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Genetic polymorphisms in vitamin D receptor, vitamin D-binding protein, Toll-like receptor 2, nitric oxide synthase 2, and interferon-γ genes and its association with susceptibility to tuberculosis

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Mycobacterium tuberculosis kills more people than any other single pathogen, with an estimated one-third of the world's population being infected. Among those infected, only 10% will develop the disease. There are several demonstrations that susceptibility to tuberculosis is linked to host genetic factors in twins, family and associated-based case control studies. In the past years, there has been dramatic improvement in our understanding of the role of innate and adaptive immunity in the human host defense to tuberculosis. To date, attention has been paid to the role of genetic host and parasitic factors in tuberculosis pathogenesis mainly regarding innate and adaptive immune responses and their complex interactions. Many studies have focused on the candidate genes for tuberculosis susceptibility ranging from those expressed in several cells from the innate or adaptive immune system such as Toll-like receptors, cytokines (TNF-α, TGF-β, IFN-γ, IL-1b, IL-1RA, IL-12, IL-10), nitric oxide synthase and vitamin D, both nuclear receptors and their carrier, the vitamin D-binding protein (VDBP). The identification of possible genes that can promote resistance or susceptibility to tuberculosis could be the first step to understanding disease pathogenesis and can help to identify new tools for treatment and vaccine development. Thus, in this mini-review, we summarize the current state of investigation on some of the genetic determinants, such as the candidate polymorphisms of vitamin D, VDBP, Toll-like receptor, nitric oxide synthase 2 and interferon-γ genes, to generate resistance or susceptibility to *M. tuberculosis* infection.

Key words: Tuberculosis; Vitamin D; Vitamin D-binding protein; Toll-like receptor; Nitric oxide synthase 2; IFN-γ

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Introduction

Tuberculosis, primarily caused by *Mycobacterium tuberculosis*, continues to be an important public health problem despite the existence of national and international tuberculosis control programs. Recent data from the World Health Organization show that about 8-10 million new cases arise annually and eventually 2-3 million die of the disease every year. Tuberculosis is one of the major infections that cause disease and death worldwide. It is estimated that one-third of the World's population is infected with *M. tuberculosis*, but that only one in ten (10%) of those infected ever develop clinical disease.

The central question is if tuberculosis patients are

inherently susceptible to the disease or if disease development is caused by specific environmental factors. Clearly, environmental factors such as poor economic conditions, malnutrition, stress, and overcrowding play a role in determining the susceptibility to tuberculosis in human populations. It is known that genetic and non-genetic factors of both the bacterium and the host have impact on the immune response to *M. tuberculosis.* The interaction between these different factors is unknown as well as the impact on disease development. Thus, the increasing interest in understanding the role of human genetic factors in the immune system controlling susceptibility/resistance to infectious diseases is of great importance for tuberculosis research because this will allow a genetic dissection of anti-mycobacterial immunity and should open new possibilities for preventive and therapeutic measures.

Vitamin D

When skin is exposed to sufficient UVB light (290 to 320 nm), the hormonal cascade for the endogenous production of vitamin D is activated. The prohormone (7-dehydrocholesterol) is converted into pre-vitamin D_3 (25-hydroxy-VD3 or $25(OH)_2D_3$) and then to 1,25 dihydroxy-D3 ($1,25(OH)_2D_3$ or vitamin D_3) within keratinocytes. Sunlight also induces the expression of vitamin D receptors (VDR). Circulating $25(OH)_2D_3$ is the major form of vitamin D, with concentrations 1000 times higher than $1,25(OH)_2D_3$, the active form of vitamin D. The conversion of $25(OH)_2D_3$ to $1,25(OH)_2D_3$ is controlled mainly by parathyroid hormone, calcium and phosphate levels. The extent of this conversion regulates the effect of vitamin D on bone, gut and kidney. The importance of sunlight has been observed throughout the history of tuberculosis treatment as an alternative treatment of pulmonary and cutaneous tuberculosis since as early as 1849. During the 13th Workshop consensus for vitamin D nutritional guidelines, held in Victoria, British Columbia in 2007, it was pointed out that half of the elderly in North America and two-thirds of the elderly in the rest of the world do not have enough circulating vitamin D to maintain healthy bone density through calcium metabolism, thus increasing their risk of fractures, major cancers, problems of tooth attachment, and of reduced muscle strength. Research reported at that meeting showed that adults must maintain a much higher blood vitamin D level than children, i.e., approximately 75 nM/L. Low levels of circulating vitamin D were reported for an elderly Brazilian population, which has a deficiency (25 nM/L; 15.4%) or insufficiency (25-50 nM/L; 41.9%) of vitamin D (1). To date, there are no reports regarding the serum level of vitamin D in the Brazilian population comparing age, gender, sun exposure, food intake, and related disease. However, the general consensus is that a desirable serum concentration of vitamin D is 50 nM (or 20 ng/mL). A recent nutritional analysis of food intake demonstrated vitamin D deficiency in all major geographic regions of Brazil that could result in up to a 6-fold decrease in presumable normal serum levels of vitamin D, independent of socio-economic level (2). The correlation between serum level of vitamin D and risk for tuberculosis latent infection or disease development has been described in African immigrants living in Australia (3). Lower mean vitamin D levels were observed in latent infection compared with individuals with no *M. tuberculosis* infection and lower in tuberculosis patients. Higher vitamin D levels were associated with lower probability of any *M. tuberculosis* infection (4). These results and other studies in sunny countries have shown that the existence of vitamin D deficiencies might be related to some risk factors, such as inadequate food source, sunshine exposure, sun block use, religion, pollution and, finally, skin color (ethnicity). These factors explained how it was possible in sunny countries to detect a vitamin D deficiency (4). This deficiency associated with the existent environmental conditions and parasitic infection seemed to explain the increased risk for tuberculosis development (4).

