ABOUT THE AUTHOR

Johanneke Kleinnijenhuis was born in Den Ham on March 21th, 1979. After completing secondary school (Augustinus College, Groningen and Menso Alting College, Hoogeveen) she studied Biomedical (Health) Science at the Radboud University, Nijmegen from 1997-2001. She obtained research experience during two internships in the Radboudumc at the departments of Pathology and Clinical Pharmacy. In 2001 she started with Medicine and went abroad for two international internships during that study. The first one was in the Hospital Dr. Humberto Alvarado Vasquez, Masaya, Nicaragua. She finished her medical degree in the Sengerema Designated District Hospital, Tanzania in 2006. Later that same year she began her residency Internal Medicine at the Radboudumc (prof. dr. Jos van der Meer, Prof. dr. Jacqueline de Graaf, and Prof. dr. Jan Nuij). In 2008 she continued her residency at the Slingeland Hospital in Doetinchem (Dr. A Mudde). During that time she started to discuss the possibility of doing a PhD project with Prof. Dr. Mihai Netea which resulted in a personal grant from the Radboudumc in 2009. She started the project “Host response to mycobacteria: innate recognition and memory” in 2010 and alternated research and clinical work until 2015. During her PhD project she visited Seph Borrow’s group at Oxford University for four months to work on a joined project. From November 2012 till August 2013 she continued her medical training as registrar infectious diseases (Prof. dr. B.J. Kuilberg) and worked during that time four months in the Academic Hospital Paramaribo, Suriname.

HOST RESPONSE TO MYCOBACTERIA: INNATE RECOGNITION & MEMORY

JOHANNEKE KLEINNIJENHUIS
HOST RESPONSE

TO MYCOBACTERIA:

INNATE RECOGNITION

& MEMORY

Johanneke Kleinnijenhuis
HOST RESPONSE TO MYCOBACTERIA:
INNATE RECOGNITION AND MEMORY

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The cure for anything is salt water: sweat, tears or the sea.

Isak Dinesen (1885-1962)
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INTRODUCTION
INTRODUCTION

A historical perspective
It seems likely that mycobacterial existence, including its most famous and infamous family member, Mycobacterium tuberculosis (MTB), goes back as far as 40,000 years (1). Throughout Egyptian dynastic times paintings were made depicting angular spinal deformities characteristic of tuberculous spondylitis. In mummies from several Egyptian sites skeletal changes typical of tuberculosis were observed, further supporting the notion that the skeletal changes were due to tuberculosis (TB). (2) In ancient times TB was known as ‘consumption’ and ‘white plague’. Hippocrates, the ‘father of the western medicine’, identified ‘phthisis’ (consumption) as the most widespread and fatal disease of his time.

Another important moment in the history of tuberculosis occurred on the evening of 24 March, 1882. For the first time the causal agent of tuberculosis was discovered and presented that evening in Berlin. Robert Koch described the importance of this discovery: ‘If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera and the like. One in seven of all human beings dies from tuberculosis’. For his investigations and discoveries regarding tuberculosis he was awarded the Nobel Prize in Physiology or Medicine in 1905. (2) Now, more than 130 years later, Robert Koch’s words are, unfortunately, still valid. TB is second only to human immunodeficiency virus (HIV) as the most fatal single infectious agent, comprising in 1.3 million deaths in 2012. A third of the world’s population is latently infected with MTB, which led to 8.6 million active infections in 2012 (3). In Koch’s time there was no way of preventing TB. With his knowledge of the causative agent, the means of transmission was identified in the following decades. In addition, breakthroughs in immunology research resulted in a vaccine that is still in use. Bacille Calmette Guerin (BCG) was first implemented in the 1920s and is now one of the most used vaccines worldwide. It is still widely used in high burden countries, leading to an enormous decrease in both MTB-related and unrelated infant mortality and morbidity. Unfortunately, this vaccine is not particularly effective at preventing the main disease presentation of TB, adult pulmonary TB.

When preventive strategies are lacking, managing the disease becomes even more important. In the case of many infectious diseases, to conquer them one needs besides antimicrobial agents a solid immune response. The importance of an intact host immune system in fighting TB is underlined in fact that the HIV epidemic led to an explosive increase in both incidence and mortality of TB. Additionally, TB risk groups in low incidence areas include patients who are deliberately being immunocompromised as an (un)intended part of their treatment of other diseases (e.g. transplantation, autoimmune diseases, and cancer). The following paragraph will elaborate on the important processes in the immune response to MTB.
Host defence against mycobacteria

Host response to microbial threats comprises of a few vital steps. In order to pose a threat the first barrier for microorganisms to overcome is to enter the body. When present in the tissue they will come into contact with the immune system, with resident macrophages being among the first types of cells. MTB enters the body through the respiratory system, resulting in a major role for the alveolar macrophages. The following crucial step is recognition; innate immune cells sense pathogen associated molecular patterns (PAMPs) through an array of pattern recognition receptors (PRRs) in and on these cells. Recognition leads to activation of that cell, triggering events such as the production and release of chemokines and cytokines, phagocytosis and destruction of the pathogen, and attraction of other cells to the inflammation site (Figure 1). The monocyte/macrophage is a pivotal cell in the host response to MTB as it is involved in phagocytosis and killing of the mycobacteria, as well as in the initiation of the adaptive immune response through antigen presentation.

There are a few distinct outcomes possible after that first encounter: the macrophage successfully
kills the mycobacterium in that instance (early clearance) (4); however, mycobacteria possess strategies to escape the initial intracellular destruction that will lead to multiplication of the bacteria and disruption of the harbouring cell. Outside the cell, mycobacteria can grow even more rapidly, resulting in attraction of other monocytes and macrophages that will subsequently be infected (5). When thereafter T cells are attracted, the adaptive response to TB starts. When this infection cannot be contained, primary TB will occur. Besides monocytes and T cells, B cells, NK cells, and neutrophils are attracted to the inflammation site forming a 'granuloma' (Figure 2). This granuloma symbolizes an equilibrium state for mycobacteria and the immune system, resulting in the hallmark of TB infection: its latency. Which way the balance is tipped during life is largely dependent on the quality of the immune system, resulting in either postprimary TB or only a detectable adaptive immune response to MTB.

Figure 2. Schematic overview of a granuloma. When macrophages fail to degrade engulfed mycobacteria these mycobacteria can multiply in the cells resulting in disruption of the harbouring cell. Attracted to this inflammation site immune cells like monocytes, T cells, B cells, NK cells, and neutrophils will try to wall off this potential threat. This organised structure of cells surrounding mycobacteria is called a 'granuloma'. This is an efficient way of trimming down the immune response however, it also forms a fertile environment for mycobacteria to grow. This equilibrium state for mycobacteria and the immune system can last for several years.
Essential components of innate immune response to MTB

Macrophages and monocytes are some of the most important cells in the innate immune response to MTB. They are the first cell types coming into contact with the mycobacteria and have capabilities of phagocytosis and destruction of the pathogen through either acidification of phagolysosomes, oxidative stress from reactive nitrogen intermediates, or autophagy (a process in which cytoplasmatic cargo is targeted for degradation in specialized structures called autophagosomes). In addition, they produce important cytokines: tumour necrosis factor α (TNFα), interleukin 1β (IL1β), interleukin 12 (IL12), and interleukin 18 (IL18). The other vital cytokine interferon γ (IFNγ) is produced by T cells and NK cells, enabling macrophages to kill mycobacteria probably partly by inducing autophagy. Autophagy, the cellular machinery for targeting intracellular components for lysosomal degradation, has an essential role in antimycobacterial host defence (6). Besides inhibiting intracellular mycobacterial survival, autophagy facilitates antigen presentation and T cell activation, and modulates production of proinflammatory cytokines (6, 7). Although it is considered an essential component of host defence against MTB, what drives autophagy and what influences it is not completely known. One cytokine that can activate autophagy is IFNγ that subsequently activates macrophages to kill and eliminate the mycobacteria. It also enhances macrophage expression of MHC class II molecules, which results in improved antigen presentation to T cells. IFNγ is secreted by NK, CD4+, and CD8+ T cells upon release of endogenous IL12 and IL18 by macrophages and dendritic cells. The crucial importance of IFNγ for human antmycobacterial defence is demonstrated by the increased susceptibility to mycobacterial infections in patients with IFNγ receptor or IL12 receptor deficiencies (8-10). The importance of other cytokines is proved by murine models deprived of these cytokines or signalling pathways (TNFα (11, 12), IL1β (13, 14)), leading to increased bacterial numbers, defective granuloma formation, and poorer survival. Knowing the importance of these cytokines raises the questions of how these cytokines are produced, what influences their production, and whether we can identify groups at risk with defects in this production.

Therefore, we focus in part one of this thesis on cytokine production, the role of autophagy, and their interplay in host response to MTB in order to provide insight in TB pathogenesis and crucial immune processes which may eventually translate in TB treatment improvement.

One of the key cytokines in TB infection IL1β (13) is produced in two steps. The transcription results in pro-IL1β that needs to be processed to IL1β by caspase-1. Which steps in recognition and signalling are vital in MTB-induced IL1β production are not known. In Chapter 2 we therefore investigate the mechanisms responsible for IL1β activation and processing in MTB infection in human monocytes and alveolar macrophages.

In Chapter 3 we study the effect of autophagy on production of monocyte and T cell-derived cytokines induced by MTB. In addition, we examine the impact of two functional single nucleotide polymorphisms (SNPs) in autophagy genes on MTB induced cytokine production.

In Chapter 4 we elaborate on autophagy and its role in MTB. We examine twenty-two polymorphisms in fourteen autophagy genes in a large cohort of Indonesian TB patients and controls. In these patients we furthermore investigate a possible association between these SNPs and the MTB genotype. In addition, we search for differences in cytokine production in another cohort that is genotyped for
these SNPs. In Chapter 5 we provide a general overview of the innate immune recognition of MTB, the first step in the host response.

Innate memory
All immune responses are divided into an innate part and an adaptive part, and although the influence of one on the other is generally recognised, they remain two distinct processes. The innate immune system is the first line of defence; it is fast but does not adapt. The adaptive immune cells take a longer time but produce an immune response specific to the microbial threat, and are able to remember this.

However, this traditional dichotomy is now being challenged. More and more reports are being published lately on the memory properties of innate immune cells, recently termed ‘trained immunity’ (15). However, the subject is by no means novel; this phenomenon has been acknowledged in plant and invertebrates immune systems (16, 17) for decades, and the first papers on the memory properties of innate immune cells in mammals were also written years ago (18, 19). BCG could protect mice deprived of functional T and B cells from a subsequent infection with *Schistosoma mansoni* (18). In addition, macrophages were found to have increased functions after BCG exposure, leading in an *in vivo* model to decreased outgrowth of *Candida albicans* in that infection (19).

Besides these observations in mice, large epidemiological studies showed a survival benefit in children vaccinated with BCG beyond the disease burden of TB (20–22). These non-specific effects have been described for years; however, without a biological explanation they have been met with scepticism. Therefore, the aim of the second part of this thesis is to elucidate the biological mechanism of nonspecific effects of BCG on the innate immune system.

Trained immunity is a process of improved reaction of innate immune cells after an initial stimulus (for example BCG) to reinfection with the same or a different pathogen. In Chapter 6 we aim to describe the process of BCG-induced trained immunity in detail. We study monocytes of healthy volunteers that have been BCG vaccinated in their response to reinfection in order to describe which receptors and intracellular signalling pathways are responsible for this phenomenon. In addition, we decipher the involved molecular mechanisms. As this phenomenon has potentially major (clinical) implications, its longevity is a key element and consequently is our focus in Chapter 7. Using the same population of BCG vaccinated persons as in the previous chapter, we study the described changes over a longer period. While the previous chapters focus on monocytes, trained immunity is a description of improved responses of innate immune cells, and could therefore potentially occur in all the innate immune cells.

Hence, we question in Chapter 8 what the role of NK cells would be in BCG induced trained immunity. Using the same methods as in the previous two chapters, we investigate whether we can extend our conclusions about monocytes to other innate immune cells.

In Chapter 9 we give an overview of the available literature on the heterologous effects of vaccines centred around the effects of BCG.
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TRANSCRIPTIONAL AND INFLAMMASOME-MEDIATED PATHWAYS FOR THE INDUCTION OF IL-1B PRODUCTION BY *MYCOBACTERIUM TUBERCULOSIS*
ABSTRACT

Proinflammatory cytokines of the IL1 family play an important role for the anti-mycobacterial host defence mechanisms. In the present study we have deciphered the pathways leading from recognition of Mycobacterium tuberculosis to the production and release of IL1β, the most important member of the IL1 family. By stimulating cells defective in various pattern recognition receptors, we could demonstrate that IL1β production is induced by M. tuberculosis through pathways involving TLR2/TLR6 and NOD2 receptors. In contrast, TLR4, TLR9 and TLR1 receptors are not involved in IL1β induction. Recognition of M. tuberculosis by TLR and NOD2 leads to transcription of proIL1β through mechanisms involving ERK, p38 and Rip2, but not JNK. Interestingly, although caspase-1 is necessary for the processing of proIL1β, activation of caspase-1 is not dependent on the stimulation of cells by M. tuberculosis. Monocytes expressed constitutively active caspase-1. The secretion of IL1β is dependent on the activation of P2X7-induced pathways by endogenously released ATP. In conclusion, we have dissected the molecular mechanisms responsible for IL1β production by M. tuberculosis, and that may contribute to a deeper knowledge of the mechanisms of cell activation by M. tuberculosis.
INTRODUCTION

It is estimated that approximately one-third of the world population is infected with *Mycobacterium tuberculosis*, the agent causing tuberculosis (TB), although only 5% of these individuals will eventually develop clinical disease. The WHO estimated that 1.6 million deaths resulted from TB in 2005, equalling 4400 deaths a day (1). The highest rates of infection and especially the mortality are seen in the developing countries, and rates continue to climb in countries where the prevalence of HIV infection is high, despite the implementation of TB control programs (2). Furthermore, the increased prevalence of multidrug-resistant strains of *M. tuberculosis* represents a challenge for treatment programmes (3).

The first line of defence against *M. tuberculosis* is formed by alveolar macrophages, which ingest and sequester the bacilli within granulomatous structures. The control and resolution of the infection involve the activation of macrophages, in which activated T lymphocytes play a crucial role (4). The release of proinflammatory cytokines such as IL1β, TNFα, IL12 or IL18 from monocytes and monocytederived macrophages induces the production of T cell-derived cytokines, most importantly IFNγ, which in turn will activate macrophages for the killing and elimination of the microorganisms (5,6). Patients with defects in receptors for IL12 and IFNγ have an increased susceptibility to mycobacterial infections (7–9), while the association between low CD4 counts and TB is well-known in HIV-infected patients (2).

While most of the research has focused on the mechanisms responsible for the IFNγ induction, relatively little is known about the mechanisms leading to the production and release of the first wave of cytokines that are needed for the activation of T cells. While TNFα is known to be important for the integrity of the granuloma, two of the cytokines of the IL1 family, IL1β and IL18, are considered to be crucial for the induction of IFNγ. IL1-mediated signals have been recently demonstrated to be an essential component of the MyD88-dependent innate response to *M. tuberculosis* infection, while IL18-dependent pathways seemed to be dispensable (10).

In contrast to other proinflammatory cytokines, IL1β lacks a signal peptide (11). After transcription and translation, pro-IL1β is processed by the cysteine protease caspase-1, followed by secretion from the cell (12). Activation of caspase-1 requires generation of a protein complex called the inflammasome, which recognizes specific ligands such as muramyl dipeptide, ATP or uric acid leading to caspase-1 activation (13). Detection of the PAMP or danger signals in the inflammasome is achieved by proteins of the NOD-like receptor family such as NALP3, NALP1 or IPAF, leading to a conformational change and caspase-1 activation (14). Caspase-1 activity is essential in host defence against infection with *Francisella tularensis* (15), *Legionella pneumophila* (16), *Shigella* (17) and *Pseudomonas aeruginosa* (18). The fact that IL1β and IFNγ play an important role during infection with *M. tuberculosis* and are regulated by caspase-1 activity suggests that inflammasome activation may be important for anti-mycobacterial defence mechanisms. In the present study, we investigate the mechanisms responsible for IL1β activation by *M. tuberculosis* in human monocytes and alveolar macrophages, including the receptor pathways leading to pro-IL1β mRNA transcription and the mechanisms of caspase-1 activation.
MATERIALS AND METHODS

Volunteers
Blood was collected from ten healthy, non-smoking volunteers who had no infectious or inflammatory disease at the time of the study. In addition, blood was collected from 74 patients with Crohn's disease, and the presence of the 3020insC NOD2 polymorphism was analyzed by Genescan analysis on an ABI-Prism 3100 Genetic Analyzer, as previously described (19). Four patients with Crohn's disease were found to be homozygous for the 3020insC mutation, and they were further investigated in the cytokine studies. None of the patients with Crohn's disease used immunosuppressive medication at the time of the study. The study was approved by the Ethics Committee of the Radboud University Nijmegen. In addition, alveolar macrophages collected from healthy volunteers by bronchoalveolar lavage were suspended to a concentration of 5×10^6 cells/mL and stimulated for cytokine production.

Microorganisms and reagents
Cultures of M. tuberculosis H37Rv were grown to mid-log phase in Middlebrook 7H9 liquid medium supplemented with oleic acid/albumin/dextrose/catalase (Difco, Becton-Dickinson, Palo Alto, CA), washed three times in sterile saline, and resuspended in RPMI 1640 medium at various concentrations. Separate culture suspensions were sonicated for 10 min on ice, in order to obtain cell lysates. The irreversible caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-2,6 dimethylbenzoyloxymethylketone was purchased from AlexisBiochemicals (San Diego, CA). The inhibitor was reconstituted in 10 mM DMSO and subsequently diluted to the desired concentration in RPMI. SB202190, p38/Rip2 MAPK inhibitor (p38-i); SP600125, JNK1/2/3 inhibitor (JNK-i) and U0126 MEK1/2 inhibitor (Erk-i) were purchased from Superarray Bioscience Corporation (Bethesda, MD). In experiments using pharmacological inhibitors, control cells were treated with an equivalent concentration of vehicle (0.01–0.1% DMSO).

Isolation of PBMC and stimulation of cytokine production
After informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10mL EDTA tubes (Monoject, s-Hertogenbosch, Netherlands). Isolation of PBMC was performed as described elsewhere (47), with minor modifications. The PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll–Paque (Pharmacia Biotech, Uppsala, Sweden). Cells were washed twice in saline and suspended in culture medium (RPMI 1640 DM) supplemented with 10μg/mL gentamicin, 10 mM L-glutamine and 10 mM pyruvate. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, Netherlands), and the number was adjusted to 5×10^6 cells/mL.

PBMC (5×10^5) were added in a 100μL volume to round-bottom 96-well plates (Greiner, Alphen, Netherlands), incubated with 100μL of culture medium (negative control) or M. tuberculosis bacteria (1×10^3 cfu/mL), with or without caspase-1 inhibitor at different concentrations. In some blocking experiments, PBMC were preincubated for 1h with the inhibitors p38-i 20 nM, Erk-i 10 nM, or JNK-i 25 nM, before the stimulation with M. tuberculosis. The role of the endogenous ATP release for the stimulation of IL1β was investigated by blocking P2X7 receptors with oxATP (300μM) during the stimulation of cells for 24 h with LPS (20). The stimuli were checked for the contamination with LPS in
the Limulus amoebocyte lysate assay and was found to be negative. In addition, alveolar macrophages collected from healthy volunteers by bronchoalveolar lavage were suspended to a concentration of $5 \times 10^6$ cells/mL and stimulated for cytokine production.

Cytokine production by murine peritoneal macrophages

Resident peritoneal macrophages were harvested from either control mice or mice deficient for the various TLR or adaptor molecules. TLR1-/-, TLR2-/-, TLR4-/-, TLR6-/-, TLR9-/- and MyD88-/- mice back-crossed to the seventh generation into the C57Bl/6J background were kindly provided by S. Akira (Osaka University, Japan). TRIF-defective mice (Lps2 strain) were a kind donation of B. Beutler (Scripps Institute, San Diego). After centrifugation and washing, the cells were resuspended in RPMI 1640 containing 1mM pyruvate, 2 mM L-glutamine, 100 μg/mL gentamicin and 2% fresh mouse plasma. Cells were cultured in 96-well microtiter plates (Greiner) at $1 \times 10^5$ cells/well, in a volume of 100 μL. The cells were stimulated with M. tuberculosis ($1 \times 10^5$ mo/mL). After 24 h incubation at 37°C, the supernatants were collected and stored at -20°C until cytokine assays were performed.

Cytokine and ATP measurements

Human and murine cytokine concentrations were measured by commercial ELISA kits (Pelikine Compact, CLB, Amsterdam, Netherlands), according to the instructions of the manufacturer. ATP concentrations in the supernatants were assessed using a firefly luciferase assay (ATP determination kit, Invitrogen, Carlsbad, CA).

Caspase-1 colorimetric assay

In order to assess the functional activity of caspase-1, PBMC were incubated for 2h with either RPMI or various stimuli. Cells were lysed and caspase-1 activity was assessed using a commercial colorimetric assay (R&D Systems, Minneapolis, MN).

Western blot assays

After stimulation with either RPMI or M. tuberculosis for 2h, PBMC (10^7 cells/well in a total volume of 1mL) were lysed in 100 μL lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 40 mM glycerophosphate, 50 mM sodium fluoride, 200 μM sodium vanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 μM pepstatin A and 1 mM phenylmethylsulfonyl fluoride). Following centrifugation (10 000 rpm, 5 min), the protein content was determined by BCA protein assay (Pierce) and equal amounts of protein were loaded on 12% SDS-PAGE and transferred onto nitrocellulose membranes. For caspase-1 quantitations, membranes were incubated with specific caspase-1 p10 polyclonal antiserum (1/500) and procaspase-1 polyclonal antiserum (1/500) (Santa Cruz). β-actin was quantified as an internal control using specific anti-β-actin polyclonal antiserum (1/1000) (Santa Cruz). Blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1/1000 dilution). After washing the blots three times with TBS-T the blots where developed with hyperECL according to manufacturer's instructions (GE Healthcare). In addition, supernatants of PBMC stimulated for 24 h with either RPMI or M. tuberculosis and were assessed for the presence of pro-IL1β and IL1β. Membranes were incubated with anti-(pro)IL1β polyclonal antibody (1/1000 dilution) (Cell Signaling), while the rest of the procedure was similar with that employed for caspase-1 Western blots.
Quantitative PCR
PBMC were stimulated as described under the section Isolation of PBMC. After 4 h, the supernatant was removed and the cells were resuspended in 200 μl RNAzolB RNA isolation solvent (Campro Scientific) and stored at -80°C. mRNA was isolated following the manufacturer’s protocol. The amount and quality of mRNA were determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). cDNA was synthesized from using Superscript Reverse Transcriptase (Invitrogen). Relative mRNA levels were determined using the BioRad I-Cycler and SYBR Green method (Invitrogen). The following primers were used: IL1β forward (5' -TGGCCCAGGCAGTCAGA-3'), and reverse (5' -GGTTTGCTACAACATGGGCTACA-3'); β2M forward(5' -ATGAGTATGCTGCGCGGCTG-3')and reverse(5' -CCAAATGCGGCATCTTTCAAC-3') (Biolegio, Malden, Netherlands).

Statistical analysis
The differences between groups were analyzed by the Mann–Whitney U test, and where appropriate by the Wilcoxon rank test to determine differences between groups where p < 0.05. All experiments were performed at least twice, and the data presented as the cumulative result of all experiments performed. Data are given as means ±SD.

RESULTS

*M. tuberculosis* induces IL1β production through caspase-1-dependent mechanisms
PBMC produced significant amounts of IL1β when stimulated with TLR2 (Pam3Cys) and TLR4 (LPS) stimuli, as well as with *M. tuberculosis* (Figure 1A). Both heat-killed and live *M. tuberculosis* stimulated comparable amounts of IL1β. Primary alveolar macrophages isolated from healthy volunteers also released IL1β after stimulation with *M. tuberculosis* (Figure 1B). The transcription of pro-IL1β mRNA was stimulated by mycobacteria (Figure 1C), and this resulted in the release of active IL1β in the supernatant (Figure 1D). The release of the mature form of IL1β from the cell was dependent on caspase-1 (Figure 1E).

Both TLR2/TLR6 and NOD2 pathways mediate induction of IL1β by *M. tuberculosis*
When murine macrophages isolated from the various mouse knock-out strains were stimulated with *M. tuberculosis*, cells from TLR2-/- and TLR6-/- mice released significantly less IL1β compared with wild-type controls (Figure 2A and B), similar to stimulation induced by the specific TLR2 agonist Pam3Cys (not shown). In contrast, macrophages from TLR4-/- and TLR1-/- mice released normal amounts of IL1β (Figure 2C and D). TLR9 had no role in the stimulation of IL1β after challenge with *M. tuberculosis* (Figure 2E). LPS and CpG induced cytokine production only in wild-type macrophages, but not in cells isolated from TLR4-/- or TLR9-/- mice, respectively. Interestingly, PBMC from individuals defective in NOD2 function (Crohn’s disease patients homozygous for the 3020insC NOD2 mutation) (19) stimulated with *M. tuberculosis* released significantly less IL1β than either healthy volunteers or Crohn’s disease patients homozygous for the wild-type NOD2 allele (Figure 2F).
Figure 1. *M. tuberculosis* induces IL1β production through caspase-1-dependent mechanisms. (A) Human PBMC were stimulated with Pam3Cys (1 μg/mL), LPS (10 ng/mL), heat-killed, or live *M. tuberculosis* (1×10⁵ mo/mL) for 24 h at 37°C. IL1β in supernatant was measured by ELISA. (B) Human primary alveolar macrophages isolated from healthy volunteers were stimulated with *M. tuberculosis* (1×10⁵ mo/mL) for 24 h at 37°C. IL1β in supernatant was measured by ELISA. (C) Human PBMC were stimulated with *M. tuberculosis* (1×10⁵ mo/mL) for 4 h at 37°C. The transcription of pro-IL1β mRNA was assessed by RT-PCR. (D) Western blot for pro-IL1β and IL1β of the supernatants of PBMC stimulated for 24 h with either RPMI or *M. tuberculosis* (1×10⁵ mo/mL). (E) Human PBMC were stimulated with *M. tuberculosis* (1×10⁵ mo/mL), in the presence or absence of a caspase-1 inhibitor (10 mM) for 24 h at 37°C. IL1β and IL6 in the supernatant were measured by ELISA (n=10, mean±SEM, *p<0.05 by Wilcoxon signed rank test.

The intracellular pathways leading to IL1β mRNA transcription involve MyD88 and ERK/p38 kinases
To assess the role of the intracellular pathways involved in the induction of IL-1β by *M. tuberculosis*, cells from MyD88−/− and TRIF-defective mice were stimulated with the microorganisms for 24 h. IL1β production was strongly dependent on MyD88, but not on TRIF (Figure 3). In addition, stimulation of IL1β in PBMC by *M. tuberculosis* was mediated by the ERK, as well as p38 and/or Rip2 kinases, but not by JNK (Figure 4A). Quantitative PCR analysis demonstrated that p38 and ERK inhibitors decreased IL1β mRNA expression (Figure 4B). In line with these data, pro-IL1β intracellular concentrations were decreased by 55–70% by addition of p38 and/or ERK inhibitors (not shown). Similar effects were observed for the induction of IL6 production. In contrast, the induction of TNFα was regulated through ERK and JNK-dependent pathways, while blockade of p38 had no effect (Figure 4A).
Figure 2. The role of TLR and NOD2 pathways for the induction of IL1β by M. tuberculosis. Murine macrophages isolated from TLR2−/− (A), TLR6−/− (B), TLR4−/− (C), TLR1−/− (D) and TLR9−/− (E) mice were stimulated for 24 h at 37°C with 1×10^5 mo/mL M. tuberculosis. The experiments were performed in twice with ten mice/group. IL1β and TNFα in the supernatant were measured by ELISA. (F) PBMC from individuals defective in NOD2 function (n=4, NOD2 def, white bars), Crohn’s disease patients with wild type NOD2 (n=4, NOD2wt, gray bars) and healthy volunteers (n=10, black bars) were stimulated for 24 h at 37°C with 1×10^5 mo/mL M. tuberculosis (mean±SEM, *p<0.05 by Wilcoxon signed rank test).

Figure 3. The role of MyD88 and TRIF pathways for the induction of IL1β by M. tuberculosis. Murine macrophages isolated from MyD88−/− (A) and TRIF−/− (B) mice were stimulated for 24 h at 37°C with 1×10^5 mo/mL M. tuberculosis. IL1β and TNFα in the supernatant were measured by ELISA (n=10, means ±SEM, *p<0.05 by Wilcoxon signed rank test).
Figure 4. The role of intracellular kinase pathways for the induction of IL1β by M. tuberculosis. (A) PBMC were preincubated for 1 h with the inhibitors for the kinases p38 (20 nM), Erk (10 nM), or JNK (25 nM), before the stimulation for 24 h at 37°C with M. tuberculosis (1×10^5 mo/mL). IL1β, IL6 and TNFα in the supernatant were measured by ELISA (n=6, means ±SEM, *p <0.05 by Wilcoxon signed rank test). (B) Inhibitors of p38 and ERK kinases decreased the expression of IL1β mRNA after 4 h stimulation of PBMC with M. tuberculosis, as assessed by quantitative PCR (one representative experiment out of three performed).

