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Mechanisms of Defense against Intracellular Pathogens Mediated by Human Macrophages

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ABSTRACT The key question our work has sought to address has been, "What are the necessary and sufficient conditions that engender protection from intracellular pathogens in the human host?" The origins of this work derive from a long-standing interest in the mechanisms of protection against two such paradigmatic intracellular pathogens, *Mycobacterium tuberculosis* and *Mycobacterium leprae*, that have brilliantly adapted to the human host. It was obvious that these pathogens, which cause chronic diseases and persist in macrophages, must have acquired subtle strategies to resist host microbicidal mechanisms, yet since the vast majority of individuals infected with *M. tuberculosis* do not develop disease, there must be some potent human antimicrobial mechanisms. What follows is not a comprehensive review of the vast literature on the role of human macrophages in protection against infectious disease, but a summary of the research in our two laboratories with collaborators that we hope has contributed to some understanding of mechanisms of resistance and pathogenesis. While mouse models revealed some necessary conditions for protection, e.g., innate immunity, Th1 cells and their cytokines, and major histocompatibility complex class I-restricted T cells, here we emphasize multiple antimicrobial mechanisms that exist in human macrophages that differ from those of most experimental animals. Prominent here is the vitamin D-dependent antimicrobial pathway common to human macrophages activated by innate and acquired immune responses, mediated by antimicrobial peptides, e.g., cathelicidin, through an interleukin-15- and interleukin-32-dependent common pathway that is necessary for macrophage killing of *M. tuberculosis in vitro*.

SOME NECESSARY CONDITIONS FOR PROTECTION

While animal models of human disease have provided great insights that would have been difficult to achieve in studies in humans, our initial experiments on protection against *Mycobacterium tuberculosis* were designed to take advantage of transgenic knockout mice. While animal models have contributed enormously to our understanding, it must be noted that none faithfully reproduces the pathology or course of disease of human tuberculosis (TB) or leprosy. With that caveat, we explored the question of immunologically necessary conditions for protection of mice against *M. tuberculosis* infection. We found that mice lacking the gene for gamma interferon (IFN-γ) died from *M. tuberculosis* challenge in a matter of 2 to 3 weeks after intravenous challenge and within a month after aerosol challenge (1). We hypothesized that the pathology observed in the lungs would likely be mediated by local production of tumor necrosis factor alpha (TNF-α) and were quite surprised to learn that TNF-α-depleted mice succumbed with precisely the same time to death as the IFN-γ knockouts (2). Additionally, we (3) and others (4, 5) found that mice whose major histocompatibility complex (MHC) class I presentation to cytotoxic T lymphocytes (CTLs) was deficient, e.g., β_2-microglobulin deficient or TAP (transporter associated with antigen

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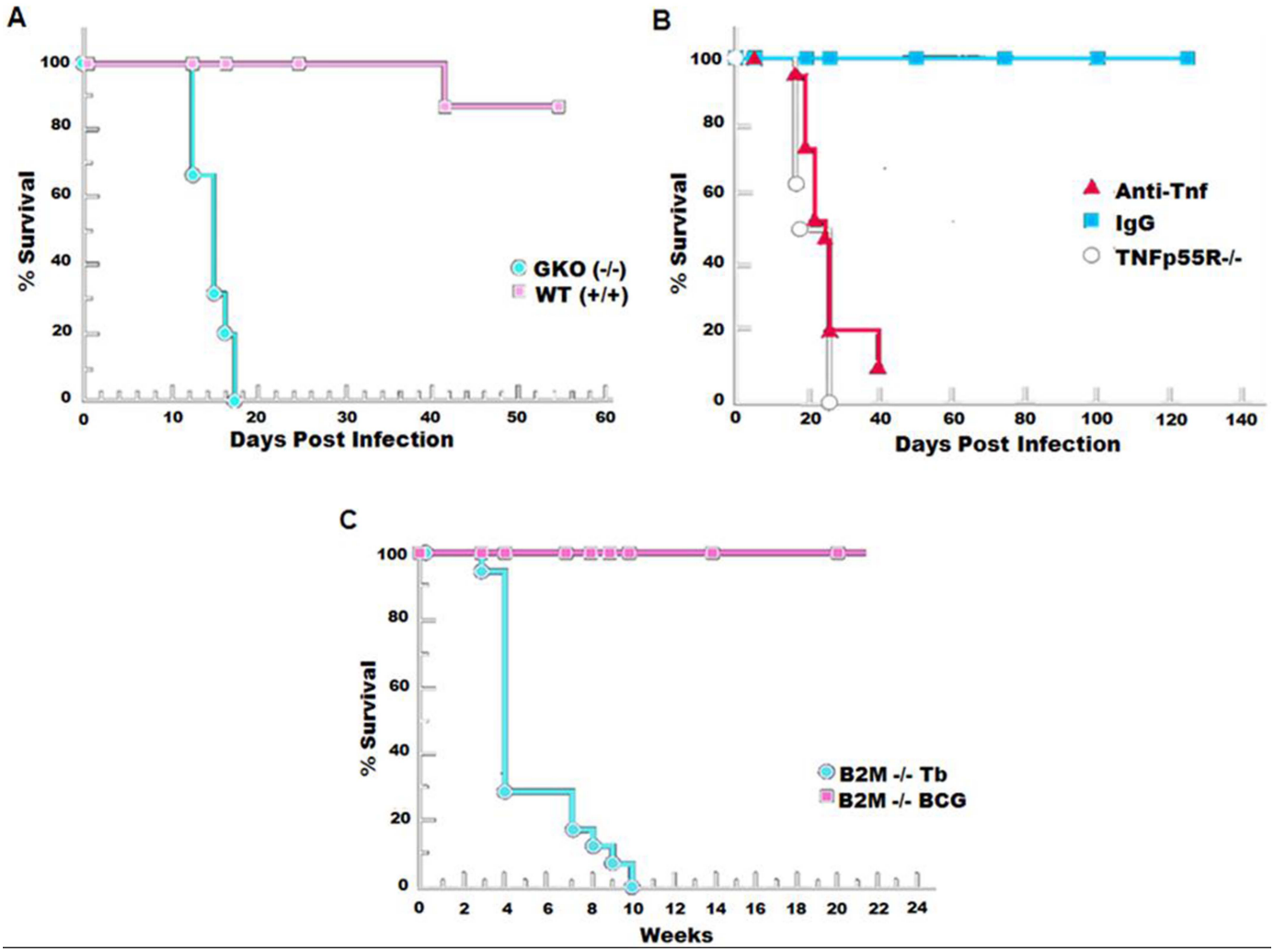
processing) deficient, succumbed to TB infection far earlier than control mice, but many weeks later than the IFN-γ- and TNF-α-deficient mice (1) (Fig. 1). These results established that IFN-γ and TNF-α are necessary for initial protection in mice, likely mediated by innate immunity and cytokine-activated macrophages, and suggested that CTLs may play a role later in infection.

