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# Tuberculosis Bacilli Still Posing a Threat. Polymorphism of Genes Regulating Anti-Mycobacterial Properties of Macrophages

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger
on the tenth anniversary of his passing away

Abstract

One third of the earth·s population is infected with *Mycobacterium tuberculosis*, but only 5–10% of the infected individuals will develop active disease over their lifetime. To identify the genes responsible for the variation in the human susceptibility/resistance to tuberculosis (TB) we determined the polymorphisms of three genes crucial for the function of macrophages, in TB patients and healthy controls with no past history of TB. We found no association between the polymorphisms of the *NRAMP-INT4*, *MBL* (codons 52, 54, 57) and *CD14-159* genes and TB in a Caucasian Polish population. However, we have suggested a possible involvement of CD14 and MBL molecules in the host-mycobacteria interactions on the basis of the significant increase in the serum CD14 and MBL in TB patients compared to healthy controls.

Key words: tuberculosis, *CD14*, *MBL*, *NRAMP-1*, polymorphism

## Introduction

Tuberculosis (TB) carries serious health and economic implications in today's society. The global burden of tuberculosis remains enormous, mainly because of poor control in Southeast Asia, sub-Saharan Africa and eastern Europe, and because of high rates of *Mycobacterium tuberculosis* and Human Immunodeficiency Virus (HIV) coinfection in some African countries (Dye *et al.*, 1999). It has been estimated that *M. tuberculosis* infects about one-third of the world population and causes 8 million active cases of TB per year (WHO 2004). About 2 million people die of TB every year. The highest mortality of TB is notified in some African countries with high HIV rates. A new increasing problem is the appearance of multidrug-resistant strains of *M. tuberculosis* and this represents a failure in case management. The bacilli Calmette – Guèrin (BCG), a live attenuated strain of *M. bovis*, is administered to approximately 100 million people every year. This vaccine is effective in preventing TB in children, however, it failed to reduce the incidence of the disease in the adult population. New drugs, more effective vaccine and better understanding of *M. tuberculosis* – host interactions are needed to counter TB.

Epidemiological data show that most of *M. tuberculosis* infected population neither develops disease nor becomes infectious, and clinical disease occurs in less than 10% of infected subjects. It suggests that genetic

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differences in the host determine the immunological response, disease severity, and ultimate outcome of infection with mycobacteria. As intracellular pathogens, *M. tuberculosis* bacilli have the ability to survive within the host macrophages. Thus, we may expect that in the majority of *M. tuberculosis* infected subjects, the interaction between *M. tuberculosis* and phagocytic cells results in a dynamic balance between the host defence system and the virulence factors of mycobacteria allowing the persistence of *M. tuberculosis* in the absence of disease. Unfortunately, in about 10% of *M. tuberculosis* infected subjects, the interaction between macrophages and *M. tuberculosis* bacilli may result in tissue damage characterised by tissue necrosis with the formation of cavities and dissemination of the disease. To identify the genes responsible for differences in the human susceptibility to TB we investigated the polymorphism of three genes, *NRAMP-1*, *MBL*, *CD14*, which encode the proteins crucial for the functions of macrophages, in TB patients and healthy subjects who had no past history of tuberculosis. The *NRAMP-1* gene (Natural Resistance Associated Macrophage Protein-1) is a human homologue of mouse *Nramp-1* gene which confers increased resistance to infection with *Salmonella typhimurium*, *Leishmania donovani* and *M. bovis* BCG (North *et al.*, 1996). The *MBL2* gene encodes mannose-binding lectin (MBL) a calcium-dependent serum lectin that acts as an opsonin to promote phagocytosis and activates complement *via* the classical pathway. The co-dominant single-base substitutions in codons 52, 54 and 57 result in reduced serum MBL concentrations (Turner, 1996). The *CD14* gene encodes a glycosylphosphatidylinositol-linked cell surface molecule CD14 which mediates mycobacteria induced activation of macrophages *via* Toll-like receptors (TLR), particularly TLR2 (LeBouder *et al.*, 2003). The concentration of soluble CD14 (sCD14) and MBL in the sera from TB patients and controls was also determined.