Figure 5. Mechanisms involved in the processing and secretion of IL1β. (A) Western blots of the inactive (p45) and active (p10) caspase-1 fragments in monocytes isolated from healthy volunteers, either not stimulated (RPMI), or stimulated with LPS or M. tuberculosis (MTB). One representative experiment out of three experiments is presented. (B) The role of the endogenous ATP release for the stimulation of IL1β was investigated by blocking P2X7 receptors with oxATP (300 μM) during the stimulation of cells for 24 h with M. tuberculosis. Mature IL1β concentrations were measured by ELISA in both PBMC lysates and supernatants. (n=6, means ±SEM, *p <0.05 by Wilcoxon signed rank test). (C) Diagram depicting the pathways leading to IL1β production and release induced by M. tuberculosis.
Posttranslational mechanisms involved in the processing and secretion of IL1β

Active caspase-1 is important for the processing and release of IL1β. Surprisingly, unstimulated monocytes expressed both the inactive (p45) and the active forms of caspase-1 (p10) even before stimulation, while LPS and M. tuberculosis had little additional activating effects (Figure 5A). These data were supported using a functional colorimetric assay that showed that in contrast to control medium alone (11±3 conventional units/mL), lysates of freshly isolated PBMC contained 91±15 units/mL caspase-1. The secretion of IL1β has been reported to be mediated by the interaction between endogenous ATP and P2X7 receptor (20). This was also the case for the induction of IL1β by M. tuberculosis, as intracellular IL1β was present in high concentrations in PBMC stimulated with mycobacteria (2780±765 versus 20±10 pg/mL in unstimulated cells, p<0.01), while blockade of P2X7 with oxATP significantly inhibited the IL1β release (Figure 5B). This suggested that endogenous ATP was present in the supernatants: indeed, endogenous ATP was released in the supernatants by primary human monocytes (3.5±0.7 nM).

DISCUSSION

Cytokines of the IL1 family play an important role for the anti-mycobacterial host defence mechanisms. In the present study we have deciphered the pathways leading from recognition of M. tuberculosis to the production and release of IL1β, one of the most important proinflammatory cytokines. By stimulating cells defective in various pattern recognition receptors, we demonstrate that IL1β production is induced by M. tuberculosis through pathways involving TLR2/TLR6 and NOD2 receptors. This leads to transcription of IL1β through mechanisms involving ERK, p38 and Rip2. Interestingly, although an active caspase-1 is required for the release of active IL1β, its activation was only moderately dependent on the stimulation of cells by M. tuberculosis, due to the presence of constitutively active caspase-1 in human primary monocytes.

The importance of understanding the mechanisms responsible for IL1β production by M. tuberculosis is underlined by the fact that an intact IL1-mediated signal is an essential component of the host defence to mycobacteria (10,21,22). Aerogenic infection with M. tuberculosis proved fatal for IL-1RI-/- mice, resulting in a mycobacterial load increased by 2 logs in the lungs and necrotizing pneumonia (21,22). Defective granuloma formation was seen, and splenocytes of IL-1RI-/- mice produced less IFNγ (21). These data are in contradiction with a recent study by Master et al., suggesting that M. tuberculosis prevents inflammasome activation and IL1β secretion (23): if mycobacteria would not induce IL1β secretion and this pathway would be absent during the anti-mycobacterial host defence, IL1-deficient mice could not be more susceptible to TB (21,22). Our study also clearly demonstrates the capacity of M. tuberculosis, either alive or heat-killed, to stimulate IL1β synthesis in human and murine primary monocytes or macrophages. In contrast, the study of Master et al. has shown defective IL1β production only in the murine macrophage cell-lines RAW 264.7 and J774A (23). This demonstrates the importance of using primary cells when the inflammasome activation and IL1β induction is investigated.

Production and release of IL1β are regulated at several levels: the transcription of the gene, processing from the inactive 31kDa pro-IL1β into the active 17kDa IL1β form, and secretion from the cells (24). The transcription of the genes encoding proinflammatory cytokines is under control of signals induced
by pattern recognition receptors on the immune cells. TLRs have been shown to be pivotal in the recognition of *M. tuberculosis* and the induction of proinflammatory cytokines (25). In our study, the induction of IL1β by *M. tuberculosis* is mediated by pathways involving TLR2 and TLR6. These data are in line with the studies involving TLR2 for the recognition of mycobacteria (26,27), and the increased susceptibility to *M. tuberculosis* infection of TLR2-/- mice (28). TLR2 is a receptor for bacterial lipopeptides, which are recognized by TLR2/TLR1 or TLR2/TLR6 heterodimers (29,30). Cooperation of TLR2 and TLR6 has been described to be involved in recognition of mycobacterial components (31). In line with this, IL1β production induced by *M. tuberculosis* was dependent on TLR2 and TLR6, but not TLR1 (Figure 2). Moreover, TLR4 also does not seem to mediate the induction of IL1β by *M. tuberculosis*, although it is involved in the general recognition of mycobacteria. Differences in the transcription mechanisms responsible for the induction of TNFα and IL1β are well known, and this may be one of the factors explaining why TLR4 does not modulate induction of IL1β, while influencing other proinflammatory cytokines (26,32). Alternatively, other pathways apart of TLR4 (e.g. TLR2/TLR6) may be sufficient for redundant induction of IL1β by *M. tuberculosis*. TLR9 was not significantly engaged in the induction of IL1β. TLR9-/- mice have shown only minor defects in pathogen clearance, while enhancing the effects of TLR2 deficiency (33).

Interesting new information is the involvement of NOD2 as a receptor mediating IL1β production by *M. tuberculosis*. NOD2 is a receptor of bacterial peptidoglycans (34) and only recently we demonstrated its role in the recognition of mycobacteria (35,36). It has also been recently shown that common polymorphisms of NOD2 are strongly associated with TB in African-American patients (37). These data argue that TLRs and NOD2 collaborate for an effective recognition of *M. tuberculosis* and induction of IL1β production.

We further investigated the mechanism of IL1β production by identifying the signalling pathways leading to transcription of the IL1β gene. TLR2 induces intracellular signals through Mal/MyD88, while TLR4 is able to activate a secondary TRIF/TRAM-dependent pathway. In line with the data obtained in the various TLR knock-out mice, IL1β induction was dependent on MyD88, but not on TRIF. MyD88-/- mice are also known to be more susceptible to mycobacterial infection (38,39). The distal intracellular pathways induced by TLR involve the MAPK ERK, JNK and p38. Recently, it has been shown that the blockade of LPS-induced p38 kinase activation inhibits the IL1β production in rat pulmonary interstitial macrophages (40), but no data on the role of MAPK on IL1β induction by mycobacteria are available. Our data show that both the ERK and p38 and/or Rip2 pathways are part of the intracellular pathway of IL1β gene activation induced by *M. tuberculosis*. The pharmacological inhibitors available cannot discern between the TLR/p38 pathways and the NOD2/Rip2-mediated pathways, and future experiments are needed to determine the relative importance of these two mechanisms. In contrast, no role for the JNK kinases for the induction of IL1β by *M. tuberculosis* has been observed.

The processing of IL1β is assumed to be mediated by caspase-1 activation by a protein complex formed mainly by receptors of the NOD-like receptor family, called the inflammasome. Several inflammasomes activate IL1β during bacterial infections, such as the NALP3 inflammasome that recognizes bacterial ligands including muramyl dipeptide (41), the NALP1b inflammasome that recognizes anthrax lethal
toxin (42), and the Salmonella-induced Ipaf inflammasome (42). Although *M. tuberculosis* contains peptidoglycans, the bacterial structures containing muramyl dipeptide, it is not known whether inflammasome components recognize and are activated by mycobacterial PAMP. We have shown that IL1β production by human monocytes stimulated with *M. tuberculosis* is caspase-1 dependent. When we assessed the capacity of *M. tuberculosis* to activate caspase-1, we observed that caspase-1 was already present in its active form in freshly isolated human PBMC, and only moderately upregulated by stimulation with mycobacteria. These data were supported by the demonstration of functional caspase-1 in human primary monocytes using a functional colorimetric assay. This implies that in human primary monocytes the inflammasome is constitutively active, and bacterial products are able only to moderately increase caspase-1 activation and the processing of pro-IL1β. Therefore, although inflammasome components such as NALP3 and ASC are central for caspase-1 activation, this process does not necessarily require activation by microbial products in human monocytes. This is in contrast with the literature from which the concept was proposed that inflammasome activation by danger molecules (either bacterial or not) is an essential step for the induction of IL1β production (43). However, this concept has been built upon a body of evidence derived from monocyte/macrophage cell-lines (e.g. THP-1 cells) or murine cells, and not from primary cells (41,44). Important differences between THP-1 cells (no active caspase-1) and primary human monocytes (constitutively active caspase-1) for the activation of caspase-1 have been recently demonstrated by our group (45). Thus, the present study cautions against the extrapolation of data obtained in cell-lines with the physiology of human primary cells.

The release of IL1β from the cells was dependent on endogenous ATP production from the monocytes (46), and the blockade of the P2X7 receptor with oxATP significantly reduced IL1β production.

IL1β is one of the important proinflammatory cytokines with anti-mycobacterial activities, and in this study we show that *M. tuberculosis* is a potent inducer of IL1β secretion from human primary PBMC and alveolar macrophages. Interestingly, the main regulatory step of IL1β production is at the level of transcription, and PBMC contain constitutively active caspase-1. These data question the necessity for inflammasome activation during recognition of mycobacteria by these cells. Upon recognition of *M. tuberculosis*, TLR2/TLR6 and NOD2 receptors induce IL1β transcription through pathways involving Erk, p38 and Rip2 (Figure 5C). In conclusion, we have dissected the molecular mechanisms responsible for IL1β production by *M. tuberculosis*, and this contributes to a better understanding of the mechanisms leading to host cell activation by mycobacteria.
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AUTOPHAGY MODULATES THE MYCOBACTERIUM TUBERCULOSIS-INDUCED CYTOKINE RESPONSE

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ABSTRACT

Both autophagy and proinflammatory cytokines are involved in the host defence against mycobacteria, but little is known regarding the effect of autophagy on *Mycobacterium tuberculosis* (MTB)-induced cytokine production. In the present study, we assessed the effect of autophagy on production of monocyte and T cell derived cytokines, and examined whether two functional polymorphisms in autophagy genes led to altered cytokine production. Blocking autophagy inhibited TNFα production, while enhancing IL1β production in peripheral blood mononuclear cells stimulated with MTB. Induction of autophagy by starvation or interferon had the opposite effect. The modulation of both TNFα and IL1β production by autophagy was induced at the level of gene transcription. Functional polymorphisms in the autophagy genes ATG16L1 and IRGM did not have a major impact on MTB-induced cytokine production in healthy volunteers, although a moderate effect was observed on IFNγ production by the ATG16L1 T300A polymorphism. These data demonstrate the interplay between autophagy and inflammation during host defence against mycobacteria, and future studies to investigate the clinical implications of these effects for the susceptibility to tuberculosis are warranted.
INTRODUCTION

Tuberculosis (TB) is a major health problem with 10 million new cases diagnosed each year, causing a death toll of nearly 2 million victims [1]. However, from the estimated 2 billion individuals that have been initially infected with Mycobacterium tuberculosis (MTB), most develop asymptomatic infection, also called 'latent tuberculosis'. The pathogen is often not eliminated and may persist for years inside macrophages, due to its capacity to evade the host immune response. This persistence allows progression to active tuberculosis either as the primary disease or, years later, as reactivation when cellular immunity fails.

The interaction between MTB and cells of both the innate and adaptive immune system results in secretion of proinflammatory cytokines such as tumour necrosis factorα (TNFα), interleukin (IL)1β, IL18, IL12 and IFNγ. TNFα, produced by mononuclear cells, is crucial for host defence as TNFα deficient mice succumb rapidly after MTB infection, with significantly higher mycobacterial outgrowth in different organs compared to wild-type animals [2]. TNF is important for formation of granuloma, a mechanism for containing and restricting the replication of the bacilli [2, 3]. The crucial role played by TNFα in human TB is also demonstrated by the increased susceptibility to TB in patients treated with anti-TNFα therapy [4].

IL1β is an essential component of the host defence to mycobacteria [5-7]. MTB infected IL1 receptor type 1 knock-out mice exhibit lower production of IFNγ, defective granuloma formation, and decreased survival [6]. IFNγ is secreted by natural killer (NK), CD4+, and CD8+ T cells upon release of endogenous IL12 and IL18 by macrophages and dendritic cells. IFNγ activates macrophages to kill and eliminate the mycobacteria. It also enhances their expression of major histocompatibility complex (MHC) class II molecules, which results in improved antigen presentation to T cells. The crucial importance of IFNγ for human anti-mycobacterial defence is demonstrated by the increased susceptibility to mycobacterial infections in patients with IFNγ receptor or IL12 receptor deficiencies [8-10]. As cytokines are therefore crucial for host defence against MTB, it is essential to understand the molecular pathway of the MTB-induced cytokine production.

Autophagy is a pathway through which cytoplasmic components, including organelles and intracellular pathogens, are sequestered in a double-membrane-bound autophagosome and delivered to the lysosome for degradation [11, 12]. Elimination of dysfunctional cell components is one of the main roles of autophagy, besides the recycling of cytoplasmic material, allowing the cell to maintain macromolecular synthesis and energy homeostasis during starvation and other stressful conditions. In addition to its role in cell survival, autophagy is also involved in host defence, with important effects on both innate and adaptive immune system [13, 14]. Autophagy has an essential role for anti-mycobacterial host defence by fusing the autophagosomes containing mycobacteria with lysosomes [15, 16], leading to antigen presentation and T cell activation [17, 18]. Recent experiments in mice suggest that autophagy also modulates cytokine production: macrophages from ATG16L1 knockout mice with no functional autophagy exhibit elevated IL1β production, suggesting an inhibitory role of autophagy on the production of this cytokine [19]. However, so far this has not been studied in humans: although both autophagy and proinflammatory cytokines have an important role in host defence against mycobacteria, there are no reported data regarding the effect of autophagy on MTB-
induced cytokine production. Therefore, in the present study, we assessed the effect of autophagy on production of monocyte and T cell derived cytokines induced by MTB, and examined if two functional polymorphisms lead to altered MTB-induced cytokine production.

MATERIAL AND METHODS

PBMC stimulation assays
After obtaining informed consent, venous blood was drawn from the cubital vein of healthy volunteers into 10 ml EDTA tubes (Monoject). The mononuclear cell fraction was isolated by density centrifugation of blood, diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, PA, USA). Cells were washed twice in saline and resuspended in culture medium (RPMI, Invitrogen, CA, USA) supplemented with gentamicin 10 μg/ml, L-glutamine 10 mM, and pyruvate 10 mM. Cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to 5x10⁶ cells/ml. A total of 5 x 10⁵ mononuclear cells in a 100μl volume was added to round-bottom 96-wells plates (Greiner) in duplicate. Cells were incubated with either RPMI or increasing doses of 3-methyl adenine (3-MA, Sigma) ranging from 1-10 mM for 24, 48 hours or 7 days at 37°C (incubation time was dependent on the cytokines measured). Cytokine concentrations were assessed in the supernatants using enzyme-linked immunosorbent assay (ELISA).

For autophagy inhibition and induction experiments, cells were pre-incubated for 1 hour at 37°C in RPMI or in the presence of 3MA (10mM) or IFNγ (400IE/ml, Immukine, Boehringer Ingelheim), respectively. After pre-treatment, 100 μl of RPMI (negative control) or sonicated MTB H37Rv (1μg/ml end concentration) was added to the cells. Cells were incubated for 24 hours and cytokine production was measured in supernatant using ELISA.

Autophagy was also induced by incubation of PBMCs in starvation medium (Earle’s Balanced Salt Solution, EBSS, Invitrogen, Carlsbad, CA, USA). In some wells, PBMCs were pre-incubated for 1 hour at 37°C in the presence of 3MA (10 mM), to block the induction of autophagy. Subsequently, cells were stimulated for 3 hours with sonicated MTB H37Rv (1μg/ml). Thereafter, supernatants were discarded and TRIzol reagent was added to the cells, after which they were stored at -80°C until analyzed.

To investigate the effect of 3MA on the ATP-dependent IL1β release, PBMCs were pre-treated for 1 hour with either RPMI or 3MA (10mM). Subsequently, cells were stimulated with RPMI or MTB H37Rv (1μg/ml) for 3 hours. After stimulation, supernatants were discarded and refreshed with RPMI containing 1 mM ATP, after which the cells were incubated for another 15 minutes. The ATP-dependent IL1β secretion after the additional 15 minutes was assessed in the supernatant.

Inhibition of autophagy by siRNA
As a complementary approach to inhibit the autophagy PBMC’s were transfected with control (scrambled) or ATG7 siRNA with nucleofector kit using an Amaxa electroporation chamber (Lonza, Basel). After 24h incubation at 37°C, cells stimulated with MTB H37Rv (1μg/ml), and after an additional incubation of 24h supernatants were collected and stored at -80°C until analysis.
Cytokine measurements

Cytokine measurements of TNFα, IL1β, IL6, IFNγ, IL10, IL22 and IL23 were performed in the supernatants using commercial ELISA kits (R&D Systems, MN, USA (TNFα, IL1β, IL22), Sanquin, Amsterdam, The Netherlands (IL6, IL10, IFNγ) or E-Bioscience, San Diego, USA (IL23)).

Real-Time PCR for IL-1β, TNFα and ATG7

RNA purification was performed from TRIzol according to manufacturer's instructions. Isolated RNA was transcribed into complementary DNA using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) followed by quantitative PCR using SYBR Green (Applied Biosystems, CA, USA). Following primers were used: IL1β forward 5'-GCCCTAACAGATGAAGTGCTC-3' and reverse 5'-GAACCAAGCATTTCCCTAG-3', TNFα forward 5'-TGGCCAGGCGACTCAGA-3' and reverse 5'-GGTTGCTACAACATGGGCTACA-3' and ATG7 forward 5'-CAGTTTGCCCTTTTAGTAGTG-3' and reverse 5'-CCTTAATGTCTTGGAGGTCTTCA-3'. Data were corrected for expression of the housekeeping gene β2-microglobulin, for which the primers forward 5'-ATGAGTATGCCTGCCGTGTG-3' and reverse 5'-CCAAATGCGGCATCTTCAAAAC-3' were used.

Genotyping for ATG16L1 Thr300Ala and IRGM polymorphisms

DNA was isolated from whole blood by using the Puregene isolation kit (Gentra Systems, MN, USA), according to the manufacturer's protocol. Genotyping for the presence of the ATG16L1 Thr300Ala (rs2241880), and IRGM promoter polymorphisms rs4958847 and rs13361189 polymorphisms was performed using TaqMan single nucleotide polymorphism (SNP) assay C_9095577_20, C_1398968_10 and C_31986315_10 respectively, on the 7300 ABI Real-Time polymerase chain reaction system (Applied Biosystems, CA, USA). These genotyping assays were performed in a 25 μL total reaction volume, containing 2 μL of genomic DNA as well as primers, two specific probes (with either VIC or FAM label) and Universal PCR 2x Master mix (Applied Biosystems). Cycling conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycli of 95°C for 15 sec and 1 min at 60°C. Fluorescence intensities were corrected using a post-read / pre-read method for 1 min at 60°C before and after the amplification. The software automatically plotted genotypes based on a two-parameter plot with an overall success rate of >95%. Intermediate samples were excluded from the analysis.

Two cohorts of healthy volunteers without known TB contact have been assessed (N=73 and N=104), consisting of foresters from the ‘Geldersch Landschap’ and ‘Kroondomein’ departments in the Netherlands. The individuals were between 23-73 years old, and 77% were male. All volunteers gave written informed consent, and the study was approved by the Ethical Committee of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. The cytokine production induced by sonicated MTB H37Rv in cells isolated from volunteers bearing various ATG16L1 and IRGM genetic variants was compared.

Statistical analysis

Differences were analyzed using the Wilcoxon signed rank test or Friedman test for paired samples and Mann-Whitney or Kruskal-Wallis test for unrelated samples. P<0.05 was considered statistical significant. Data are shown as cumulative results of level obtained in all volunteers (means + SEM).
RESULTS

Inhibition of autophagy enhances MTB-induced production of IL1β and IL6, but decreases production of TNFα.

Inhibition of autophagy with 3MA (a blocker of the Beclin1 complex that is crucial for the initiation of autophagy) decreased TNFα production induced by MTB H37Rv (Friedman test p=0.003). In contrast, autophagy inhibition increased the production of IL1β and IL6 (Friedman test p=0.017 and p=0.035 respectively) in a dose dependent manner (Figure 1). No changes were observed in the induction of IL10 (range 46-50 pg/ml) or IL18 (range 2-4 pg/ml) production when autophagy was inhibited (data

Figure 1. The effect of the autophagy inhibitor 3MA on MTB-induced cytokine production. Freshly isolated human PBMCs were pre-incubated with increasing doses of 3MA (1, 2, 5, or 10 mM) for 1 hour at 37°C in culture medium and subsequently stimulated with sonicated MTB H37Rv (1μg/ml). After 24 hours of incubation, TNFα (A), IL1β (B) and IL6 (C) were measured in the supernatant using specific ELISA. Data are presented as means ±SEM, n=4 *p<0.05 compared to RPMI.
not shown). The T cell derived cytokines IFNg, IL17, IL22 and IL23 levels were not detectable when cells were stimulated with MTB H37Rv, most likely due to the naïve TB status of the individuals tested.

**Induction of autophagy enhances TNFα production.**
In line with the experiments showing that inhibition of autophagy leads to decreased production of TNFα, induction of autophagy with IFNg enhanced TNFα production in MTB stimulated PBMCs. This effect was abolished when autophagy was inhibited by 3MA (p<0.01), suggesting that normal autophagy is necessary for the effects of IFNg on TNFα production (Figure 2a). In contrast, treatment of PBMCs with IFNg did not significantly modify IL1β production (Figure 2b).
Inhibition of autophagy using siRNA against ATG7 influences both TNF\(\alpha\) and IL1\(\beta\) production.

Figure 3 shows inhibition of autophagy with siRNA against ATG7. In line with experiments using a pharmacological agent (3MA) to inhibit autophagy, inhibition of autophagy by ATG7 siRNA showed the same trends of diminished production of TNF\(\alpha\) (Figure 3a) and increased production of IL1\(\beta\) (Figure 3b).

Modulation of IL1\(\beta\) and TNF\(\alpha\) production is regulated at the transcriptional level. In the following set of experiments we assessed the level at which autophagy regulates cytokine production. Both induction (with starvation medium EBSS) and inhibition (with 3MA) of autophagy modulated the transcription of proinflammatory cytokines. TNF\(\alpha\) mRNA levels significantly increased...
Figure 4. The modulatory effect of autophagy on cytokine production is exerted at a transcriptional level. Cells pre-treated for 1 hour with RPMI or starvation medium, in the presence or absence of 3MA (10mM), were stimulated for 3 hours with sonicated MTB (1μg/ml). RT-PCR was performed and relative levels of TNFα (A, C) and IL1β (B, D) mRNA were determined. Data are presented as fold change, n=6, *p<0.05, **p<0.01.

Separately, PBMCs were pre-incubated for 1 hour in the presence or absence of 3MA (10mM) and were stimulated for 3 hours with MTB (1μg/ml) at 37°C. After the stimulation, supernatants were discarded and refreshed with RPMI or RPMI containing 1mM ATP, and cell were incubated for additional 15 minutes. ATP-dependent IL1β production was measured with ELISA (E). Data are presented as means ±SEM, n=6.
when incubating stimulated PBMCs in starvation medium (EBSS) (Figure 4a), while induction of autophagy seemed to decrease IL1β mRNA (Figure 4b). The opposite effect was exerted by autophagy inhibition through 3MA: TNFα mRNA was decreased (Figure 4c), while a trend towards an increase in IL1β mRNA was observed (Figure 4d). In contrast, inhibition of autophagy had no effect on IL1β processing and release induced by the inflammasome activation with ATP [20] (Figure 4e).

Figure 5. ATG16L1 polymorphisms and MTB-induced cytokine production. Cytokine production capacity of TNFα (A), IL1β (B) and IFNγ (C) by PBMCs obtained from healthy volunteers after stimulation for 24 hours (TNFα and IL1β) or 48 hours (IFNγ) with sonicated MTB stratified for ATG16L1 Thr300Ala genotype (A allele equals 300Thr allele, G allele equals 300Ala allele). Data are presented as means + SEM, n=73 (13 AA, 37 AG, 23 GG).
Figure 6. IRGM1 polymorphisms and MTB-induced cytokine production. Cytokine production capacity of TNFα (A, D), IL1β (B, E) and IFNγ (C, F) by PBMCs obtained from healthy volunteers after stimulation for 24 hours (TNFα and IL1β) or 48 hours (IFNγ) with sonicated MTB stratified for IRGM genotypes. Data are presented as means ± SEM. n=104 (2 AA, 25 GA, 77 GG for rs4958847, and 2 CC, 17 TC, 83 TT for rs13361189).
Autophagy gene polymorphisms and cytokine production induced by MTB

Polymorphisms in two genes involved in the process of autophagy, ATG16L1 and IRGM, have been associated with auto inflammatory disorders [21-23], and IRGM polymorphisms also with susceptibility to tuberculosis [24-26]. Considering the possible role of autophagy in modulating cytokine production induced by MTB, we examined if ATG16L1 and IRGM polymorphisms lead to altered cytokine production. However, no statistically significant differences in MTB-induced cytokine production were observed when PBMCs were used from healthy volunteers with different ATG16L1 genotypes, although PBMCs from individuals bearing the ATG16L1 300G variant tended to produce less IFNγ (Figure 5). In addition, no significant differences in cytokine production were observed between individuals bearing the different IRGM alleles (Figure 6). Although the cohorts were relatively large (57-104 subjects), only one (in the case of TNFα and IFNγ) or two (in the case of IL1β) individuals were homozygous for the IRGM rs4958847 AA and rs13361189 CC allele, precluding a definitive conclusion regarding the effect of these two IRGM SNPs on cytokine production.

DISCUSSION

Autophagy is essential for host defence against MTB [15, 16, 24]. In the present study, we show that autophagy modulates pro-inflammatory cytokine production after stimulation with MTB. Autophagy showed differential effects on MTB-induced cytokine production; autophagy stimulated TNFα production and inhibited IL1β and IL6 production. These effects of autophagy on MTB-induced proinflammatory cytokines were exerted at the transcriptional level.

Activation of autophagy resulted in opposite effects on TNFα and IL1β, exerting an increase of TNFα production and a decrease in IL1β production. This is an important, yet puzzling, effect considering the role of both TNFα and IL1β in host defence against MTB [11, 12, 15, 16, 27, 28]. On the one hand, the increase in the production of TNFα is likely to increase anti-mycobacterial innate immunity. On the other hand, one may speculate that the decrease in the production of IL1β may result in decreased Th17 responses [29], and thus indirectly shifting the response towards protective Th1 cellular immunity, as Th1 and Th17 are known to negatively regulate each other [30]. We were not able to study directly the effect of autophagy on Th17 responses for two reasons. Inhibition of autophagy through 3MA inhibited T cell proliferation, while prolonged incubation of cells, necessary for optimal Th17 responses, led to cell death when cells were kept for long periods of time in the EBSS (starvation) medium. Another mechanism that might influence cytokine levels in these experiments is IFNγ-induced TLR2 and NOD2 expression [31, 32], which in turn can lead to more cytokine production. However, induction of autophagy with starvation medium instead of IFNγ led to similar results, while the effects of IFNγ on cytokine production were abolished when 3MA was added. Therefore, the effects of IFNγ on cytokine production are at least partly mediated through autophagy.

In a separate set of experiments, we investigated the level at which the effects of autophagy on MTB-induced cytokines are exerted. Autophagy clearly influenced transcription of both TNFα and IL1β, while no post-transcriptional effect was observed for modulation of IL1β synthesis. However, a different effect was seen on TNFα versus IL1β transcription. There are several pathways that induce pro-inflammatory cytokines after MTB stimulation. The most important receptors involved in MTB recognition and
subsequent induction of cytokine responses are TLRs (TLR2 in combination with TLR1 or TLR6, TLR4 and TLR9) and NOD2. We have previously shown that NOD2- but not TLR-responses are modulated by autophagy [33]. TNFα and IL1β transcription are differently regulated, with IL1β transcription being mediated by ERK and p38, but not JNK kinase. In contrast, TNFα transcription is mediated through ERK and JNK-dependent pathways, with little effects through p38 kinase [34]. Our data therefore suggest that differential modulation of the MAP kinase transcriptional pathways by autophagy may be responsible for the differences in TNFα and IL1β production, although this remains to be confirmed in future studies.

IL1β is processed and secreted after activation of a protein complex called the inflammasome, leading to activation of caspase-1 and processing of inactive pro-IL1β into the active cytokine. ATP acting on the P2X7 receptor is a known activator of the inflammasome. No effect of autophagy on the IL1β processing and secretion induced by ATP was observed, providing an additional argument that autophagy modulates cytokine production in humans at the transcriptional, rather than the post-transcriptional level [33]. This is in contrast to studies in murine cells, in which inhibition of the inflammasome by autophagy seems to play an important role [19, 35]. This argues that important differences exist between the effects of autophagy in mice and humans.

