiScan program (www.nubiscan.unibas.ch) was used. A −1071-bp *HAMP* gene promoter sequence was analyzed using the general weighted matrix for nuclear receptor half-sites (including the VDR canonical half-sites), enabling direct-repeat 3 sites characteristic of VDR to be acquisitioned. For the search parameter, an automatic scan with a raw score threshold of 0.5 was used, where the optimal match would have a raw score of 1.

To assess physical binding of the VDR and associated transcriptional machinery to the *HAMP* gene promoter, ChIP was carried out. PBMC monocytes were treated with 1,25D (5 nM, 24 hours) and chromatin extracts prepared as previously described.[47] ChIP-qPCR assays were performed using the ChIP-IT Express kit (Active Motif) and the ChampionChIP kit (SABiosciences, Inc., Valencia, CA). ChIP-grade antibodies were used to detect VDR (sc-13133) and RNA polymerase II (SABiosciences, Inc.) interactions. The resulting enriched genomic DNA was purified (QIAquick kit; Qiagen, Inc., Valencia, CA) and measured by qPCR using the EpiTect ChIP-qPCR Primer Assay for *CYP24A1* (GPH021775(−)01A (−1 kb from the transcription start site) and GPH021775(−)02A (−2 kb from the transcription start site), for *CAMP* (NM_004345.3, −1 kb: GPH1009280(−)01A), for *HAMP* (NM_021175.2, −1 kb: GPH1006776(−)01A), and for the negative control sequence *IGX1A* (all Qiagen). The qPCR program was 10 minutes at 95°C, followed by 15 seconds at 95°C and 1 minute at 60°C for 40 cycles. A dissociation curve analysis was run to monitor the specificity of amplification and lack of primer dimers. Data were shown first as arbitrary units for qPCR amplification of DNA associated with RNA Pol II or VDR in cells treated with vehicle or 1,25D. In addition, fold-change values were calculated to show the change in RNA Pol II or VDR binding to DNA after treatment with 1,25D.

Luciferase Reporter Analysis of Vitamin D–Mediated Regulation of the *HAMP* Promoter

A pGL4.17 firefly luciferase reporter vector containing the entire proximal promoter (2997 bp) DNA sequence was used for analysis of 1,25D/25D-regulated *HAMP* transcription using previously reported protocols.[48] All transient transfections were performed using Lipofectamine 2000 Reagent (Invitrogen) at a ratio of 1:1 (total DNA to lipofectamine) according to the manufacturer's recommendations. Briefly, cells were seeded into 96-well plates at a density of 2×10^4 cells per well 24 hours before transfection. Each transfection was performed using 200 ng *HAMP* construct cotransfected with 50 ng *Renilla* luciferase plasmid to normalize transfection efficiency. Transfection recipient MC3T3 cells, which express *VDR* but do not express *CYP27B1*, were treated with vehicle (<0.2% ethanol), 25D (100 nM), or 1,25D (10 nM) 24 hours before harvest with 30 μl Passive Lysis Buffer (Promega, Fitchburg, WI). Luciferase activity in cell lysates was assessed using the Dual Luciferase assay kit (Promega) according to the manufacturer's protocol and measured using a FLUOstar Omega instrument (BMG Labtech, Cary, NC). Specifically, 20 μl of each sample was transferred into a white 96-well plate. The FLUOstar was programmed to inject 90 μl Luciferase Assay Reagent II followed by 90 μl Stop & Glo. Transcriptional activity was expressed as luciferase activity relative to *Renilla* activity. Each treatment was performed in triplicate and repeated on at least three separate occasions. Results were expressed as fold-change over untreated cells by dividing the firefly/*Renilla* ratio of treated cells by the firefly/*Renilla* ratio of untreated cells.

Statistical Analyses

RT-PCR data for *in vitro* and *ex vivo* studies were compared statistically using an unpaired *t* test. Where indicated, multifactorial data involving 25D/1,25D treatments were compared using one-way ANOVA with the Holm–Sidak method used as a *post hoc* multiple comparison procedure. Statistical analyses were carried out using raw ΔCt values and fold-changes. Spearman correlation test was used for bivariate analyses.

For the vitamin D supplementation in healthy volunteers study, clinical data are presented as the mean ± SD for variables with normal distributions, or median (range) for variables with skewed distribution. A paired *t* test was used to compare baseline samples, and

baseline 1 with post-treatment samples. All statistical tests were performed at the two-sided 0.05 level of significance. Analyses were performed using the SPSS software (version 19.0; SPSS, Inc., Chicago, IL) for Windows.

