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

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SUPPLEMENTARY MATERIALS

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Fig. S1. Expression of IL-32 in adherent PBMC microarray.
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Fig. S3. IL-32 induction of CYP27b1 is dose-dependent.
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Author contributions: D.M. conceived, designed, and performed the experiments and wrote the paper. M.S.I. performed computational data analysis. P.T.L. performed virulent TB experiments. S.R. helped to perform siRNA knockdown studies. R.M.B.T. helped to interpret TB data. P.V. and M.A.M. performed the experiments. M.S. provided general advice. W.R.S. provided cell type–specific scoring tool. R.C., K.Z., M.H., and J.S.A. performed metabolism experiments. S.H. aided analysis of WGCNA data. B.R.B. provided supervisory support and wrote the paper. M.P. and R.L.M. provided supervisory report, designed the experiments, and wrote the paper.

Competing interests: U.S. provisional patent “Use of biomarker of protection against TB as a therapeutic and diagnostic marker” has been filed serial no. 61/974,258. The other authors declare no competing interest.

Data and materials availability: Macrophage gene expression files containing array data are available under the accession no. GSE59184 in the Gene Expression Omnibus (GEO) database.

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Abstract

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 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.

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 *e tal*. [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 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 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 phagolysosmal 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 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 exportNetworkToVisANTQ 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 *735g* 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 16S 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 16S ribosomal RNA and genomic element DNA levels were assessed from the cDNA and DNA using quantitative PCR (qPCR), and the relative 16S 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 16S 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 PCRMaster 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-01598–800–0005) 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 Material

Refer to Web version on PubMed Central for supplementary material.

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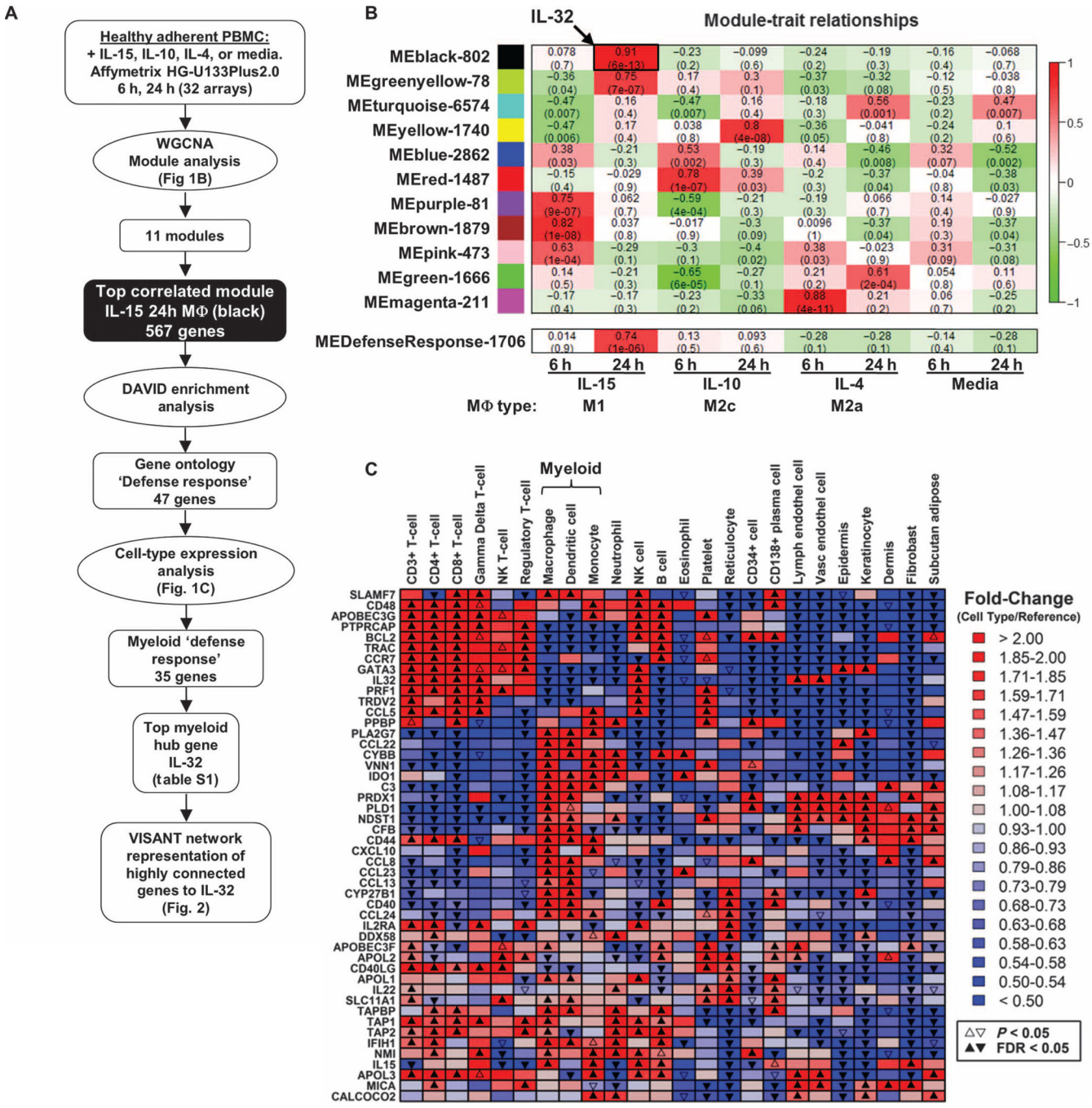