Single nucleotide polymorphisms in the autophagy genes ATG16L1 and IRGM have been associated with Crohn’s disease [21-23], and an association of IRGM SNPs and TB has also been reported [24-26]. ATG16L1 is a component of a large protein complex formed together with ATG5 and ATG12 that is essential for autophagosome formation. IRGM is a downstream effector of IFNγ and has been implicated in the process of autophagy induction in macrophages [16]. Subsequently, reduction in IRGM1 levels are associated with decreased autophagy [24]. However, these polymorphisms did not influence the induction of TNFα or IL1β production by cells isolated from healthy volunteers and stimulated with MTB. While the T300A ATG16L1 polymorphism has been shown to modulated NOD2-induced IL1β production, it does not affect TLR-induced cytokine synthesis [33]. Although we should be careful interpreting these data, given the small number of subjects with carrying the functional SNPs, this suggests that modulation of cytokine production by NOD2 ligands in MTB may be compensated by the unaffected TLR-dependent induction. Interestingly, a trend towards lower IFNγ production was observed in cells isolated from individuals homozygous for the 300A allele of ATG16L1, and this may affect anti-mycobacterial host defence in the patients. Whether the T300A ATG16L1 polymorphism also modulates susceptibility to TB remains unclear. The effects of this ATG16L1 SNP may be exerted not only through IFNγ production, but also through modulation of antigen presentation at the level of MHC class II, a process known to be influenced by autophagy [17, 18].

Our findings might have been strengthened by examination of M. tuberculosis genotype in relation to host genotype. Another SNP in the IRGM gene, -261T, has been shown to be associated with protection from certain MTB genotypes but not with protection against Mycobacterium africanum [25]. This supports the concept of co-evolution of M. tuberculosis and the human immune system, similar to a previous study [36]. In this study we looked at the relation between host genotype and autophagy-mediated modulation of cytokine production, but in future patient studies we hope to investigate whether there is a relation between certain autophagy host-genotypes and M. tuberculosis genotypes.
In conclusion, the process of autophagy influences cytokine production induced in human peripheral blood mononuclear cells by MTB. These effects are different on TNFα versus IL1β induction, and are induced at the transcriptional level. The next step will be to relate these effects on cytokine production to the direct anti-mycobacterial defence mechanisms in macrophages. Finally, an assessment of the clinical implications of this autophagy-inflammation interaction is needed, with the important question whether (partial) defects in autophagy may increase susceptibility to TB.
REFERENCES


POLYMORPHISMS IN AUTOPHAGY GENES AND SUSCEPTIBILITY TO TUBERCULOSIS

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ABSTRACT

Recent data suggest that autophagy is important for intracellular killing of *Mycobacterium tuberculosis*, and polymorphisms in the autophagy gene *IRGM* have been linked with susceptibility to tuberculosis (TB) among African-Americans, and with TB caused by particular *M. tuberculosis* genotypes in Ghana. We compared 22 polymorphisms of 14 autophagy genes between 1022 Indonesian TB patients and 952 matched controls, and between patients infected with different *M. tuberculosis* genotypes, as determined by spoligotyping. The same autophagy polymorphisms were studied in correlation with ex-vivo production of TNFα, IL1β, IL6, IL8, IFNγ and IL17 in healthy volunteers.

No association was found between TB and polymorphisms in the genes *ATG10*, *ATG16L2*, *ATG2B*, *ATG5*, *ATG9B*, *IRGM*, *LAMP1*, *LAMP3*, *P2RX7*, *WIPI1*, *MTOR* and *ATG4C*. Associations were found between polymorphisms in *LAMP1* (p=0.02) and *MTOR* (p=0.02) and infection with the successful *M. tuberculosis* Beijing genotype. The polymorphisms examined were not associated with *M. tuberculosis* induced cytokines, except for a polymorphism in *ATG10*, which was linked with IL8 production (p=0.04). All associations found lost statistical significance after correction for multiple testing. This first examination of a broad set of polymorphisms in autophagy genes fails to show a clear association with TB, with *M. tuberculosis* Beijing genotype infection or with ex vivo proinflammatory cytokine production.
INTRODUCTION

*Mycobacterium tuberculosis* (M. *tuberculosis*), the main cause of tuberculosis (TB) worldwide, is an intracellular pathogen that primarily infects macrophages (1, 2). This pathogen resides and multiplies within a host-derived phagosome where it persists through interference with phagosome-lysosome biogenesis (3, 4). Recent studies suggest that autophagy, a homeostatic process involved in nutrient regeneration and immune responses, is involved in intracellular killing of *M. tuberculosis* (3, 5, 6), and that physiological or pharmacological induction of this process *in vitro* (i.e.: with ATP, IFNγ, vitamin D3) promotes fusion of phagosomes containing *M. tuberculosis* with lysosomes and subsequent killing of the pathogen in autophagic characteristic double-membrane autolysosomes (1, 3, 7). In addition, intracellular survival of *M. tuberculosis* was shown to depend on its ability to escape or inhibit autophagy (5, 8), and a study by Kumar et al. found that genes that regulate intracellular survival of *M. tuberculosis*, regardless of its genotype, are in the autophagy pathway itself or in pathways that affect autophagy (9).

Susceptibility to TB is partly genetically determined and variations in genes involved in the autophagic pathway may affect the host response to *M. tuberculosis* infection. Indeed, mice deficient in autophagy and autophagy related genes were found to be more susceptible to infection with *M. tuberculosis* (10, 11) and human mononuclear cells with certain polymorphisms in autophagy related genes displayed an impaired ability to control *M. tuberculosis* growth (12, 13), thus suggesting that polymorphisms in autophagy and autophagy related genes may be associated with TB. This appears to be the case as various polymorphisms in one autophagy gene IRGM, a downstream effector of IFNγ, have been associated with increased protection against *M. tuberculosis* infection in African-American (14) and Chinese individuals (15) and infection by particular *M. tuberculosis* genotypes in Ghana (16). In addition, polymorphisms in a number of genes which affect autophagy, such as *P2RX7*, have also been associated with TB (17, 18). However, to our knowledge, besides IRGM no other gene of the autophagy pathway itself has been examined in TB patients. We have therefore examined a selection of autophagy genes in a large cohort of TB patients and healthy controls in Indonesia. Since susceptibility to TB may depend on the interplay between host and mycobacterial genotype (2, 9, 19), we also grouped patients’ *M. tuberculosis* isolates into W-Beijing genotype strains, which account for one-third of all *M. tuberculosis* infections in Indonesia (20, 21), and non-W-Beijing genotypes. Furthermore, in a Caucasian cohort that was genotyped for the 22 SNPs, we measured cytokine production in peripheral blood mononuclear cells (PBMCs) stimulated with *M. tuberculosis*.

MATERIAL AND METHODS

Subject recruitment

We previously recruited consecutive TB patients diagnosed in two outpatient clinics and two hospitals in Jakarta and Bandung (Indonesia) from January 2001 to December 2006, for a series of genetic studies examining host susceptibility to TB (19, 22, 23).

Diagnosis of pulmonary TB (PTB) was done according to World Health Organization criteria by clinical presentation and chest radiograph examination, followed by confirmation with microscopic detection
of acid-fast bacilli in Ziehl-Neelsen-stained sputum smears and positive culture of M. tuberculosis on 3% Ogawa medium. For M. tuberculosis genotype analysis, mycobacterial DNA was extracted by bringing 2 loops of bacterial mass from an M. tuberculosis culture in saline solution and subsequently heating it at 95ºC for 5 min. M. tuberculosis genotype was determined by using a commercially available Spoligotyping kit (Isogen Bioscience, Maarssen, The Netherlands) as previously described (20). M. tuberculosis Beijing genotype was defined as a spoligo-pattern showing hybridization to at least 3 of the 9 spacers 35–43 and absence of hybridization to spacers 1–34. Spoligotyping was done at the Hasan Sadikin Hospital, Bandung, Indonesia. In addition, for quality control purposes, spoligotyping of 10% of the isolates and of all isolates lacking hybridization were also done at Gelre Hospital, Apeldoorn, The Netherlands.

We excluded from the genetic studies patients with a confirmed diagnosis of extra-pulmonary TB (n=93), diabetes mellitus (fasting blood glucose >126mg/dL) (n=139) and HIV-positive subjects (n=10). Standard regimen for treatment of TB consisted of isoniazid, rifampin, pyrazinamide, and ethambutol (2HRZE/4H3R3) was administered free of charge to all patients according to the Indonesian National TB program.

During the above mentioned period we also recruited 1000 randomly selected age and gender matched, but genetically unrelated control subjects from the same, mostly poor and densely populated areas where TB is abundant. All control individuals were subjected to the same physical examination, blood tests and chest radiography as the TB patients. A total of 952 control subjects were enrolled in the study after excluding individuals with symptoms or chest X-ray abnormalities suggesting active TB or a history of TB.

A structured questionnaire was used for patients and control subjects to record clinical information, age, gender, self and parental ethnicity, socio-economic status and concurrent medical history.

Ethics statement
All individuals recruited signed a written informed consent. The study protocol was reviewed and approved by the local institutional review boards of the medical faculty of university of Indonesia, the Eijkman institute for molecular biology in Jakarta in Indonesia and the Medical Ethical Committee Arnhem-Nijmegen in The Netherlands.

Genotyping
Using NCBI SNP database we selected SNPs in autophagy genes previously associated with TB (P2RX7- rs2393799 [17]), other diseases (ATG16L1- rs2241880 (24, 25), ATG5- rs2245214 (26) IRGM rs72553867 (27) rs4958847 (28) or with a minor allele frequency of at least 5% (Table 1).

Blood samples were obtained by venapuncture. Genomic DNA was isolated from EDTA blood of patients, controls and a cohort of healthy volunteers using standard methods, and 5 ng of DNA was used for genotyping. Multiplex assays were designed using Mass ARRAY Designer Software (Sequenom) and genotypes were determined using Sequenom MALDI-TOF MS according to manufacturer’s instructions (Sequenom Inc., San Diego, CA, USA). Briefly, the SNP region was amplified by a locus-specific PCR reaction. After amplification a single base extension from a primer adjacent to the SNP was performed to introduce mass differences between alleles. This was followed by salt removal
and product spotting onto a target chip with 384 patches containing matrix. MALDI-TOF MS was then used to detect mass differences and genotypes were assigned real-time using Typer 4 software (Sequenom Inc. San Diego, CA, USA). As quality control, 5% of samples were genotyped in duplicate and each 384-well plate also contained at least 8 positive and 8 negative controls, no inconsistencies were observed. DNA samples of which half or more of the SNPs failed (N=90) were excluded from analyses. Variants with call-rates below 90% were also excluded from further analyses (n=0).

For quality control purposes the genotype of at least two samples for each homozygous genotype were confirmed by sequencing using Sanger method with Big Dye Terminator version 3 (Applied Biosystems). After the cycle sequence reaction, the samples were purified by ethanol precipitation and analysed on a 3730 Sequence Analyzer (Applied Biosystems).

Previously polymorphisms in various genes were genotyped on a two-stage genome-wide association study (GWAS) using Illumina’s GoldenGate Assay according to manufacturer instructions, aiming to discover genes relevant in pulmonary TB susceptibility in the same Indonesian cohort involved in the current study (29). Among the SNPs studied, five were in autophagy genes and were included in our data analysis (Table 1). The overlap of study subjects between the current study and the GWAS is shown in Figure 1.

Cytokine production by M. tuberculosis stimulated PBMC
Cells isolated from healthy Caucasian volunteers bearing various genotypes were examined for cytokine production induced by sonicated M. tuberculosis H37Rv (n=67). These individuals were aged 23-73 years, 77% was male and none had a known TB contact. All gave written informed consent, and the study was approved by the Ethical Committee of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

Blood samples were obtained by venapuncture. The mononuclear cell fraction was isolated from blood by density centrifugation of blood, diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, PA, USA). Cells were washed twice in saline and resuspended in culture medium (RPMI, Invitrogen, CA, USA) supplemented with gentamicin 10 μg/ml, L-glutamine 10 mM, and pyruvate 10 mM. Cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to 5x10⁶ cells/ml. A total of 5 x 10⁵ mononuclear cells in a 100 μl volume of RPMI was added to round-bottom 96-wells plates (Greiner) with or without sonicated M. tuberculosis H37Rv (final concentration: 1 μg/ml). After 24 hours, 48 hours (both without serum) or 7 days of incubation (in the presence of 10% serum), supernatants were stored at -20°C. Cytokine concentrations were assessed in the supernatants using enzyme-linked immunosorbent assay (ELISA).

Cytokine measurements of TNFα, IL1β, IL6, IL8 (after 24 hours incubation); IFNγ (after 48 hours incubation), and IL17 (7 days incubation) were performed in the supernatants using commercial ELISAs from R&D Systems, MN, USA (TNFα, IL1β, IL8, and IL17) or Sanquin, Amsterdam, The Netherlands (IL6 and IFNγ).
Table 1. Polymorphisms in autophagy genes studied

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene ID</th>
<th>SNP</th>
<th>Heterozygosity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Disease associated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Asians&lt;sup&gt;b&lt;/sup&gt;</td>
<td>All populations</td>
</tr>
<tr>
<td>ATG10</td>
<td>83734</td>
<td>rs1864183</td>
<td>19% (49%)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs3734114</td>
<td>38% (25%)</td>
<td>N.A.</td>
</tr>
<tr>
<td>ATG16L1</td>
<td>55054</td>
<td>rs2241880</td>
<td>47% (48%)</td>
<td>Inflammatory bowel disease (24) and Crohn’s disease (25)</td>
</tr>
<tr>
<td>ATG16L2</td>
<td>89849</td>
<td>rs11235604</td>
<td>N.D. (18%)</td>
<td>N.A.</td>
</tr>
<tr>
<td>ATG2A</td>
<td>23130</td>
<td>rs77228473</td>
<td>N.D. (N.D.)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs77833427</td>
<td>8% (0.3%)</td>
<td>N.A.</td>
</tr>
<tr>
<td>ATG2B</td>
<td>55102</td>
<td>rs9323945</td>
<td>42% (16%)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs74719094</td>
<td>N.D. (0.3%)</td>
<td>N.A.</td>
</tr>
<tr>
<td>ATG5</td>
<td>9474</td>
<td>rs22345214</td>
<td>62% (50%)</td>
<td>Systemic lupus erythematosus (26)</td>
</tr>
<tr>
<td>ATG9B</td>
<td>285973</td>
<td>rs61733329</td>
<td>17% (4%)</td>
<td>N.A.</td>
</tr>
<tr>
<td>IRGM</td>
<td>345611</td>
<td>rs72533867</td>
<td>29% (22%)</td>
<td>Crohn’s disease (27)</td>
</tr>
<tr>
<td>LAMP1</td>
<td>3916</td>
<td>rs9577229</td>
<td>17% (16%)</td>
<td>N.A.</td>
</tr>
<tr>
<td>LAMP3</td>
<td>27074</td>
<td>rs482912</td>
<td>42% (51%)</td>
<td>N.A.</td>
</tr>
<tr>
<td>P2RX7</td>
<td>5027</td>
<td>rs2393799</td>
<td>48% (48%)</td>
<td>Tuberculosis [17]</td>
</tr>
<tr>
<td>WIPI1</td>
<td>55062</td>
<td>rs883541</td>
<td>49% (35%)</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

GWAS

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MTOR</td>
<td>2475</td>
<td>rs6701524</td>
<td>21% (30%)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs10492973</td>
<td>18% (20%)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs10493328</td>
<td>5% (15%)</td>
<td>N.A.</td>
</tr>
<tr>
<td>ATG4C</td>
<td>84938</td>
<td>rs10493327</td>
<td>40% (48%)</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs10493329</td>
<td>4% (19%)</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

<sup>a</sup>data from dbSNP http://www.ncbi.nlm.nih.gov/projects/SNP/; <sup>b</sup>data from HapMap-HCB (Han Chinese from Beijing); <sup>c</sup>data from low coverage pilot panel CHB+JPT (Han Chinese from Beijing and Japanese from Tokyo); N.D. = no data available, N.A. = no known association

Statistical analysis
All data collected from the questionnaires and genotyping were analysed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The Hardy-Weinberg equilibrium (HWE) was checked for each SNP using the program HWE Version 1.10 (Rockefeller University, New York). The program Conting was used to calculate the $\chi^2$ and the associated values for a contingency table. Patient data were stratified for the M. tuberculosis genotype with which they were infected; Beijing or non-Beijing strains and the $\chi^2$ was calculated with SPSS. Differences in cytokine production were analyzed using the Wilcoxon signed rank test. All statistical analyses were 2-sided, and $P < 0.05$ was considered to be statistically significant.

The available number of study subjects allowed us to observe a 5% allele difference between patients and controls for the SNP in IRGM (rs4958847), based on previously reported allele distribution in the general population, a power ($\beta$) of 0.80 and a significance level ($\alpha$) of 0.05.
RESULTS

Study subjects
A total number of 1022 confirmed pulmonary TB patients and 952 age- and gender matched community controls were included in the data analysis. As shown in Table 2, 78% of patients and control subjects were Javanese (a population group with relatively low genetic variance in Indonesia (30)) with similar age, gender distribution, and likelihood of having a BCG scar. Furthermore, both groups also had a similar socioeconomic status (not shown) and previous analysis in this cohort (29) showed that population stratification was minimal.

Table 2. Demographic information of the study population

<table>
<thead>
<tr>
<th></th>
<th>TB patients</th>
<th>controls</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>33</td>
<td>33</td>
<td>0.7</td>
</tr>
<tr>
<td>Gender male (%)</td>
<td>53.4</td>
<td>53.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Self reported ethnicity</td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>Javanese</td>
<td>78.5%</td>
<td>78.3%</td>
<td></td>
</tr>
<tr>
<td>Mixed (either parent Javanese)</td>
<td>11.1%</td>
<td>11.8%</td>
<td></td>
</tr>
<tr>
<td>Non-Javanese</td>
<td>8.1%</td>
<td>8.2%</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>2.3%</td>
<td>1.7%</td>
<td></td>
</tr>
<tr>
<td>BCG scar present</td>
<td>44%</td>
<td>49%</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Association between polymorphisms in autophagy genes and susceptibility to TB
Polymorphisms rs11235604 (in ATG16L2), rs77228473 and rs77833427 (in ATG2A), rs74719094 (in ATG2B), rs72553867 (in IRGM), rs10493328 and rs10493329 (in ATG4C) were rare in the study subjects. With the exception of the SNP rs3759601 in ATG2B (HWE: 2p = 0.034), all polymorphisms were in Hardy–Weinberg equilibrium in the healthy controls. The distribution of the alleles for all polymorphisms analyzed in the current study is presented in Table 3. After Chi-square testing we did not detect significant associations between any genetic polymorphism and susceptibility to TB. This was also the case when the largest group (Javanese) was analysed separately (data not shown).

Figure 1: The overlap of study subjects between the current study (n=1974) and the previous genome-wide association study (GWAS, n=1139) (29).
### Table 3. Distribution of polymorphism allele frequencies in cases and controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Allele</th>
<th>Frequency in cases (%)</th>
<th>Frequency in controls (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autophagy specific genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG10</td>
<td>rs1864183</td>
<td>A</td>
<td>1450 (86)</td>
<td>1400 (83.3)</td>
<td>0.094</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>236 (14)</td>
<td>280 (16.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>625 (74.1)</td>
<td>583 (69.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>200 (23.7)</td>
<td>234 (27.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>18 (2.1)</td>
<td>23 (2.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs3734114</td>
<td>C</td>
<td>491 (29.2)</td>
<td>502 (29.8)</td>
<td>0.489</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>1189 (70.8)</td>
<td>1180 (70.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>71 (8.5)</td>
<td>84 (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TC</td>
<td>349 (41.5)</td>
<td>334 (39.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>420 (50)</td>
<td>423 (50.3)</td>
<td></td>
</tr>
<tr>
<td>ATG16L1</td>
<td>rs2241880</td>
<td>C</td>
<td>449 (26.6)</td>
<td>453 (26.8)</td>
<td>0.876</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>1237 (73.4)</td>
<td>1235 (73.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>62 (7.4)</td>
<td>59 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TC</td>
<td>325 (38.6)</td>
<td>335 (39.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>456 (54.1)</td>
<td>430 (53.3)</td>
<td></td>
</tr>
<tr>
<td>ATG16L2</td>
<td>rs1135604</td>
<td>CC</td>
<td>835 (99)</td>
<td>839 (99.2)</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TC</td>
<td>9 (1)</td>
<td>6 (0.8)</td>
<td></td>
</tr>
<tr>
<td>ATG2A</td>
<td>rs77228473</td>
<td>C</td>
<td>1676 (100)</td>
<td>1680 (100)</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
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Association between polymorphisms in autophagy genes and M. tuberculosis genotype
To examine a possible association between host and mycobacterial genotype, autophagy gene polymorphisms were compared between patients infected with M. tuberculosis Beijing genotype and other M. tuberculosis genotypes. One hundred and sixty-one patients (33%) were infected with M. tuberculosis Beijing genotype strains, 322 with a non-Beijing strain, while no strain information was available for the remainder (n=540). Patients infected with M. tuberculosis Beijing and non-Beijing strains were not significantly different in terms of age, sex, or history of previous tuberculosis treatment. The distribution of the alleles for all polymorphisms among patients infected with M. tuberculosis Beijing and non-Beijing strains is shown in Table 4. The polymorphism in LAMPI (rs9577229) showed an association with TB caused by M. tuberculosis Beijing strains, when the TC was combined with the low
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<td>59 (31.6)</td>
<td>154 (40.5)</td>
<td></td>
</tr>
</tbody>
</table>
**Gene** | **SNP** | **Allele** | **Frequency in Beijing (%)** | **Frequency in non-Beijing (%)** | **P value**
--- | --- | --- | --- | --- | ---
**Autophagy specific genes**

LAMP3 | rs482912 | A | 149 (46.3) | 309 (47) | 0.831
|  | G | 163 (50.6) | 349 (33) |  |
|  | AA | 32 (19.9) | 72 (21.9) |  |
|  | GA | 85 (32.8) | 165 (50.2) |  |
|  | GG | 44 (27.3) | 92 (28) |  |

P2RX7 | rs2393799 | C | 170 (52.5) | 301 (45.9) | 0.164
|  | T | 154 (47.5) | 355 (54.1) |  |
|  | CC | 46 (28.4) | 74 (22.6) |  |
|  | TC | 78 (48.1) | 153 (46.6) |  |
|  | TT | 38 (23.5) | 101 (30.8) |  |

WIP11 | rs883541 | A | 124 (38.3) | 265 (40.3) | 0.547
|  | G | 200 (61.7) | 393 (59.7) |  |
|  | AA | 24 (14.8) | 47 (14.3) |  |
|  | GA | 76 (46.9) | 171 (52) |  |
|  | GG | 62 (38.3) | 111 (33.7) |  |

**GWAS**

MTOR | rs6701524 | A | 219 (95.2) | 417 (91) | 0.023
|  | G | 11 (4.8) | 41 (9) |  |
|  | AA | 105 (91.3) | 188 (82.1) |  |
|  | AG/GG | 10 (8.7) | 41 (17.9) |  |

rs10492975 | A | 205 (89.1) | 416 (90.6) | 0.84
|  | G | 25 (10.9) | 44 (9.4) |  |
|  | AA | 92 (80) | 189 (82.5) |  |
|  | AG | 21 (18.3) | 37 (16.2) |  |
|  | GG | 2 (1.7) | 3 (1.3) |  |

ATG4C | rs10493328 | A | 10 (4.3) | 22 (4.8) | 0.85
|  | G | 220 (95.7) | 436 (91.7) |  |

rs10493327 | A | 158 (68.7) | 307 (67) | 0.81
|  | G | 72 (31.3) | 151 (33) |  |
|  | AA | 53 (46.1) | 103 (45) |  |
|  | AG | 52 (45.2) | 101 (44.1) |  |
|  | GG | 10 (8.7) | 25 (10.9) |  |

rs10493329 | A | 220 (95.7) | 432 (95.2) | 0.84
|  | G | 10 (4.3) | 22 (4.8) |  |

n.a.- not analysed; SNP not polymorphous in this population

prevailing TT genotype (p=0.02). The same was true for the polymorphism in MTOR (rs6701524); when combining the AG with the low prevalent GG genotype, MTOR was significantly associated with infection with *M. tuberculosis* Beijing strains (p=0.02). However, both associations lost statistical significance after correction for multiple testing.

Polymorphisms in autophagy genes and *M. tuberculosis* induced cytokine production

Association between host genotype and *M. tuberculosis* induced cytokine production by PBMC was examined in healthy Caucasian individuals. **Table 5** shows the difference in *M. tuberculosis* induced production of TNFα, IL1β, IL6, IL8, IFNγ and IL17 by PBMC isolated from individuals stratified for different genotype of autophagy related genes. Six of these polymorphisms showed no polymorphic distribution in the Caucasian individuals and could therefore not be analysed. With the exception of
ATG10 (rs1864183), for which a significant difference was found in IL8 production between individuals with an AA and GG genotype (p=0.04), no associations were observed between the investigated cytokines and the autophagy related polymorphisms. Figure 2 presents scatter plots of TNFα, IFNγ, and IL17 stratified for genotypes of both investigated polymorphisms in IRGM which was previously linked with susceptibility to TB.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>IL1β</th>
<th>IL6α</th>
<th>IL8α</th>
<th>IL17α</th>
<th>TNFαα</th>
<th>IFNγα</th>
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<tr>
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<td>1.2b</td>
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<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>rs3734114</td>
<td>1.4</td>
<td>1.1</td>
<td>1.1</td>
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<td>1.8</td>
<td>1.2</td>
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<td>1.1</td>
<td>1.1</td>
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<td>1.4</td>
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<tr>
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<td>1.4</td>
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<tr>
<td></td>
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<td>1.3</td>
<td>1.7</td>
<td>1.8</td>
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<td>2.1</td>
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<td>n.a.</td>
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</tr>
<tr>
<td>LAMP3</td>
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<td>1.2</td>
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<td>1.8</td>
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<td>WIP1</td>
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<td>1.1</td>
<td>1.2</td>
<td>2.9</td>
<td>2.0</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Values are expressed as the ratio of cytokine production associated with different genotypes (median for all individuals bearing the same genotype), using the genotype groups with the highest and lowest cytokine production. Significantly differences (P<0.05) in genotype groups with highest and lowest cytokine production calculated by Wilcoxon signed rank test.

n.a. not analysed; SNP not polymorphous in this population

**DISCUSSION**

In vitro data strongly support a role for autophagy in control of *M. tuberculosis*, and a study involving 2010 patients with pulmonary TB and 2346 control subjects from Ghana has previously reported an association between a polymorphism in the autophagy gene IRGM and TB (16). To further explore a role of autophagy in TB we examined polymorphisms in a number of autophagy genes in TB patients and matched controls from Indonesia. Among almost 2000 subjects, no association was found between TB and 22 SNPs in 14 different autophagy and autophagy related genes, including IRGM and P2RX7 which were previously associated with TB. When TB patients were stratified according to *M. tuberculosis* genotype, associations were observed between SNPs in LAMP1, MTOR and infection with *M. tuberculosis* Beijing genotype, but statistical significance was lost after correction for multiple testing. No significant correlation was found between *M. tuberculosis* induced cytokine production and genotype of autophagy related genes in a separate cohort of healthy Caucasian volunteers.

IRGM, a downstream effector protein of IFNγ, induces autophagy and subsequent generation of
large autolysosomal organelles as a mechanism for the elimination of intracellular *M. tuberculosis* (31).

In a cohort of 2010 Ghanaian patients and 2346 controls a polymorphism (rs9637876) in IRGM was associated with decreased susceptibility to TB caused by *M. tuberculosis* Euro-American (EUAM) lineage, although not for *M. tuberculosis* East-African-Indian (EAI), Beijing, Delhi, *M. africanum* and *M. bovis* lineages (16). In a study in the US, a polymorphism in IRGM (rs10065172) was more common in 370 African-American TB patients compared to controls, but not in 177 Caucasian patients compared to 110 Caucasian controls (14). We did not find an association between TB, which in Indonesia is mainly caused by the *M. tuberculosis* Beijing lineage, and two different polymorphisms in IRGM.

*P2RX7* is an autophagy related gene. It encodes for the P2X7 receptor, a plasma membrane receptor which mediates ATP-induced autophagy and subsequent intracellular killing of *M. tuberculosis* upon upregulation in mature macrophages (32-34). *P2RX7* displays a high genetic heterogeneity (12), and a polymorphism with a C allele at position -762 in the *P2RX7* promoter region was found to have a protective effect against TB in over 300 TB patients and 160 ethnically matched controls subjects from The Gambia (18). However, no association was found between the same polymorphism and TB in our cohort of Indonesian subjects. It is noteworthy that the protective effect of this polymorphism in Gambian subjects was weak and that it did not correlate with altered receptor expression or activity, suggesting the effect of this SNP might be influenced by other host and pathogen factors (18). In addition, the relative importance of the role of P2X7 receptor in the control of *M. tuberculosis* growth is still debated since mice deficient for P2X7 receptor displayed a similar ability to control pulmonary *M. tuberculosis* infection compared to wild-type mice (35). Unfortunately, studies on the effect of *P2RX7* polymorphisms in susceptibility to pulmonary TB in humans have not yet been done either in vivo or in other ethnic groups.

Polymorphisms in various genes have been associated with TB, but only polymorphisms in *VDR* (36-38), *NRAMP1* (39, 40) and *MBL* (41, 42) were found to be associated with TB in different geographic regions and ethnic groups. However, the effect of SNPs in these genes varies among racial groups. SNPs in *NRAMP1* were associated with an increased risk of PTB in Gambians (39) but were found to have a protective effect in Cambodians (40), polymorphisms in *MBL* were associated with protection against TB in South Africans (41) but in South Indians increased susceptibility to this disease (42), while SNPs in *VDR* were found to increase susceptibility to PTB in three African countries (36) but to have no effect in Cambodians (40). As suggested by Fernando et al, these contrasting findings between different ethnic groups may be due to differences in allele frequencies (17). In addition, the phylogeography of mycobacteria implies that *M. tuberculosis* lineages have become differentially adapted to genetic variations among racial groups (2).