MOUSE MACROPHAGE MICROBICIDAL MECHANISMS

The experiments *in vivo* were followed by *in vitro* experiments on mouse macrophages to learn how the cytokines IFN-γ and TNF-α contributed to protection. Activation by IFN-γ and TNF-α revealed that mouse macrophages were able to kill *M. tuberculosis*, but the mechanism was mediated by the action of reactive oxygen intermediates, which were also effective against many other pathogens (6). In 1991, Chan et al. established that while the killing of *M. tuberculosis* by mouse macrophages was not affected by inhibitors of reactive oxygen intermediates, the killing was dramatically reduced by inhibitors of nitric oxide synthase (NOS2) and that *M. tuberculosis* was directly killed by NO itself (7). In elegant experiments, MacMicking et al. established the importance of macrophage killing by NO *in vivo* by demonstrating that NOS2 knockout mice died rapidly from *M. tuberculosis*. Of interest, the time of death was not quite as rapid as in the case of the IFN-γ knockouts (8). These results indicated that the major mediator of

FIGURE 1 Necessary conditions for protection in mice. Depletion of IFN-γ **(A)**, TNF-α **(B)**, or MHC class I **(C)** results in increased susceptibility and death following *M. tuberculosis* challenge in C57BL/6 mice. B2M, beta-2 microglobulin; BCG, Bacillus Calmette–Guérin; GKO, IFN-gamma gene knock out; WT, wild type. Sources: references 1–3.



macrophage killing of *M. tuberculosis* in the mouse was reactive nitrogen intermediates. Mycobacteria have evolved multiple mechanisms to resist oxygen radicals (6, 9) and nitrogen radicals (10–12), including scavenging of radicals by surface carbohydrates, and enzymatic mechanisms.