Fig. 1. Identification of IL-32–correlated gene module during differentiation of Ml macrophages

(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 Ml, 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.

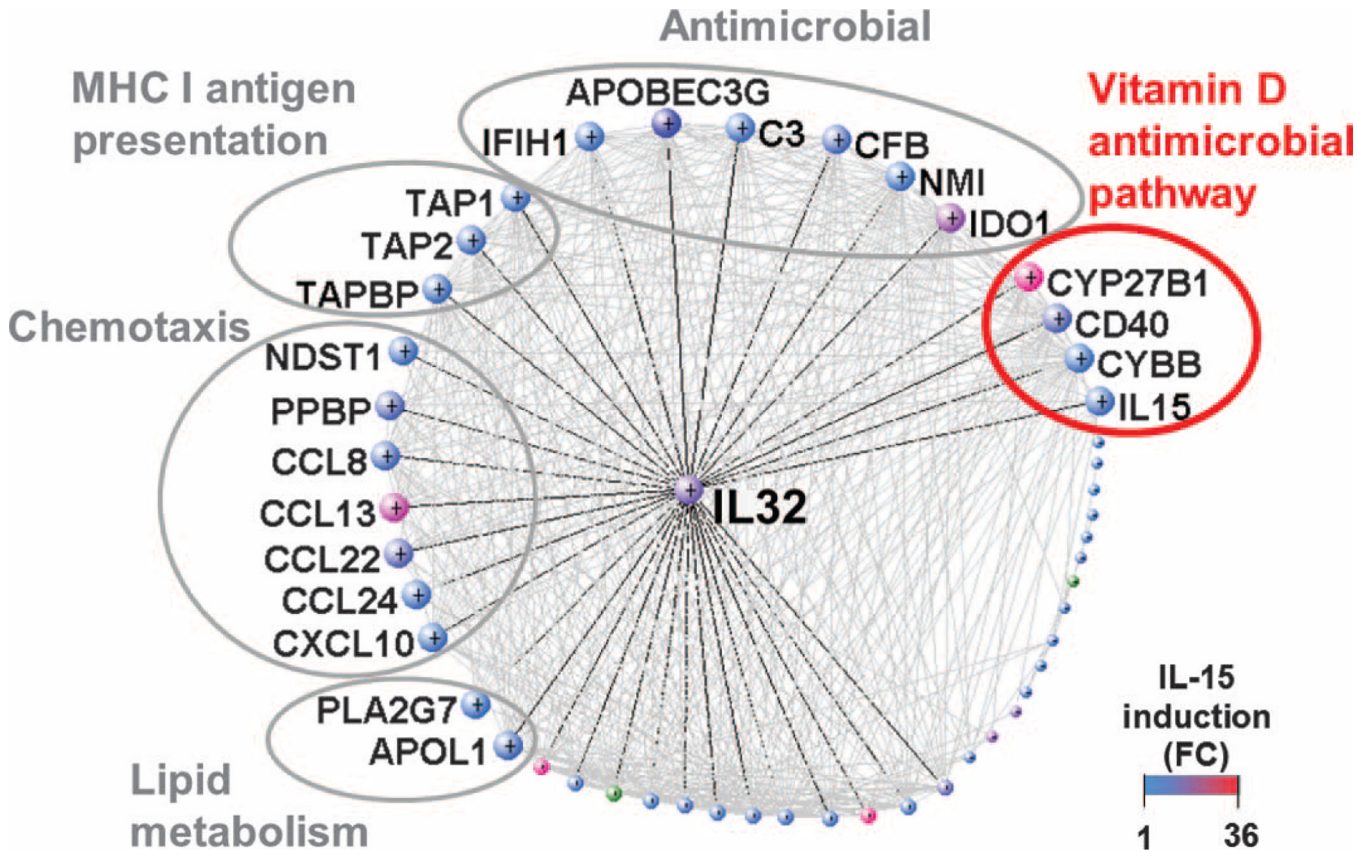

Fig. 2. IL-15 defense response network links IL-32 to the vitamin D antimicrobial pathway

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.