The development of TB is the result of a complex interaction between the host and pathogen influenced by environmental factors (43). After stratification according to *M. tuberculosis* genotype, we found a suggestive association between TB caused by *M. tuberculosis* Beijing genotype and a polymorphism in *LAMP1*, similar to what we have previously shown for polymorphisms in *SLC11A/NRAMP1* (19). However, nine major *M. tuberculosis* genotypes have been previously identified in Indonesia (20) and some polymorphisms analysed here may be associated with TB caused by other genotypes not identified in this study.
LAMP1 and LAMP2 are two major protein components of late endosome and lysosome membranes, thought to form a protective barrier against degradation by hydrolytic enzymes (44, 45). Mice lacking Lamp2 display impaired autophagy and lysosome biogenesis, while deletion of both Lamp1 and Lamp2 is embryonically lethal (44). However, the contribution of these two lysosomal membrane proteins to phagosomal maturation and killing of intracellular pathogens still needs to be clarified.

Our group recently showed that inhibition of autophagy (genetically or with either siRNA or 3MA) increased IL1β production (46-48). However, with the exception of a SNP in ATG10 and IL8, no differences in cytokine production were observed in M. tuberculosis stimulated PBMCs of healthy volunteers stratified for genotype of autophagy related genes.

Our paper has several limitations. First and most importantly, no tuberculin skin testing was performed in the control population. However, exposure to tuberculosis must be common in this group, as the majority of controls lived in households of tuberculosis patients, who mostly had a productive cough (98%) for a median of 3 months before first presentation at the TB clinic (49). Especially because of the high incidence of TB in Indonesia, 128 per 100,000 in 2005 (50). Second, as we powered our study on an expected 5% difference in allele frequency between the groups the study lacks power to exclude any association amongst SNPs that are below this frequency.

This is the first paper to investigate the relation of different SNPs in a broad set 14 autophagy genes with susceptibility to TB, as well as with the infecting M. tuberculosis genotype and ex vivo cytokine production. These data further supports the belief that susceptibility to TB has a polygenic nature and polymorphisms in more than one gene may be required to render individuals more or less susceptible to develop active disease.

Figure 2: Scatter plots of TNFα, IFNγ, and IL17 produced after stimulation of PBMC with M. tuberculosis stratified for genotypes of both investigated polymorphisms in IRGM.
REFERENCES


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INNATE IMMUNE RECOGNITION OF 
MYCOBACTERIUM TUBERCULOSIS

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Marije Oosting 
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Mihai G. Netea 
Reinout van Crevel
ABSTRACT

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is a major health problem, with 10 million new cases diagnosed each year. Innate immunity plays an important role in the host defense against *M. tuberculosis*, and the first step in this process is recognition of MTB by cells of the innate immune system. Several classes of pattern recognition receptors (PPRs) are involved in the recognition of *M. tuberculosis*, including Toll-like receptors (TLRs), C-Type lectins (CLRs), and Nod-like receptors (NLRs). Among the TLR family, TLR2, TLR4, and TLR9 and their adaptor molecule MyD88 play the most prominent roles in the initiation of the immune response against tuberculosis. In addition to TLRs, other PRRs such as NOD2, Dectin-1, Mannose receptor and DC-SIGN are also involved in the recognition of *M. tuberculosis*. Human epidemiological studies revealed that genetic variation in genes encoding for PRRs and downstream signalling products influence disease susceptibility, severity and outcome. More insight into PRRs and the recognition of mycobacteria, combined with immunogenetic studies in TB patients, does not only lead to a better understanding of the pathogenesis of tuberculosis, but may also contribute to the design of novel immunotherapeutic strategies.
Tuberculosis (TB) is a major public health problem, with 10 million new cases diagnosed each year, causing a death toll of 2 million victims. However, from the estimated 2 billion persons individuals that have been initially infected with *Mycobacterium tuberculosis*, only 5 to 10% develop symptomatic TB. The reason why some infected individuals develop active disease while others do not, is not yet entirely understood. The role of inborn variability in susceptibility to tuberculosis has been accidentally proven by an episode that occurred almost a century ago, when in 1926 newborn infants from the town of Lübeck in Germany received live *Mycobacterium tuberculosis* (MTB) instead of the vaccine bacillus Calmette-Guérin (BCG). Some of the children became gravely ill, while others were unaffected [1]. This finding indicates that at least some individuals display an effective immune response to MTB and that this plays an important part in determining the outcome of the infection. In addition, this episode in young infants known to have immature adaptive immunity also suggests that the innate host defense is an important arm of anti-mycobacterial host defence.

Much has been learned during the last decade on the mechanisms through which the immune response to MTB is initiated. The first step is recognition of mycobacteria as invading pathogens, followed by activation of innate host defence responses, and the subsequent initiation of adaptive immune responses. Knowledge about these processes is crucial for understanding the pathophysiology of tuberculosis on the one hand, and for the development of novel strategies of vaccination and treatment such as immunotherapy on the other hand. This review focuses on the first step of the immune response, the recognition of mycobacteria by cells of the innate immune system.

Initiation of the innate immune response starts with pattern recognition of microbial structures called pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs is performed by germline-encoded receptors expressed mainly on immune cells termed pattern recognition receptors (PRRs) [2]. The first step in understanding the mechanisms of recognition of pathogenic bacteria is a solid knowledge of the structure of the cell wall of the microorganism, which is the first structure to come in contact and to be recognized by the cells of the immune system.

The mycobacterial cell wall

MTB is a slow-growing intracellular pathogen that can survive inside the macrophage of the host. MTB is an acid-fast bacterium due to the fact that the cell wall mainly consists of hydrophobic mycolic acids. This is a specific component of mycobacterial cell wall and makes up 50% of its dry weight. Due to this thick layer of mycolic acids, the entry of nutrients is impaired, which causes slow growth of mycobacteria, but it also increases cellular resistance to degradation through lysosomal enzymes [3]. The mycolic acids are distributed as a thick layer mostly at the external portions of the cell wall, while the internal layers of mycobacteria consist mostly of arabinogalactan, phosphalidyl-myoinositol mannosides (PIMs), and peptidoglycans (Figure 1) [4]. Next to the mycolic acid layer, other components include mannose-containing biomolecules including mannose-capped lipoarabinomannan (Man-LAM), the related lipomannan (LM), and manno-glycoproteins [4]. Mannan and arabinomannan are present on the surface and form the outer capsule of this bacterium. Man-LAM, LM and PIMs all share a conserved mannosyl-phosphatidylinositol (MPI) domain that presumably anchors the structures into the plasma membrane [5].
Man-LAM, one of the most abundant mannans present on the cell surface, is an important virulence factor of MTB [6]. Man-LAM is a heterogeneous lipoglycan with a characteristic tripartite structure of a carbohydrate core, the MPI anchor and various mannose-capping motifs. These mannose-capped motifs are characteristic for all pathogenic mycobacteria, and they are not present on fast-growing mycobacterial strains which are significantly less pathogenic. These strains have either uncapped LM or have phospho-myoinositol caps (PILAM), which are known to display more robust immunostimulatory effects. PIMs can be divided into two groups dependent on the mannose content, which determines its immunogenic effect [7,8]. Also present on the cell surface are the manno-glycoproteins, which can also be secreted during growth.

Figure 1. The structure of the *Mycobacterium tuberculosis* cell wall. This figure shows schematic the major components of the cell wall and their distributions. The inner layer is composed of peptidoglycan which is covalently linked to arabinogalactan layer. The outer membrane contains mycolic acids, glycolipids like (mannose-capped) lipomannan, and manno-glycoproteins.

Innate immunity and host defence
After the inhalation of infected aerosols into the lungs of the host, the first encounter of mycobacteria is with alveolar resident macrophages. Mycobacteria that escape the initial intracellular destruction can multiply and disrupt the macrophage, after which chemokines are released, attracting monocytes and other inflammatory cells to the lung. Inflammatory monocytes will differentiate into macrophages which readily ingest but do not destroy the mycobacteria [9]. In this stage of the infection the mycobacteria grow logarithmically and blood-derived macrophages accumulate, but little tissue damage occurs. Two to three weeks after infection, T cell immunity develops and antigen-specific T lymphocytes arrive, proliferate within the early lesions or tubercles, and release proinflammatory cytokines such as interferon γ (IFNγ) that will activate macrophages to kill the intracellular mycobacteria. Subsequently,
the early logarithmic bacillary growth stops, and central solid necrosis in these primary lesions or granuloma inhibits extracellular growth of mycobacteria. Several scenarios may follow, with infection becoming stationary or dormant in some individuals, or progressive in the lung or with hematogenous dissemination in a minority of patients. In addition, reactivation can occur months or years afterwards, under conditions of failing immune surveillance [9]. Granuloma often contain central caseous necrotic tissue which gives rise to cavities, and aerogenic spread of mycobacteria.

The macrophage is a pivotal cell in these events, as it is involved in phagocytosis and killing of mycobacteria as well as in the initiation of adaptive T cell immunity. Phagocytosis of MTB involves different receptors such as the scavenger receptors, the mannose receptor (MR) and complement receptors [10-13]. Phagocytosis can involve both uptake of the bacilli after opsonization with complement factors, or it can be initiated as a non-opsonic event. In vitro experiments have shown that complement receptor 3 (CR3) mediates approximately 80% of complement-opsonized MTB phagocytosis [12]. Non-opsonic phagocytosis is an important process in the primary infection of the lung, because complement factors are largely absent in the alveolar space [14].

Macrophages can eliminate mycobacteria through different mechanisms, such as production of reactive oxygen and nitrogen species, acidification of the phagosome, and phagosome fusion with the lysosomes [9]. The fate of intracellular mycobacteria is also influenced by autophagy, a cellular process through which cytoplasmic components, including organelles and intracellular pathogens, are sequestered in a double-membrane-bound autophagosome and delivered to the lysosome for degradation [15]. Activation of autophagy leads to phagosome maturation, an increased acidification in the phagosome, and killing of mycobacteria in macrophages [16]. However, once inside the cell, MTB often evades destruction by the innate microbial machinery [17], one of the main mechanisms being the inhibition of phagosome-lysosome fusion [18].

The interaction between MTB and cells of both the innate and adaptive immune system results in the secretion of chemokines and cytokines, the most important being tumor necrosis factor α (TNFα), cytokines of the interleukin 1 family (IL1β, IL18), IL12 and IFNγ. TNFα deficient mice succumb rapidly after MTB infection, with significantly higher mycobacterial outgrowth in different organs compared to wild type animals [19]. TNFα is also important for formation of granuloma, an important mechanism for containing and restricting the replication of the bacilli [19,20]. The importance of IL1β production is underlined by the fact that an intact IL1-mediated signals are an essential component of the host defence to mycobacteria [21-23]. Infection of IL1 receptor type 1 knock-out mice with MTB is associated with lower production of IFNγ, defective granuloma formation, and lower survival [22]. IFNγ activates macrophages to kill and eliminate the mycobacteria. It also enhances their expression of MHC class II molecules, which results in improved antigen presentation to T cells. IFNγ is secreted by NK, CD4+, and CD8+ T cells upon release of endogenous IL12 and IL18 by macrophages and dendritic cells. The crucial importance of IFNγ for human anti-mycobacterial defence is demonstrated by the increased susceptibility to mycobacterial infections in patients with IFNγ receptor or IL12 receptor deficiencies [24-26].

Various macrophages subsets have been identified with different potential functions. For example
alveolar macrophages, usually the first encounter with the mycobacterium, have an immune suppressive and poor antigen presenting ability [27,28]. Two main subtypes are described; the classical and the non-classical or alternative phenotypes. The classical route of differentiation induced by microbial products or IFNγ leads to induction of antimicrobial effects and production of proinflammatory cytokines as TNFα, IL1β and IL12(p40) and IL23 [29,30]. This in contrast to the nonclassical macrophages subsets, which lack antimicrobial activity and production of IL12. These subsets have a poor antigen presenting capacity and can suppress cellular immunity by production of IL10 [30]. The macrophages subset polarization may determine the outcome of the host response in skewing the pro and anti-inflammatory immune response and subsequently in elimination of mycobacteria.

The first step in the activation of innate host defence begins with the pattern recognition of the pathogen. The PAMPs of MTB are sensed by specific PRRs, which in turn trigger production of proinflammatory cytokines and chemokines, phagocytosis and killing of the mycobacteria, and antigen presentation. This review focuses on the role of the PRRs and downstream signalling for the recognition of MTB, including the intracellular mechanisms activated by PRRs. First, we will review specific evidence from in vitro studies and animal research. Then, we will discuss the human genetic studies done to assess the role of variation in PRR genes for the susceptibility to tuberculosis.

RECOGNITION OF MYCOBACTERIUM TUBERCULOSIS – EXPERIMENTAL STUDIES

The interaction between MTB and host cells is complex and, although extensively studied, not yet completely elucidated. Here we will focus on the PRRs that recognize specific PAMPs of mycobacteria and induce intracellular signals leading to cytokine production and initiation of adaptive immunity. A schematic representation is presented in Figure 2. Host receptors which are mainly involved in bacterial phagocytosis rather than immune recognition, such as complement receptors and scavenger receptors, go beyond the scope of this review.

Toll-like receptors
Toll-like receptors (TLRs) are a family of PRRs consisting of 12 members in mammals. TLRs are expressed on the surface of the cell membrane or on the membrane of endocytic vesicles of mainly immune cells including macrophages and dendritic cells (DCs). Although the interaction of MTB with TLRs leads to phagocyte activation, the interaction itself does not lead to immediate ingestion of the mycobacteria. After the interaction of specific mycobacterial structures with TLRs, signalling pathways are triggered in which adaptor molecule myeloid differentiation primary response protein 88 (MyD88) plays an important role [31]. Subsequently, IL1 receptor associated kinases (IRAK), TNF receptor associated factor (TRAF) 6, TGFβ-activated protein kinase 1 (TAK1) and mitogen-activated protein (MAP) kinase are recruited in a signalling cascade leading to activation and nuclear translocation of transcription factors such as the nuclear transcription factor (NF)-κb [32]. This leads to the transcription of genes involved in the activation of the innate host defence, mainly the production of proinflammatory cytokines as TNFα, IL1β and IL-12 and nitric oxide [33].
MyD88 plays a central role in the activation of the innate immune response to *M. tuberculosis*; compared to wild-type mice, MyD88 knockout mice are more susceptible to infection [21]. In addition to MyD88, TLR4 can induce intracellular signals through a second pathway, which is mediated by the adaptor molecule Toll/IL-1R (TIR) domain-containing adapter inducing interferon (IFN) β (TRIF). Recently, this MyD88-independent, TRIF-dependent TLR4 signalling cascade was shown to be involved in the LPS-induced autophagy [34]. As the TLR4-induced activation of autophagy plays an important role in the phagosome-lysosome fusion, a process counteracted by MTB [34], it is tempting to speculate that the interaction between TRIF and autophagy is an important component of the innate host defence to mycobacteria.

The TLRs known to be involved in recognition of MTB are TLR2, TLR4, TLR9, and possibly TLR8 [35-40]. TLR2 forms heterodimers with either TLR1 or TLR6. These heterodimers have been implicated in recognition of mycobacterial cell wall glycolipids like LAM, LM, 38-kDa and 19-kD mycobacterial glycoprotein, and phosphatidylinositol mannoside (PIM), and triacylated (TLR2/TLR1) or diacylated (TLR2/TLR6) lipoproteins [39,41,42]. TLR2 is believed to be important in the initiation of innate host defence through its stimulatory effects on TNFα production in macrophages [31,38]. In turn, an important role for TLR2 and TLR6, but not TLR4 or TLR9, was found for the stimulation of IL1β production [43]. TLR2 is also important for IL12 release in macrophages, but not in DCs [44]. TLR2−/− mice show defective granuloma formation, and when infected with high doses of MTB, they have a greatly enhanced susceptibility to infection compared to the WT mice [45,46]. In addition, TLR2−/− mice display defects in controlling chronic infection with MTB [46].

TLR4 is activated by heat shock protein 60/65 [37,47], a protein that is secreted by a variety of MTB species. Studies with TLR4 transfected CHO cells and murine macrophages showed the importance of TLR4 in recognition of MTB [36,39]. Macrophages of TLR4 deficient mice showed less, but not completely abolished TNFα production. *In vivo* murine studies on the role of TLR4 in the recognition of MTB have shown conflicting results, even when the same mouse strain was used. Reiling et al showed that TLR4 deficient mice, in contrast to TLR2 deficient mice, showed similar susceptibility to MTB infection compared to wild-type animals [45]. In contrast, Abel et al reported higher mycobacterial outgrowth in lungs, spleen and liver, and a lower survival following infection compared to wild type animals [48]. More studies are necessary to elucidate the source of these discrepancies, and the role of TLR4 for MTB infection.

TLR9 recognizes unmethylated CpG motifs in bacterial DNA. *In vitro* studies showed that MTB-induced IL12 release in dendritic cells was TLR9-dependent [38,44]. *In vivo* experiments showed that when mice were infected with a high infectious dose of MTB, animals lacking TLR9 succumb earlier to infection than wild type animals [38].

TLR8 is able to recognize single stranded RNA from pathogens such as RNA viruses. Interestingly, Davila et al. demonstrated upregulation of TLR8 protein expression in macrophages after infection with BCG [40]. Until now, this is the only study addressing a potential role of TLR8, but the mechanism through which TLR8 recognizes MTB and signals intracellular remains unknown.

A partially redundant role of TLRs for the host defence against mycobacteria has been suggested, and it
has been hypothesized that defects in multiple TLRs are necessary to unveil the role of these receptors for antitycobacterial defence. Indeed, TLR2 and TLR9 double knock-out mice display greater defects of IL12 and IFNγ production in comparison with both single TLR knock-out mice, and they succumb earlier to infection, even when infected with a low inoculum of MTB [38].

NOD like receptors
The NOD like receptors (NLR) family of proteins highly resembles the family of plant R (resistance) proteins, which have a crucial role in the defence against plant pathogens. The mammalian NLR family consists out of more than twenty members with a conserved structure. The core of the molecule is formed by the nucleotide-binding domain, named NACHT (NAIP, CIITA, HET-E, TP-1 [49]) or NOD (nucleotide oligomerization domain) domain. The C-terminal part consists of a series of leucin-rich

Figure 2. Pattern recognition receptors in the recognition of mycobacteria and downstream signaling pathways. Mycobacteria can be recognized through different pattern recognition receptors (PRRs) of the host. Both intracellular and extracellular receptors are involved in this process. After recognition of mycobacteria, intracellular signalling cascades are activation which eventually will lead to the activation of transcription of NF-kB. After transcription, production of pro- and anti-inflammatory cytokines and chemokines is induced. The type of signalling cascade induced depends mainly on the type of PRR that recognizes (components of) MTB.
repeats, which are thought to recognize the PAMPs of the pathogen and initiate activation of the molecule. The N-terminal portion of the molecule contains an effector domain of CARD (caspase activation and recruitment domain), PYRIN, or BIR (baculovirus inhibitor of apoptosis repeat domain) [50]. CARD-containing NLRs such as NOD1 and NOD2 are thought to form oligomers and then to recruit receptor-interacting protein 2 (RIP2) (or CARD containing kinase - RICK) through CARD-CARD interactions, which leads to the recruitment of NF-κB [51].

A major signalling pathway for the activation of the antimycobacterial host defence is represented by the inflammasome, that through activation of caspase-1 leads to processing of pro-cytokines of the IL1 family into the bioactive IL1β and IL18. Several PYRIN-domain containing NLRs (NALPs) can form different variants of the inflammasome containing either NLRP1, NLRP3 (cryopyrin) or NLRC4 (Ipaf) [52], as well as the adaptor protein ASC [53,54]. A fourth type of inflammasome formed by the intracellular protein AIM2 is activated by intracytoplasmic DNA [55]. A recent study has shown that induction of IL1β production by MTB is mediated by TLR2/TLR6 and NOD2 receptors, while caspase-1 is constitutively activated in human primary monocytes [43]. This is in contrast with the study of Master et al. that suggested that MTB inhibits inflammasome activation and IL1β production [56]. However, these studies are not completely comparable as the latter study has used murine macrophage cell-lines, in contrast to the human primary cells used by the former study. In addition, if MTB would inhibit IL1β production even in normal hosts, that could not explain the increased susceptibility to infection of IL1R-deficient mice [22].

NOD2 is an intracellular receptor mediating stimulation of proinflammatory cytokine production by MTB. NOD2 is a receptor for bacterial peptidoglycans [57] and recently we demonstrated its role in the recognition of mycobacteria [58,59]. NOD2 deficient mice showed impaired production of proinflammatory cytokines and nitric oxide when infected with MTB. However, the susceptibility to MTB infection of NOD2-deficient mice is variable [60,61].

C-type lectins
C-type lectins are a family of PRRs involved in the recognition of polysaccharide structures of pathogens. The mannose receptor (MR, CD206) consists of eight linked carbohydrate recognition domains and one cystein rich domain. MR is highly expressed on alveolar macrophages [62]. Mycobacterial stimulation through MR leads to production of the antiinflammatory cytokines IL4 and IL13, inhibition of IL12 production, and failure to activate oxidative responses [63,64]. Man-LAM and other major components of the MTB cell wall like PIMs are natural mycobacterial ligands for MR. In addition, binding of MTB to MR induces phagocytosis, but phagosome-lysosome fusion is limited [65-67].

Differences at the level of mannosylation between MTB strains may also contribute to recognition by C-type lectins. Torrelles et al. showed that differences in virulence between MTB strains could be related to expression of Man-LAM on the cell wall [4]. Virulent MTB strains with less surface mannosylation do not use MR for phagocytosis, but rely primarily for recognition and phagocytosis on CR3 after opsonisation. These strains are virulent because they display more other cell envelope components (like phenolic glycolipids and triacylglycerols) [68,69]. These cell components regulate
the cytokine response, and demonstrate rapid intracellular growth and marked tissue damage [70,71]. On the contrary, heavily mannosylated MTB strains such as the laboratory strain H37Rv use the MR receptor during invasion of the cell and are associated with a higher survival within the macrophage and an anti-inflammatory cytokine response. It is speculated that this type of recognition might lead to a latent stage of infection [4]. This might not be the case for all mycobacterial species; a mutant *Mycobacterium bovis* strain, which entirely lacked surface mannosae showed a comparable cytokine profile as the non mutant did [72].

**DC-SIGN**

Dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN, CD209) plays an important role in MTB-DC interaction. This receptor is mainly expressed on DCs and serves as both a PRR and an adhesion receptor, due to its functions in DC migration and DC-T-cell interactions [73,74]. The carbohydrate recognition domain of DC-SIGN recognizes Man-LAM and lipomannans and the amount of Man-LAM determines the binding strength [64]. Recently it was shown that α-glucan (a dominant capsular polysaccharide) is also a ligand for DC-SIGN [75]. After engagement of mycobacterial structures, DC-SIGN promotes an anti-inflammatory immune response by maturation of infected DCs and induction of IL10 production [64]. Later, it was shown that DC-SIGN exerts its immunosuppressive effects through induction of acetylation of the NF-kb subunit p65 via Raf-1, but only in the presence of simultaneous TLR stimulation [76].

**Dectin-1**

Dectin-1 is a receptor with an extracellular carbohydrate recognition domain and an intracellular ITAM domain. This receptor is mainly expressed on macrophages, DCs, neutrophils and a subset of T cells. Dectin-1 mainly recognizes β-glucans present in fungal pathogens, but it has been suggested to play an important role in MTB recognition as well. The precise PAMP that leads to the recognition through dectin-1 is not known, although some species of MTB express α-glucan on the cell surface [77] as a ligand for dectin-1. Murine bone-marrow derived macrophages infected with either virulent or avirulent mycobacteria produce TNFα and IL6 in a dectin-1-independent or dectin-1-dependent manner, respectively [78]. A study with DCs isolated from spleens showed that dectin-1 triggers the production of IL12 [79]. Several studies have shown synergistic effects between TLR2 and dectin-1 for the recognition of fungal pathogens [80,81], but this remains to be demonstrated in case of mycobacteria. Finally, a recent report showed that dectin-1, independent of TLR2 recognition, is important for the innate immunity recognition of MTB and for inducing Th1 and Th17 responses [82].

**RECOGNITION OF MYCOBACTERIUM TUBERCULOSIS - HUMAN GENETIC STUDIES**

In order to have a complete picture of the role of PRRs for the host defence to MTB, the results of in vitro and animal studies need to be corroborated with studies in patients. The association of host genetic factors with susceptibility or resistance to TB has been studied extensively with candidate gene approaches and genome-wide association studies. These analyses have revealed several important
candidate genes for susceptibility to TB [83,84]. For the scope of this review this section is limited to PRRs and their signalling pathways only. Table 1 shows an overview of investigated SNPs with or without association with TB.

<table>
<thead>
<tr>
<th>receptor/signalling pathway</th>
<th>gene</th>
<th>amino acid</th>
<th>association</th>
<th>no association</th>
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<tr>
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<td>[101]</td>
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<td>-</td>
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<td>[108,110]</td>
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<tr>
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<td>[108,109,122]</td>
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* I/D insertion/deletion

Toll like receptors
The TLR2 gene is located on chromosome 4q32 and is composed of two non-coding exons and one coding exon [85]. More than 175 SNPs for the human TLR2 have been reported. In a Turkish cohort an association between Arg753Gln and susceptibility to TB [86] was reported, while this was not confirmed in two Asian cohorts due to the absence of this particular polymorphism in these populations [87,88]. Arg753Gln seems to be present only in Caucasian populations, with percentages ranging from 0 to 0.49% in East Asian populations [87,89-91]. In a Tunisian cohort Arg677Trp showed an association with susceptibility to TB [92], but these results were put in doubt by the discovery of a pseudogene on which this SNP seems to be located [93].

The TLR2 genotype 597CC has been correlated with susceptibility to TB, especially with disseminated forms of the infection (miliary and meningitis) caused by a particular MTB genotype family (“the Beijing genotype”), in a cohort of patients from Vietnam [94,95]. A highly polymorphic guanine-thymine repeat, located 100 base pairs upstream of the TLR2 translation start site in intron 2, was correlated
with promoter activity and the expression of TLR2 on CD14+ PBMCs (the shorter the repeat, the weaker the promoter activity and the lower the expression of TLR2) for both tuberculosis and non-tuberculosis mycobacterial lung infections in a Korean cohort [96,97]. However, these data could not be reproduced in a Taiwanese population [98]. Another variation in genotype that seems to influence TLR2 expression is -196 to -174 insertion/deletion, with a recent study displaying an association with TB, while another study showed a possible effect on development of systemic symptoms [98,99]. Many other polymorphisms in human TLR2 are examined for their association with enhanced susceptibility to TB, but this requires further confirmation [99].

Since TLR1 and 6 form heterodimers with TLR2, SNPs in these receptors might influence TLR2 signalling as well. One example is Ile602Ser SNP in TLR1, which leads to aberrant TLR1 cell trafficking, no functional TLR1 on the cell surface, and which might influence the mycobacterial recognition [100]. The 602I variant is over expressed in African-Americans infected with TB [101]. In addition, an association between the TLR6 SNPs Ser249Pro and Thr361Thr and MTB-induced cytokine production has been shown [102].

Immunogenetic studies have reported in two other TLR genes: TLR4 and TLR8. In these genes the genetic variation associated with susceptibility to TB seems to be less pronounced.

TLR4 Asp299Gly SNP showed an association with TB in HIV positive Caucasians and Tanzanians, but not in a Gambian population [103-105]. TLR8 has always been linked with recognition of viral PAMPs, but in an immunogenetic study in Indonesia, the TLR8 gene, which is located on the X chromosome, was the only gene showing an association with TB. This finding was confirmed in a second much larger cohort from Russia, and supported by functional data, as discussed above [40]. Further studies are needed to confirm these findings.

Besides the PRR receptor polymorphisms, SNPs in the TLR signalling pathways may also influence susceptibility to MTB. Khor et al. proposed that the Ser180Leu SNP in the gene coding for TIR domain containing adaptor protein (TIRAP) was associated with a higher susceptibility to TB in a cohort from West-Africa [106], although the frequency of the mutant allele was very rare. However, this association could not be confirmed in a study involving 9000 individuals from Ghana, Russia and Indonesia [107].

Regarding the other PRRs important for MTB recognition, the 871G and 336A variants located in the promoter region of DC-SIGN were associated with protection against tuberculosis in a South-African cohort of patients [108]. This finding was however not confirmed in a Tunisian cohort [109], while a later study even showed an association in opposite direction (a protective effect of 336G) [110]. Furthermore, genetic variation of the neck-region of DC-SIGN (which supports the carbohydrate recognition domain) failed to show an association with tuberculosis susceptibility [109,111].

**CONCLUSIONS AND FUTURE RESEARCH**

Pattern recognition of MTB is a complex process in which a multitude of receptors recognize specific PAMPs of the microorganism. Recognition by specific receptors is followed by different intracellular signalling pathways, in order to integrate and induce an efficient activation of the innate host defence
mechanisms. While activation through TLRs, NLRs and dectin-1 initiates essentially a proinflammatory response, signalling through the C-type lectins DC-SIGN or MR have mainly a modulatory function. The interplay between these pathways lead to finely tuned response of the immune system during the encounter with MTB.

One has to acknowledge that both in vitro and in vivo studies suffer from specific limitations, which may at least partly explain some discrepancies between experimental and immunogenetic studies in TB patients. In vitro studies use various cell types, murine macrophages (bone-marrow derived or alveolar), DCs or PBMCs. This can influence the outcome due to the preferential expression of specific receptors on different cell types. A second limitation is that in most in-vitro studies only a single receptor is examined, isolated from its physiological environment, while the interplay between different pathways is probably one of the most relevant aspects of pathogen recognition. The role of the innate immune receptors involved in MTB recognition has often been studied in transfected cell lines, while animal models deficient of specific receptors show that these receptors can compensate for each other and sometimes display redundant roles [112-114].