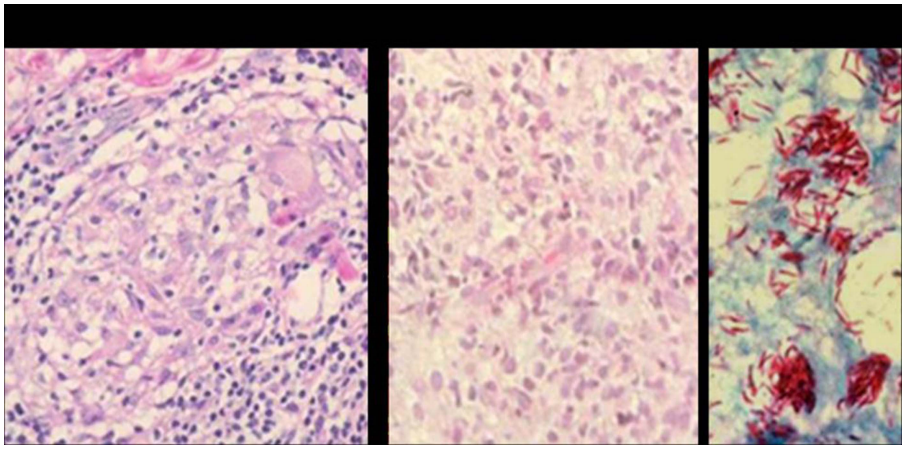
LEARNING FROM HUMAN LESIONS

The battle between the tubercle or leprosy bacillus and the host macrophages is fought at the site of disease in the tissue lesions. Establishing what is happening in the lungs of TB patients at any stage in the disease has been extremely difficult. In contrast, leprosy is essentially a skin disease, accessible for study by biopsy used for diagnosis, staging, and assessing prognosis. Consequently, we sought to characterize the events occurring in lesions of human leprosy. One of the fascinations of leprosy is that it does not exist as a single clinical entity, but rather is a spectrum. At one pole of the spectrum, tuberculoid leprosy, there are well-organized granulomas and well-differentiated macrophages but almost no acid-fast bacilli. At the other pole, lepromatous leprosy, there are macrophages full of acid-fast *Mycobacterium leprae*, very few lymphocytes, and the granulomas are disorganized (Fig. 2). Most patients are classified as "borderline" and show lesions with mixed characteristics. Leprosy forms are not always static and patients can undergo reactional states moving toward either pole. Tuberculoid lesions have a preponderance of CD4 Th1 cells, while lepromatous lesions have few CD4 and CD8 Th2 cells (13). Analysis of gene expression in lesions revealed that in tuberculoid lesions IFN-γ, interleukin-2 (IL-2), and lymphotoxin predominate, while in polar lepromatous lesions IL-4, IL-5, and IL-10 predominate (14, 15), which was the first example of the Th1 and Th2 dichotomy, previously described in mice (16), existing in human disease lesions.

THE INNATE AND ACQUIRED VITAMIN D-DEPENDENT ANTIMICROBIAL MECHANISM OF HUMAN MACROPHAGES

If, as is generally assumed, the infectious dose of *M. tuberculosis* required to cause infection and disease in humans is of the order of 1 to 400 bacilli, our first approach was to explore the ability of cells of the innate immune system to respond to the pathogen. This is of particular interest in light of the finding of many in the field that there are people with high levels of exposure to *M. tuberculosis* who remain healthy and fail to convert their tuberculin skin test. That suggests that the innate immune system may have the capability of effectively killing the initial low level of transmitted bacilli so rapidly that the bacilli are unable to multiply to a level required to engage the acquired T-cell response. In that context, it seemed important to pursue innate mechanisms of killing of intracellular mycobacteria by human monocytes and monocyte-derived macrophages stimulated through the Toll-like receptors (TLRs) through *in vitro* studies. Human peripheral blood precursors can be differentiated *in vitro* into macrophages or dendritic cells (DCs) when cultured in different cytokine-containing growth media. Macrophages were derived from cultures with macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage CSF (GM-CSF), and DCs were

FIGURE 2 Macrophages in leprosy lesions. Cellular infiltrates in tuberculoid and lepromatous leprosy lesions. The third panel shows an acid-fast stain, indicating the abundance of bacilli in lepromatous lesions. Courtesy of Thomas H. Rea.

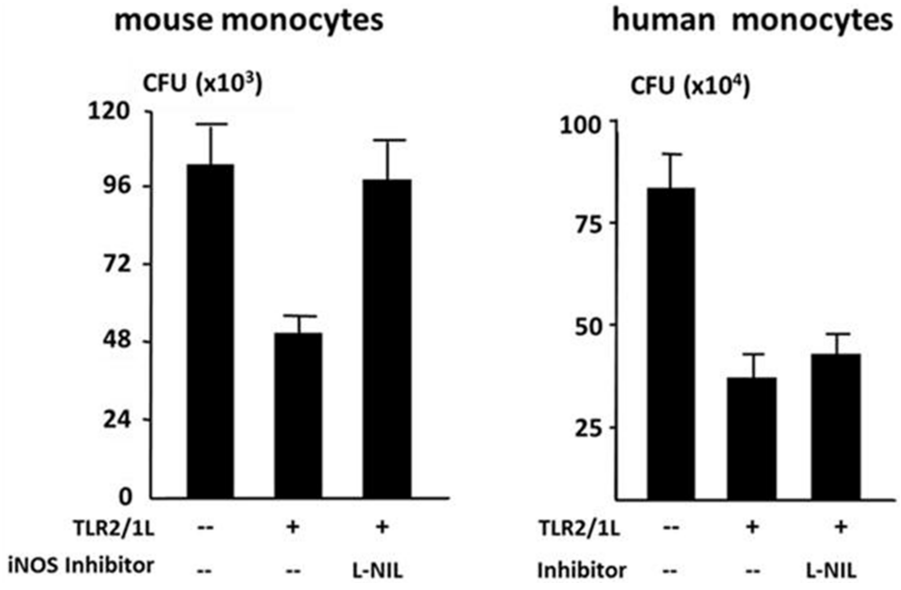


differentiated with GM-CSF + IL-4, and each possessed characteristic surface markers. When these cells were cultured in appropriate human sera for 4 days followed by activation via the innate immune receptor TLR2, we found a striking reduction in *M. tuberculosis* CFU by macrophages but not by the DCs (17, 18). Surprisingly, while the NOS2 inhibitor L-NIL was able to almost completely block killing of *M. tuberculosis* by TLR2-activated mouse macrophages, it had almost no effect on killing by activated human macrophages (Fig. 3). This was the first evidence that human macrophages, at least *in vitro*, kill *M. tuberculosis* by a mechanism different from that of mouse macrophages.