Vitamin D metabolites are transported in the blood by vitamin D-binding protein (VDBP) and are cleared from the blood by the liver, where they are converted into 25-hydroxyvitamin D_3 (calcidiol) and released back into the blood as the major circulating form of vitamin D. $25(OH)_2D_3$ is taken up by the kidneys, where it undergoes further conversion into the most active hormonal form of vitamin D ($1,25(OH)_2D_3$), and is released back into the blood.

Recently, the role of vitamin D has been emphasized not only in bone mineralization and in anti-tumor activity but also, a large body of work has accumulated supporting an important role for vitamin D in multiple organ systems as well as physiologic and molecular processes. VDR are distributed ubiquitously, and by binding with its receptor, vitamin D initiates a series of events that can affect cellular proliferation and differentiation, inflammation, the immune and endocrine systems, insulin resistance, and lipid metabolism (5). Vitamin D metabolites are important immunomodulatory hormones, which activate monocytes and suppress lymphocyte proliferation, immunoglobulin production, and cytokine synthesis. Moreover, this hormone has been shown to act as both an up-regulating agent during natural immunity via the enhancement of phagocytosis by monocyte/macrophage populations and a down-regulator during acquired immune response via an inhibitory effect on MHC class II expression by professional antigen-pre-

senting cells (6). Apparently $25(OH)_2D_3$ has no direct antimycobacterial action, but its active metabolite $1,25(OH)_2D_3$ is able to block the maturation of myeloid dendritic cells (DCs) by reducing the expression of co-stimulatory molecules. This inhibits secretion of IL-12 and increases IL-10 production as well as inhibits the secretion interferon-γ (IFN-γ) and IL-2 by T cells and stimulates Th2 cell development with increased production of IL-4, IL-5, and IL-10 (7). Thus, these data indicate that the $1,25(OH)_2D_3$ does not mediate protection via those cytokines. It is known that the modulation of $1,25(OH)_2D_3$ by binding to nuclear VDR up-regulates protective innate host responses, including the induction of nitric oxide synthase (NOS2). However, the mechanisms that regulate the expression of NOS2 and the role of $1,25(OH)_2D_3$ in tuberculosis infection are not known. Some investigators have reported that the human macrophage-like cell line acquires the ability to produce substantial amounts of nitric oxide (NO) on stimulation with lipopolysaccharide or $1,25(OH)_2D_3$ in the absence of activating factors such as IFN-γ. This suggests that $1,25(OH)_2D_3$ acts to suppress the growth of *M. tuberculosis* in these cells and that vitamin D-induced NO production may play a role in the host defense against human tuberculosis (8). In addition to the NO production induced by $1,25(OH)_2D_3$, this metabolite also up-regulates the expression of 1α-hydroxylase, the enzyme that metabolizes $25(OH)_2D_3$ to $1,25(OH)_2D_3$ due to ligation of Toll-like receptor (TLR)2/1 in macrophages stimulated by *M. tuberculosis* antigens and up-regulates the cathelicidin gene. $1,25(OH)_2D_3$ regulates the expression and activation of antimicrobial peptides in keratinocytes and monocytes in the epidermis and provides a stimulus for rapid production of a chemical antimicrobial shield with the expression of LL-37, a human antimicrobial peptide belonging to the cathelicidin family (9). Cathelicidins are relevant to defense against microbes including Gram-positive and -negative bacteria as well as fungi and certain viruses and it is increased with increasing concentrations of $1,25(OH)_2D_3$ (9). The addition of a VDR antagonist VAZ (ZK159222) inhibited the induction of cathelicidin mRNA by greater than 80%, and consequently the antimicrobial activity was reduced by about 70% (10). The increase in TLR2/1 enabled the cells to respond to microbial stimulation and also amplify the synthesis of $1,25(OH)_2D_3$ (Figure 1). The active metabolite of vitamin D, $1,25(OH)_2D_3$, suppresses growth of *M. tuberculosis in vitro* and this effect may be facilitated by TLR *in vivo* (10). Thus, there is an elegant system of control of innate immunity by $1,25(OH)_2D_3$. The most reasonable explanation suggests that vitamin D stimulates the immune cells to resist *M. tuberculosis* infection by mediating protection against tuberculosis by 'nonclassical' mechanisms.

Vitamin D receptor

Vitamin D exerts its action through VDR, a nuclear hormone receptor when activated by its ligand $1,25(OH)_2D_3$, that is present on monocytes and activated T and B lymphocytes (Figure 1) (5). Extensive data indicate that there are two mechanisms of action. One involves the activation of nuclear VDR and transcriptional regulation of many vitamin D-responsive genes. The other involves activation of non-genomic signal transduction pathways in target cells. This second mechanism is likely to involve a membrane VDR. A candidate form of VDR that is a 64.5-kDa protein from chick epithelium has been described recently, which specifically binds $1,25(OH)_2D_3$ and is responsible for some of the rapid cellular actions of $1,25(OH)_2D_3$ (11). Is it possible that this membrane receptor for $1,25(OH)_2D_3$ acts with ligand-binding properties that are different from those of the nuclear/cytosol receptor? Studies suggest that the ligand-receptor complex mediates the signal transduction of the hormone via opening of voltage-gated Ca^{21} channels to initiate the biological response(s) (12).