In vivo animal studies have the disadvantage that the most commonly used murine models do not represent human TB; granuloma are not formed in these models, which is a crucial step in the latency of this disease. Rabbit and monkey models which are more similar with human TB are rarely used. Even human genetic studies have limitations, in terms that these studies often lack the translation at the level of protein function, while in other situations an important gene is highly conserved and lacks functionally-relevant genetic variants that can be assessed.

While pattern recognition is an important component of the host response to infection with MTB, other factors are relevant as well, including the intrinsic capacity of macrophages to kill MTB, the distribution and function of different T cell subsets, and regenerative and fibrotic tissue responses. These particular aspects were beyond the scope of this review.

Humans and MTB have co-evolved for millennia, and it is likely that a close relationship exist at the genomic level. Indeed, two studies have shown a direct association between the genetic characteristics of patients with tuberculosis and their mycobacterial isolates [95,115]. Both. Polymorphisms in respectively TLR2 and SLC11A1 (NRAMP1) were associated with higher change of be infected with strains belonging to the evolutionary successful M. tuberculosis Beijing genotype. Globally, M. tuberculosis shows strong geographical differences [116,117], and this might be triggered by evolutionary pressure from the innate immune system ('co-evolution'). Besides M. tuberculosis also host immune gene polymorphisms show strong geographical differences. The studies of Caws et. al and van Crevel et. al [95,115] provide support for the hypothesis that evolutionary adaptation of particular M. tuberculosis lineages to certain human populations. For instance, in the case of TLR2 in the study of Caws, a certain M. tuberculosis genotype family might have a higher or lower affinity for TLR2 expressed in individuals with a particular TLR2 genotype, leading to differences in downstream signalling and subsequent events after recognition of M. tuberculosis. Clearly, this concept needs to be investigated in terms of innate immune recognition by examining a number of PRR genes in TB patients in relation to their infective M. tuberculosis genotypes. Comparing host-mycobacterial genotype relationships of more successful M. tuberculosis genotypes like the Beijing family and less successful genotypes will help
increase the understanding of the concept of 'co-evolution', virulence and innate host defence to M. tuberculosis.

Other important new areas of research related to innate immunity have been initiated recently, and their relationship with tuberculosis remains to be answered. One of the important cellular responses associated with antimycobacterial defence has been suggested to be the process of autophagy. Autophagy has been also shown to modulate the inflammation [118], especially through its interaction with the peptidoglycan receptor NOD2 [119,120]. One important question to be answered is whether there is a role for autophagy in the induction of an inflammatory response by MTB? What is the explanation for the apparent redundancy in the pattern recognition, and which PRR is most important in which stage of the disease? Answers to these questions are needed in order to develop rational immunotherapeutic interventions like addition of TLR-agonists to candidate vaccines.

More can also be learned from studies in human patients. For instance: patients with advanced HIV-infection have virtually no T cell immunity. However, even in settings which are hyperendemic for TB, some HIV-infected patients will never develop TB. Certainly, these individuals must have a very effective innate host response against MTB. A pivotal approach will be to combine genetic with functional studies; what does a SNP associated with susceptibility to TB mean in terms of the function of the immune response?

Another suggestion is to study an increased number of SNPs in more PRRs in the same population, and to assess the cumulative effects of various combinations of SNPs to obtain a stronger association with disease. A striking observation is that only loss-of-function mutations are investigated. Could it be that gain-of-function mutations of PRRs might influence the immune response to MTB as well?

Finally, one of the most important challenges for the coming years is to translate the knowledge gained in the basic science of immune responses to mycobacteria into improved or novel immune-based treatment strategies, ranging from a better vaccine to immunotherapy.
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BCG INDUCES NOD2-DEPENDENT NON-SPECIFIC PROTECTION TO REINFECTION VIA EPIGENETIC REPROGRAMMING OF MONOCYTES

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ABSTRACT

Adaptive features of innate immunity, recently described as 'trained immunity', have been documented in plants, invertebrate animals and mice, but not yet in humans. Here we show that Bacillus Calmette-Guérin (BCG) vaccination in healthy volunteers leads to phenotypic changes in circulating monocytes, that persist for at least three months after vaccination. Monocytes of BCG-vaccinated individuals display increased activation markers, and produce higher amounts of proinflammatory cytokines in response to unrelated bacterial and fungal pathogens. These training effects are induced through NOD2 and are mediated by increased histone methylation. In experimental studies, BCG vaccination induced T and B lymphocyte-independent protection of SCID mice from disseminated candidiasis. In conclusion, BCG induces trained immunity and non-specific protection to infections through epigenetic reprogramming of innate immune cells.
INTRODUCTION

The general perception in immunology is that innate immunity, as opposed to adaptive immunity, is static and does not adapt to an enhanced functional state. There is, however, an increasing body of scientific literature indicating enhanced non-specific protection against infections after previous exposure to certain microbial components in plants (1), invertebrates (2, 3), as well as mice (4). In our recent review of the relevant literature we have proposed the term ‘trained immunity’ for this effect (5). Prominent microbial components that are able to induce this enhanced effector function are mycobacterial components such as Bacillus Calmette-Guérin (BCG), complete Freund’s adjuvant and muramyl dipeptide (6-9).

BCG, the live attenuated vaccine against tuberculosis (TB) is one of the world’s most widely used vaccine (10). It is usually given to newborns protecting them especially against severe forms of TB (e.g. TB meningitis, disseminated TB) (11). Soon after its introduction in the 1930’s, epidemiological studies surprisingly demonstrated that BCG also protects against childhood mortality independent of its effect on TB (12-15). Recent studies corroborated these findings and suggested a reduction of the burden of infection other than TB (16-20). For example, in a case-control study in Brazil, BCG reduced the risk of death from pneumonia by 50% (17). Little is known about the mechanisms responsible for these protective effects of BCG, although macrophages from BCG-vaccinated mice displayed a higher release of oxygen radicals and intracellular fungal killing (8), suggesting an important role of innate immune mechanisms.

In the present paper, we explored the mechanisms of the enhanced immune function induced by BCG both in vitro and in vivo.

MATERIALS AND METHODS

Subjects

Subjects (age range 20-36 years) who were scheduled to receive a BCG vaccination at the public health service, because of travel or work in TB-endemic countries, were asked to participate in this trial. Twenty healthy individuals were included between August and November 2010. Blood was drawn before the vaccination, two weeks after, and three months after the BCG vaccination. The study was approved by the Arnhem-Nijmegen Ethical Committee.

In vitro cytokine stimulation experiments were performed with PBMCs isolated from buffy coats obtained from healthy volunteers (Sanquin Bloodbank, Nijmegen, the Netherlands).

PBMC stimulation assays

The mononuclear cell fraction was isolated from blood by density centrifugation of blood, diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, PA, USA). Cells were washed twice in saline and resuspended in culture medium (RPMI, Invitrogen, CA, USA) supplemented with gentamicin 10 μg/ml, L-glutamine 10 mM, and pyruvate 10 mM. Cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to 5x10⁶ cells/ml. A total of 5 x 10⁴ mononuclear cells in
a 100μl volume was added to round-bottom 96-wells plates (Greiner) with RPMI or with sonicated MTB H37Rv (1μg/ml end concentration), heat-killed Candida albicans, heat-killed Candida albicans hyphae 1x10^5 microorganisms/ml strain UC820), Staphylococcus aureus (1x10^6 microorganisms/ml), or E. coli lipopolysaccharide (LPS, Sigma-Aldrich, 1ng/ml). After 24 hours or 48 hours, supernatants were stored at -20°C. Cytokine concentrations were assessed in the supernatants using enzyme-linked immunosorbent assay (ELISA).

For ChIP analysis, adherent monocytes from the subjects were obtained in 6 wells plate by incubating 15 x10^6 PBMCs for 1 hour. Then, adherent monocytes were collected before further treatment for chromatin immunoprecipitation.

Buffy coats of blood from anonymous blood donors were used for the in vitro ‘training’ experiments. For the isolation of PBMCs the same procedure was used as described above. Monocytes were used after adherence for one hour in flat-bottom 96-wells plates. For the training of monocytes, RPMI, BCG (1μg/ml, BCG vaccin SSI from the Netherlands vaccine institute, MDP (10 μg/ml), Pam3Cys (10 μg/ml) or LPS (1 ng/ml) were added for 24h incubation at 37°C. Thereafter, supernatant was discarded and fresh RPMI with 10% serum was replaced. After 7 days at 37°C, supernatants were discarded and the cells were stimulated with either E. coli LPS (10ng/ml), sonicated MTB H37Rv (1μg/ml), Staphylococcus aureus (1x10^6 microorganisms/ml) or RPMI as a control for an additional 24h. Subsequently, supernatants were stored at -20°C until ELISA was performed. In some representative experiments, the final cell number after initial training with either control medium, BCG or MDP was assessed using Hoechst 33342 Fluorescent Stain and showed little variation. The normalized cytokine levels based on the number of cells actually present in the well showed the same trend as the non-normalized cytokines levels.

In the “inhibition” experiments, before the priming with BCG, the adherent monocytes were pre-incubated for 1 hour with the inhibitors: Bartonella LPS (1μg/ml), anti-TLR2 antibody and anti-IgG (10 μg/ml, eBioscience), Rip2/p38 inhibitor (1μM, Sigma-RBI), Pargyline (3μM, Sigma-Aldrich), MTA (1mM, Sigma-Aldrich).

Cytokine measurements
Cytokine measurements of TNFα, IL1β and IFNγ were performed in the supernatants using commercial ELISA kits from R&DSystems, MN, USA (TNFα and IL1β) or Sanquin, Amsterdam, The Netherlands (IL6 and IFNγ). In a small proportion of baseline samples in which cytokine concentrations were beyond the detection limit, these outliers were excluded from the analysis.

FACS analysis
Cells were phenotypically analyzed by ten-colour and five-colour flow cytometry using a Coulter Navios and Coulter Cytomics FC 500 respectively (Beckman Coulter, Fullerton, FL) and evaluated using Kaluza 1.1 software (Beckman Coulter). Cells were washed with PBS with 1% bovine serum albumin before being labeled with fluorochrome-conjugated mAbs. After incubation for 30 min at 4°C in the dark, cells were washed twice to remove unbound antibodies and analyzed. For cell surface staining, the following mAbs were used: CD11b (IM25814), CD14-ECD (IM2707U), CD45-PC7 (IM3548) (all from Beckman Coulter Corporation), CD284-PE (TLR 4) (312806), CD206-PE (321106)
and CX3CR1-FITC (341606) (all form Biolegend), CD282-APC ((558319 BD biosciences) and dectin-1-APC ((FAB1859A) R&D systems).

Quantitative reverse-transcription PCR
For qRT-PCR, adherent monocytes were primed with either culture medium or β-glucan and subjected after 7 days to a second stimulation with LPS 10ng/ml, Pam3Cys 10μg/ml, or heat-killed C. albicans 1x10⁵/mL for 4 hours. Samples were treated with TRIzol Reagent (Invitrogen) and total RNA purification was performed. Isolated RNA was reverse-transcribed into complementary DNA using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using the SYBR Green method. The following primers were used in the reaction (5'→3'): TNFa forward: CAGGCCAGGCTTCGTCAGA and TNFa reverse: GCTTTCAGTCATACCTGTCTGA, IL1β forward: GCTTTCAGTCATACCTGTCTGA and IL1β reverse: GCTTTCAGTCATACCTGTCTGA, β2-microglobulin forward ATGAGTATGCCTGCCGTGTG and β2-microglobulin reverse CCAAATGCGGCATCTTCAAAC (Biolegio). Each sample was analyzed following a relative quantification model with efficiency correction, and β2-microglobulin was used as housekeeping gene. The mRNA expression level of non-primed and non-stimulated sample was used as reference. The results are presented as the ratios of mRNA production by β-glucan in primed vs. non-primed monocytes.

Chromatin immunoprecipitation
For Chromatin immunoprecipitation (ChIP), adherent monocytes were cultured as described above (cf. “Stimulation experiments”). ChIP was performed using antibodies against H3K4me3 (Diagenode). ChIPed DNA was further processed for qPCR analysis. The following primers were used in the reaction (5'→3'): TNFa forward: CAGGCCAGGCTTCGTCAGA and TNFa reverse: GCTTTCAGTCATACCTGTCTGA, IL6 forward: TCGTGCATGACTTCAGCTTT and IL6 reverse: GCGCTAAGAAGCAGAACCCAC, TLR4 forward: GTCCCTGCTCTGCTACCTTG and TLR4 reverse: TTGAAAGGAGCAGGGTGACT, myoglobin forward: AGCATGGTGCCACTGTGTG and myoglobin reverse: GGCTTAATCTCTGCCTCATGAT.

Mouse experiments
PrkdcScid mice (abbreviated SCID) were obtained from Charles River Wiga (Sulzfeld, Germany). Female mice between 6 and 8 weeks of age were used. The mice were fed with sterilized laboratory chow (Hope Farms, Woerden, The Netherlands) and water ad libitum. Mice were housed in a pathogen-free facility. The experiments were approved by the Ethics Committee on Animal Experiments of the Radboud University, Nijmegen. Mice were first injected with BCG vaccine SSI (750μg/mouse) in a 100μL volume of sterile pyrogen-free phosphate-buffered saline (PBS) or with PBS alone. 14 days later, mice were infected intravenously with a lethal dose of C. albicans blastoconidia (1x10⁴ CFU/mouse). Survival was then monitored and kidney fungal burden was assessed 3 and 14 days after the C. albicans injection. To assess cytokine production, splenocytes from BCG or vehicle- vaccinated mice were retrieved 7 days after the intravenous infection with C. albicans and stimulated in vitro with LPS (10 ng/ml). The measurement of cytokines concentrations was performed using a specific radioimmunoassay, as described previously (21) in supernatants collected after 48 hours of incubation at 37°C in 5% CO₂ in a 48-well plate.
Statistical analysis
Differences were analyzed using the Wilcoxon signed rank test or Friedman test for paired samples. P<0.05 was considered statistically significant. Otherwise stated, data are shown as cumulative results of level obtained in all volunteers (means ± SEM).

RESULTS

Monocyte phenotype is modified upon BCG vaccination.
In the first series of experiments, blood was collected from twenty naïve (non-exposed) volunteers, before and after (2 weeks and 3 months) vaccination with BCG (Figure 1A).

As expected, two weeks after BCG vaccination, IFNγ production induced by MTB was seven-fold higher than the production before vaccination, and this effect remained present after at least three months (Figure 1B). Surprisingly however, IFNγ production was also significantly increased when cells were exposed to unrelated pathogens such as yeasts (7.7-fold increase, Figure 1B) or hyphae (5.2-fold increase, Figure S1A) of C. albicans, and S. aureus (3.5-fold induction, Figure 1B). Exposure of PBMC to LPS induced production of IFNγ in very low amounts that did not differ before and after BCG vaccination (Figure S1B).

Interestingly, in the same series of experiments, the production of the proinflammatory cytokines TNFα and IL1β was also found to be enhanced when cells isolated from the volunteers after BCG vaccination were exposed to mycobacterial and non-mycobacterial stimuli (Figure 1C,D). Of note, the increased production of cytokines remained present 3 months after the BCG vaccination (Figure 1B-D).

These data demonstrate that BCG alters the functional state of circulating mononuclear cells. To further explore this, we investigated the expression of known cell surface activation markers of monocytes. We observed a slight increase in the population of CD14+ monocytes after BCG vaccination (Figure 2A), which all express the differentiation markers CX3CR1 and CD11b (Figure S2C, D, E). Interestingly, the proportion of CD14+ monocytes expressing TLR4 changed significantly after vaccination (Figure S2B). In addition, the expression of CD14 (Figure S2A), TLR4 (Figure 2B-D) and CD11b (Figure 2E, F) was enhanced on monocytes isolated 2 weeks and 3 months after BCG vaccination, compared with their expression before vaccination. The mean fluorescence intensity of the CX3CR1 marker was not changed (Figure S2F). No changes could be observed in the proportion or expression of pattern recognition receptors such as dectin-1, TLR2 or mannose receptor (Figure S2G–K). These observations show that BCG vaccination can induce long-term changes in the phenotype of circulating monocytes, in line with the higher proinflammatory cytokine production after BCG. To examine the level at which the enhanced monocyte function is exerted, we assessed whether the increased cytokine production was due to enhanced transcription. Indeed, mRNA expression for tnfα and il1β was increased after BCG vaccination (Figure 2G).
Increased H3K4 trimethylation in monocytes after BCG vaccination.

As there is accumulating evidence that histone modifications (both acetylation and methylation) are crucial for long-term transcriptional regulation during inflammation (22-24), the involvement of epigenetic mechanisms was assessed. Increased trimethylation of histone H3 at lysine 4 (H3K4), which
has been previously associated with an increased transcription of proinflammatory cytokine genes (23), could represent the mechanism responsible for the long-term modulation of monocyte-derived cytokines. In line with this hypothesis, H3K4 trimethylation was found significantly increased at the level of cytokine and TLR4 promoters in the circulating cells collected three months after vaccination with BCG, compared to the values before BCG vaccination (Figure 2H).

BCG-induced protection is T and B cell-independent. To establish whether BCG-induced trained immunity is indeed mediated by innate immune cells (e.g., monocytes) and independent of T and B lymphocytes, we injected either BCG or saline in two groups of severe combined immunodeficiency (SCID) mice that lack both T and B cells. Two weeks later, both groups of mice were injected with a lethal inoculum of *C. albicans*. The BCG-vaccinated mice had a significantly better survival than the saline-injected animals (Figure 3A), and this was accompanied by a decreased fungal burden in the kidneys, the target organ of disseminated candidiasis in mice.
These data strongly support the notion that BCG can induce non-specific protection against non-mycobacterial infections through functional reprogramming of innate immune cells such as monocytes. In order to demonstrate that the “trained immunity” function of monocytes can also be found after BCG vaccination of SCID mice in vivo, we have performed an additional experiment in vaccinated mice. As previously, SCID mice lacking functional T and B cells and vaccinated with BCG (or with control PBS) were infected with C. albicans, and one week later spleen monocytes were re-stimulated in vitro with LPS, a classical inducer of innate immunity responses that is not related to mycobacteria. Innate proinflammatory cytokines production induced by LPS was significantly higher in the BCG-vaccinated mice (Figure 3C).

Training effects of BCG vaccination are NOD2 and Rip2 dependent. The mechanisms responsible for the training by BCG was further explored in vitro using human cells. Freshly isolated human monocytes were pre-incubated with either culture medium or BCG for 24 hours, followed by washing of the cells. After a washout period of 7 days, during which cells were incubated solely with culture medium, various secondary stimuli were added to the cell culture (Figure 4A). During the first week monocytes had a viability > 95% either when trained with culture medium or BCG, but extension of the experiments beyond 10 days could not be done due to death of primary monocytes in longer-term cultures (based on morphology and trypan blue exclusion). Pre-incubation of cells with BCG markedly increased the production of the proinflammatory cytokine TNFα after the secondary stimulus (Figure 4B), reproducing the effects observed during BCG vaccination. Remarkably, BCG increased production of cytokines induced by a secondary non-mycobacterial challenge, such as purified Toll-like receptor (TLR) ligands or whole heat-killed bacteria.

In a following set of experiments we assessed the receptors and signaling pathways responsible for these effects. Blockade of TLR2 or TLR4 receptors by specific inhibitors, or the use of monocytes from a dectin-1 deficient individual, did not abolish the training ability induced by BCG (Figure 4C-E). In contrast, monocytes isolated from patients with a complete NOD2 deficiency due to homozygous

Figure 3: BCG vaccination protects mice against lethal Candida albicans infection through a T/B lymphocyte-independent mechanism. (A) Survival rate of SCID mice to an infection with live C. albicans (2×10⁶ CFU/mouse) injected intravenously. Mice were either vaccinated intravenously with PBS (Control) or BCG, 14 days prior to inoculation of lethal C. albicans dose (n=15 per group, 2 independent experiments). (B) Fungal burden of kidneys from control and BCG vaccinated SCID mice 3 and 14 days after the lethal C. albicans infection (n=5). (C) TNFα production of spleen monocytes after re-stimulation in vitro with LPS from control and BCG vaccinated SCID mice 7 days after the lethal C. albicans infection (n=5). *p<0.05, **p<0.01, ***p<0.005. vs control (PBS) animals.
Figure 4: BCG primes the production of pro-inflammatory cytokines. (A) Diagram showing the course of the in vitro pre-incubation experiment. Cells were pre-exposed to culture medium or BCG vaccine for 24 hours (1st stimulation - training). After the first stimulus was washed, the cells were incubated for 7 days in culture medium supplemented with serum. Afterwards, a second in vitro stimulation (2nd stimulation) of cytokine production with various PRR ligands was performed for an additional 24 hours. (B) BCG training in vitro using freshly isolated adherent monocytes (C, D) Inhibition of TLR4 or TLR2 does not affect the training effects induced by BCG. (E, F) BCG training of monocytes is severely affected in cells obtained from NOD2-deficient volunteers (F) but not from Dectin1-deficient volunteer (E). (G) The training effects induced by BCG could be reproduced with MDP but not with Pam3Cys or LPS. (H, I) TNFα production in BCG-primed monocytes in the absence or presence of Rip2/p38 inhibitor (I), the histone demethylase inhibitor pargyline, or of the histone methyltransferases inhibitor MTA (H). (C–I) The ratios of cytokine production in BCG-primed vs. non-primed monocytes are presented. Data presented in (E) are mean of two independent experiments (n=1+1). Data presented in (F) were each obtained from 12 healthy volunteers (control) and two different NOD2-deficient individuals (NOD2−/−). (B–D, G–I) *p<0.05, **p<0.01, ***p<0.005. Data are presented as mean ± SD (n ≥ 10). Wilcoxon signed rank test was used to detect significant differences.
presence of the 1022insC frameshift mutation were not able to mount an increased cytokine response upon training with BCG (Figure 4F). This is a strong argument that NOD2, but not TLRs or the C-type lectin receptor dectin-1, is required for the training of monocytes by BCG. This conclusion is further supported by experiments showing that only the NOD2 specific ligand muramyl dipeptide (MDP), but not the TLR4 ligand LPS or the TLR2 ligand Pam3Cys, was able to mimic the effects of BCG pre-incubation on cytokines production by monocytes (Figure 4G). In line with this effect of MDP, an inhibitor of the Rip2 kinase also impaired the BCG induced training (Figure 4H). These data are further supported by a recent study demonstrating priming effects of NOD2 engagement for stimulation of human macrophages with mycobacteria (25).

Finally, as H3K4me3 was increased after BCG vaccination in vivo, we investigated whether blocking histone methylation may reverse monocyte training in vitro. Indeed, inhibition of histone methylation by the methyltransferase inhibitor MTA (5′-deoxy-5′-methylthio-adenosine) almost completely reversed the training induced by BCG (Figure 4I). In contrast, the histone demethylase inhibitor pargyline did not influence the training effects induced by BCG.

DISCUSSION

The non-specific adaptive features of innate immunity (26) that have been demonstrated in plants, invertebrates and mice and that we termed “trained immunity” (5) have not been demonstrated until now to be active in vivo in humans, and the molecular substrate of “trained immunity” has not yet been identified. In the present study, we demonstrate that monocytes can be functionally reprogrammed, or “trained”, to exhibit an enhanced and lasting phenotype after vaccination with BCG. These data point to the mononuclear phagocyte as the cell that mediates the non-specific protection against reinfection after BCG vaccination. Our data also imply that vaccination with BCG induces two types of immune response: on the one hand it induces classical specific immune response involving antigen-specific T cells and memory leading to protection against TB; while on the other hand BCG induces adaptive trained immunity based on functional reprogramming of mononuclear phagocytes that induces protective effects not only against tuberculosis, but also against other infections. In a combination of in vivo and in vitro experiments, we demonstrate that a NOD2-mediated epigenetic change at the level of histone methylation (H3K4me3) is the mechanism through which BCG enhances innate immune responses.

The mechanisms activated by BCG vaccination are almost certainly multiple and complex: classical adaptive immune memory, epigenetic reprogramming (“training”) of innate immunity as described here, but possibly also secondary non-specific effect of adaptive immunity on innate immune responses (e.g. increased cytokines, as shown in the study of Strutt et al. (27)). Conceptually, it is important to discern between the classical adaptive immunity mediated by T and B lymphocytes and the trained immunity mediated by innate immune cells. In this respect, trained immunity reflects an increase in non-specific antimicrobial capacity through epigenetic reprogramming, close to the concept of adaptive characteristics of innate immunity, as described by Mantovani (26, 28). Moreover, whereas the adaptive immune cells mediated responses take some time to become robust, innate (trained) immunity becomes apparent much sooner. This was also recently demonstrated in epidemiological
studies in which the beneficial effects of BCG in newborn children are apparent already within days of the vaccination (29). The rapidity of the non-specific adaptive characteristics of innate immunity underlines that heterologous adaptive responses cannot fully explain trained immunity.

The impact of the training effects on innate immune responses becomes apparent in our experiments in which SCID mice are vaccinated with BCG: these mice were protected from mortality, and the kidney burdens were reduced to 20-40% of the levels in the unvaccinated mice after a secondary lethal infection with C. albicans. Old studies with BCG in experimental schistosomiasis demonstrated similar protection that was at least partially T cell-independent (30). It is an obvious thing to link these experimental results to the non-specific protection against non-mycobacterial disease conferred by BCG (8,9,12-20). In the present study, we provide for the first time mechanistic insights into the processes mediating the adaptive features of innate immunity, or \textit{trained immunity}, in humans. Both the modified methylation status of cytokine promoters after BCG vaccination in human monocytes, as well as the blockade of the in-vitro training effects with methyltransferase inhibitors, suggest that the innate immune response in humans can be reprogrammed epigenetically. Similar mechanisms have been demonstrated before in plants, during the so-called “systemic acquired resistance” (1), and in invertebrates (31), however, both classes of organisms are devoid of adaptive immune responses. The present study teaches us that also in the presence of adaptive immunity – as is the case in mammals - training of innate immunity is operational and serves to enhance resistance to infection.

One important point for discussion is also whether the training effects of BCG vaccination are exclusively due to epigenetic reprogramming of innate immune cells, or whether long-term BCG infection may also play a role. Little is known on how long BCG stays viable in the human body. Fortunately however, a very recent study by Minissian et al. has reported that four weeks after vaccination, only 50% of individuals still display viable BCG at the site of vaccination (32). Although we cannot fully exclude a low persistence of BCG in a minority of the volunteers, it is to be expected that in the majority of them, all the microorganisms have been cleared after three months. Moreover, in our \textit{in vitro} experimental setting of trained immunity, monocytes were trained with live BCG as well as with inert muramyl dipeptide, highlighting that live BCG persistence is not mandatory for the training.

It should be stressed that trained immunity in mammals is not solely induced by BCG. Indeed, murine CMV infection can induce protection to reinfection in a T/B cell-independent and NK cell-dependent fashion (4, 33); and systemic infection with \textit{C. albicans} induced T/B cell independent non-specific protection in mice (34). The role of epigenetic reprogramming was not investigated in these studies.

In conclusion, in this study we provide for the first time firm evidence that innate immunity in humans has adaptive features, and it has the capacity to display an enhanced response upon reinfection. This process of trained immunity is likely to represent a paradigm shift in immunity, as it demonstrates the existence of (non-specific) immunological memory in the absence of adaptive immune responses. Better insights into the relative role of adaptive immune memory and epigenetic reprogramming of innate immunity (\textit{trained immunity}) may have important consequences for vaccine design, more specifically with regard to selection of antigens and the development of adjuvants. In this respect, answers remain to be provided regarding the strength and duration of epigenetically-induced trained immunity. In this
context, it is important to note that BCG and the NOD2-ligand MDP have long been known to induce non-specific protective effects against infections (7, 15) and neoplasms (35), providing the hope that trained immunity can indeed be harnessed for preventive and therapeutic purposes.
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SUPPLEMENTARY MATERIAL

Figure S1: Non-specific production of INFg after BCG vaccination. The production of INFg was assessed by ELISA in the supernatants collected after in vitro stimulation with heat-killed C. albicans pseudohyphae (A) or LPS (B). *p<0.05.
Figure S2: BCG alters the phenotype of circulating monocytes. (A) Flow cytometry analysis of CD11b within CD14 in cells isolated from 1 volunteer before and after BCG vaccination. (B, C, D) Histograms show the expression level of the specific monocyte differentiation marker CD11b (B), CX3CR1 (C) and TLR4 (D) within the CD14+ monocytes population isolated from 20 volunteers before and after BCG vaccination. (E, F) Histograms show the average surface expression level of CD14+ (E), CX3CR1 (F) and dectin-1 (G) on the cells isolated from 20 volunteers before and after BCG vaccination. (H, I) Histograms show the expression level of the pattern recognition receptors TLR2 (H) and mannose receptor (I) within the CD14+ monocytes population isolated from 20 volunteers before and after BCG vaccination. (J, K) Histograms show the average surface expression level of TLR2 (J) and mannose receptor (K) on the cells isolated from 20 volunteers before and after BCG vaccination. *p<0.05, **p<0.01, ***p<0.005.
LONG-LASTING EFFECTS OF BCG VACCINATION ON BOTH HETEROLOGOUS TH1/TH17 RESPONSES AND INNATE TRAINED IMMUNITY

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ABSTRACT

We have recently shown that BCG vaccination in healthy volunteers induces epigenetic reprogramming of monocytes, leading to increased cytokine production in response to non-related pathogens for up to three months after vaccination. This phenomenon was named ‘trained immunity’. In the present study we assessed whether BCG was able to induce long-lasting effects on both trained immunity and heterologous T-helper 1 (Th1) and Th17 immune responses one year after vaccination. The production of TNFα and IL1β to mycobacteria or unrelated pathogens was higher after two weeks and three months post-vaccination, but these effects were less pronounced one year after vaccination. However, monocytes recovered one year after vaccination had an increased expression of pattern recognition receptors such as CD14, TLR4, and mannose receptor, and this correlated with an increase in pro-inflammatory cytokine production after stimulation with the TLR4 ligand lipopolysaccharide. The heterologous production of Th1 (IFNγamma) and Th17 (interleukin (IL)17 and IL22) immune responses to non-mycobacterial stimulation remained strongly elevated even one year after BCG vaccination. In conclusion, BCG induces sustained changes in the immune system associated with non-specific response to infections both at the level of innate trained immunity, as well as at the level of heterologous Th1/Th17 responses.
INTRODUCTION

Non-specific beneficial effects of BCG have been described for many decades. Soon after its introduction in the 1920s, epidemiological studies surprisingly demonstrated that BCG protects against infant mortality independent of its effect on tuberculosis (TB) (1-5). Recent studies supported these findings and suggested a reduction of the burden of infections other than TB (6-10). For example, BCG decreased by half the neonatal mortality of children in several recent trials in West-Africa (8, 9, 11-14), while in a case-control study in Brazil, BCG reduced the risk of death from pneumonia by 50% in children less than one year of age (7). In light of these data, the question arises which mechanisms mediate these non-specific immune effects of BCG, and how long do they last.