Those findings led to experiments seeking to learn which genes were expressed by macrophages but not expressed by DCs following TLR2 stimulation (18). Using two-way analysis of variance, there were three genes predominantly expressed in the microbicidal macrophages and not in DCs: S100A12; the vitamin D receptor (VDR); and Cyp27B1, the enzyme that converts 25-hydroxyvitamin D to the active 1,25-dihydroxyvitamin D. That, for the first time, suggested a role for vitamin D in the microbicidal process. When cathelicidin and β-defensin 2 (DEFB4), known downstream products of Cyp27B1 related to antimicrobial activity, were screened for in macrophages activated by TLR2 ligand alone, neither of the mRNAs for these antimicrobial peptides was expressed. However, when the macrophages were stimulated by TLR2 ligand in the presence of 25-hydroxyvitamin D, mRNAs for cathelicidin and DEFB4 were stimulated 5- to 20-fold (19). We also demonstrated induction of the active form of cathelicidin, a 37-amino-acid amphipathic α-helical antimicrobial peptide cleaved from a large precursor, encoded by the CAMP gene (18). We were able to establish that the cathelicidin peptide itself was capable of killing *M. tuberculosis* in culture. Further, although it is well known that *M. tuberculosis* in macrophage phagocytic vacuoles is able to block fusion of lysosomes and is refractory to many cellular proteins, including the NADP oxidase, we were able to observe that the cathelicidin peptide in TLR2-activated cells was colocalized with green fluorescent protein-labeled mycobacteria within the phagocytic vacuole (18). The formal demonstration that cathelicidin and DEFB4 were critical for killing of *M. tuberculosis* in human macrophages *in vitro* was the demonstration that small interfering RNA to cathelicidin essentially eliminated the antimicrobial activity (20) (Fig. 4).

Multiple reports in the literature concluded that, in contrast to murine macrophages, IFN-γ, the best-studied T-cell mediator of macrophage activation, was not able to activate human monocyte-derived macrophages *in vitro* to kill *M. tuberculosis* (21–28). However, we were able to show that the vitamin D-dependent antimicrobial pathway, with expression of cathelicidin and DEFB4, is induced in human macrophages by the acquired immune response *in vitro* and mediated by

FIGURE 3 Inducible NOS2 (iNOS) is essential for killing of *M. tuberculosis* by activated mouse macrophages *in vitro* but not for human monocyte-derived macrophages. L-NIL is an inhibitor of NOS2. Source: reference 17.



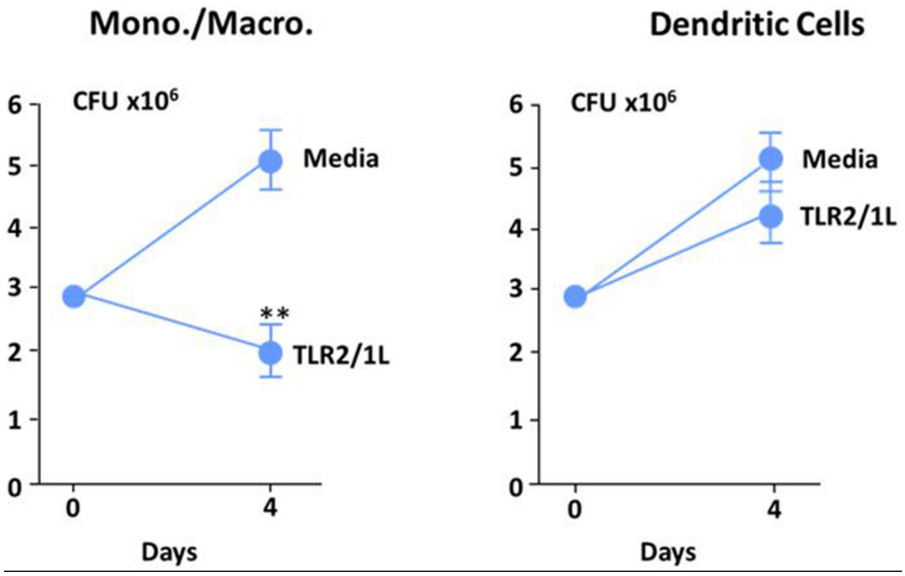
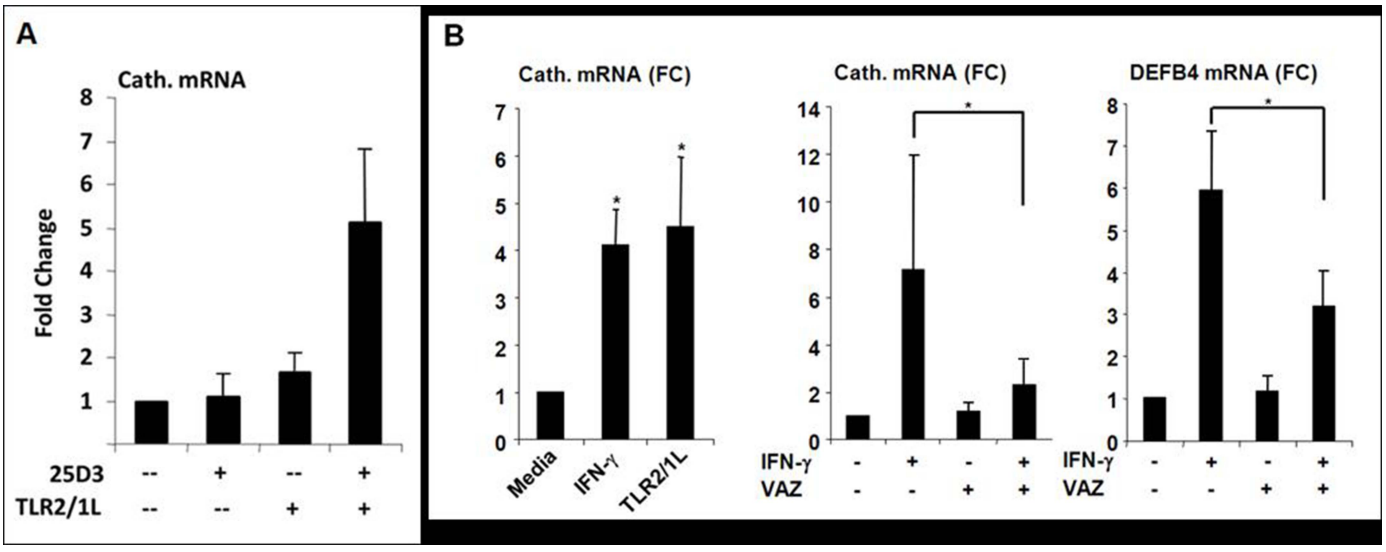