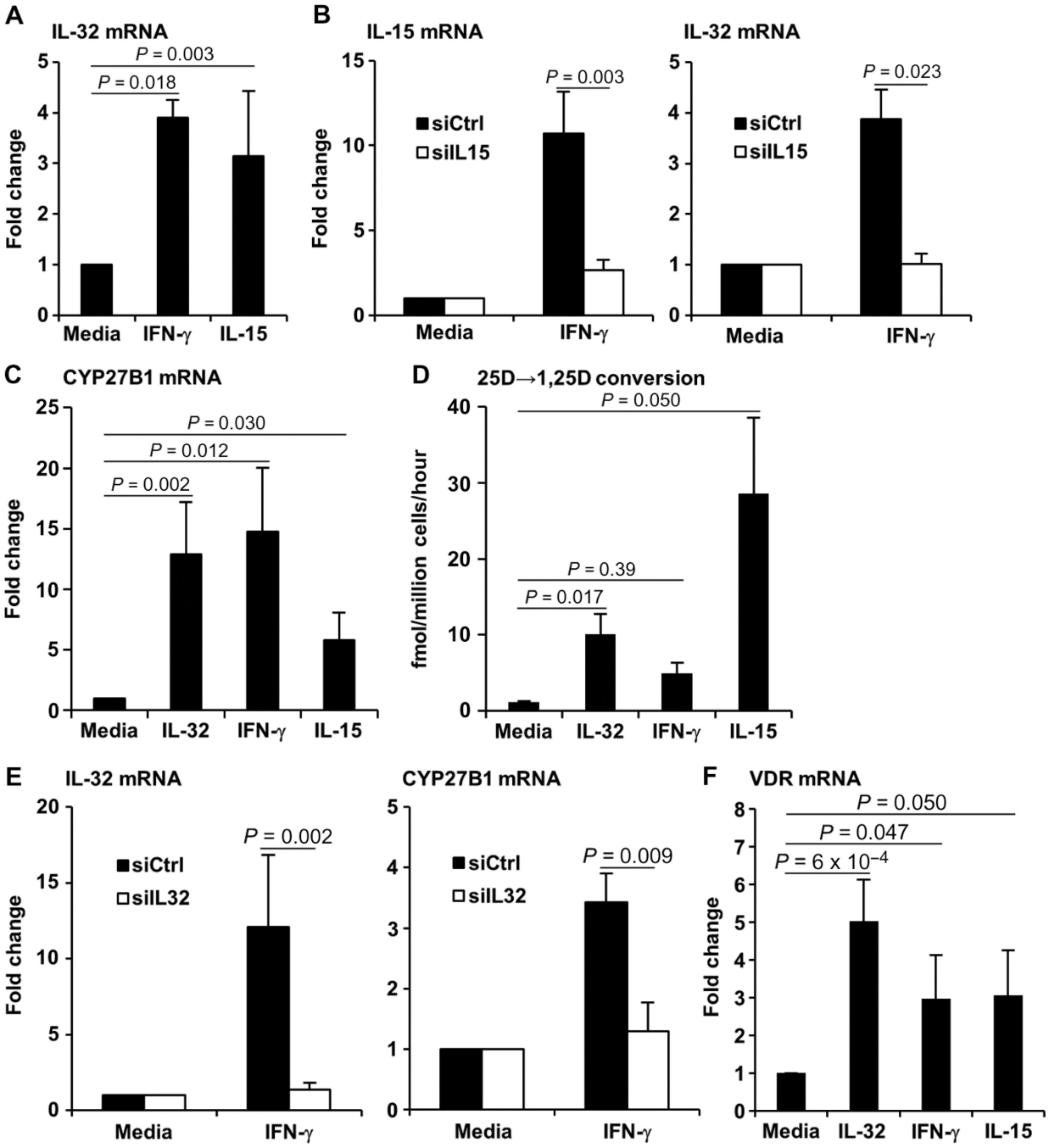

Fig. 3. IL-32 is necessary and sufficient for the induction of the IFN-γ–dependent vitamin D pathway

(**A**) Adherent PBMCs were treated with IFN-γ (1.3 rig/ml) or IL-15 (200 rig/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* (silLI 5) 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 