The *VDR* gene is located on the long arm of chromosome 12 (12q12-14) and is composed of 10 exons, the first of which is not transcribed (13). The *VDR* gene is a candidate locus for susceptibility to different diseases, such as prostate cancer, inflammatory bowel disease, osteoporosis and tuberculosis, due to allelic variation that affects the activity of the receptor and subsequent downstream vitamin D-mediated effects such as calcium absorption, excretion, and modulation of cellular proliferation and differentiation. The variation in the ability to synthesize vitamin D, including polymorphisms in the *VDR* gene, may be a contributing factor to increase tuberculosis susceptibility. Four of these *VDR* polymorphisms can be distinguished by digestion with restriction enzymes. Three of them do not change the translated protein (*Taq*I, *Apa*I and *Bsm*I). The polymorphisms recognized by *Bsm*I (*BB*, *Bb* and *bb*) and *Apa*I (*AA*, *Aa* and *aa*) are located in intron 8 of the *VDR* gene and the one recognized by *Taq*I (*TT*, *Tt* and *tt*) is located in exon 9, that leads to a silent codon change, with ATT and ATC, both coding for isoleucine and has been associated with increased *VDR* mRNA stability. The genotype *tt*, detected with the *Taq*I restriction enzyme, is associated with decreased risk of tuberculosis. The fourth VDR polymorphism, recognized by *Fok*I restriction enzyme (*FF*, *Ff* and *ff*), results from a C→T transition that creates an alternative initiation codon (ATG), three codons from the downstream start site. The *VDR* encoded by the *f* allele from the *Fok*I restriction enzyme recognition is increased in length by three amino acids, and the transcription of this allele is 1-7 times less efficient than the *F* allele and can

alter the amount of VDR produced (13).

The association or not of *VDR* polymorphism with tuberculosis in several geographic areas was discussed by Lewis et al. (14). They evaluated *VDR* gene polymorphism in different ethnic population studies published prior to August 2004. The *tt* genotype was underrepresented in tuberculosis patients from Gambia (15) and India (16), but in patients from Peru (17) the *Tt* and *FF* genotypes were associated with faster sputum culture conversion after initiation of therapy. The same was seen in China (18), where the *FF* genotype was associated with resistance to tuberculosis. After this meta-analysis description other results appeared describing *VDR* polymorphism in Chinese Tibetans (19) in which *Fok*I polymorphism of the *VDR* gene might be associated with tuberculosis, but there was no evidence that the *Taq*I polymorphism was associated with the disease in this population. A larger study conducted by Babb et al. (20) with a South African population reported that the *VDR* genotypes might affect the severity of disease or the ability of the treated cases to recover (positive sputum conversion or smear stain to negative), but no association was demonstrable between tuberculosis and *VDR* polymorphisms. When they used diplotypes and haplotypes to analyze the results, only a weak association was detect between the *VDR* haplotypes and tuberculosis susceptibility. They found that the *Fok*I-*Apa*I-*Taq*I haplotype ('FAT') tended to be associated with tuberculosis and may be a risk factor, whereas the 'FaT' haplotype is probably protective. Regarding the role of *VDR* polymorphisms in disease caused by *Mycobacterium leprae*, Goulart et al. (21) reported that there was a higher risk of leprosy when individuals carry the combination of *tt* genotype from *Taq*I polymorphisms in the *VDR* gene and negative Matsuda test, demonstrating a possible synergistic role of these two variables in leprosy susceptibility via effects on cellular immunity in a Brazilian population.

While there is evidence that vitamin D promotes macrophage killing of *M. tuberculosis*, the effector mechanisms are not clear, and the association of *VDR* polymorphisms with susceptibility to tuberculosis remains unproved. As described in Lewis et al. (14), larger studies are required to determine whether *VDR* polymorphisms play a role in genetic susceptibility to tuberculosis worldwide. A sample size of more than 2000 cases and controls is required to demonstrate if *VDR* polymorphism is associated with tuberculosis. This large sample size would be necessary to have enough power to detect specific polymorphisms related to tuberculosis, because any association will be diluted due to the extended linkage disequilibrium with the specific functional allele. Most studies conducted until now were in ethnically and geographically different populations, thus, they were underpowered and impossible to reach any conclusions by examining the alleles separately. The *VDR* polymorphisms contain many more single nucleotide polymorphisms (SNPs) than the four normally measured and it is necessary to conduct family-based studies that will allow research to construct reliable haplotypes and eliminate the possibility of population stratification and other risk factors such as gender, HIV status, occupation, and environment issues.