FIGURE 4 TLR2-activated human monocyte-derived macrophages are able to kill *M. tuberculosis*, whereas activated DCs (derived by culture of monocytes with GM-CSF and IL-4) were unable to kill under the same conditions. $^{**}P \leq 0.01$. Source: reference 18.

IFN-γ (Fig. 5B) (29). Thus both innate and acquired immune-activated human macrophages, through TLR2 triggering of the MyD88 signaling pathway or through activation by the type II IFN IFN-γ of a STAT1-mediated pathway, were able to kill *M. tuberculosis*. It is perhaps significant that the same antimicrobial pathway is conserved through evolution of the innate and adaptive immune system, though induced by two different immune mechanisms of activation acting through two different signaling pathways (29).

FIGURE 5 **(A)** Failure of TLR2-activated human macrophages to induce cathelicidin (Cath.) mRNA in culture with sera from African Americans can be reversed by addition of 25-hydroxyvitamin D. **(B)** IFN-γ activates human macrophages to produce cathelicidin to a comparable extent as the innate response to TLR2 agonists. Production of cathelicidin mRNA is dependent on vitamin D and inhibited by VAZ, a VDR antagonist. Vitamin D is essential for human macrophages activated by either TLR2 or IFN-γ to kill *M. tuberculosis in vitro*. FC, fold change. Sources: references 18, 29.



In all these experiments, inhibition of the VDR by a chemical VDR antagonist, VAZ, blocked the induction of cathelicidin, DEFB4, and the antimicrobial activity. Thus the killing of *M. tuberculosis* in human macrophages *in vitro* was mediated by the vitamin D antimicrobial mechanism, not by oxygen or nitrogen radicals. The mechanism does not appear to be important in mouse macrophages, which have a CAMP homolog, but one which lacks any vitamin D responsive elements (30). This makes sense since mice are essentially nocturnal animals that have little exposure to sunlight. Only some primates and humans have the vitamin D responsive elements in their CAMP. The failure of previous studies to observe killing of *M. tuberculosis* in human macrophages is most likely due to the culture of the cells either in fetal calf serum, which has negligible levels of 25-hydroxyvitamin D, or in human sera that lacked sufficient levels. While our experiments clearly demonstrate a novel vitamin D-dependent antimicrobial mechanism used by human macrophages in culture, we would caution that there may well be additional mechanisms for killing intracellular pathogens, including *M. tuberculosis*, by the macrophages of the human lung and other tissues.

THE QUESTION OF THE ROLE OF VITAMIN D IN HUMAN TB AND LEPROSY

Vitamin D is produced in human skin exposed to UV light. The circulating form is 25-hydroxyvitamin D, where the hydroxyl group is added by the liver. The biologically active form is 1,25-dihydroxyvitamin D, which we found to be produced by the action of Cyp27B1 in activated human macrophages (31). It had long been thought in the pre-antibiotic era that exposure of TB patients to sunshine and fresh air was helpful for recovery, and this was the rationale for the sanatorium movement in Europe and the United States. Clearly exposure to UV light would be expected to increase vitamin D levels, which might have been helpful in recovery. There is a fascinating historical study of cod liver oil, which is rich in vitamin D, in TB patients at Brompton Hospital in London in 1848 (32). The results indicated that while cod liver oil cured none of the patients, it arrested the disease in 18% of patients and reduced mortality from 33 to 19%.

It is well known that African Americans have a greater susceptibility to *M. tuberculosis* infection (33) and that in the pre-antibiotic era they developed more virulent forms of TB (34). Since melanin is known to absorb UV light, this history suggested to us that dark-skinned individuals might not have sufficient levels of vitamin D. In fact, a literature existed reporting that African Americans had vitamin D levels at 10 to 20% of what is considered normal. Consequently, we tested the ability of human sera from African Americans and white individuals to support the induction of cathelicidin in TLR2-stimulated macrophages. The result was striking: only 20% of the sera from African Americans had levels of 25-hydroxyvitamin D, in the normal range and were able to induce expression of cathelicidin mRNA. Addition of 25-hydroxyvitamin D, to the cultures enabled the induction of cathelicidin mRNA to levels comparable to those in the serum of white individuals and corrected the defect (Fig. 5).