Two possible immunological mechanisms have been proposed to explain non-specific beneficial effects of vaccination. The first such mechanisms is heterologous immunity, in which cross-protection is mediated by heterologous T cell memory responses (15). However, heterologous immunity needs at least a couple of weeks to develop, and it is thus highly unlikely to be responsible for the very rapid effects observed on perinatal immunity (14). A second mechanism of protection has been recently proposed in the form of epigenetic reprogramming of immune cells, a phenomenon conferring non-specific immune memory to innate immune responses and termed ‘trained immunity’ (16). Many species of organisms lacking adaptive immunity, such as plants (17) or insects (18, 19), manifest robust immune memory after previous exposure to infections or certain microbial components, and adaptive features of innate immunity have also been demonstrated in mice devoid of functional adaptive immune responses (20, 21). Prominent microbial components that are able to potentiate the effector function of innate immune cells and protect against infections are mycobacterial stimuli such as Bacillus Calmette-Guérin (BCG), complete Freund’s adjuvant and muramyl dipeptide (22-25). Recently, we have shown that BCG vaccination in healthy volunteers led to NOD2-dependent epigenetic reprogramming of monocytes, which resulted in an increased expression of cellular receptors on the monocytes, accompanied by higher cytokine production in response to non-related pathogens (26). This newly described mechanism might represent the explanation for the rapid non-specific protective effects induced by BCG vaccination. However, it is not known how long trained immunity and/or heterologous immunity persist after BCG vaccination.

Therefore, in the present study we investigated the long-term non-specific effects of BCG vaccination in healthy volunteers, by assessing the responses of both innate and adaptive immune cells to non-mycobacterial stimulation in a period extending up till one year after the vaccination.
MATERIALS AND METHODS

Subjects
Individuals (age range 20-36 years) scheduled to receive a BCG vaccination at the public health service because of travel or work in TB-endemic countries were asked to participate in this trial. Twenty healthy individuals were included between August and November 2010. Blood was drawn before the vaccination, and two weeks, three months, and one year after the BCG vaccination. Two persons were lost to follow-up after three months. The study was approved by the Arnhem-Nijmegen Ethical Committee.

PBMC stimulation assays
For the PBMC stimulation assays the mononuclear cell fraction was isolated by density centrifugation from blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, PA, USA). Cells were washed twice in saline and resuspended in culture medium (RPMI, Invitrogen, CA, USA) supplemented with gentamicin 10 μg/ml, L-glutamine 10 mM, and pyruvate 10 mM. Cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to 5x10⁶ cells/ml. A total of 5x10⁵ mononuclear cells in a 100μl volume was added to round-bottom 96-wells plates (Greiner) with RPMI or with sonicated Mycobacterium tuberculosis (MTB) H37Rv (1μg/ml end protein concentration), heat-killed Candida albicans (1x10⁶ microorganisms/ml, strain UC820), Staphylococcus aureus (1x10⁶ microorganisms/ml, clinical isolate), or E. coli lipopolysaccharide (LPS, Sigma-Aldrich, Ing/ml). After 24 hours, 48 hours, or 7 days supernatants were stored at -20°C. Cytokine concentrations were assessed in the supernatants using enzyme-linked immunosorbent assay (ELISA).

Cytokine measurements
Circulating IFNγ, IL17 and IL22 was measured in plasma with Sanquin Pelikine ELISA kits (IFNγ) or R&D Quantikine ELISA kits (IL17 and IL22), respectively following the description of the manufacturer.

Cytokine measurements of TNFα, IL1β, IFNγ, IL17, and IL22 after PBMC stimulation were performed in the supernatants using commercial ELISA kits from R&D Systems, MN, USA (TNFα, IL1β, IL17 and IL22) or Sanquin, Amsterdam, The Netherlands (IFNγ). In a small proportion of baseline samples in which cytokine concentrations were beyond the detection limit, these outliers were excluded from the analysis.

FACS analysis
Cells were phenotypically analyzed by ten-colour and five-colour flow cytometry using a Coulter Navios and Coulter Cytomics FC 500 respectively (Beckman Coulter, Fullerton, FL) and evaluated using Kaluza 1.1 software (Beckman Coulter). In order to guaranty reliable result the flow cytometry was calibrated with flow set pro beads (Beckman Coulter). Cells were washed with PBS with 1% bovine serum albumin before being labeled with fluorochrome-conjugated mAbs. After incubation for 30 min at 4°C in the dark, cells were washed twice to remove unbound antibodies and analyzed. For cell surface staining, the following mAbs were used: CD3-PECy7 (737657), CD4-PB (A82789),
CD8-APC-A700 (A66332), CD45-PO (A96416), CD11b-PE (IM25814), CD14-ECD (IM2707U), CD45-PC7 (IM3548) (all from Beckman Coulter Corporation), CD284-PE (TLR 4) (312806) and CD206-PE (321106) (both form Biolegend), CD16-FITC (335035) and CD282-APC (TLR2) (558319) (both from BD biosciences) and dectin-1-APC ((FAB1859A) R&D Systems).

Statistical analysis
Differences were analyzed using the Wilcoxon signed rank test or Friedman test for paired samples. P<0.05 was considered statistically significant. Unless otherwise stated, data are shown as cumulative results of data obtained in all volunteers (means + SEM).

RESULTS
The effect of BCG vaccination on heterologous Th1 and Th17 responses
FACS analyses of T-cell subpopulations did not show major shifts in CD4 and CD8 lymphocytes (Supplementary Figure 1). Circulating concentrations of the T cell derived cytokines IFNγ, IL17 and IL22 were below detection limit at all time points (data not shown). In contrast, two weeks and three months after BCG vaccination, IFNγ production induced by MTB was seven-fold higher than the production before vaccination, as also reported previously (26). Interestingly, this effect remained present for at least one year (Figure 1A). A similar increase in cytokine production after two weeks and three months was observed when cells were stimulated with unrelated pathogens (Candida albicans; Figure 1B or Staphylococcus aureus; Figure 1C). The increased production persisted until one year after vaccination in case of S. aureus, while it largely returned to pre-vaccination levels after C. albicans stimulation.

In addition to its effects on Th1 responses, we sought to investigate the effect of BCG vaccination on the production of Th17-derived cytokines, namely IL17 and IL22. MTB-stimulated IL17 production was significantly higher in cells retrieved after BCG vaccination (Figure 2A). Moreover, this effect was independent of the stimulating pathogen, as a persistently increased IL17 production was also observed upon stimulation with C. albicans (Figure 2B) and S. aureus (Figure 2C). The increased heterologous Th17 immunity was also reflected by the potentiated IL22 production after BCG vaccination. This was apparent after stimulation of cells with MTB, C. albicans or S. aureus (Figure 2D-F).

The effect of BCG vaccination on trained immunity
Previously, we reported that BCG affected production of proinflammatory cytokines produced by monocytes, a prototypic innate immune cell population (26). While TNFα and IL1β production was higher two weeks and three months after vaccination, it largely returned to normal one year after BCG vaccination. This waning effect on TNFα and IL1β release was observed upon stimulation of the cells with MTB, as well as with the non-related pathogen C. albicans (Figure 3A, B) or S. aureus (not shown). Similar data were obtained on IL1β production (data not shown). However, LPS-induced TNFα (Figure 3C) and IL1β (Figure 3D) production one year after vaccination remained significantly higher compared to pre-vaccination levels.
The effect of BCG vaccination on receptor expression on monocytes

The expression of several pattern recognition receptors and activation markers on monocytes showed a sustained rise in expression over time. Presented in Figure 4 are mean fluorescent indexes (MFI) of either markers of activity of monocytes (CD14; Figure 4A and CD11b; Figure 4B), Toll-like receptor 4 (TLR4) and TLR2 (Figure 4C and 4D), and C-type lectins (CLRs) (Mannose receptor (MR); Figure 4E, and dectin-1; Figure 4F). The increase in MFI was receptor specific, as not all TLRs or CLRs have

Figure 1. BCG vaccination increased the heterologous Th1 responses. (A–C) PBMCs isolated from eighteen volunteers before and after (2 weeks, 3 months, and 1 year) vaccination were stimulated in vitro with sonicated M. tuberculosis (A), heat-killed C. albicans yeast, (B), and S. aureus (C). IFNγ production was assessed in the supernatants by ELISA. *p<0.05, **p<0.01.
Figure 2. BCG induces long lasting heterologous Th17 responses. (A–F) PBMCs isolated from eighteen volunteers before and after (2 weeks, 3 months, and 1 year) vaccination were stimulated in vitro with sonicated M. tuberculosis (A,D), heat-killed C. albicans yeast, (B,E), and S. aureus (C,F). IL17 (A–C) and IL22 (D–F) production was assessed by ELISA in the supernatants. *p<0.05, **p<0.01, ***p<0.005.
been influenced by the vaccination. The activity markers CD14 and CD11b showed persistent higher levels of expression on monocytes that lasted for at least one year. Also TLR4 and MR expression on monocytes were increased after BCG vaccination one year after vaccination. No effect was seen on the expression of TLR2 or dectin-1 (Figure 4D and F).

Supplementary Figure 2 shows the gating strategy for monocytes used in Figure 4. In addition, supplementary Figure 3 presents representative histograms of all receptors shown in Figure 4.

DISCUSSION

BCG has beneficial effects on infections other than tuberculosis, yet the immunological mechanisms responsible for these effects remain obscure. Recently, two immunological mechanisms have been suggested to mediate these effects: the short-time effects are most likely mediated by epigenetic reprogramming of innate immune cells, a process called ‘trained immunity’ (26), while long-term effects may be mediated by heterologous Th1/Th17 immunity (15), possibly in combination with residual trained immunity effects. In the present study we show that BCG vaccination induces non-
specific potentiation effects of both innate trained immunity and heterologous T-helper responses that are clearly present for at least one year after vaccination.

The long-term effects after BCG vaccination are most prominent on heterologous Th1 and Th17 responses. An explanation for this could be the process of ‘cross-protection’ described five decades ago (27), or termed later as ‘heterologous immunity’ (15, 28). The classical form of cross-protection is mediated by lymphocytes that release IFNγ after stimulation with a first-encountered pathogen and subsequently activate bystander macrophages, generating a state of temporary heightened innate immunity against a secondary infection, which wanes rapidly once the primary pathogen is eliminated (29). The second form is long-lived and therefore maybe more comparable with our findings: Berg et
al. described that both effector and memory CD8+ cells have the potential to secrete IFNγ in response to IL12 and IL18 in the absence of a related antigen (30). The IL12 and IL18 are produced in the initial stages of a secondary infection. One additional possible explanation can be epitope sharing as described previously although this is unlikely to play a major role for explaining the increased responses to *C. albicans* or *S. aureus* after BCG vaccination.

One could speculate that the increased reactivity of Th17 cells occurs in a similar fashion. The general principle of the induction of Th17 responses is similar to that of Th1 immunity, with the difference that IL1β and IL23 (rather than IL12 and IL18) are the cytokines responsible for Th17 differentiation. The increased expression of pattern recognition receptors known to induce Th17 responses such as MR (31), and proinflammatory cytokines such as IL1β(32), may at least partly account for the induction of heterologous Th17 immunity. The increase in these typical innate immune responses during the induction of trained immunity could thus contribute to heterologous T-helper responses, but this direct relationship remains to be formally demonstrated by future studies. In addition, one may also speculate regarding potential deleterious effects of BCG-induced heterologous immunity during autoimmune processes. An important difference between BCG vaccination and autoimmune disorders is that while autoimmunity induces spontaneous IL17 production with autoimmune tissue damage, BCG vaccination only induces a primed status of the cells to respond stronger to secondary microbial stimulation. Without stimulation, no higher production of these cytokines was seen. In line with this, one study even reported a beneficial effect of BCG vaccination on type 1 diabetes mellitus (33), and beneficial effects on asthma and allergies have also been reported (34).

In addition to the effects on heterologous immunity, BCG vaccination also shows long-term effects on innate trained immunity. This is revealed by the persistent increase in LPS-mediated proinflammatory cytokine production, and expression of pattern recognition receptors (PRRs) and monocyte activation markers. One may hypothesize that these two effects are related, as it is mainly the expression of the LPS receptors TLR4 and CD14 (35) that remained high one year after BCG that are responsible for enhanced cytokine responses after LPS stimulation. In contrast, the expression of other PRRs such as dectin-1 and TLR2 was not affected by BCG vaccination. While alterations in *S. aureus* and *C. albicans*-mediated cytokine production were clearly present 2 weeks and 3 months after vaccination, these effects waned after one year. This suggests that at least for some innate immune pathways, the epigenetic reprogramming responsible for the trained immunity effects (26) are relatively short-lived and present for months, rather than years.

In conclusion, in the present study we characterize the long-term non-specific immunological effects of BCG vaccination. We have previously shown that innate immunity has adaptive features named as trained immunity (16). While a maximum duration of trained immunity effects of three months has been reported to date (26), in the present study we show that the non-specific training effects of BCG vaccination on receptor expression and LPS-induced proinflammatory cytokines is long-standing and present for at least one year. In addition, strongly increased heterologous Th1 and Th17 responses have been observed one year after the vaccination. These double effects of BCG vaccination on both trained immunity and heterologous T cell responses provide a likely explanation for both the short-term and long-term non-specific effects of BCG, which are already known for decades and may have important implications for vaccine development and healthcare policy.
REFERENCES


Figure S1. No differences in T cell population after BCG vaccination. Histograms show the expression level of CD4, CD8 or both receptors as percentage of total cells (A, B, and C, respectively) and within the CD3+ population (D, E and F, respectively) isolated from 18 volunteers before and after BCG vaccination.
Figure S2. Flow cytometry analysis of peripheral blood cells of one volunteer as example of the gating strategy forward scatter against side scatter and CD14 against CD16 positive cells.
Figure S3. BCG alters the phenotype of circulating monocytes in healthy volunteers. Flow cytometry analysis of surface markers within CD14+ cells isolated from 1 volunteer before and 1 year after BCG vaccination, activation markers CD14+ (A) and CD11b (B), Toll like receptors TLR4 (C) and TLR2 (D), and C-type lectin receptors Mannose receptor (MR) (E) and Dectin-1 (F).
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BCG-INDUCED TRAINED IMMUNITY IN NK CELLS: ROLE FOR NON-SPECIFIC PROTECTION TO INFECTION

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ABSTRACT

Adaptive features of innate immunity, also termed ‘trained immunity’, have recently been shown to characterize monocytes of BCG vaccinated healthy volunteers. Trained immunity leads to increased cytokine production in response to non-related pathogens via epigenetic reprogramming of monocytes. Recently, memory-like properties were also observed in NK cells during viral infections, but it is unknown if memory properties of NK cells contribute to trained immunity due to BCG vaccination. BCG vaccination of healthy volunteers increased proinflammatory cytokine production following ex vivo stimulation of NK cells with mycobacteria and other unrelated pathogens up until at least three months after vaccination. In addition, in a murine model of disseminated candidiasis, BCG vaccination led to an increased survival in SCID mice, which was partially dependent on NK cells.

These findings suggest that NK cells may contribute to the non-specific (heterologous) beneficial effects of BCG vaccination.
INTRODUCTION

The traditional paradigm in immunology is that innate immunity - as opposed to adaptive immunity - is static, and does not adapt after encountering an external stimulus to an enhanced functional state. However, we have recently shown that Bacillus Calmette Guerin (BCG) (1) and Candida albicans (2) can induce enhanced non-specific protection to infections though epigenetic programming of monocytes, and we proposed the term ‘trained immunity’ for this effect (3).

Natural killer (NK) cells are an important cellular component of innate immune system. Interestingly, recent studies have shown that mouse and human NK cells exhibit adaptive memory-like properties, as they can be primed for enhanced IFNγ production upon restimulation (4-7). O’Leary et al. were the first to report that mouse NK cells may have recall responses to haptens during a delayed hypersensitivity response, a phenomenon previously attributed to T cells (4). Later it was shown that initial stimulation of murine and human NK cells with interleukin 12 and 18 (IL12 and IL18) leads to increased IFNγ production after restimulation with cytokines or activating receptor ligation up to three weeks afterwards (5, 6). In addition, Sun et al. described murine ‘memory’ NK cells with enhanced reaction upon restimulation, which are present after an initial infection with murine Cytomegalovirus (MCMV) (7).

These studies have shown adaptive characteristics of NK cells, with increased IFNγ production upon re-exposure to cells with the same stimulus. In trained immunity, however, we observe that re-exposure to both the same or unrelated stimuli induce an enhanced secondary response, due to the non-specific nature of epigenetic priming of the cells (1, 2). Moreover, we have recently shown that trained immunity of monocytes induced by BCG vaccination lasts up to one year after the vaccination (8), and it is tempting to regard this effect as a mechanism for the non-specific (heterologous) protective effects of BCG vaccination (9). Considering the memory-like characteristics described for NK cells, in the present study we tested the hypothesis that NK cells also contribute to trained immunity after BCG vaccination in both human volunteers and in experimental murine disseminated candidiasis.

MATERIAL AND METHODS

Subjects
Twenty-nine individuals (age range 20-36 years) scheduled to receive a BCG vaccination at the public health service because of travel or work in TB-endemic countries (starting after the study was finished) were included in the study. Blood was drawn before BCG vaccination, as well as two weeks and three months afterwards. In the first twenty volunteers the cytokine measurements were done on total PBMCs, therefore an extra nine volunteers were recruited to determine the role of NK cells. There are no demographic differences between the groups. The study was approved by the Arnhem-Nijmegen Ethical Committee, and written informed consent was given by all the participants.

Cellular stimulation assays
The mononuclear cell fraction was isolated from blood by density centrifugation of blood, diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, PA, USA). Cells were washed twice in saline
and resuspended in culture medium (RPMI, Invitrogen, CA, USA) supplemented with gentamicin 10 μg/ml, L-glutamine 10 mM, and pyruvate 10 mM. CD14+ (monocytes) and CD56+ (NK cells) subsets were purified from freshly isolated PBMCs using MACS microbeads, according to the instructions of the manufacturer (Miltenyi Biotec). Purity was checked with FACS and was >90%. Cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to 5x10^5 cells/ml. A total of 1 x 10^5 monocytes or NK cells in a 100μl volume was added to round-bottom 96-wells plates (Greiner) with RPMI (with a additions as previously mentioned) or with sonicated *Mycobacterium tuberculosis* H37Rv (1μg/ml final concentration), heat-killed *C. albicans* (1x10^6 microorganisms/ml, strain UC820), *Staphylococcus aureus* (1x10^6 microorganisms/ml), or *Escherichia coli* lipopolysaccharide 1ng/ml (LPS, Sigma-Aldrich, Ing/ml). After 48 h supernatants were stored at -20°C. Cytokine concentrations were assessed in the supernatants using enzyme-linked immunosorbent assay (ELISA).

**Cytokine measurements**

Cytokine measurements of TNFα, IL1β, IL6, IFNβ and IFNγ were performed in the supernatants using commercial ELISA kits from R&D Systems, MN, USA (TNFα and IL1β) PBL assay science, Piscataway, USA (IFNβ) or Sanquin, Amsterdam, The Netherlands (IL6 and IFNγ).

**Flow cytometric analysis**

Cells were phenotypically analyzed using a Navios™ instrument with 10-color PMTs and three solid-state lasers (Beckman Coulter, Fullerton, FL). The list mode data files were further analyzed using Kaluza™ software (Beckman Coulter). In order to guarantee that the optics, laser, fluidics and fluorescence intensity were stable during all measurements calibration was performed using Flow Check Pro Fluospheres (Beckman Coulter) and Cyto-Cal Multifluor + Violet Fluorescence Alignment Beads (Thermo Scientific, Fremont, CA). Cells were washed with PBS with 1% bovine serum albumin before being labelled with fluorochrome-conjugated mAbs. After incubation for 30 min at 4°C in the dark, cells were washed twice to remove unbound antibodies and analyzed. For cell surface staining, the following mAbs were used: CD3-ECD (A07748), CD16-FITC (IM0814U), CD45-Krome Orange (A96416), CD56-APC-Alexa Fluor750 (custom made), CD158a-APC-Alexa Fluor700 (custom made), CD158b-PC7 (A66901), CD158e1/e2-APC (A60795), CD159a-PC5.5 (custom made) (all from Beckman Coulter, Marseille, France) and CD159c-PE (FAB138P; R&D).

**Mouse experiments**

Non-obese diabetic (abbreviated NOD) -Prkdc^scid^ mice (abbreviated SCID) lacking functional B and T cells, and NOD -Prkdc^scid^Il2rg^tm1Wjll^ (abbreviated NOD/SCID/IL2Rg, NSG) mice lacking B, T and NK cell, were obtained from Charles River Wiga (Sulzfeld, Germany). Female mice between 6 and 8 weeks of age were used. The mice were housed in a pathogen-free facility and were fed with sterilized laboratory chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. The experiments were approved by the Ethics Committee for Animal Experiments of the Radboud University, Nijmegen. Mice were first injected with BCG vaccine SSI (750 μg/mouse) in a 100μL volume of sterile pyrogen-free phosphate-buffered saline (PBS) or with PBS alone. Two weeks later, mice were infected intravenously with a lethal dose of *C. albicans* blastoconidia (2•10^6 CFU/mouse). Survival was monitored for 28 days after the *C. albicans* injection.
Statistical analysis
Differences were analyzed using the Wilcoxon signed rank test or Friedman test for paired samples. P<0.05 was considered statistically significant. Otherwise stated, data are shown as cumulative results of level obtained in all volunteers (means ± SEM).

RESULTS

BCG does not change the proportion of NK cell subsets
In a first set of experiments, we examined possible changes in NK cell subsets following BCG vaccination. So far, no specific phenotypic marker has been described for memory or memory-like NK cells. For increased IFNγ production there are some candidate markers that have an established correlation with human NK cell IFNγ production, such as CD94, CD69, CD159a and CD159c (6). We decided to include some of these markers and in addition some novel activity and inhibitory markers of NK cells. NK cells can be divided into CD56dimCD16+ and CD56brightCD16- cell subsets, which differ in their homing properties. Because approximately 90% of peripheral blood NK cells are CD56dimCD16+, we decided to assess the differential expression of activation or inhibitory markers in this most prominent subset. The CD56brightCD16- NK cells dwell mainly in the lymph nodes and tonsils; hence their number in our samples is too low to observe changes in activity and inhibitory cell markers.

Significant changes were noted neither in the number and distribution of NK cell subsets following BCG vaccination (Table 1), nor in the mean fluorescence indexes of these markers (MFI; Table 2).

| Table 1. Percentage of total cells of 20 volunteers before and after BCG vaccination |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                  | Before BCG    | 2 weeks        | 3 months       |                  | p         |
| CD56dimCD16+                    | 2.25 ± 1.56   | 2.24 ± 1.01    | 2.30 ± 1.40    | 0.97            |
| CD56brightCD16                  | 0.07 ± 0.06   | 0.07 ± 0.04    | 0.05 ± 0.04    | 0.09            |
| CD58a+ (KIR2DL1)                | 25.8 ± 6.91   | 24.9 ± 5.86    | 25.0 ± 6.92    | 0.76            |
| CD58b+ (KIR2DL2)                | 32.5 ± 9.76   | 31.1 ± 8.15    | 31.7 ± 10.1    | 0.25            |
| CD58c+ (KIR3DL1)                | 15.7 ± 12.2   | 14.1 ± 10.9    | 15.6 ± 11.1    | 0.76            |
| CD59a+ (NKG2a)                  | 46.1 ± 14.1   | 46.9 ± 13.9    | 45.2 ± 13.3    | 0.07            |
| CD59c+ (NKG2c)                  | 4.40 ± 2.72   | 4.30 ± 2.60    | 4.25 ± 2.57    | 0.83            |
|                                  |               |                |                |                  |           |

BCG vaccination does not increase interferon-gamma production by NK cells
Two weeks and three months after BCG vaccination, isolated monocytes displayed slightly increased IFNγ production in response to unrelated stimuli including *S. aureus* and *C. albicans* (Supplementary Figure 1), in accordance with previous observations (1). In contrast, isolated NK cells did not display an increase in IFNγ production following BCG vaccination (Figure 1). IFNβ concentrations were below detection limits in stimulated NK cells and in monocytes in these experiments (data not shown).
Table 2. Mean fluorescence indexes of 20 volunteers before and after BCG

<table>
<thead>
<tr>
<th>CD56dimCD16+</th>
<th>Before BCG mean</th>
<th>Before BCG sd</th>
<th>2 weeks mean</th>
<th>2 weeks sd</th>
<th>3 months mean</th>
<th>3 months sd</th>
<th>p</th>
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<tr>
<td>CD56highCD16+</td>
<td>30.0</td>
<td>3.55</td>
<td>30.6</td>
<td>3.37</td>
<td>28.5</td>
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<td>0.25</td>
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<tr>
<td>CD158a+ (KIR2DL1)</td>
<td>13.5</td>
<td>3.28</td>
<td>13.4</td>
<td>3.59</td>
<td>12.9</td>
<td>4.08</td>
<td>0.31</td>
</tr>
<tr>
<td>CD158b+ (KIR2DL2)</td>
<td>8.66</td>
<td>1.89</td>
<td>8.52</td>
<td>2.06</td>
<td>7.95</td>
<td>2.58</td>
<td>0.45</td>
</tr>
<tr>
<td>CD158c+ (KIR3DL1)</td>
<td>8.56</td>
<td>5.85</td>
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<tr>
<td>CD159a+ (NKG2a)</td>
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<td>2.58</td>
<td>10.3</td>
<td>2.46</td>
<td>9.73</td>
<td>2.23</td>
<td>0.21</td>
</tr>
<tr>
<td>CD159c+ (NKG2c)</td>
<td>6.56</td>
<td>3.31</td>
<td>5.99</td>
<td>1.22</td>
<td>5.89</td>
<td>1.26</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* MFI of CD16
* MFI of CD56
* MFI on CD56dimCD16+ for the activity markers (CD158a,b,e and CD159a,c)

**Figure 1.** BCG vaccination does not increase interferon-gamma production by NK cells. NK cells isolated from naïve (non-exposed) volunteers, before and after (2 weeks and 3 months) vaccination with BCG were stimulated *in vitro* with sonicated *M. tuberculosis*, heat-killed *S. aureus* and *C. albicans* blastoconidia. INFγ production was assessed by ELISA in the supernatants. Data are presented as mean ± SEM (n = 9).

**Figure 2.** BCG enhances NK cell production of proinflammatory cytokines. BCG induces trained immunity in monocytes resulting in an increased production of proinflammatory cytokines (1). We examined whether BCG induces similar changes in NK cells. Indeed, NK cells isolated 2 weeks and 3 months after BCG vaccination produced more pro-inflammatory cytokines upon stimulation (Figure 2). This was especially true for IL1β, which showed after BCG vaccination a marked increase after stimulation with either MTB, *C. albicans* or *S. aureus* (Figure 2A, D, and G, respectively). Similar changes were noted, although not significantly in all cases, for IL6 and TNFα (Figure 2B, E, and H and Figure 2C, F, and I, respectively).
BCG-induced protection against disseminated *C. albicans* infection is partially NK cell dependent

We have previously demonstrated that BCG vaccination protects SCID mice, which lack functional T and B cells, against systemic lethal candidiasis (1). We examined the role of NK cells for this BCG-induced protective effect, by comparing SCID mice with NOD/SCID /IL2Rg (NSG) mice that both lack functional T and B cells, the latter lacking functional NK cells as well. In two separate experiments, we injected i.v. NSG and SCID mice with either BCG or saline. Two weeks later, all groups of mice were challenged with a lethal intravenous inoculum of *C. albicans*. Saline-injected SCID and NSG animals were similarly susceptible to the lethal infection, succumbing at the same rate to the candidiasis (Figure 3). As previously demonstrated, all BCG vaccinated SCID mice survived. This protective effect was partially lost in NSG mice, suggesting a role for NK cells in the protection conferred by BCG (Figure 3).
DISCUSSION

In the present study we show that BCG vaccination in healthy volunteers leads to an increased proinflammatory cytokine production by NK cells in response to mycobacteria, as well as to unrelated bacterial and fungal pathogens. This effect, most profoundly seen with IL1β, lasts for at least three months, in contrast to classical non-specific activation of innate immunity, but is not accompanied by changes in NK cell subset distribution or expression of cell surface markers. These effects of BCG on NK cells are biologically relevant, as we demonstrate a functional role for the trained NK cells in a murine model of lethal systemic candidiasis, in which the protection induced by BCG vaccination in SCID mice seems partially dependent on NK cells. Surprisingly, this improved response does not involve an increase of IFNγ production, which is described in literature as an important memory-like property of NK cells (4-7).