Vitamin D-binding protein

VDBP is a multifunctional, highly expressed, polymorphic serum protein that was initially named the group-specific component of serum (Gc-globulin). VDBP, a 458-amino acid polymorphic serum protein, is the major plasma carrier of vitamin D_3 and its metabolites and ensures that vitamin D is transported to the liver, $25(OH)_2D_3$ to the kidney, and $1,25(OH)_2D_3$ to target cells and organs. VDBP is encoded by the *Gc* gene, a member of a multigene cluster that includes albumin and α-fetoprotein genes, located at chromosome 4q11-q13. Sequence variations at codons 416 and 420 in exon 11 of the *Gc* gene give rise to major electrophoretic variants of VDBP, termed Gc1 fast (Gc1F), Gc1 slow (Gc1S) and Gc2 (22). These variants differ by amino-acid sequence, as well as by attached polysaccharides. Combinations of the three VDBP or Gc variants result in six common circulating phenotypes: Gc1F/Gc1F, Gc1F/Gc1S, Gc1S/Gc1S, Gc1F/Gc2, Gc1S/Gc2, and Gc2/Gc2 (23). *DBP* polymorphism (Gc phenotype) is related to the VDBP concentration and vitamin D status, as described by Lauridsen et al. (23). These authors showed a strong correlation of higher, intermediate and lower circulating levels of $25(OH)_2D_3$ and $1,25(OH)_2D_3$ with Gc1-1, Gc1-2 and Gc2-2 phenotypes, respectively, in Danish Caucasian postmenopausal women population. In addition to storing and transporting vitamin D_3 hormones, VDBP also affects their pharmacokinetics (24) and plays a role in the sustained effective conversion of cutaneously derived vitamin D_3 into $25(OH)_2D_3$. In addition to transporting vitamin D_3, VDBP has multifunctional properties, including an important role in macrophage activation that is distinct from its vitamin D-binding ability. Variations in this property could affect the functioning of the immune system, as shown for *DBP* knockout mice that exhibited an impaired immune response to bacterial infections (24). VDBP has been shown to enhance the leukocyte chemotactic activity of activated complement peptides, which are the precursor of the macrophage-activator factor (MAF). Indeed Gc-MAF influences macrophage chemotaxis (24).

A role of *DBP* polymorphism in autoimmune diabetes

mellitus and infectious disease in Polynesia and Japan (25) has been suggested. Until now, only one study evaluated the *DBP* phenotype in tuberculosis patients and no differences were seen among patients and the control group. In that study, a 33% frequency of Gc2 in tuberculosis patients was slightly but not significantly higher than in the control group (26%), and this elevation was at the expense of both Gc1F and Gc1S alleles (26). Further studies are necessary to understand the physiological role of VDBP and its phenotypes on susceptibility to tuberculosis and other diseases.

The Toll-like receptors

The TLRs represent a group of single-pass transmembrane receptors observed from *Drosophila* to mammals. They are expressed on innate immune cells and are the key sensors for a broad spectrum of pathogen-derived molecules, and are critical in shaping host-pathogen interactions (Figure 1). All bacteria contain ligands for many TLRs and the ligation of TLRs by these pathogen-specific ligands initiates a signal transduction pathway in the host cell that culminates in the activation of nuclear factor-kappa B (NF-κB) and the induction of cytokines and chemokines (27), which are crucial for eliciting the adaptive immune response against pathogens. The production of IL-12, TNF-α and NO is induced mostly by macrophages soon after innate recognition of mycobacteria through TLRs (28). TLRs have been recognized to be the principal mediators of the pro-inflammatory signal, TNF-α, induced by whole *M. tuberculosis* (28). Indeed, TLR2 has been shown to be involved in response to mycobacteria, specifically to the 19-kDa lipoprotein and lipoarabinomannan from *M. tuberculosis*. Interestingly, the triacylated mycobacterial lipopeptide is recognized by dimmers of TLR2 and TLR1, whereas similar diacylated compounds are recognized by dimmers of TLR2 and TLR6 (29). However, recent data point to the deactivation of macrophage cytokine induction by *M. tuberculosis* proteins. ESAT-6, a member of a unique family of proteins present in *M. tuberculosis*, probably attenuates the innate immune response by dampening the production of the IL-12 p40, TNF-α and NO (30). Recently, it has been shown that ESAT-6 and some peptides were able to dampen TLR2 signaling by preventing assembly of the cytosolic MyD88-dependent signaling scaffold (31). However, other members of the TLR family could be activated by *M. tuberculosis*, with or without TLR2 interaction. DNA from mycobacteria contains stimulatory CpG motifs that activate TLR9 (31) and up-regulate Th1 response.

The role of TLR in resistance to *M. tuberculosis* was suggested initially by the fact that MyD88-deficient mice are more susceptible to *M. tuberculosis* infection (31) and by the observation that TRL2/TLR1 reduced the viability of intracellular *M. tuberculosis* in human monocytes and macrophages, but not in monocyte-derived DCs (10). Liu et al. (10) also reported that TLR induces up-regulation of the *VDR*, 1α-vitamin D hydroxylase (enzyme that converts inactive to active vitamin D) and *CYP27B1* gene expression in monocytes and macrophages. These actions permit the macrophages to internalize serum VDBP-bound $25(OH)_2D_3$ from the extracellular fluid by facilitated endocytosis and use that $25(OH)_2D_3$ as substrate for the upregulated CYP27B1 (expression gene of 1α-vitamin D hydroxylase). Because VDR is functional when exogenous $1,25(OH)_2D_3$ is added, it was hypothesized that TLR2/1 induction of CYP27B1 and the conversion of $25(OH)_2D_3$ to $1,25(OH)_2D_3$ by 1α-vitamin D hydroxylase could represent a key step of the TLR pathway. Because TLR affects the interaction of innate and adaptive immune response against *M. tuberculosis*, a TLR2 agonist has been used as "natural adjuvant" to enhance Th1-induced response, inducing pulmonary IFN-γ secreting-$CD4^+$ T cell (32), or in prime-boost vaccine, inducing antigen specific IFN-γ and IL-2 responses in mice (33). The structural integrity of TLR signaling components is essential for immunological protection from infection. Changes in structure of TLR signaling molecules that result from SNPs are often associated with susceptibility to various infectious diseases. The fact that TLR2-deficient mice are highly susceptible to *M. tuberculosis* suggests that polymorphisms that affect TLR2 expression or structure may impair the susceptibility to tuberculosis in the human host. Approximately 89 SNPs have been reported in the TLR2 gene; 26 of them are in the 5'-untranslated region, 17 are in the 3'-untranslated region, 29 are located in intronic parts of the gene, and 17 modify bases of the third exon of TLR2. Six non-synonymous SNPs of the *TLR2* gene change amino acids in the cytosolic part of this receptor. However, only two have been linked to reducing NF-κB activation and increasing risk of infection. The first consists in a change of C to T replacing arginine (Arg; R) with tryptophan (Trp, W) at position 677, abolishing the binding with MyD88 with TLR2. This specific polymorphism located within the *bb* loop of *TLR2* (Arg677Trp) abolishes activation of NF-κB in response to *M. tuberculosis* (34), resulting in decreased IL-12 serum level production by 677W carriers. The second functional *TLR2* variant consists of a G to A substitution, which substitutes an arginine for glutamine at position 753. The *TLR2* 753Q seems to be associated with an increased risk of developing tuberculosis for carriers the AA and AG genotypes (35). Recently, Thuong et al. (36) described a