These results suggest the possibility that the increased susceptibility of dark-skinned individuals from Africa or Asia to TB might be related to insufficient levels of vitamin D. Consistent with these findings are time-series epidemiological findings showing a parallel seasonal variation in vitamin D levels and notifications of TB in South Africa (35). There have been several *in vivo* studies of vitamin D supplementation, with sometimes conflicting results. A few contemporary clinical studies have described a potential benefit for vitamin D supplementation as an adjuvant to chemotherapy, measuring various endpoints including clinical and radiological improvement (36–38), sputum conversion (37, 39), and immune responses (38, 40, 41). However, these studies were generally inconclusive due to a number of study design confounders. While some studies showed shortening of the time to sputum clearance with chemotherapy, others found no beneficial effect. The studies were subject to many confounding factors, including being underpowered and being characterized by a low baseline prevalence of vitamin D deficiency; seasonality and increased levels of vitamin D in placebo controls; inadequate levels of supplementation due to dose and/or time; lack of sputum cultures in some studies; and the absence of agreed-upon, clearly defined clinical endpoints. However, a recent study in which patients were treated with high doses of vitamin D found clinical and radiological improvement, including a reduction in the number of cavities (38).

It is clearly challenging to ascertain a beneficial effect of vitamin D in patients who have active disease and are of necessity receiving antimycobacterial drugs. Vitamin D is not itself an antimicrobial drug, and it is asking a great deal to see dramatic effects in patients with active TB in which the tubercle bacillus has escaped protective mechanisms such that there are enormous numbers of bacilli in their tissues. If, as is believed, humans with disease are infected with between 1 and 400 CFU of

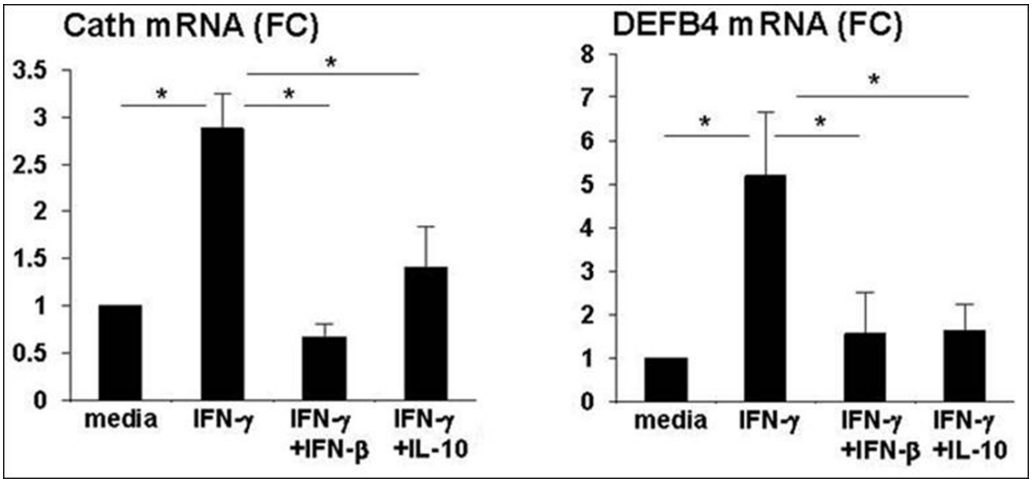
tubercle bacilli, it is possible that the greatest effect of vitamin D would be in raising the threshold of innate resistance to infection, and the effects of vitamin D supplementation might be best found in healthy contacts or patients with latent infection.

THE PARADOXICAL ROLE OF IFNs

From a variety of animal and human studies it has long been known that type I IFNs (IFN-α and IFN-β) can engender protection against many viral infections but little protection against bacterial infections. As described above, in both humans and mice, IFN-γ is essential for protection against *M. tuberculosis*. Using analysis of gene expression arrays on peripheral blood of TB patients in Europe and in Africa, several laboratories have identified sets of genes that distinguish individuals with active TB disease from those with latent infection (42, 43). The most striking characteristic of the "signature" for active TB thus far is the increase in a set of genes regulated by IFNs, in particular the type I IFNs IFN-β and IFN-α (42, 44). The induction of the type I IFN gene program was associated with the extent of disease (42), and reversed completely by 2 months of effective treatment (45). At the same time, studies have shown that *M. tuberculosis* induces type I IFNs in macrophages, requiring the ESX-1 secretion pathway (46) for *M. tuberculosis* double-stranded DNA to gain access to the cytoplasm, where it activates DNA sensors including STING (47). In a mouse model, the hypervirulent *M. tuberculosis* strain HN878 induced high levels of type I IFN expression and low levels of IL-12p40 (48, 49), which augments Th1 responses. The spectrum of leprosy offered a unique opportunity to investigate the involvement of type I and type II IFNs in lesions of the human disease. The results were striking: in tuberculoid leprosy, type I IFN-associated mRNAs were hardly detectable, but there was evidence of type II IFN-induced genes. In contrast, in lepromatous leprosy lesions, there was marked elevation of type I IFN-induced genes, particularly IL-10 (50). In parallel, mRNAs for Cyp27B1 and the VDR were elevated in tuberculoid leprosy lesions.

When human macrophages were stimulated by IFN-γ *in vitro*, there was elevation of mRNAs for the microbicidal peptides cathelicidin and DEFB4. This stimulation of the antimicrobial response was found to be vitamin D dependent (29). In contrast, treatment of peripheral blood adherent cells with IFN-β induced IL-10. Simultaneous addition of IFN-γ and IFN-β resulted in suppression of cathelicidin and DEFB4 mRNAs. This inhibition of type II IFN activation was blocked by anti-IL-10 antibody, indicating that the suppressive effect of type I IFNs was mediated by IL-10 (50). When macrophages were activated by IFN-γ in serum sufficient for vitamin D, they were able to kill and reduce the viability of *M. leprae* and *M. tuberculosis*. The process required autophagy, and surprisingly, vitamin D was required for autophagy as well (29). When type I IFN or IL-10 was added, IFN-γ-induced killing was abrogated, and treatment with anti-IL-10 restored the antimicrobial effect (Fig. 6). These results demonstrate that type I and type II IFNs have opposing effects on the microbicidal activity of human macrophages and identify IL-10 as the mediator of type I IFN suppression of antimicrobial activity (Fig. 7).