Several recent studies have provided arguments for the existence of adaptive characteristics of NK cells, implying a paradigm shift in our understanding of the function of this innate cell population (4-7, 10). Several pathways have been described that may lead to functional reprogramming of NK cells. Firstly, infection with specific pathogens such as the virus MCMV results in an antigen-specific recall response of memory NK cells after reinfection. This process results in increased IFNγ production and enhanced cytotoxicity (7). Secondly, proinflammatory cytokines such as IL12, IL15 and/or IL18 can induce memory characteristics in NK cells. These NK cells do not possess a distinct surface phenotype, nor does restimulation result in enhanced cytotoxicity (5, 6). Thirdly, hapten or viral antigens can induce liver-restricted memory NK cells with increased cytotoxicity, an effect that is dependent on CXCR6 (4, 11). In the present study we uncover an additional dimension to the adaptive capabilities of NK cells in contrast with memory in the classical sense what is antigen specific: we demonstrate that a vaccination such as BCG induces non-specific priming of NK cells, resulting in an increased inflammatory cytokine response to unrelated stimuli, as well as enhanced resistance against an...
unrelated fungal infection.

The mechanisms that mediate the protective effects induced by NK cells during reinfection vary between the different models. Murine CMV infection induces NK cell expansion and contraction, reminiscent of T cell activation, and the protection depends on increased expression of the Ly49H NK cell receptor, on enhanced degranulation, and on increased production of IFNγ (7). Interestingly, as we show here, BCG-induced activation results in an improved production of proinflammatory cytokines such as IL1β and TNFα, rather than IFNγ. Altogether, these studies suggest that NK cells have the capacity to react differently to various stimuli, and to adapt their memory function depending on the infectious agent they encounter. Whether epigenetic changes are at the basis of the increased expression of Ly49H after MCMV or the increased cytokine production after BCG, similarly to the trained immunity in monocytes, remains to be investigated.

The low IFNγ-production capacity reported in our experiments after stimulation with microbial stimuli is in line with earlier data in literature (12), which showed that NK cell production of IFNγ only occurs after co-stimulation of a viral or mycobacterial stimulus and a stimulatory cytokine (IL2, IL12 or IL15 and IL18) (13-15). Another explanation for the lack of IFNγ production in our experiments could be the fact that IFNγ is mainly produced by CD56brightCD16- NK cells, which dwell in the lymph nodes however, have a low frequency in peripheral blood. Different from IFNγ, we did see increased ex-vivo production of IL1β, TNFα, and IL6, both after stimulation with mycobacteria and unrelated pathogens, up until at least three months after BCG vaccination. Despite the relatively small number of individuals who participated in the study, and the known biological variability in cytokine production, a large number of the parameters describing the proinflammatory cytokine production capacity differed significantly before and after BCG vaccination.

Considering the memory-like features of NK cells described in the literature and in this study, one could reasonably hypothesize that the differences in protection induced by BCG between SCID and NSG mice are due to the NK cells. However, defects of differentiation and function of antigen presenting cells (APCs) have been also reported in NSG mice (16), and therefore we cannot exclude that the BCG effects observed here are due to combined effects of NK cells and APCs such as macrophages and/or dendritic cells.

An important observation of the present study is the long-term effect on NK cell function exerted by BCG vaccination. Long-lasting effects have been previously reported in studies showing that pre-activated NK cells that underwent cell divisions had similar degrees of enhanced IFNγ production, indicating heritable memory-like properties (5, 6). Others have also shown that in mice memory-like NK cell responses persist for at least one month in vivo after adoptive transfer (4, 5). Similarly, epigenetic reprogramming leads to enhanced inflammatory properties in monocytes for up to 3 months (1) or even one year (8) after BCG vaccination. One could speculate on the mechanisms inducing these effects. The process of trained immunity in monocytes is mediated by epigenetic regulation (1, 2), and similar processes have been reported to mediate at least some aspects of NK cell maturation and adaptive change (17). Although with the currently available technology it is not yet possible to
investigate epigenetic profiles of circulating NK cells, given the low numbers of circulating NK cells in normal individuals, studies using an improved methodology in the near future are warranted.

In conclusion, we demonstrate that BCG vaccination enhances the cytokine production by human NK cells after re-challenge with an unrelated microbial stimulus, a process earlier termed ‘trained immunity’. This effect is long lasting, and demonstrates important adaptive characteristics of NK cells, a prototype innate immune population. These findings have important consequences on the one hand for our better understanding of innate immunity, and on the other hand, to explain and better exploit the effects of vaccines. Non-specific effects of BCG vaccination are widely known in literature (18) and our results with NK cells may explain some of these non-specific protective effects of BCG. Better insights into the role of trained immunity may thus have important consequences for vaccine design, more specifically with regard to selection of antigens and the development of new adjuvants that boost both specific immunity and the adaptive properties of innate immune cells.
REFERENCES


Supplementary figure 1. BCG vaccination increases interferon-gamma production by monocytes. Monocytes isolated from naïve (non-exposed) volunteers, before and after (2 weeks and 3 months) vaccination with BCG were stimulated in vitro with sonicated *M. tuberculosis*, heat-killed *S. aureus* and *C. albicans* blastoconidia. INF production was assessed by ELISA in the supernatants. Data are presented as mean ± SEM (n = 9).
9

TRAINED IMMUNITY: CONSEQUENCES FOR THE HETEROLOGOUS EFFECTS OF BCG VACCINES

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Mihai G. Netea
ABSTRACT

A growing body of evidence from epidemiologic and immunologic studies have shown that in addition to target disease-specific effects, vaccines have heterologous effects towards unrelated pathogens. Like some other vaccines, BCG has shown in observational studies and randomised clinical trials to increase survival beyond the disease burden of the target disease. The immunologic substrate for these non-specific protective effects have been ascertained to heterologous T cell effects on the one hand, and to priming of innate immunity on the other hand. The term ‘trained immunity’ has been proposed to describe these potentiating effects of vaccines on innate immune responses. This latter process can explain the rapid effects of BCG vaccination and has been suggested to be mediated by epigenetic programming of monocytes or macrophages. This novel concept has important implications for the possible use of vaccines, for vaccination policy, and even for the design of novel immunotherapeutic approaches.
INTRODUCTION

Bacille Calmette-Guerin (BCG), the vaccine that is used for the prevention of tuberculosis (TB) has continuously been in use for more than 90 years. Beside the protection against TB, BCG has non-specific immunological effects that might be valuable for prevention or treatment of other diseases, the best example being the use of intravesical BCG in the treatment of bladder cancer (1). However, many more reports of non-specific effects of BCG have been published, such as treatment of other forms of cancer (2-6), protection against asthma (7), effects on severity of auto-immune diseases like type 1 diabetes (8), or multiple sclerosis (9) and use as treatment for infectious diseases like warts (10, 11). There is an increasing amount of evidence from old and recent studies on these heterologous effects of BCG.

Historical observations

Shortly after the introduction of BCG in the 1920s, reports were published on the ‘non-specific’ effect of this vaccine (12). In a report from Sweden in 1931, BCG improved survival of infants, (12) and this effect exceeded the disease burden of tuberculosis in that age group. It was therefore concluded that the nearly three-fold lower mortality of the BCG-vaccinated children was due to protection against other diseases. Similar observations were made around the world in the following decades (13-16). Although these observations cannot definitively prove a causal relationship between BCG vaccination and the decreased mortality, due to the lack of information needed to exclude biases, they are suggestive for an important unexplored beneficial effect of BCG vaccination.

Besides the beneficial effects on survival in general, BCG was successfully used as treatment against different forms of cancer since the sixties and seventies of the previous century (2-6). These treatments could be either locally (intralesionally in prostate cancer (2) and melanomas (3) intrapleurally in the case of lung cancer (4) or intravesically in bladder cancer (5)) or intradermally, as in patients with Hodgkin disease (6).

BCG is not the only vaccine with potential beneficial non-specific effects. The positive side effects of the widely distributed vaccinia virus vaccine were already noticed by doctors in the nineteenth century (17). There were claims that vaccinated persons were less susceptible to measles, scarlet fever, whooping cough and syphilis. Furthermore, observations of cure of leprosy or whooping cough by smallpox vaccination were reported (18, 19).

After much attention and debate on non-specific effects of BCG and other vaccines following their introduction, this topic became somewhat forgotten. However, a second wave of interest on this phenomenon emerged in the last two decades, when a series of studies analyzed and reported the effects of vaccines in countries with high infectious disease pressure. Again, starting from large observational studies, measles vaccine (another live preparation) was suspected to have capabilities beyond its target disease. Shortly after its introduction in Guinea-Bissau, the group of Aaby et al reported a massive reduction in childhood mortality, also beyond measles mortality (20). This first observation let to a large-scale analysis of non-specific effects of measles vaccine in developing countries, (21) and in addition, to analysis of possible non-specific protective effects of other vaccines such as BCG (22) and vaccinia (23). Several large epidemiological studies have suggested that BCG reduces the
incidence of asthma; a recent meta-analysis of these studies indeed showed an protective effect of BCG on developing childhood asthma (odds ratio 0.86, 95% CI 0.79-0.93) (7). Similarly, recent data suggest that vaccinia and/or BCG may reduce the incidence of melanoma (24), and improve its prognosis (25).

Interestingly, not all the heterologous effects of vaccines appear to be beneficial, deleterious effects for diphtheria-tetanus-pertussis (DTP) vaccine have been reported (26). It seemed that mainly the sequence of the vaccines (DTP being the latter) explained this effect. Some of the factors influencing heterologous effects of vaccines are summarized in Box I.

**Box I. Factors that may influence the heterologous effects of vaccines**
- Timing of the vaccine (newborn, infants, adults, elderly)
- Gender
- Geographic location
- Sequence of vaccinations
- Other immune modulators (high dose vitamin A, iron and zinc)

EVIDENCE FROM RANDOMISED CLINICAL TRIALS
Observational studies are prone to selection bias: healthier children may be vaccinated more often, or children from parents who provide better care are probably also more likely to be vaccinated (‘good parenting’). Therefore, doubts remained about the non-specific/heterologous effects of vaccines (27). Strong arguments for heterologous effects of vaccines were however provided thereafter by randomised clinical trials (RCTs) performed with BCG (28) and measles (29) vaccines. Because these vaccines have established effects on its target disease, denying infants this vaccine would be unethical. In low birth-weight children however, BCG vaccination is mostly postponed, providing an opportunity to perform a randomised trial in these children. The observed survival benefit of BCG was significant at 1 month of age before the control group was vaccinated, with less neonatal sepsis and respiratory infections (28). Similarly, advancing measles vaccination in children from 9 months to 4.5 months resulted in less non-measles hospitalization, again decreasing respiratory infections (29). With regard to the possible beneficial effects of BCG on allergies, one RCT failed to show a significant effect at 18 months, however a trend was observed for lower rates and treatment needs of eczema (30). In addition, the efficacy of BCG installation on bladder cancer was proven by multiple RCTs (31, 32) and a pooled analysis of controlled trials from 1966-1992 suggested a limited benefit of intralesional BCG in melanoma (33). Additional RCTs are currently under way in high-income countries such as Denmark (ClinicalTrials.gov Identifier: NCT01694108) and Australia (NCT01906853) to examine the benefit of BCG in settings with a lower infectious disease burden, and to address additional questions like those related to possible reduction of allergies. However, in the absence of a biological explanation, many investigators remained sceptical regarding to heterologous effects of vaccines.

HETEROLOGOUS EFFECTS OF VACCINES - ADAPTIVE OR INNATE IMMUNITY?
Shortly after the first observations were made on heterologous effects of vaccines, researchers started exploring biologically plausible mechanisms. In 1928 Louise Pearce was the first to publish the beneficial effects of vaccinia vaccine on syphilis in an experimental setting with rabbits. She speculated that ‘it appears that the influence of a preexisting vaccinal immunity is mainly concerned with the properties of host
reaction and resistance’ (34). No comments were made about the distinction of either innate or adaptive immunity as the dichotomy was not established by then. Nowadays, we consider both to play a role in heterologous effects of vaccines.

An adaptive immune mechanism of non-specific effects could be heterologous immunity (35); vaccines can give rise to T cell cross-reactivity and antibody production that may confer some protection against unrelated pathogens (35). This concept is reminiscent of the phenomenon of ‘original antigenic sin’ first described with influenza. Different strains and variants of influenza virus are commonly cross-reactive as was already recognized decades ago (36). Vaccination with a specific subtype of influenza leads to an antibody response to a previous encountered subtype and this effect can be transferred to naïve animals. Besides cross-protection between somewhat related viral species the same phenomenon was also observed with BCG, a mycobacterial vaccine, and vaccinia virus (37). This protection was CD4 T cell dependent, as was confirmed in adoptive transfer, and these cells were the main IFNγ-producing cells after challenge with vaccinia virus. The effects of heterologous adaptive immunity have been reviewed extensively (35) and its influence on non-specific effects of vaccines is subject of a recent review (38).

Still, adaptive immunity cannot fully explain the heterologous effects of BCG. In fact, experiments performed simultaneously with the original antigenic sin experiments showed that some effects are independent of T and B cells. BCG-vaccinated mice could be protected against a later challenge with Candida albicans and Staphylococcus aureus, even after a strong suppression of T and B cell activity by cyclophosphamide prior to BCG vaccination (39). Similarly, BCG conferred non-specific protection against Schistosoma mansoni in nude mice, which have no mature T cells (40). Finally, in a recent study it was shown that BCG vaccination protects SCID mice, which lack both T and B cells, from a lethal challenge with C. albicans (41). These experiments clearly show that these non-specific effects of vaccines may be independent of adaptive immunity, and that innate immunity plays an important role.

In addition to T and B cells, monocytes or macrophages also seem essential for heterologous effects of BCG. In an experimental model, BCG vaccination protected mice against a subsequent ectromelia virus infection, but this effect was abolished by use of anti-macrophage serum (serum from rabbits injected with murine macrophages) (42). A key role of macrophages was also supported by an older study that examined the effect of BCG vaccination on in vitro challenge with Brucella melitensis. In these experiments, bacterial clearance and cell survival were much higher in vaccinated animals (43). Also in bladder cancer, monocytes play an important role. Depleting these cells, in an in vitro setup, abolished the cytotoxicity of the T cells, while addition of only 1% of monocytes was enough to restore this effect (44).

BCG vaccine-induced alterations of innate immune cell function
Different studies examined how BCG vaccination could alter function of monocytes or macrophages, showing effects on phagocytosis, as well as on reactive oxygen species (ROS) production and intracellular killing. Already in 1959 a correlation was found between resistance against Salmonella enteritidis infection and improved phagocytic functions in BCG vaccinated mice (45). A causal relationship, however likely, was not shown. A few years later it was observed that macrophages
from BCG-vaccinated animals were in a more active state, with rapid movements and packed with lysosomal structures (46). Confirmation of this effect of BCG on macrophages came almost 25 years later, when macrophage activity was assessed by increased production of ROS to an unrelated stimulus, leading to increased intracellular killing of intracellular fungi compared to macrophages of non-vaccinated mice (47). In addition, more recently it was shown that BCG increases expression of pattern recognition receptors and other activation markers on human monocytes (41). In the same study the BCG vaccination induced higher production of proinflammatory cytokines when stimulated with non-related pathogens.

BCG may also have an effect on other innate cells. There is clear support for memory-like characteristics of natural killer (NK) cells, and it is tempting to speculate about their role in heterologous effects of vaccines. Indeed, BCG vaccination appears to have an effect on NK cells in healthy human volunteers. Purified NK cells from these individuals produced higher amounts of pro-inflammatory cytokines to unrelated bacterial and fungal pathogens after vaccination. In addition, an animal model using NSG mice (severely immune compromised mice lacking T-, B-cells, and NK cells) demonstrated a clear role that NK cells play in the BCG-induced protection against *C. albicans* (48). This may be a direct effect, as it is known that NK cells can directly recognize BCG without involvement of antigen-presenting cells (49). Importantly, the well-known effect of BCG on bladder cancer is at least partly dependent on NK cells as NK-deficient mice could not be protected with BCG immunotherapy in contrast to control mice (50). Collectively, these data clearly support a role for NK cells in heterologous immunity of BCG vaccination.

Epigenetic reprogramming explaining ‘trained immunity’

Considering these data on the role of innate immune cells in the heterologous effects of vaccines, an important question remains concerning the molecular mechanism mediating these adaptive characteristics of innate immune cells. Recently, we proposed the term ‘trained immunity’ for a state of improved reaction of innate immune cells to reinfection with the same or a different pathogen (51), and some characteristics of this phenomenon are shown in Box 2. The fact that innate immune cells have capabilities of improving their responses is generally accepted in plant immunology for decades, under the term ‘systemic acquired resistance’ (52, 53) and is much studied in invertebrates (54), both of which lack adaptive immune cells. A process of epigenetic reprogramming through histone modification

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**Box 2. Characteristics of trained immunity**

- Induced after a primary infection or vaccination, and confers protection against a secondary infection through mechanisms independent of T and B cell adaptive responses.
- Increased non-specific resistance of the host to reinfection, and thus provides cross-protection to other infections.
- The cellular mechanisms that mediate trained immunity involve innate immune cells such as macrophages and natural killer (NK) cells, and lead to improved pathogen recognition by pattern recognition receptors (PRRs) and an enhanced protective inflammatory response.
- Molecular mechanisms that induce trained immunity involve epigenetic reprogramming (e.g. histone modifications, miRNA), rather than gene recombination that characterizes adaptive immune memory.
underlies trained immunity, with increased transcription of innate immune genes due to methylation of histones in regions increasing chromatin availability. We have recently shown that epigenetic reprogramming of monocytes is responsible for the heterologous effects of BCG vaccination, as displayed in Figure 1 (41). A known mark of transcriptionally active genes, trimethylation of histone 3 lysine at position 4 (H3K4me3) (55), was increased at the promoter regions of the genes encoding immune receptors and cytokines. NOD2 is essential for this effect, with NOD2-deficient cells lacking the capacity of being ‘trained’ by BCG. Epigenetic reprogramming has also been demonstrated to be the mechanism of the systemic acquired resistance in plants (56). In plants these epigenetic changes can be transferred to their progeny through seeds, with a central role of acetylation of H3K9 in this process (56).

Figure 1. Schematic presentation of ‘trained immunity’ in heterologous effects of Bacille Calmette Guerin. 

a) Naïve monocytes with its pattern recognition receptors (PRRs) and detailed presentation of histones (insert). 

b) Trained monocyte: BCG vaccination (1) signals through NOD 2 and RIP 2 (2) resulting in increased trimethylation of H3K4 (H3K4me3); (insert;3) and, increased expression of PRRs on the cell surface (4). 

c) Trained monocyte; restimulated with pathogen X: Recognition of X through PRRs (5) leads to increased cytokine production (6) by increased accessibility of transcription of certain genes (insert;7).

The effect of BCG on human monocytes last at least 3 months and some protective effects even up to 1 year after vaccination (57). This is puzzling given the short half-live of these cells in the circulation. One explanation might be, in-line with the observations in ‘memory’-NK cells, that a pool of
epigenetically-changed monocytes remain elsewhere in the body with the possibility of being attracted whenever needed: the spleen has been proposed as such a site. Another possibility could be that monocyte precursors (stem cells) could become ‘trained’ while still in the bone marrow, through yet undetermined factors, and would give rise to the longevity of this process. An argument for this process is represented by a recent study demonstrating functional reprogramming of bone marrow myeloid cell precursors by TLR2 ligands (58). In addition, a recent publication showed that monocytes have the most conserved chromatin state during differentiation (59).

CLINICAL AND PUBLIC HEALTH IMPLICATIONS

With a wealth of evidence on heterologous effects of vaccines, it is important to consider possible implications of this phenomenon for public health or clinical practice (summarised in Box 3).

Box 3. Clinical and public health implications of heterologous effects of vaccines

- Changing vaccine police (sequence, timing etc.)
- Including heterologous effects in discussions on new (tuberculosis) vaccines
- Discussion about unintended effects of abrogation of vaccines after eradication of its target disease
- Potential to booster low-efficiency vaccines

Firstly, these findings may have implications for vaccination policy; as it is clear that the outcome of non-specific effects are influenced by many factors (see Box 1), these factors should be involved in planning the vaccination strategy. Sequence of vaccines, its importance mainly shown in the studies on DTP (26), and timing of vaccination (28, 29), could have enormous impact on childhood mortality without changing the vaccines in use. The importance of this subject in potentially changing vaccine policy is supported by the WHO in the strategic advisory group of experts (SAGE) (http://www.who.int/immunization/policy/sage/en/).

Second, data on the heterologous effects of vaccines should be incorporated in evaluating potential vaccine replacements. Soon after introduction of BCG debates were raised not only about the non-specific effects of BCG, but also about the protective effect against TB (12, 60). Many questioned the degree and length of specific immunity against TB and calls for an improved vaccine were made (13). Significant efforts have been made in this field (61, 62) however a valuable addition would be to include the non-specific effects of BCG while evaluating its potential successor.

Another point of consideration regarding the optimal use of the heterologous effects of vaccines is the abrogation of some vaccines when the target disease is eradicated, like in the case of smallpox. Depriving people from this vaccine might have more repercussions than previously considered.

Similarly, heterologous effects of vaccines might help booster the response to other vaccines with a low efficiency like Salmonella typhi vaccine or influenza vaccine (a trial concerning the latter has begun; NCT02114255) as several reports suggest a valuable role for BCG (63-66). BCG may also act as booster
in groups who have a low vaccination response, like HIV–infected individuals, however the fact that these are live vaccines limits its use in this group.

Moreover, the heterologous effects of vaccines through trained immunity might also have implications beyond vaccination. For example, vaccines might help ‘training’ a person’s innate immune system in waning immunity in old age or other immunocompromising states. More research needs to be done to demonstrate whether this may be a valid approach.

Concluding remarks and research challenges
Heterologous effects of vaccines are a long-described phenomenon with recent progress in describing its biological mechanisms. Both heterologous adaptive immunity and trained immunity are likely to be involved as biologically-plausible mechanisms. More research is warranted to assess these mechanisms in vivo and their possible implications. Questions regarding the longevity of this phenomenon were already raised and are of vital importance in determining its clinical implications. Another question waiting to be answered is whether trained immunity can be generalised to all innate immune cell types, with a focus on monocytes/macrophages and NK cells, but also dendritic cells and neutrophils.

The major impact of vaccines is illustrated by their worldwide coverage. To improve vaccine usefulness, both their specific and heterologous effects should be considered.
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Studying host response to mycobacteria can help us to understand this complex disease tuberculosis in all its presentations, and inform our method of treatment. The vital role of an intact immune system is proven in increased susceptibility of different immunocompromised patients to TB. The immune response consists of numerous different components, each playing a role in a combined defence against mycobacteria.

The present thesis has two objectives: firstly, to elucidate the host response to MTB with a focus on the innate immune system, in order to provide insight in TB pathogenesis and crucial immune processes which may eventually translate to an improvement in TB treatment; secondly, to explore the role of BCG on the recently described phenomenon of innate memory or ‘trained immunity’. This paradigm shift in immunology will have numerous implications beyond this thesis subject. When considering its role in this thesis we hope to contribute to the development of immunomodulatory strategies in the management of infectious diseases as well as to provide grounds for improving vaccination strategies.

This chapter addresses the main findings in this thesis, puts them in context with other literature, describes the possible implications of these findings, and finally addresses the future research challenges.

Innate immunity and tuberculosis

In the last few decades huge endeavours have led to revolutionary breakthroughs in innate immunity in general and in TB research specifically. The field has changed enormously with the establishment of the role of TLRs in humans, which led to better understanding of recognition of pathogens and underlined the important role of innate immune cells in host response. The interaction of the innate and adaptive immune system has also been an area of increasing knowledge. That both are indispensable and influence each other has been the subject of many papers. Finally, another ground-breaking discovery has redefined basic principles of immunology: innate memory has become an established phenomenon in the last few years.

In host defence against TB the main focus in the previous decades was the adaptive immune response. However, the role of innate immune system is proven in experiments were T cell memory could protect mice against disseminated TB but not against the preceding pulmonary infection (1, 2). In humans we know that vaccination induced memory does prevent disseminated TB but not pulmonary TB, (3) and the same holds true for naturally acquired memory (4). T cell-independent mechanisms therefore have important implications, also in early clearance wherein MTB is eliminated without development of T cell memory (5). Knowledge of innate host response against MTB has developed apace in the recent years. The indispensable role of cytokines has become clearer in absence of these cytokines. Patients lacking IFNγ, IL12, or its receptors appeared to be very susceptible to mycobacteria, implying a crucial role for these cytokines (6). However, only anecdotal case reports show treatment with IFNγ in patients with disseminated mycobacterial infections unresponsive to other treatments with variable success (7-9). Similar observations were made with the introduction of TNFα blocking agents in the treatment of rheumatoid arthritis. Progression to active TB in these patients was exceeding the background incidence starting as soon as two weeks after treatment, strongly suggesting a role of TNFα in protection against active TB (10).
Autophagy is another main player in the host response whose role has been recently established recently. It is a process for the degradation of cellular components, recently shown to be able to degrade microorganisms as well. Mycobacteria escape the immune system, hiding away in macrophage phagosomes, and can influence the phagolysosomal process in inhibiting their fusion with lysosomes. Activation of autophagy (with for example IFNγ) can overcome this blockage, (II) leading to degradation and subsequently antigen processing for antigen presentation.

These important parts of the innate immune response to mycobacteria were the subject of the first part of this thesis, and the results will be summarized in the following paragraph.

Summary of research findings
In chapter 2 we described the production of IL1β in infection with MTB from beginning till end. We showed in murine knock-out models that TLR2/6 and NOD2 are indispensable in recognition of MTB, leading to transcription of pro-IL1β, this in contrast to other receptors known to be involved in the recognition of mycobacteria (TLR1, TLR4, and TLR9). Intracellularly MyD88, ERK, RIP2, and p38 make up the signalling pathway leading to IL1β transcription with no role for TRIF and JNK. The last step in producing active IL1β is cleavage of pro-IL1β through caspase-1. We showed in this chapter that in contrast to what was previously believed, there is no need for a second signal in order to activate caspase-1 or the inflammasome as monocytes already possess activated caspase-1. Stimulation of these cells with bacterial pathogens hardly increased the amount of active caspase-1. Finally, we confirmed that release of IL1β is dependent on P2X7 and endogenous ATP as the release could be significantly reduced by blocking P2X7, as was shown before (12).

Autophagy and cytokines are closely linked; IFNγ can activate this process, and autophagy in turn can influence cytokine production as well (13). We explored this interaction in Chapter 3. We showed that autophagy has important immunomodulatory effects on the MTB-induced cytokine production. Blocking autophagy inhibited TNFα mRNA production while enhancing IL1β transcription. Induction of autophagy with either starvation medium or IFNγ logically showed the opposite effect. The effect of IFNγ on cytokine production could be eliminated through inhibition of autophagy, showing that the known IFNγ-induced effect is at least partly dependent on autophagy. In addition, we examined the effect of functional polymorphisms in autophagy genes ATG16L1 and IRGM, involved in susceptibility to Crohn’s disease (14), on cytokine production. Due to the fact that there were only a limited number of individuals carrying the functional SNPs, conclusions were difficult to draw. However, a trend in lower production of IFNγ was seen in individuals homozygous for the 300A allele of ATG16L1, suggesting a role in host defence against MTB.

To investigate the possible clinical implications of autophagy on TB susceptibility, we searched for differences in autophagy gene-related SNPs in a cohort of 1000 Indonesian TB-patients and a matched control group. In Chapter 4 we showed that these fourteen genes do not influence the susceptibility to TB in this large population. Furthermore, we studied the effect of these SNPs on MTB-induced cytokine production, which showed no differences in the studied Caucasian cohort. This set of SNPs included two genes previously associated with susceptibility to TB. IRGM, a downstream effector protein of IFNγ, induces autophagy and was shown to influence susceptibility to TB caused by MTB
Euro-American lineage but not for East-African-Indian, Beijing, Delhi, *M. africanum* and *M. bovis* lineages in an African cohort (15). The most common MTB lineage in our Indonesian cohort is the evolutionary successful *M. tuberculosis* Beijing genotype; however, no influence of any of the investigated SNPs on infecting lineage could be found. The other gene that has previously been linked to susceptibility to TB in an African cohort is P2RX7, which codes for the P2X7-receptor that mediates ATP-induced autophagy. No effect was observed in our Indonesian population. This underlines the importance of validating a correlation of SNPs and disease in cohorts of different ethnic background.

In Chapter 5 we gave an overview of the recognition of MTB by the innate immune system. In this review, we describe the importance of the different classes of pattern recognition receptors in the recognition of MTB. Their importance is underlined in the fact that in human epidemiological studies, genetic variation in genes encoding for these receptors influence susceptibility, severity, and outcome of the disease. Pattern recognition of MTB is a complex process in which a multitude of receptors recognize specific PAMPs of the microorganism. Recognition by specific receptors is followed by different intracellular signalling pathways, in order to integrate and induce an efficient activation of the innate host defence mechanisms. While activation through TLRs, NLRs and dectin-1 initiates essentially a proinflammatory response, signalling through the C-type lectins DC-SIGN or MR have a mainly modulatory function. The interplay between these pathways leads to a finely tuned response of the immune system during the encounter with MTB.