strong association of SNP T597C *TLR2* with susceptibility to military tuberculosis patients from Vietnam. Further association was described among Koreans regarding the microsatellite polymorphisms in intron II or *TLR2* (37). In addition, *TLR1* polymorphism in a non-synonymous region (I602S) could be associated with TLR1/2 heterodimer binding sites to mycobacterial lipopeptide, since individuals with 602II genotype produced substantially more IL-6 than those with the 602SS variant (38). However, these preliminary studies need to be repeated on a large scale in distinct populations. Currently, the polymorphism in *TLR2* might be an important risk factor for disease progression, and that the functional consequences of different alleles may explain some of the variation in cytokines and NO

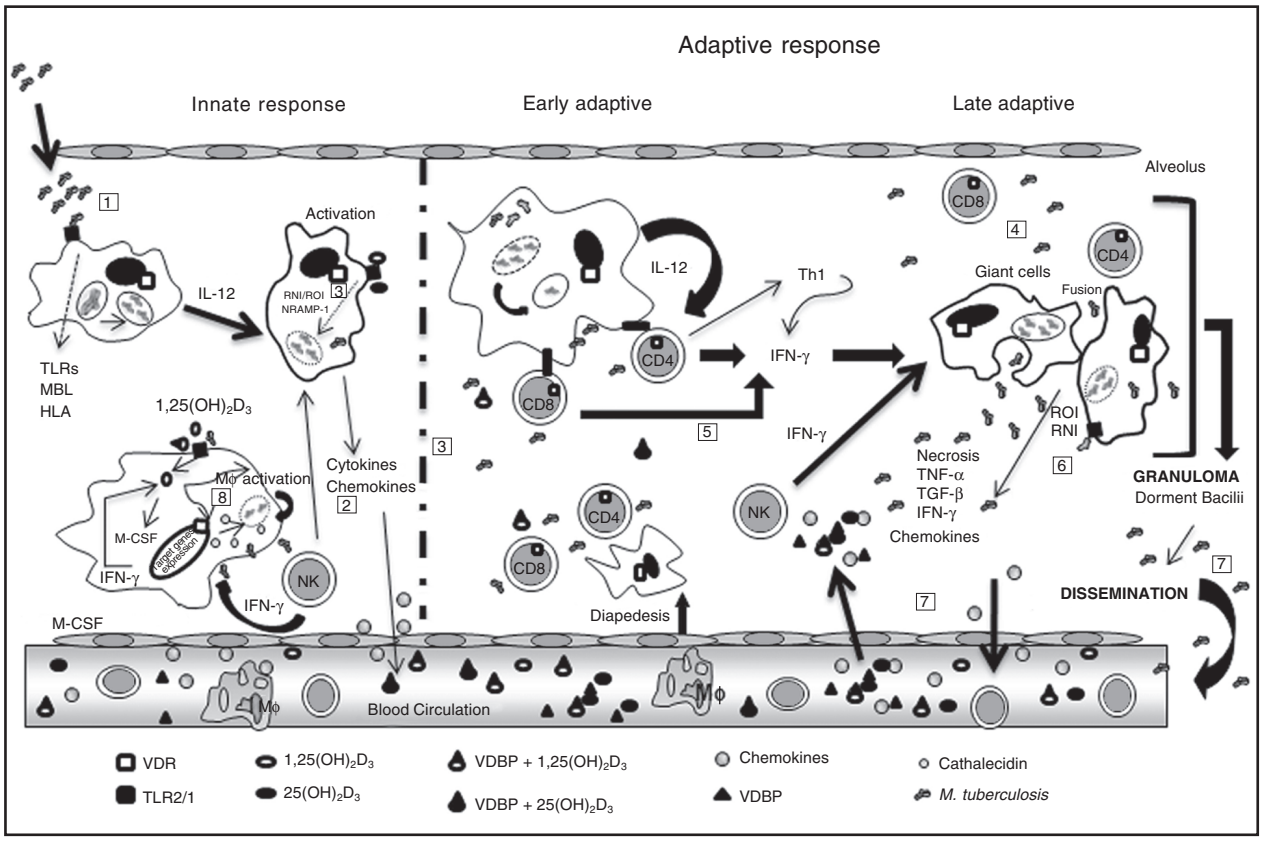

Figure 1. Scheme of the innate and adaptive immune response phases against mycobacteria. The figure identifies all polymorphic proteins involved during and after entrance of mycobacteria into the cell. The first step achieved by the mycobacteria is entering the macrophages (Mϕ) (1). After this step, the Mϕ are activated by cytokines (IFN-γ) to kill the mycobacteria (2). This phase is marked by important polymorphisms like nitric oxide production inside the phagocyte (3). If the Mϕ survives, the adaptive immune system is activated. This system establishes the granuloma, an important structure formed to contain the dissemination of the mycobacteria. The diagram illustrates the Mϕ, giant cells (GC-epithelial cells fusion), CD4, CD8 T and natural killer cells (NK) harboring intracellular mycobacteria from the centre of granuloma (4 and 5). The IFN-γ activates monocytes from circulation to Mϕ, which starts to kill the intracellular mycobacteria via reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (6). The Mϕ associated with the other antigen presenting cells (GC) present antigens to T cells and activate them to produce a variety of cytokines and chemokines. The chemokines recruit additional cells from circulating to kill the mycobacteria. Granulomas are a result of CD-mediated delayed type hypersensitivity reaction within parenchymal tissues (7). Accordingly, the same system that is responsible for bacterial growth decrease (host defense) is also intrinsically associated with tissue damage through granuloma formation and necrosis. If you have any disturbance in this system, you could be more susceptible to tuberculosis development. $1,25(OH)_2D_3$ and the vitamin D receptors (VDR) then together induce the expression of the gene encoding the human antimicrobial peptide LL-37. Circulating monocytes are activated by TLR2/1 agonists present on specific microbes. The genes encoding VDR and CYP27B1 are induced. CYP27B1 converts $25(OH)_2D_3$ from the circulation to $1,25(OH)_2D_3$, joins with VDR and activates the gene encoding LL-37, leading to an increase in cellular LL-37 and enhanced microbicidal activity of the phagocyte (8). IL = interleukin; IFN-γ = interferon-γ; TNF-α = tumor necrosis factor α; TLR = Toll-like receptor; MBL = mannose binding lectin; HLA = human leukocyte antigen; Th1 = T helper 1; TGF-β = tumor growth factor β; VDBP = vitamin D-binding protein; NRAMP-1 = natural resistance-associate macrophage protein 1. M-CSF = macrophage colony-stimulating factor.

expression and clinical forms of the disease. Understanding gained from knowledge of the effects of different alleles can contribute to the design of new therapeutic strategies including vaccines.

Nitric oxide synthase 2