silL-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.

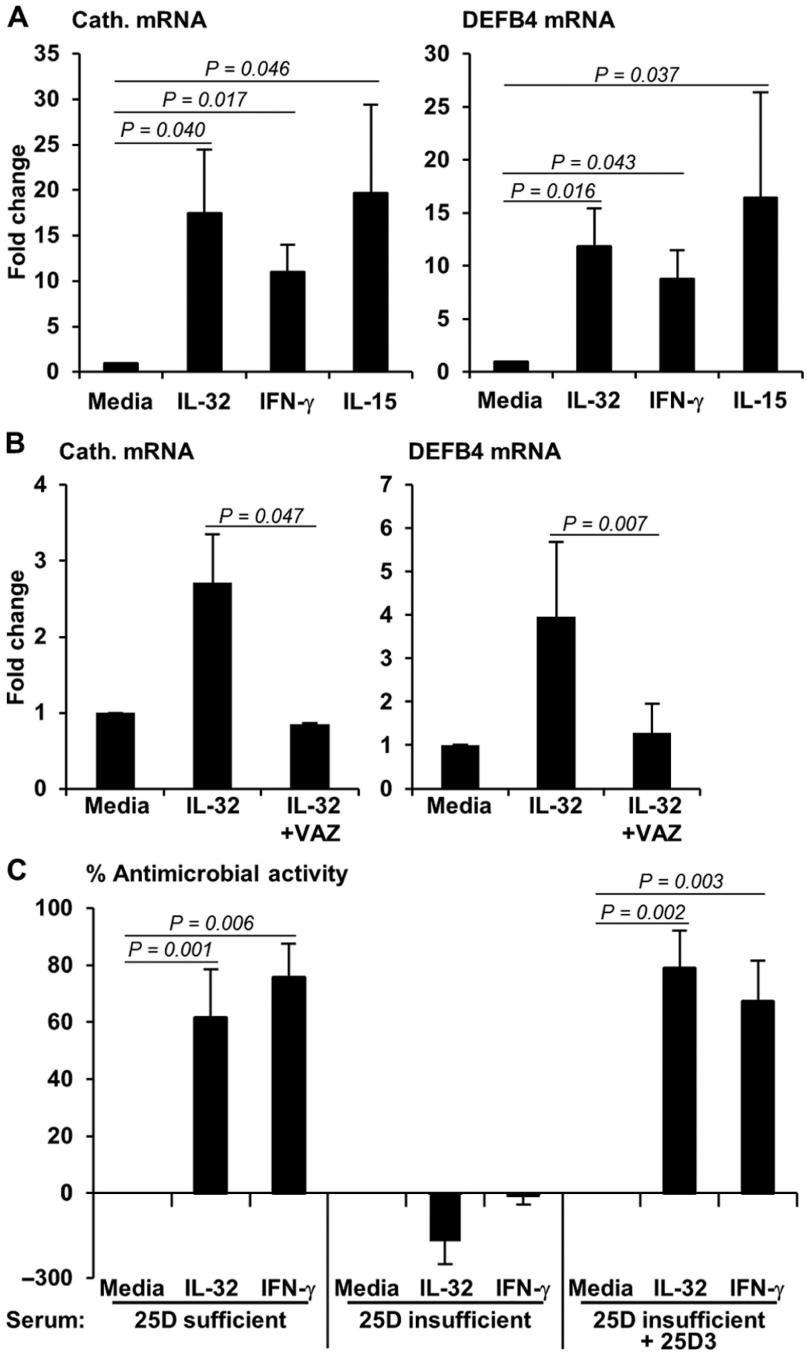

Fig. 4. IL-32 triggers a vitamin D–dependent antimicrobial response against *M. tuberculosis*

(**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 16S 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.