**BCG-induced trained immunity**

As mentioned before, old studies described non-specific beneficial effects of BCG, the well-known vaccine against tuberculosis, in both animal models and epidemiological studies (16-19). Mice could be protected against a subsequent infection with another pathogen, and in these studies innate immune cells played a prominent role (16, 17). In large cohort studies of BCG vaccinated children, a survival benefit, larger than the disease burden of TB, suggested non-specific effects as well (18, 19). While heterologous immunity was considered as a possible explanation, it could not explain the animal studies lacking adaptive immune cells and the suggestive protective effect shortly after vaccination (20). We show in this second section of this thesis an additional biological explanation for these effects: trained immunity (21). Besides this possible explanation of BCG survival benefit, we prove another more fundamental scientific point: the existence of innate memory in humans.

**Summary of research findings**

In Chapter 6 we demonstrate the validity of this concept of trained immunity for the first time in mammalian hosts. In human volunteers vaccinated with BCG we showed enhanced responses to nonmycobacterial stimuli, accompanied by phenotypic changes of circulating monocytes, even three months after vaccination. We developed an *in vitro* model for this concept, training monocytes with BCG in order to decipher the molecular mechanisms of this process. With this model we discovered that a process of epigenetic reprogramming through histone modification underlies these changes, resulting in increased chromatin availability. A known mark of transcriptionally active genes, trimethylation of lysine at position 4 of histone 3 (H3K4me3) (22), was increased at the promoter regions of the genes encoding immune receptors and cytokines. In addition, we showed that NOD2 is essential for
this effect as cells of NOD2-deficient patients lack these improved actions. Furthermore, the clinical relevance was shown in an in vivo murine model of BCG-vaccinated SCID mice. After vaccination, these mice, which are devoid of T and B lymphocytes, showed a strong protection against disseminated candidiasis, proving the role of innate immune cells.

As a novel and potentially vital concept, the duration of trained immunity would be of major importance. We therefore assessed this in Chapter 7 using the same model, BCG vaccinated healthy volunteers. The phenotypical changes of monocytes described in Chapter 6 were present until one year after vaccination. However, some other effects of trained immunity wane over time; proinflammatory cytokines produced by monocytes returned to prevaccinated levels, with the exception of TLR4 induced responses. In contrast, the skewed Th1 and Th17 responses were still present after one year even in response to unrelated pathogens.

Another important question regarding trained immunity is whether this concept can be extended to other cell types of the innate immune system. Therefore, the role of trained immunity in NK cells was the subject of Chapter 8. Circulating NK cells from BCG vaccinated healthy volunteers displayed increased production of pro-inflammatory cytokines in response to both mycobacterial and other bacterial and fungal pathogens, similar to what we saw with monocytes. No changes were observed in the surface markers of these NK cells. In addition, we demonstrated a functional role for BCG-trained NK cells as NSG mice, lacking in addition to T and B cells also NK cells, could not be protected against lethal systemic candidiasis as well as SCID mice.

In Chapter 9 we gave a historical perspective on heterologous effects of vaccines and discussed the recent progress in describing their biological mechanisms. The review mainly focussed on BCG, as most literature is on this vaccine, and because it is the world's most-used vaccine.

General discussion

This thesis clearly shows the complexity of the immune response to TB; while certain processes are known to play a role in host defence against MTB, not all can be clinically confirmed. This redundancy in immune processes is a challenge in implications of research findings.

We described the crucial steps in producing IL1β upon stimulation with MTB, which is an important cytokine in murine TB. However, its role in disease susceptibility or severity in humans is not that clearly shown. For example, patients receiving anakinra, an IL1 receptor antagonist, do not show an increased incidence of TB; this is in contrast to patients on TNFα blockers. In addition, the clinical implications of autophagy, a vital part in many immune processes from cytokine production to degradation of the bacteria facilitating antigen presentation, are difficult to confirm. While results described in this thesis underline these biological mechanisms of autophagy in host defence against TB, SNPs in autophagy genes lack an association with TB susceptibility.

The main models used in these papers are in vitro models, animal models (mainly mice), and association studies combining certain genetic profiles and susceptibility to TB. One has to acknowledge that both in vitro and in vivo studies suffer from specific limitations, which may at least partly explain some discrepancies between experimental and immunogenetic studies in TB patients.
In vitro studies use various cell types, murine macrophages (bone-marrow derived or alveolar), DCs, or PBMCs. This can influence the outcome due to the preferential expression of specific receptors on different cell types. A second limitation is that in most in vitro studies only a single receptor is examined, isolated from its physiological environment. In both situations the interplay between different pathways is probably one of the most relevant aspects of the immune response.

Animal models have different limitations concerning disease entities of TB. Murine models cannot present all the aspects present in human TB. Mice are relatively resistant to TB, and are therefore infected with sublethal amounts of bacilli. They can control the infection with a high but stable burden of bacilli until senescence or immunosuppression and subsequently progressive respiratory disease occurs (23). Therefore, one of the characteristics of TB, the formation of granulomas, is not present in murine models. There are animal models that better present the human pathology of TB, namely the guinea pig and rabbit. Latency is not investigated in the guinea pig model, but besides that, the similarities with human TB in route of infection, course of disease, granuloma formation, and vaccination response to BCG is remarkable (24). Another animal model used frequently since the 1930s is the rabbit. Lurie determined the different stages of pulmonary infection in this model, stages also present in humans. (25). Although these animals better represent the human disease activity of TB a disadvantage is the higher costs in comparison with rodents. In addition, as most research is performed in murine models, knowledge of that immune system is greater and commercially accessible tools for this research are widely available.

Association studies in TB are influenced by differences in infecting genotypes as they differ geographically, which could be a result of evolutionary selection in specific ethnic groups. Humans and MTB have co-evolved for millennia, and it is likely that a close relationship exists at the genomic level. Indeed, two studies have shown a direct association between the genetic characteristics of patients with tuberculosis and their infecting mycobacterial strains (26, 27). Both polymorphisms studied in these papers, TLR2 and SLC11A1 (NRAMP1), were associated with a higher chance of being infected with the MTB Beijing strain. Globally, MTB shows strong geographical differences (28, 29), and this might be triggered by evolutionary pressure from the innate immune system (‘co-evolution’) as besides the pathogen, host immune gene polymorphisms also show strong geographical differences.

These observations further complicate research on TB, as there is not one ‘MTB strain’ nor one representative host. Usually these differences are not taken into account when SNPs are studied in host defence against TB. Another limitation of human genetic studies is that the already mentioned focus on one SNP ignores the redundancy in the system composed of a complicated interplay of innate and adaptive immune response. The value of one SNP in host response to TB would be more adequately shown in combination with functional assays on a protein level. Showing these SNPs to be functionally relevant could improve the scientific value of these studies.

Innate memory
Trained immunity is a novel description of the immune system; the traditional dichotomy—where the innate immune cells are, as first line defenders, thought to be incapable of tailoring their response in contrast to the slower, specific, and, more importantly, educational adaptive immune cells—is in need
of revision. Trained immunity is a process of improved reaction of innate immune cells after an initial stimulus (for example BCG) to reinfection with the same or a different pathogen.

Adaptive immune responses also have non-specific effects, termed heterologous immunity. Vaccines can give rise to T cell cross-reactivity and antibody production that may confer some protection against unrelated pathogens (30). A suggested mechanism is that CD8+ cells have the potential to secrete IFNγ in response to IL12 and IL18 in the absence of a related antigen (31). The IL12 and IL18 are produced in the initial stages of a secondary infection. Heterologous immunity was previously observed with BCG and vaccinia virus (32). This protection was CD4 T cell dependent, as was confirmed in adoptive transfer, and these cells were the main IFNγ-producing cells after challenge with vaccinia virus. In our experiments we did not see differences in number or subtypes of T cells, but the function was not assessed in adoptive transfer. No observations have been published on heterologous immunity in Th17 cells; however, one could speculate about a similar mechanism for these responses including IL1β and IL23 (instead of IL12 and IL18) necessary for the Th17 differentiation. This thesis shows that BCG potentiates both immune processes, which can explain non-specific effects of this vaccine. How these two distinct processes work together is not yet clear.

Non-specific effects of vaccines are a long-described phenomenon dating from the introduction of these vaccines. Implications of these effects are potentially enormous because of the worldwide distribution of vaccines and because of the mortality of infectious diseases, particularly in tropical areas. To underline this importance, the WHO formatted a strategic advisory group of experts (SAGE) to advise them in policy making on vaccine strategies.

Trained immunity distinguishes itself from other innate immune memory descriptions like NK cell memory by its non-specific nature. Recently, NK cell memory was described in a landmark study of Sun et al (33) which describes innate memory of NK cells in mice with MCMV infection. The NK memory Ly49H+ cells could adoptively transfer memory and subsequently protection to MCMV in naïve animals. Later publications showed different forms of NK cell induced memory: cytokine-induced (34) and hapten-antigens-induced NK cell memory (35). However, NK memory results in improved response to the same stimulus, while BCG trained immunity generates a generally improved response to any secondary stimulus.

The strength of this research lies in combining well-known data from epidemiological studies with prospective observations in humans, and in describing an in vitro model in which the mechanistic details could be investigated. Combining all these different strategies and coming to one fitting explanation makes the outcome more robust and trustworthy. However, the separate components still have their potential confounding properties.

Regarding the epidemiological studies, investigating the effects of vaccines could be confounded by ‘good parenting’: parents who vaccinated their children might take better care of them as well. In addition, healthier children are vaccinated more often, which might result in selection bias.

Randomised clinical trials with vaccines might be considered unethical because of the established value on the target disease. However, some small randomised clinical trials were performed in children who normally were not vaccinated (low birth weight children), showing similar results (20, 36).
Shortcomings of in vitro systems were previously discussed but necessary in this research to explore the role of different cell subtypes in trained immunity. Even our human in vivo model that might seem to be the ‘gold standard’ in immunology has some disadvantages. The immune cells we want to study are not necessarily restricted to the easily accessible peripheral blood. Highlighting the vital role of immune cell source is reflected in the liver-restricted CXCR6+ NK cells in hapten-induced memory.

Host response to mycobacteria starts with recognition of these pathogens. Many receptors are involved herein, such as TLRs, NLRs, and C-type lectins; however, signalling through these receptors leads to different actions. While TLR1, 4, and 9 are known to be involved in host response to MTB, they do not have a role in initiating IL1β production upon stimulation with this pathogen. In contrast, the role of TRL2 herein is indispensible. More importantly, TRL2 is involved in susceptibility to TB meningitis by the successful Beijing strain, in contrast to Euro-American and Indo-Oceanic strains in Vietnamese patients. Another receptor involved in IL1β production upon stimulation with MTB is NOD2. This receptor also has another important function: it initiates trained immunity by BCG. These different and dual functions make recognition a crucial but complicated aspect of host response to mycobacteria. In the next section I will address some of the most pressing and potential clinically relevant issues regarding immune responses to mycobacteria.

Future perspectives
Host response against MTB is complicated. Many complementary systems coexist and influence each other, intertwined in unknown ways. In addition, some parts of the system are redundant, as we see in studies where SNPs, despite their recognized role in host defence, do not influence susceptibility.

Research starts with questions on clinical dilemmas. Who will get primary tuberculosis in a population with similar exposure? Who develops latent tuberculosis? Who from those latently infected people will eventually develop post-primary tuberculosis?

Genome-wide screenings of people in one of these groups are done in order to identify important players in the immune response that might lead to explanations of why these people belong to one of these groups (37). Basic research in model systems of the human immune system like in vitro systems or animal models investigate these separate components of apparent important players in determining outcome during MTB infection. Huge breakthroughs have been detected this way: a transcriptional signature, the importance of autophagy, IFNγ, and macrophages. However, this knowledge did not lead to improvement of clinical TB. Autophagy seems vital, and therefore a stimulating agent might have effects in treating TB. However, its actions on cytokines are dual: while it stimulates TNFα it blocks IL1β. As in humans the role of TNFα is established quite firmly and the role of IL1β is less clear, future research should lean more towards a potential role of inducing autophagy. One way of stimulating it, through IFNγ, is a sporadically used regime in patients with severe mycobacterial infections, although this has variable results (7-9). Thus, it is not all that clear whether stimulating autophagy through other mechanisms might be beneficial.

A pivotal approach will be to combine genetic studies with functional studies; what does a SNP associated with susceptibility to TB mean in terms of the function of the immune response? This is especially relevant considering the importance of host and mycobacterial genetic background and
their co-evolution. SNPs differently influence susceptibility to TB caused by different mycobacterial strains. Genetic association studies should therefore focus on both the genetic profile of the host and the pathogen. Studies wherein this association is already proven could serve as a starting point for more thorough investigation on the mycobacterial side. What makes this strain so vulnerable to recognition through TRL2, for example? As a result, host-specific (or pathogen-specific) therapy might become an option within reach.

As we better understand the immune response to mycobacteria, we might be able to improve our vaccine as well. Big efforts are invested in finding an improved vaccine. However, trained immunity might have important implications in protection against TB in addition to its recognised effects on other infections. BCG might improve long-lasting innate immune responses to mycobacteria in addition to the adaptive response. Maybe this explains some of the disappointing protective effects of the initially promising MVA85A vaccine (38). It is quite possible that innate immune cells play a vital role in vaccination initiated host defence to TB in addition to the more-studied adaptive response.

Consequently, the focus should be broadened while evaluating new TB vaccine candidates on both the adaptive response and on the effect of trained immunity. In addition, the non-specific beneficial responses should be included in evaluating a vaccine that might replace BCG. Endpoints of these trials should be reduction of all-cause mortality or infection not restricted to solely TB.

Innate memory is an entirely new research area where many questions remain to be answered, from the underlining molecular foundations to the clinical implications in protection against infectious diseases and vaccine strategies. There are two further possible further clinical implications worth investigating. People who are scheduled to become immunocompromised (e.g. from stem cell transplantation or chemotherapy) might benefit from vaccination with BCG in training their innate immune system, something that could be investigated in a small pilot study. Another known effect of BCG is as booster for other vaccinations. This might be an effect of trained immunity as well, as improved innate immune effects might help in initiating the adaptive vaccination response. This is especially important for vaccines with low efficacy, like influenza and typhoid fever.

Future challenges lie in combining all these key findings done in previous decades ‘on the benches’ and transforming them to possible implications ‘at the bedside’, thereby addressing the initial clinical dilemmas. The ‘from bedside to bench’ approach for generating research questions is being used constantly, however, the attention should focus on the more challenging ‘from bench to bedside’ approach in applying this scientific knowledge in clinical implications about susceptibility to tuberculosis, its treatments, and vaccination, to improve health all over the world.
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Tuberculose (TB) is een complexe ziekte met vele verschillende uitingen veroorzaakt door Mycobacterium tuberculosis, (MTB). Het bestuderen van de reactie van het immuunsysteem tegen deze bacterie kan ons helpen de ziekte en de mogelijke behandeling beter te gaan begrijpen. We weten dat het immuunsysteem erg belangrijk is als bescherming tegen MTB omdat mensen bij wie het immuunsysteem minder goed werkt meer kans hebben om TB te ontwikkelen. Ook de immunerespons tegen MTB is erg complex, bestaat uit meerdere componenten met elk een eigen rol die samen de hele reactie van het immuunsysteem tegen MTB bepalen.

Dit proefschrift bestaat uit 2 onderzoeksdelen: in het eerste deel beschrijven we in een aantal hoofdstukken welke componenten van het aangeboren immuunsysteem belangrijk zijn in de respons op MTB. In het tweede deel focussen we op een nieuw ontdekt onderdeel van het aangeboren immuunsysteem wat we ‘trained immunity’ hebben genoemd. Deze nieuwe ontdekking werpt een nieuw licht op een van de belangrijkste fundamenten van het immuunsysteem, het vermogen tot aanpassing van de immunerespons. Ons inzicht van de mogelijkheden van het aangeboren immuunsysteem zijn hierdoor totaal veranderd. Dit heeft implicaties ver buiten dit proefschrift, maar in dit proefschrift bekijken we de rol van trained immunity in het sturen van de immunerespons in infectieziekten en in de mogelijkheid om vaccinatie strategieën te kunnen verbeteren.

Deel I: aangeboren immuunsysteem en tuberculose

De rol van het verworven immuunsysteem in de bescherming tegen MTB is beter bekend, die rol verbeteren is ook het doel van vaccineren. Dat het aangeboren immuunsysteem hierin ook een belangrijke rol speelt wordt de afgelopen jaren steeds duidelijker. Zowel uit dierstudies als uit humane epidemiologie waaruit blijkt dat het hebben van een goede verworven immunerespons (door vaccinatie of door het hebben doorgemaakt van TB) niet beschermt tegen alle vormen van TB.

Zoals gezegd bestaat de aangeboren immunerespons uit meerdere componenten (zie ook figuur 1 van de introductie blz II). De immunerespons begint zodra de (in dit geval) mycobacterie wordt herkend door de cellen van het immuunsysteem. Dit gebeurd door pathogene herkenningsreceptoren (PRRs) die nodig zijn om de cel aan te zetten tot het maken van IL1β, welke signaleringspaden zijn hiervoor nodig en hoe gaat de verwerking van IL1β. Het blijkt dat, hoewel we weten dat meerdere PRRs MTB kunnen herkennen er maar een paar nodig zijn als het gaat om het maken van IL1β, namelijk TLR2, TLR6 en NOD2. In de cellen zijn er signaleringspaden die de signalen van de PRRs doorgaven aan de celkern. We vonden dat er van deze signaleringspaden MyD88, ERK, RIP2 en p38 belangrijk waren om IL1β te maken in tegenstelling tot TRIF en JNK. De productie van IL1β is anders dan van andere cytokines omdat er een extra stap is ingebouwd, er wordt eerst niet actief pro-IL1β gemaakt en voor
die laatste stap is er een actief complex van eiwitten (inflammasome) nodig. In tegenstelling met wat er tot dan toe was beschreven in de literatuur vonden we dat in monocyten het inflammasome al actief is waardoor het produceren van IL1β niet afhankelijk is van het activeren van dit inflammasome.

Een ander belangrijk onderdeel van de aangeboren immuunrespons tegen MTB is autophagy. Autophagy is een mechanisme van de cel om oude celonderdelen af te breken en de bouwstoffen te recyclen. Dat afbreken gaat met bepaalde enzymen en datzelfde proces kan ook gebruikt worden om bacteriën of andere pathogenen af te breken. In hoofdstuk 3 bekeken we de interactie van autophagy en cytokines tijdens de immuunrespons tegen MTB. Als we autophagy blokkeerden werd er minder TNFα geproduceerd en meer IL1β, als we autophagy stimuleerden zagen we het omgekeerde. Een manier om autophagy te stimuleren is met IFNγ een voor MTB erg belangrijk cytokine. Uit de literatuur was al bekend dat IFNγ kan zorgen voor een verhoogde cytokine productie en we denken nu dat dat in ieder geval voor een deel komt door het effect op autophagy. Er zijn genafwijkingen (SNPs) bekend waardoor autophagy minder goed werkt. Als laatste keken we in dit hoofdstuk of sommige van deze SNPs een effect hebben op de hoeveelheid cytokines die er werd geproduceerd. Dit bleek niet zo te zijn, alhoewel dit moeilijk te zeggen was aangezien er in de groep mensen maar weinig waren die de bepaalde genafwijking hadden.

In hoofdstuk 4 kijken we verder naar bepaalde SNPs in autophagy maar nu in patiënten met TB. We zochten in een Indonesische populatie van 1000 TB patiënten en een vergelijkbare controlegroep naar het voorkomen van deze 14 SNPs om zo vast te stellen of het hebben van één van deze SNPs zorgt dat iemand sneller TB krijgt. Dat bleek niet het geval, ook niet als we keken naar een bepaalde soort MTB stam die in Indonesië veel voorkomt. In de literatuur was erder wel een effect van bepaalde SNPs op het krijgen van TB beschreven in populaties met een ander etniciteit. Dit geeft aan hoe complex TB en de immuunrespons tegen MTB is. Er zijn verschillende MTB stammen die zich anders gedragen en waarop de immuunrespons ook kan verschillen. Verder is het immuunsysteem van alle mensen een klein beetje anders geworden gedurende de evolutie en kunnen er daarom tussen mensen met verschillende etniciteit grote verschillen ontstaan in immuunresponsen. In hoofdstuk 5 gaven we een overzicht over alle PRRs en signaleringspaden die betrokken zijn bij de herkenning van MTB. Dat deze onderdelen in de immuunrespons belangrijk zijn blijkt uit de talloze associaties studie naar mensen met gendefecten in deze onderdelen en hun kans op het ontwikkelen van TB. Overigens worden deze associaties niet in alle studies bevestigd zoals hierboven ook reeds beschreven. Ook hebben verschillende PRRs verschillende effecten sommige (TLRs, NLRs en dectin-1) hebben met name een actieve inflammatoire respons terwijl andere (DC-SIGN en MR) de immuunrespons reguleren en in te dammen. Aangezien er meerdere receptoren tegelijk worden geactiveerd als een cel in contact komt met MTB leidt dit tot een complex geïntegreerde immuunrespons.

Deel 2: BCG geïnduceerde trained immunity

BCG is het vaccin tegen TB, het werd al in de jaren 20 ontdekt en in gebruik genomen en is een van de meest gebruikte vaccins wereldwijd. Kort na de introductie viel op dat er niet alleen minder TB voorkwam maar dat kinderen ook minder stierven door andere oorzaken. In dit deel van het proefschrift
beschreven we een mogelijke biologische verklaring voor deze niet-specifieke effecten van BCG.

De immuunrespons bestaat uit 2 onderdelen: het aangeboren en het verworven deel, ze beïnvloeden elkaar maar het blijven aparte onderdelen. Het aangeboren immuunsysteem geeft de eerste respons, reageert snel en er werd gedacht dat de respons ook altijd hetzelfde was. De verworven immuunrespons duurt langer om te vormen maar is specifiek gericht op de microbiële dreiging en deze respons vormt een geheugen om bij dezelfde dreiging de volgende keer sneller te kunnen reageren. Meer en meer artikelen worden gepubliceerd over geheugenfuncties in cellen van het aangeboren immuunsysteem. Het lijkt dus dat de oude fundamenten van de immunologie aan herziening toe zijn. Recent is er vanuit onze researchgroep de term ‘trained immunity’ geopperd om dit fenomeen te beschrijven. Trained immunity beschrijft een reactieverandering in cellen van het aangeboren immuunsysteem wanneer ze na een eerste contact met (bv) BCG anders reageren in een volgend contact met een pathogen. De zogenaamde training of leerbaarheid van deze cellen. Ze reageren dus niet continue hetzelfde maar kunnen hun respons aanpassen op basis van eerdere gebeurtenissen. In het geval met een eerder contact met BCG leidt dit tot een verbeterende respons tegen de volgende microbiële dreiging.

In hoofdstuk 6 beschreven we het concept van trained immunity voor de eerste keer in mensen. In vrijwilligers die een BCG vaccinatie kregen zagen we een verbetering van hun cytokine productie en veranderingen van receptoren (oa PRRs) op hun cellen ten opzichte van voor de vaccinatie. Dit effect hield aan tot wel 3 maanden na de vaccinatie. Om de onderliggende moleculaire mechanismes te ontrafelen ontwikkelden we een in vitro model waarbij we immuuncellen buiten het lichaam ‘trainden’. Dankzij dit model vonden we dat betere respons kwam doordat sommige immuungerelateerde genen beter bereikbaar werden en daardoor hun informatie makkelijker konden ter beschikking kondelen stellen. Verder lieten we zien dat NOD2 (een PRR) noodzakelijk is voor BCG geïnduceerde trained immunity. Als laatste bewezen we de klinische relevantie in een diermodel. Muizen zonder cellen van het verworven immuunsysteem (SCID) kregen een letale hoeveelheid Candida albicans toegediend, één groep kreeg 2 weken ervoor een BCG vaccinatie de andere groep een nep vaccinatie. De BCG gevaccineerde dieren overleefden dit allemaal terwijl de nep gevaccineerde dieren meer dan de helft stierf. Aangezien deze dieren geen functioneel verworven immuunsysteem had bewijst dit dat de effecten van BCG zoals hierboven beschreven toegeschreven kunnen worden aan het aangeboren immuunsysteem.

Aangezien trained immunity een nieuw beschreven fenomeen is, is het belangrijk te weten hoelang dit aan zou kunnen houden. Daarom was dit onderwerp van hoofdstuk 7. Opnieuw keken we in BCG gevaccineerde vrijwilligers naar hun respons op andere pathogenen voor en na de vaccinatie. De veranderingen in de celreceptoren waren nog aanwezig een jaar na vaccinatie echter sommige cytokines werden in dezelfde hoeveelheden geproduceerd als voor de vaccinatie. Dit gold niet voor alle cytokines, de cytokines geproduceerd door T cellen (IFNγ, IL17 en IL22) bleven hoger dan voor de vaccinatie en deze blijvende verhoging bleef ook te zien als TLR4 (een PRR) geactiveerd werd.

Een ander belangrijk aspect van trained immunity is of het geldt voor alle cellen van het aangeboren immuunsysteem. In de vorige hoofdstukken bekeken we de rol van monocyten; in hoofdstuk 8 werd een ander celtype van het aangeboren immuunsysteem onder de loep genomen, de natural killer cel (NK cel). Zoals bij de monocyten zagen we dat de NK cellen van mensen na de BCG vaccinatie meer
cytokines gingen produceren. Verschillen in de celreceptoren konden we niet waarnemen. Verder
herhaalden we het experiment met de muizen zonder actief verworven immuunsysteem waarbij de
voor dit hoofdstuk ook een groep muizen toevoegden die geen NK cellen hadden (NSK). Het effect
van BCG op deze NSK muizen was minder dan het effect op de SCID muizen, wat laat zien dat NK
cellen een rol spelen in deze bescherming door BCG. In hoofdstuk 9 gaven we een historisch overzicht
over niet-specifieke effecten van vaccins en bespraken de recente ontdekkingen in de onderliggende
biologisch mechanismes.

Conclusies en toekomstperspectieven
Immuunrespons tegen MTB is complex en bestaat uit vele complementaire onderdelen die ook elkaar
onderling beïnvloeden. En bij sommige van deze onderdelen is de klinische implicatie niet altijd
duidelijk; met andere woorden, ook al functioneert een onderdeel niet leidt dat niet altijd tot TB.
Gendefecten lijken ook relevanter bij sommige MTB stammen, ook dat zou een startpunt kunnen zijn
van onderzoek, wat maakt een bepaalde stam zo gevoelig voor een bepaalde PRR? MTB bestaat al
40.000 jaar en heeft zich in die tijd kunnen aanpassen aan de zwakke plekken in ons immuunsysteem.
Ook het humane immuunsysteem aan evolutie onderhevig en samen leidt dit tot co-evolutie. De mens
en de bacterie passen zich aan elkaar aan en proberen steeds aan elkaar te ontsnappen. Door meer
kennis hierover zouden we uiteindelijk misschien wel personalised medicine kunnen toepassen bij TB
voor zowel de gastheer als het pathogen.

Als we de immuunrespons beter begrijpen zouden we misschien ook in staat zijn ons vaccin te
verbeteren. Daar wordt al volop onderzoek naar gedaan maar de kandidaatvaccins die getest zijn
leveren helaas geen duidelijke verbetering op. Ondanks dat BCG niet heel goed beschermt tegen TB
weten we nu dat de duidelijke niet-specifieke effecten heeft wat zal bijdragen aan de immuunrespons.
Deze voordelen van BCG zouden meegenomen moeten worden indien er een vervanging van dit vaccin
voor handen is. Het feit dat cellen van het aangeboren immuunsysteem trainbaar zijn in hun respons
is een totaal nieuw veld van onderzoek met vele mogelijke implicaties. Bijvoorbeeld het optimaliseren
van het aangeboren immuunsysteem voor de start van medicatie die het immuunsysteem beïnvloed
(zoals chemotherapie). Ook wordt BCG wel als booster voor andere vaccins gebruikt mogelijk
werkt dit op dezelfde manier, het optimaliseren van het aangeboren immuunsysteem leidt tot beter
totstandkoming van de verworven respons.

Kortom, dit proefschrift heeft bijgedragen aan onze kennis over de immuunrespons tegen
mycobacteriën en aan de belangrijke ontdekking; trainbaarheid van cellen van het aangeboren
immuunsysteem. Door deze nieuwe informatie ontstaan er nieuwe onderzoeksuitdagingen met
belangrijke gezondheidsimplicaties wereldwijd.
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ABOUT THE AUTHOR

Johanneke Kleinnijenhuis was born in Den Ham on March 21th 1979. After completing secondary school (Augustinus College, Groningen and Menso Alting College, Hoogeveen) she studied Biomedical (Health) Science at the Radboud University, Nijmegen from 1997-2001. She obtained research experience during two internships in the Radboudumc at the departments of Pathology and Clinical Pharmacy. In 2001 she started with Medicine and went abroad for two international internships during that study. The first one was in the Hospital Dr. Humberto Alvarado Vasquez, Masaya, Nicaragua. She finished her medical degree in the Sengerema Designated District Hospital, Tanzania in 2006. Later that same year she began her residency Internal Medicine at the Radboudumc (prof. dr. Jos van der Meer, Prof. dr. Jacqueline de Graaf, and Prof. dr. Jan Smit). In 2008 she continued her residency at the Slingeland Hospital in Doetinchem (Dr. A Mudde). During that time she started to discuss the possibility of doing a PhD project with Prof. Dr. Mihai Netea which resulted in a personal grant from the Radboudumc in 2009. She started the project "Host response to mycobacteria; innate recognition and memory" in 2010 and alternated research and clinical work until 2015. During her PhD project she visited Seph Borrow’ group at Oxford university for four months to work on a joined project. From November 2012 till August 2015 she continued her medical training as registrar infectious diseases (Prof. dr. Bj. Kuijper) and worked during that time four months in the Academic Hospital Paramaribo, Suriname.