NO is a free radical and second messenger that has been shown to be important in the development of several diseases, including tuberculosis. NO plays a major role in the pulmonary host-defense mechanism in response to infections and is implicated in bacteriostatic as well as bactericidal processes. NO is formed when the guanine group of the essential amino acid L-arginine is split forming NO and L-citrulline. The reaction is catalyzed by a class of enzymes called NO synthases (NOS). NO is produced by three different NOS: NOS1, NOS2 and NOS3. NOS1 is constitutively expressed due to fluctuations of Ca^{2+} and produces relatively small amounts of NO. The alveolar macrophages induced by mycobacteria are capable of producing TNF-α, IL-1β. These cytokines along with IFN-γ produced by T-cells can induce NO via action of NOS2 (Figure 1). Thus, the NO and radical nitrogen intermediate can kill and/or inhibit intracellular pathogens like mycobacteria. In contrast to murine models of tuberculosis, there is greater controversy about the role of NO killing or limiting the growth of *M. tuberculosis* in humans. It has been proposed that NO produced by tuberculosis-infected human macrophages and by epithelial cells is also antimycobacterial against *M. tuberculosis* (10). Nevertheless, a report from our group indicates that the alveolar macrophages from the lungs of patients with tuberculosis express NOS2 in potentially mycobactericidal amounts and that this NOS2 can kill mycobacteria *in vitro* (39). NOS2 expression is not usually detected in human macrophages from cell lines or macrophages derived *in vitro* from blood monocytes of health humans. However, alveolar and tissue macrophages from the lungs of tuberculosis patients show high levels of functional NOS2. Thus, NOS2 may represent a pivotal mechanism that protects against tuberculosis. Investigation is hampered by difficulty in estimating *in vivo* production of NO, but genetic studies provide a potential means of examining the relationship between NOS2 expression and disease outcome. NOS2 is encoded by a polymorphic gene known as *NOS2A* at chromosome 17q11.2-q12. Several SNPs and microsatellite polymorphisms have been described. *NOS2* gene is located in a region that has been linked to susceptibility to tuberculosis. It is known how important the biological and genetic action of NOS2 is in the immune system, because of that; the SNPs in this gene have been reported in many populations around the world. The SNPs in the promoter region of the encoding gene (-954G→C, -1173C→T, -1659 A→T) have been shown to increase NO synthesis (40). This region in the human gene is situated from -0.7 to -2.6 kb upstream of the transcription start and contains important DNA motifs for binding of NF-κB, activator protein 1, signal transducer and activator of transcription protein 1, and NF-κB repressing factor (41). Another important region lies between -2.5 and -950 kb upstream, and is characterized by a number of polymorphic elements like SNPs and repeat regions. It is this region in which various mutations are associated with *NOS2* expression *in vivo* (40). The -954G→C variant was originally reported in a highly endemic African malaria area (42), suggesting that this mutation originated as a consequence of selective pressure of *Plasmodium*. The G allele has been shown to be absent from Caucasian populations (42) as well as from the Peruvian population (43). In Mexicans, in whom the frequency of G allele was ≈5%, this SNP was not associated with tuberculosis (44). There are no further reports regarding NOS2 polymorphism frequency in Brazilian populations. Further work is in progress to determine if *NOS2A* gene polymorphism is associated with tuberculosis in this population.