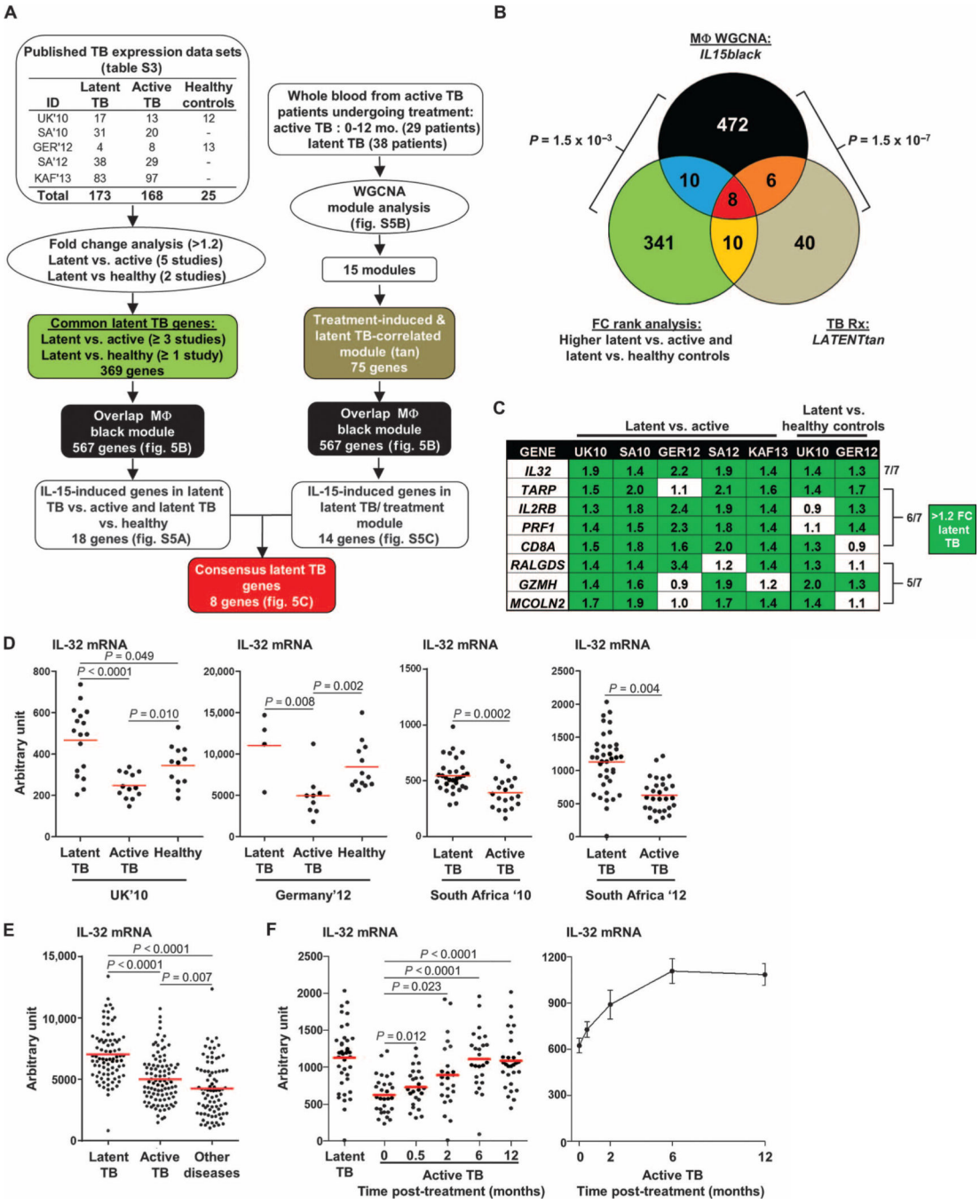

Fig. 5. IL-32 is part of an IL-15–induced gene set differentially expressed in latent TB

(**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.