## Experimental

### Materials and Methods

**Subject characteristics.** Our study group consisted of 250 Caucasian Polish subjects, 126 patients with pulmonary tuberculosis (age 17–90 years, mean 51 ± 16) and 124 healthy volunteers (age 18–85 years, mean 50 ± 14) who had no past history of TB. In the patient group (TB patients), tuberculosis was confirmed by culture of *M. tuberculosis* from sputum. The study was approved by the Human Ethics Committee. All participants signed the written informed-consent documents.

***NRAMP-1*, *CD14* and *MBL* gene polymorphism.** DNA was isolated from EDTA-anticoagulated blood using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The *NRAMP-1/INT4* (469+14G/C) and CD14/-159C/T polymorphisms were determined by PCR method. The reaction was performed in 10 µl volume that contained 50–100 ng of DNA, 0.25 mM of paired primers, 1 X PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Promega, Madison, USA). The paired primers used for CD14/–159 polymorphism were, for C allele: 5'-CTC CAG AAT CCT TCC TGT TAC GAC-3' and 5'-TTG GTG CCA ACA GAT CAG GTT CAC-3' and for T allele: 5'-TTG GTG CCA ACA GAT CAG GTT CAC-3' and 5'-TGT AGG ATG TTT CAG GGA GGG GTA-3'. The PCR conditions were: 95°C for 5 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 5 min. The primers used for *NRAMP-1/INT4* polymorphism were general primer 5'-CCT GCC TCC TCA CAG CTT CT-3' and G specific primer 5'-GGT TCT CCC TGT CCA GGC-3' or C specific primer 5'-GGT TCT CCC TGT CCA GGG-3'. The amplification consisted of a 2-min denaturation step at 94°C, 10 cycles of 10 s at 94°C and 60 s at 65°C, and 20 cycles of 10 s at 94°C and 50 s at 61° and 30 s at 72°C. PCR-amplified DNA were visualized by electrophoresis in 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized by photography under UV transillumination. In PCR for CD14/159 polymorphism, the assay yields a 381-bp band for the T allele and a 227-bp band for the C allele. Genotyping for MBL-2 variants was performed by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR). To detect the mutations at codons 54(GGC→GAC, allele B) and 57 (GGA→GAA, allele C), a 685-bp fragment was amplified by PCR using the primers: 5'-AGT CGA CCC AGA TTG TAG GAC AGA G-3' and 5'-AGT TGT TGT TCT CCT GTC CAG-3'. The PCR product was digested with BanI and MboII restriction enzymes (Promega, Madison, USA). Detection of the mutation at codon 52 (GCG®GTG, allele D) was performed by MluI and HhaI restriction enzyme digestions of the 125-bp PCR-product amplified with the following primers: 5'-CAT CAA CGG CTT CCC AGG CAA AGA CGC G-3' and 5'-AGG ATC CAG GCA GTT TCC TCT GGA AGG-3'. PCR was performed in a reaction volume of 10 µl, containing 50–100 ng of DNA, 0.25 mM of specific primers, 1 X PCR buffer with 1.5 mM MgCl2, 0.2 mM dNTP, and 0.5 U Taq DNA polymerase (Promega, Madison, USA). All PCRs were initiated by a 5-min denaturation step at 95°C and completed by a 5-min extension step at 72°C. Amplification consisted of 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. PCR products were digested with the restriction enzymes BanI, MboII, MluI and HhaI at 37°C for 2 h, separated by electrophoresis in 2% agarose gel and stained with ethidium bromide.

**Serum levels of sCD14 and MBL.** The concentration of sCD14 in the sera was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine TM sCD14, R&D, MN, USA). MBL levels in the sera were determined by using a Human MBL ELISA test Kit (HyCult Biotechnology, Uden, The Netherlands). The optical density (OD) of each sample was determined using a multifunctional counter Victor 2 (Wallac Oy, Turku, Finland) set at 450 nm.