Interferon-γ

IFN-γ is essential to a relationship between innate and adaptive immunity in combating *M. tuberculosis* infection (Figure 1). Active tuberculosis is characterized by lower peripheral blood mononuclear cell production of IFN-γ than latent infection, and local and systemic IFN-γ levels correlate with the severity of disease. Perhaps the most relevant mechanism is the activation of mononuclear phagocytes by the cytokines, particularly by IFN-γ. This cytokine is produced as a consequence of antigenic activation by natural killer (NK) and T cells, especially T helper 1 (Th1), and its production is increased by antigen-presenting cell-derived cytokines such as IL-12 and -18. IFN-γ has regulatory effects in both innate and adaptive immune responses, which include activation of mononuclear phagocytes and NK cells, increased expression of major histocompatibility complex I and II, induction of CD8+ lymphocyte maturation into cytotoxic cells, and promotion of the immunoglobulin isotype switch in B cells towards immunoglobulin G1 and G3 in humans (45).

Production of IFN-γ is genetically controlled, and there are two well-known polymorphisms in the *IFN-γ* gene. It has been reported that the 12 CA repeat microsatellite allele in the noncoding region of the first intron is associated with a high level of *in vitro* cytokine production (46). The same group has also reported complete linkage disequilibrium between the 12 CA repeat allele and the pres-

ence of the T allele at position +874 T/A *IFN-γ* from the translation start site. This polymorphism lies within a binding site for the transcription factor NF-κB, and electrophoretic mobility shift assays showed specific binding of NF-κB to the allele sequence containing the +874 *IFN-γ* T allele (47). As this transcription factor induces IFN-γ expression, +874T *IFN-γ* T and A alleles probably correlate with high and low IFN-γ expression, respectively (48). In addition, Ottenhoff et al. (49) discovered several families with Mendelian susceptibility to mycobacterial disease that has mutations in one of two subunits of the *IFN-γ* receptor gene (*IFN-γR1* and *IFN-γR2*). More recent data suggested that a more common polymorphism at position +874 is associated with risk of tuberculosis in different populations (48,50). IFN-γ acts as a regulator of gene expression through activation by a receptor complex comprising two subunits, each encoded by a different gene: *IFN-γR1* on chromosome 6, region q23-24 and *IFN-γR2* on chromosome 21 region q22.1-22.2. Homodimers of IFN-γ interact with both receptor proteins, leading to receptor dimerization. None of the three molecules (two receptor proteins and the cytokine itself) play a redundant role in ligand-activated receptor signaling.

The AA genotype in +874T/A region is associated with increased tuberculosis susceptibility in Spanish and South African populations (48,50), but not in Croatian and North American populations (51,52). Few studies have been conducted in the Brazilian admixed population regarding *IFN-γ* polymorphisms at position +874 and its correlation, if any with infectious diseases. Amim et al. (53) showed an important association in +874 AA genotype with the occurrence of active tuberculosis disease, independent of tuberculosis skin test status. However, the selection of these tuberculosis cases (from the Southwest region of Brazil) and healthy controls (from the Central-west region of Brazil) may cause a misunderstanding, because the Brazilian population is considered admixed. The genetic background between these two geographic areas should be different and may interfere in the genetic background analysis and results.

Perhaps lower IFN-γ secretion (inferred from the lack of T at +874 position) might influence replication of *M. tuberculosis*, thus influencing the outcome of the tuberculosis diagnosis (51) and disease. The association of *IFN-γ* genotype +874 AA with disease in several populations confirms a significant role of genetic variation at the *IFN-γ* locus and provides more detailed understanding of the genetic mechanisms underlying the association with disease. The absence of association with *IFN-γR2* polymorphism in the African population was interesting, but firm conclusions cannot be drawn without additional studies. Furthermore, the association of disease with *IFN-γR1* polymorphisms in the same population was novel, and together, these findings support the hypothesis that a genetically determined variation in both IFN-γ production and responsiveness influences the disease. Thus, despite the remarkable importance of IFN-γ production in *M. tuberculosis* growth control, published studies can only suggest that polymorphism in this gene might influence susceptibility to tuberculosis in some populations.

On the other hand, an important IFN-γ inducer may interfere in the multigenic tuberculosis resistance or susceptibility. IL-12, a heterodimeric pro-inflammatory cytokine produced by monocytes, macrophages, DCs and B lymphocytes, modulates Th1 differentiation, having a clear interface in IFN-γ production. The importance of IL-12 as an IFN-γ inducer lies not only in its high efficacy at low concentration but also in its synergism with many others activating stimuli. IL-12-induced IFN-γ production requires the presence of low concentrations of TNF-α and IL-1, which are usually produced in an autocrine manner by T and NK cells. The gene for the IL-12 p40 subunit is located on chromosome 5q 31.1-33.1. The SNP in the gene responsible to express this subunit was first described by Hall et al. (54). They showed a frequency of 60, 36.9, and 3.1% for genotypes AA, AB and BB, respectively, in a Caucasian population in the United Kingdom. Recently, several reports have described the *IL-12B* polymorphism in black and white North American populations with no tuberculosis susceptibility correlation. However, four SNPs described at positions 641 A-G, 684 C-T, 1094 T-C, and 1132 G-C can cause three missense variants (Q214R, M365T and G378R) and one synonymous substitution in the extracellular domain of the *IL-12Rβ1* gene (55). These investigators reported that the association of R214-T365-R378 allele (allele 2) is over-expressed in Japanese tuberculosis patients with the homozygosis for R214 - T365 - R378 (the 2/2 allele) being significantly associated with tuberculosis. In this manner, *IFN-γ* and *IL-12* gene polymorphisms and further protein expression may play an important role during the innate and adaptive immune response to *M. tuberculosis* infection and disease, driving a protective long life latent infection or disease installation. Further investigations are necessary to identify the haplotype distribution in *IFN-γ* and *IL-12* gene polymorphism.