FIGURE 6 Inhibition of the effect of IFN-γ in stimulating induction of cathelicidin (Cath) mRNA by IFN-β is mediated by IL-10. FC, fold change. *$P \leq 0.05$. Source: reference 50.



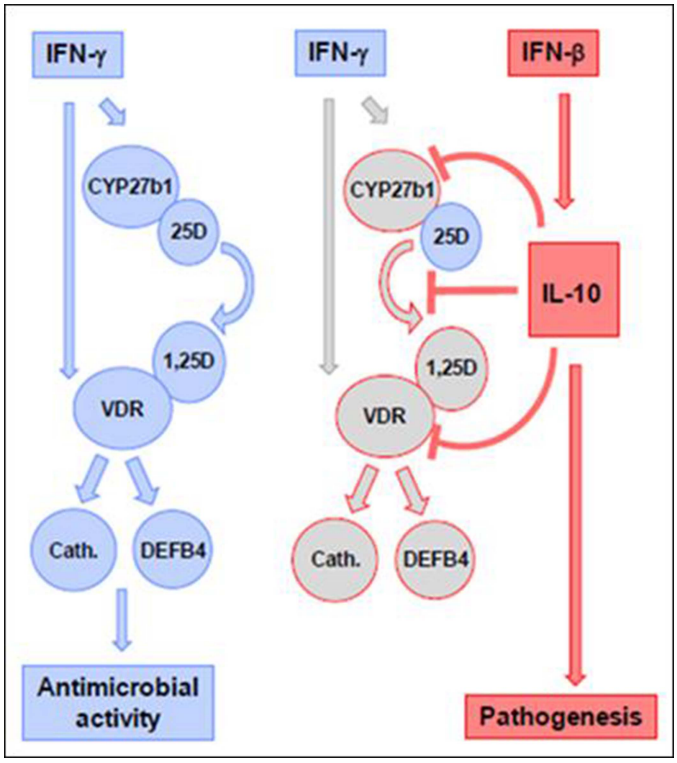


FIGURE 7 Common activation pathway for human macrophages stimulated through the innate receptor TLR2 or the acquired immune activator IFN-γ. IFN-β suppresses that activation through IL-10 by inhibiting induction of both CYP27B1 and the VDR. Source: reference 50.

OBSERVATIONS ON MAJOR DIFFERENCES BETWEEN HUMAN AND MURINE ANTIMICROBIAL RESPONSES

One general theme that has arisen in these experiments is that, despite many similarities, there are significant differences between human immune responses relating to antimicrobial activity and those of the common animal models. Some examples include the following observations. (i) Most animal models of TB fail to exhibit latency and human-like pathology. (ii) Mouse macrophages kill *M. tuberculosis* predominantly by NO, while human macrophages utilize microbicidal peptides, e.g., cathelicidin and DEFB4, and likely other mechanisms. (iii) Human macrophage killing is vitamin D dependent, while that by mouse macrophages is vitamin D independent. (iv) While the human CAMP gene, the precursor of cathelicidin, has three vitamin D responsive elements, its homolog in the mouse, a nocturnal animal, has none. (v) DEFB4 has no mouse homolog; autophagy in human *M. tuberculosis*-infected macrophages is vitamin D dependent. (vi) CD1a, -b, and -c, which present nonpeptide antigens to human T cells, have no homologs in the mouse (which does have CD1d). (vii) Human CD8 CTLs release the antimicrobial peptide granulysin, which is lacking in mice. (viii) For IL-32, no homolog has yet been found in mice.

FINDING A MARKER OF PROTECTION AGAINST TB

One of the major problems in TB, particularly affecting evaluation of new vaccines, is that we have no molecular correlate of protection. Both the last major *Mycobacterium bovis* BCG trial in south India, with 360,000 subjects followed for 15 years (51), and a recent trial of a new candidate vaccine, MVA85A, in South African infants, regrettably showed no protection against *M. tuberculosis* infection or disease (52, 53). With about 40 vaccine candidates in preclinical trials, unless there is a way to prioritize them on some rational scientific basis, it is unlikely that hugely expensive large-scale efficacy trials will be undertaken. One exciting approach to developing such correlates is systems biology approaches, specifically the study of gene expression profiles in peripheral blood of TB patients. Recently, several laboratories have identified a set of genes that distinguish individuals with active TB disease from those with latent infection (42, 43, 45, 54). The most striking characteristic of the "signature" for active TB thus far is the increase in the type I IFN-regulated genes (42, 44). Because the type I IFN gene program was associated with extent of disease (42), and response to treatment (45), the type I IFN gene signature should be considered a "correlate of risk or pathogenesis" for TB.