**Statistical analysis.** All analyses were performed using Statistica 5.0 (Statsoft). Comparisons between the various genotypes in TB patients and controls were made using the chi-square and Fisher's exact test. Comparison of mean sCD14 values between the patients and controls was made by Mann-Whitney U-test. A *P* value $<0.05$ was considered significant.

## Results

Allele and genotype frequencies of the patients with culture-proven tuberculosis (TB patients) and healthy individuals who had no past history of TB (controls) were analysed for the single nucleotide polymorphism (SNP) in the intron 4 of *NRAMP-1* gene and promoter region of *CD14*/-159 gene. Three co-dominant single-base substitutions in codons 52, 54, and 57 in *MBL* gene were also investigated in TB patients and healthy controls. The *NRAMP-1/INT4* polymorphism was determined for 126 TB patients and 114 healthy individuals. The distribution of alleles G and C as well as genotypes GG, GC and CC, was almost identical in both TB cases and control group (Table I). The *NRAMP-1* GG genotype was the most frequent in the subjects who underwent the study (range 60–61%). The distribution of GC heterozygotes was almost the same in TB patients (36%) and controls (35%). Equally low frequency (4%) of CC genotype was noticed for the control group and TB cases.

The *CD14/-159* polymorphism was determined for 126 TB patients and 122 healthy individuals. No association was found between the *CD14*/-159 gene polymorphism and the presence of TB (Table II). About half the subjects were *CD14 CT* heterozygous in both TB patients and controls (range 47–49%). Thirty eight percent of TB cases versus thirty one percent of healthy individuals were CC homozygous. This difference was not statistically significant; $P>0.05$. Also, no significant difference was found between the TB patients and control group in the frequencies of the carriers of the less frequent *CD14 TT* genotype (15 and 20% respectively).

Table I
Allele and genotype frequencies of *NRAMP-1/INT4* gene polymorphism in TB patients and healthy controls

| *NRAMP-1* | TB patients number (%) | Controls number (%) |
|---|---|---|
| Allele | | |
| G | 121 (70) | 110 (71) |
| C | 51 (30) | 44 (29) |
| Total | 172 (100) | 154 (100) |
| Genotype | | |
| GG | 75 (60) | 70 (61) |
| GC | 46 (36) | 40 (35) |
| CC | 5 (4) | 4 (4) |
| Total | 126 (100) | 114 (100) |

Table II
Allele and genotype frequencies of *CD14/C(-159)T* gene promoter polymorphism in TB patients and healthy controls

| *CD14* | TB patients number (%) | Controls number (%) | $X^2$ | p value |
|---|---|---|---|---|
| Allele | | | | |
| C | 107 (58) | 97 (54) | 0.61 | 0.41 |
| T | 78 (42) | 84 (46) | 0.67 | 0.41 |
| Total | 185 (100) | 181 (100) | | |
| Genotype | | | | |
| CC | 48 (38) | 38 (31) | 1.32 | 0.25 |
| CT | 59 (47) | 59 (49) | 0.06 | 0.81 |
| TT | 19 (15) | 25 (20) | 1.24 | 0.26 |
| Total | 126 (100) | 122 (100) | | |

Table III
Allele and genotype frequencies of *MBL* (52, 54, 57 codons) gene polymorphism in TB patients and healthy controls

| *mbl-2* alleles | TB patients number (%) | Controls number (%) |
|---|---|---|
| A | 68 (63.0) | 56 (61.0) |
| B | 23 (21.2) | 23 (25.0) |
| C | 2 (1.8) | 2 (2.1) |
| D | 16 (14.8) | 11 (11.9) |
| Total | 108 (100) | 92 (100) |

A total of 108 TB cases and 92 controls were genotyped for the *MBL* polymorphism (Table III). The frequency of the wild type *MBL* allele was not different among TB cases and controls (range 61–63%). Also, no difference was found in the distribution of the B, C and D alleles of the *MBL* gene in TB patients and healthy individuals. One TB patient possessed two mutant alleles, B and D, for *MBL* gene.