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DISCLOSURES

M.W. is a stockholder and executive officer of Intrinsic LifeSciences and has received honoraria from Janssen Research and Development. In addition, M.W. is a developer of the hepcidin assay used herein and holds United States patents involving hepcidin C-ELISA compositions and methods.

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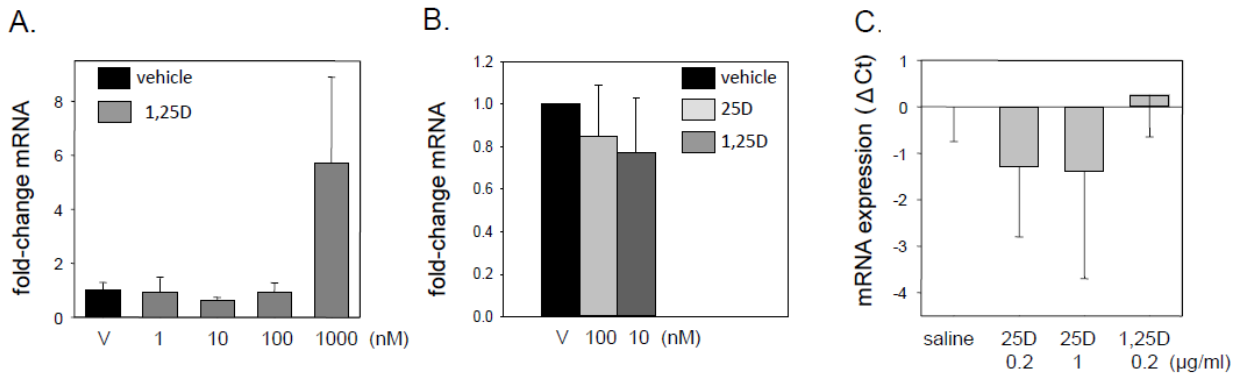
Supplemental Table 1. In silico nuclear receptor site prediction for vitamin D response elements in the *HAMP* gene promoter (-1071 bp) For general nuclear receptor target prediction the NubiScan program (www.nubiscan.unibas.ch) was used. A -1071bp *HAMP* gene promoter sequence was analyzed using the general weighted matrix for nuclear receptor halfsites (including the VDR canonical halfsites), whereby direct-repeat 3 sites were acquisitioned. For the search parameter, an automatic scan with a raw score threshold of 0.5 was used, where the optimal match would have a raw score of 1.

SEQUENCE LENGTH	POSITION	STRAND	RAW SCORE	P VALUE	SITE SEQUENCE
1071	72	-	0.602588	0.132854	AGTCCGatgAGTACA
1071	952	+	0.596919	0.259562	AGGGGAgggGGCTCA
1071	422	-	0.564744	0.580772	TGGCCAtaaATGACA
1071	903	-	0.564162	0.457681	AGATAAgcgGGAACA
1071	649	+	0.526702	0.868162	TGTGCAtgtAGGCGA
1071	193	-	0.525093	0.687983	AGCCCAggaGGCTGA
1071	10	+	0.507491	0.791509	GGCTGAgttGGTGCA



Supplemental Figure 1. **Effect of vitamin D on expression of hepcidin in mice**. S1A. Peripheral blood-derived monocytes from wild type C57BL/6 mice showed no change in mouse hepcidin (*Hamp*) gene expression following 24 hr treatment with increasing doses of 1,25D (1-100 nM). S1B. Similar results were also observed for the mouse monocyte cell line J774 following 6 hr treatment with 25D (100 nM) or 1,25D (10 nM) . S1C. To assess possible effects of vitamin D on hepatic expression of *Hamp* in vivo, 12 wk old C57BL/6 male mice were placed on a vitamin D-deficient diet for 6 weeks then transferred to a 4 parts per million (ppm) iron diet for one week. Groups of mice (n=4 in each case) were then treated with either 0.2 μg/g body weight of 25D by intraperitoneal (IP) injection, 1 μg/g 25D IP, 0.2 μg/g of 1,25D IP. A similar volume of saline IP (saline) was used as a control. Analysis of liver mRNA in these mice 24 hours after treatment showed no effect on expression of *Hamp*.