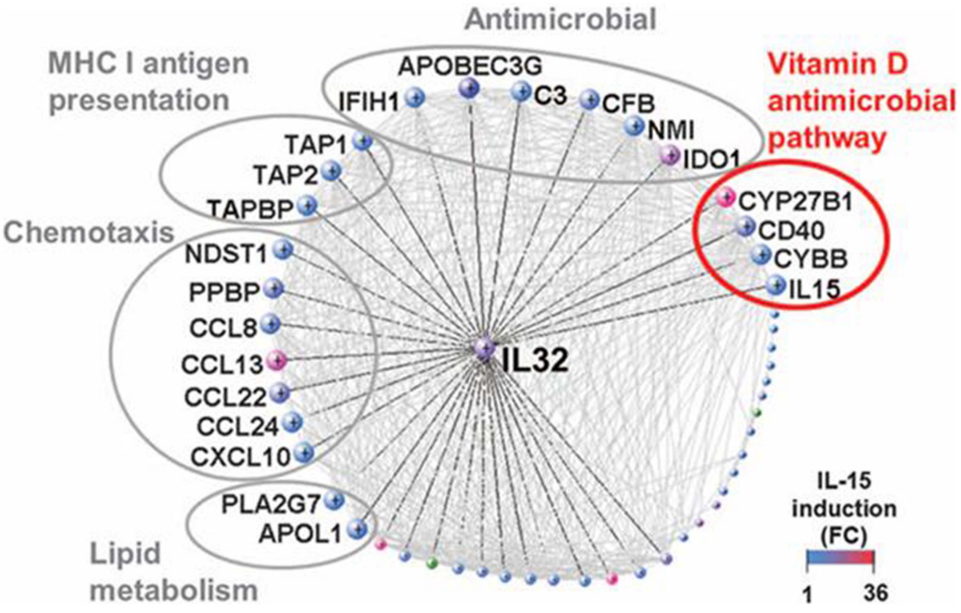
In contrast, in BCG-vaccinated children in South Africa followed for 3 years for development of TB, studies of production of cytokines by peripheral blood cells, including IFN-γ, TNF-α, IL-12, and IL-17, were unable to distinguish those who developed disease from those who did not (55). We reasoned, however, that it might be possible to gain insight into candidate molecular "correlates of protection" by focusing on individuals with evidence of *M. tuberculosis* infection who do not progress to active disease. Consequently, we sought to identify, in currently available data sets, those genes that were more highly expressed in the blood of individuals with latent TB infection, as defined by a positive blood IFN-γ release assay, compared to individuals with active TB and healthy controls. Using a sophisticated bioinformatics approach known as weighted gene co-expression network analysis, which searches for pairwise gene expression, an IL-15-induced host defense module was identified in antimicrobial macrophages,

which contained among other genes IL-32 (56). When these results were overlapped with the five existing data sets of gene expression in peripheral blood of patients with latent, active, or no TB, following informatics analysis, there were eight genes expressed to a greater extent in latent than in active patients, in healthy controls, or in sarcoidosis and other diseases in which TB was in the clinical differential diagnosis. Among those genes was IL-32, which was induced in human monocytes and macrophages by stimulation with IFN-γ (57) or activation of NOD2 by muramyl dipeptide (58) or of TLR4 by lipopolysaccharide (59). In the clinical data sets, IL-32 was elevated in latent TB, depressed in active disease, and returned to an intermediate level following successful treatment of the patients. IL-32 was found to be a central node in an IL-15-induced host defense network of 48 genes, 35 of which were expressed at baseline in the myeloid cell lineage in addition to IL-32 (Fig. 8). The IL-15-induced IL-32 gene network included vitamin D-related genes, e.g. cathelicidin, and genes related to MHC class I activity and chemotaxis, all of which could plausibly be related to antimicrobial activity.

When human monocytes or macrophages were treated with IL-32 *in vitro*, the vitamin D-dependent antimicrobial mechanism was activated to levels comparable to activation with IFN-γ or IL-15 and *M. tuberculosis* was killed by the IL-32-treated macrophages. This once again was a vitamin D-dependent process.

Since IL-32 expression correlates with the latent state of TB, which reflects a sufficient immune response to prevent the infection from progressing to active disease, we believe IL-32 should be considered as one "correlate of protection" against TB. Other biomarkers for protection are urgently needed to assess the likelihood of success of any of the many vaccine candidates before large-scale efficacy trials are undertaken. We would suggest that that may require a different scale of translational research on vaccines than has been considered previously, with multiple small-scale vaccination studies of human volunteers to develop a panel of molecular biomarkers that might be useful markers of protection. Such markers could markedly accelerate the development and testing of effective vaccines against TB. Clearly establishing any useful biomarkers for protection would require independent verification in other data sets and ideally in a longitudinal vaccine trial that provided protection to a significant number of recipients, where the correlation with protection against disease could be formally established. In the case of the vitamin D antimicrobial mechanism described here, while we do not know whether it is a necessary mechanism, we believe the evidence we have developed over the past several years clearly indicates that it is a sufficient mechanism by

FIGURE 8 IL-15 defense response network links IL-32 to the vitamin D antimicrobial pathway. The IL-15-induced host defense network reveals IL-32 as a hub gene, connected to sets of genes involved in host defense, including the vitamin D antimicrobial pathway. The color of each node depicts fold change (FC) induction by IL-15 at 24 h. Source: reference 56.



which human macrophages can kill intracellular bacterial pathogens such as *M. tuberculosis* and *M. leprae*.

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