The levels of sCD14 in the sera from 71 TB patients and 56 healthy individuals were tested by ELISA. Fig. 1 shows that there was no association between the serum sCD14 levels and *CD14/-159* promoter polymorphism, either in TB patients or in controls. However, patients with TB exhibited significantly higher levels of sCD14 compared to healthy individuals. This increase was observed for all three *CD14* genotypes (in CC 1611 ± 334 ng/ml versus 3450 ± 1646 ng/ml, $P=0.000001$; in CT 1556 ± 441 ng/ml versus

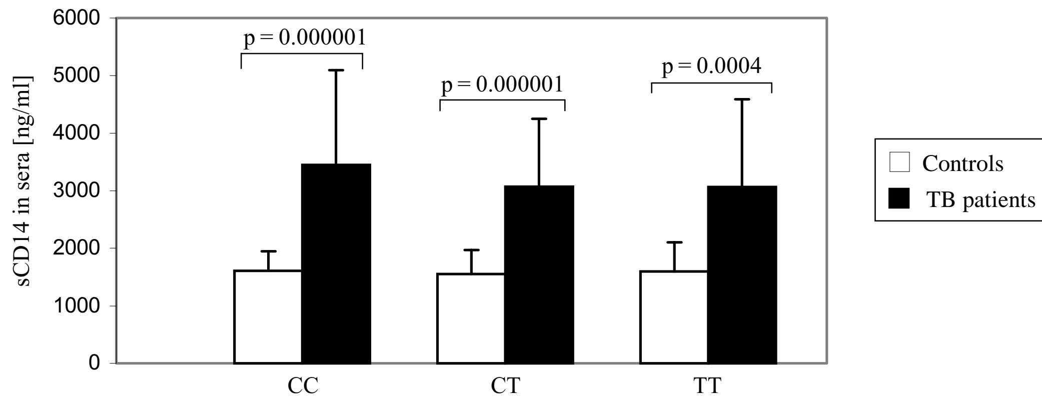


Fig.1. The significant increase in the concentration of sCD14 in TB patients and healthy controls with no history of tuberculosis. No association between the *CD14/159* gene polymorphism and serum sCD14 level.

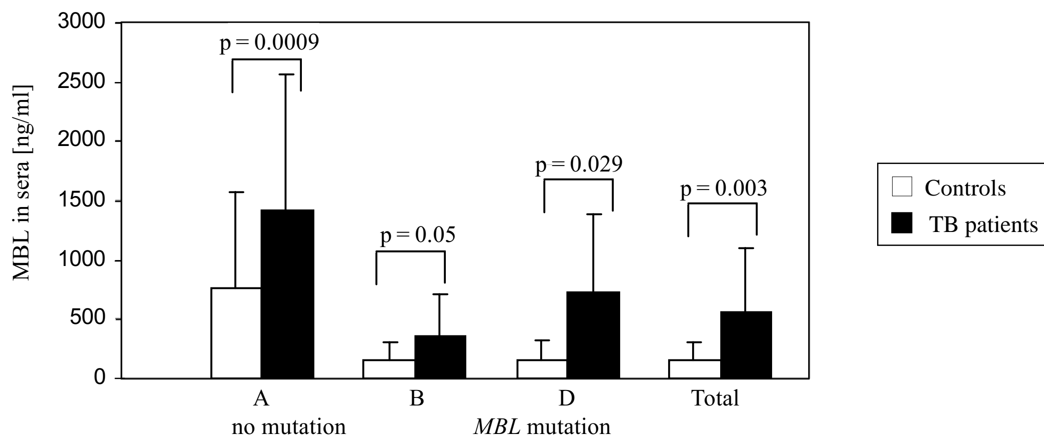


Fig. 2. The significant association between the *MBL* gene polymorphism and the serum MBL level. The significant increase in the concentration of the lectin in TB patients compared to healthy controls with no past history of TB seen in the subjects with or without *MBL* gene mutations.