Cross talk between *TLR2, NOS2, IFN-γ, VDR* and *DBP* gene expression and immunity to *M. tuberculosis*

It is known that genetic defects can lead to increased risk of mycobacterial infections (56). Thus, the identification of the genes where mutations lead to extreme suscep-

tibility will help us to identify essential components of the innate and/or adaptive human immune defense to *M. tuberculosis*. Several SNPs or defined haplotypes have been studied to identify possible candidate(s) allele(s) that may cause subtle changes in genetic function that could account for individual variation in susceptibility to tuberculosis.

For this reason, many studies described in this review have looked for an association of tuberculosis susceptibility with polymorphisms in genes encoding specific elements of the immune system thought to be important in controlling mycobacterial infection.

One of the first lines of defense of the immune response is the recognition and uptake of microorganisms by phagocytes and several different pattern recognition receptors in these cells including the TLR. Pathogen binding to specific TLRs or combinations of TLRs may recruit different adaptor proteins allowing a specific signaling cascade and gene activation programs. A central function of TLRs is to directly activate acute anti-microbial defense systems (57). These receptors, after recognition, can stimulate signalizing pathways that activate the innate immune response, cytokine production and the process of adaptive immunity. Activation of TLRs from specific mycobacterial lipoproteins can stimulate macrophages to express inflammatory cytokines (TNF-α, IL-6, IL-12 and IFN-γ) and induction of anti-microbial genes, such as the expression of *NOS2* and final production of NO (57). The activation of TLRs can also up-regulate the expression of vitamin D receptor and vitamin D-1-hydroxylase genes (enzyme that metabolizes $25(OH)_2D_3$ to $1,25(OH)_2D_3$), leading to induction of the antimicrobial peptide cathelicidin and killing of intracellular *M. tuberculosis* (8). In addition, $1,25(OH)_2D_3$, by itself, can modulate the immune response by its nuclear receptor (VDR), where it up-regulates protective immune host defense by induction of NOS (58) and cathelicidin (9) as well as down-regulation of *IFN-γ* gene expression by down-regulating its promoter (59). It is known that $1,25(OH)_2D_3$ is present 1000-fold less in peripheral blood than $25(OH)_2D_3$, and the release of these forms in the granuloma space by VDBP may modulate T cell responses. The induction of $1,25(OH)_2D_3$ can inhibit T cell proliferation and suppression of B cell immunoglobulin production initially by inhibition of IL-2, GM-CSF and IFN-γ (7). However, $1,25(OH)_2D_3$ suppresses Th1 cytokine profile favoring Th2 cells and can activate regulatory T cells (7), modulating the host response to *M. tuberculosis* infection. On the other hand, the TLR signaling in macrophages and DC leads to secretion of IL-12 that skews the resultant T-cell response towards Th1 phenotype. Th1 cells secrete IFN-γ, which has multiple functions in activating macrophages and enhances their phagocytic and microbicidal abilities (60). IFN-γ is, also, critical for further expression and production of NOS2 in adaptive response (39) (Figure 1). In addition, IFN-γ secreted by T cells inside the granuloma potentiates the up-regulation of 1α-hydroxylase and inhibits the key enzyme in $1,25(OH)_2D_3$ inactivation (24-hydroxylase) (58).

Therefore, it is possible to suggest a link between TLR, vitamin D (receptor and transporter) and specific cytokines expressed in the immune response, such as IL-12 and IFN-γ. Consequentially, possible defects in these molecules caused by specific polymorphisms might be associated with susceptibility to tuberculosis (34,35,37).

Conclusions

Several gene polymorphisms are being described in association with human tuberculosis susceptibility; however, few of them are clearly described in a genetically distinct population. Tuberculosis incidence, socio-economic status, nutritional condition, exposition grade, cultural aspects, bacilli virulence, *M. tuberculosis* strain distribution, access to healthcare and medication should also be considered. There is always a need for better understanding of the immunopathology of tuberculosis through the knowledge of the precise genetic mechanisms controlling human infectious diseases. Furthermore, SNP description alone will not be sufficient to describe susceptibility to tuberculosis in a broad diverse population, and thus, functional gene studies need to be done. A real challenge is to associate candidate genes with a biologically plausible mechanism that explains the epidemiological data for tuberculosis in which only 10% of the infected individuals will develop tuberculosis. We believe that from the genes involved in *M. tuberculosis* control, *TLR2*, *NOS2*, *IFN-γ* and vitamin D, their carrier and their receptor may generate a potent resistant haplotype and could be highlighted as powerful tools for understanding the interplay between innate and adaptive host resistance. The relationship between *M. tuberculosis* virulence strains and host genetic variability will further help us to design new vaccines and immunotherapy.

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