3073 ± 1174 ng/ml, $P = 0.000001$; in TT 1598 ± 507 ng/ml versus 3068 ± 1519 ng/ml, $P = 0.00004$). The concentration of MBL in the sera from 108 TB patients and 92 healthy individuals was determined by ELISA. In agreement with the previous report, the co-dominant single-base substitutions in codons 52, 54 and 57 of *MBL* gene resulted in reduced serum MBL concentrations (Fig. 2). The average concentration of serum MBL determined for the healthy individuals with the wild type *MBL* gene was significantly higher (755 ± 805 ng/ml) than serum MBL levels found for the healthy donors with variant alleles for this gene (157 ± 140 ng/ml, $P = 0.0002$). The pulmonary tuberculosis was associated with a significant elevation of the serum MBL concentration. The increase in serum MBL levels was noticed in TB patients with the wild type *MBL* gene (755 ± 805 versus 1423 ± 1138 ng/m, $P = 0.0009$) and those with the B (159 ± 136 versus 355 ± 345 ng/ml, $P = 0.05$) and D (153 ± 161 versus 730 ± 652 ng/ml, $P = 0.029$) variant alleles for this gene.

## Discussion

One third of the earth's population is infected with *M. tuberculosis*, but only 5–10% of the infected individuals will develop active disease over their lifetime. There is an increasing interest in the understanding of the role of genetic factors controlling susceptibility to TB in humans because this will allow us to develop effective strategies to combat this disease. A variety of studies have demonstrated that a large number of host genes are probably important in susceptibility to tuberculosis (Bellamy *et al.*, 1998a, b). The main route of infection for *M. tuberculosis* is the respiratory tract, where the bacilli are inhaled in airborne droplets. After entering lung, the first cell type encountered by the bacteria is the alveolar macrophage, which has the bactericidal ability to destroy most potential invaders. However, pathogenic mycobacteria prevent maturation of the phagosomes in which they reside inside macrophages and this is thought to be a major strategy

allowing them to survive and multiply within macrophages. The macrophages have multiple functions in *M. tuberculosis* infection including the antigen processing and presentation, production of cytokines that regulate the maturation and function of lymphocyte subsets, formation of granuloma that may retain mycobacteria inside cells and prevent their dissemination or eventually may result in tissue damage characterised by necrosis. The importance of the macrophages in the mycobacteria-host interaction prompted us to investigate the polymorphism in the *NRAMP-1*, *CD14* and *MBL* genes, which are involved in the expression of the macrophage functions. In mice, the *NRAMP-1* gene encodes a transmembrane protein that translocates to the phagocytic vacuole of macrophages following phagocytosis of bacteria and affects the intracellular survival of bacilli (Frehel *et al.*, 2002). In this study the allele and genotype frequency of *NRAMP-1*gene polymorphism was almost the same in TB patients and controls. In contrast, in case-control study in Gambia, four polymorphisms in the *NRAMP-1* gene were found associated with TB (Bellamy *et al.*, 1998a). In Denmark, Søborg *et al.*, 2002, found that variant alleles in *NRAMP-1* gene were associated with increased mycobacterial replication rather than susceptibility to TB. Also Pacheco *et al.* (2003) demonstrated no association between the *CD14/-159* polymorphism and TB among a population of Colombia. A study in Malawi showed that a heterozygosity for a newly investigated CAAA insertion/deletion polymorphism in the *NRAMP-1* gene was associated with a protection against TB (Fitness *et al.*, 2004). However, association of other variants of *NRAMP-1* gene with TB that was reported for other populations was not replicated in Malawi. This suggests that the genes and variants relevant to the susceptibility to TB may vary significantly between populations. Also, their distribution may be affected by the high rates of HIV infections in some African countries. MBL was included into our study as a key component of the innate immunity. It recognises peptidoglycan of Gram-positive bacteria *via* its C-type lectin domains and modulates the cytokine and chemokine releasing (Nadesalingam *et al.*, 2005). MBL insufficiency due to polymorphisms in the *MBL2* gene causes an opsonisation defect and predisposes to recurrent infections in children and adults (Turner *et al.*, 1996). The frequency of the *MBL* gene mutations (37%) observed by us among healthy Polish subjects was slightly higher than those reported for a Gambian population (31%) (Bellamy *et al.*, 1998b). We have also shown no association between the *MBL* gene polymorphism and a risk of TB in Caucasian Polish population. In contrast, the study among Gambiens in West Africa, and African-Americans in Texas, showed a significantly lower frequency of the C and B alleles of *MBL* gene, respectively, among TB cases compared to healthy blood donors (Bellamy *et al.*, 1998b; El Sahly *et al.*, 2004). This suggested that MBL polymorphism was protective against TB in West Africans and African-Americans. However, in agreement with our data no protective effect of *MBL* gene polymorphism was observed among a white and Hispanic population in Texas, Africans in Malawi (Fitness *et al.*, 2004) and a community in India (Selvaraj *et al.*, 1999). Thus, the ethnicity may determine the association of *MBL* polymorphism with the resistance/susceptibility to TB.

The data presented in this paper confirmed the previous reports on a significant elevation of serum MBL levels in TB patients compared to controls (Garred *et al.*, 1997; Bonar *et al.*, 2004). It is known that MBL binds mycobacteria strongly (Nadesalingam *et al.*, 2005) and it has thus been suggested that MBL may facilitate the uptake of mycobacteria by phagocytes. Because macrophages are the living environment for mycobacteria, high MBL serum levels could be a relative disadvantage for the host in relation to these bacteria. However, this suggestion contradicts the data of this study presenting the lack of association between a risk of TB and the MBL gene polymorphisms that result in serum MBL deficiency. Moreover, a previous study reported by us (Paziak-Domańska *et al.*, 2002) has shown that the variation in serum MBL level does not affect the ingestion of *M. bovis* BCG by macrophages. On the other hand, our results do not exclude a possible influence of the increased levels of serum MBL on the host immune response to *M. tuberculosis*. Thus, MBL together with peptidoglycan of Gram-positive bacteria increases the production of chemokines, IL-8 and RANTES, but reduces that of TNF-α (Nadesalingam *et al.*, 2005).

To understand the genetic background of the variation in human susceptibility to pathogenic mycobacteria we also determined the *CD14/-159* polymorphism for TB patients and healthy individuals. The macrophage CD14 plays a pivotal role in innate immunity. It functions as a multifunctional receptor for bacterial cell wall components and enhances Toll-like receptors – mediated signaling (LeBouder *et al.*, 2003). Previously, we found an association between the *CD14/-154* promoter polymorphism and development of delayed type hypersensitivity to tuberculin, in healthy volunteers vaccinated with *M. bovis* BCG vaccine. Thus it was relevant to investigate a possible relation between the *CD14/-159* polymorphism and TB. However, no difference was found in the distribution of the allele and genotype *CD14/-159* gene polymorphisms between TB patients and healthy individuals with no past history of TB. The lack of the association between the *CD14/-159* polymorphism and a risk of TB was also observed in a population of Caucasian and Mestizo ethnic groups in Colombia (Pacheco *et al.*, 2004). However, the significant increase in sCD14 has suggested

a role of the CD14 molecule in the host-mycobacteria interactions. The same increase in sCD14 concentration was observed for TB patients carrying three different CD14/-159 genotypes (CC, CT, TT). Thus there was no association between sCD14 levels and *CD14/-159* polymorphism. This finding is consistent with a previous report (Pacheco *et al.*, 2004) but in contrast with the others showing an elevation of sCD14 in the individuals with the CD14/-159 TT genotype (Baldini *et al.*, 1999; Eng *et al.*, 2004). Several reasons might account for these discrepant findings including the presence of stratification of studied groups varying by the age, ethnic origin and health status. The elevated levels of serum sCD14 were observed in several infectious and non-infectious diseases. Although the function of sCD14 in human diseases has not yet been clarified, it is believed that elevated sCD14 in infectious and non-infectious diseases results from the state of activation of monocytes and macrophages. It is known that activation of monocytes results in increased shedding of sCD14. The elevated concentration of sCD14 may also reflects an increased need of plasma clearance of inflammatory stimuli such as mycobacterial lipoarabinomannan.

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