Aus dem Institut für Mikrobiologie und Infektionsimmunologie
der Charité-Universitätsmedizin Berlin
und dem
Institut für Mikrobiologie und Tierseuchen
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Recognition of *M. tuberculosis* by innate immune receptors – A novel mechanism of TB pathogenesis revealed by genetic analysis of an Indian cohort and in-vitro studies

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
an der Freien Universität Berlin

vorgelegt von
Shruthi Thada
aus Hyderabad, Indien

Berlin 4388
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List of Abbreviations

AFB - acid-fast bacilli

AIM-2 - absent in melanoma 2

APC - Antigen presenting cells

BCG - Bacille Calmette-Guerin

BMDC - bone marrow derived dendritic cells

CD - cluster of differentiation

c-diGAMP - cyclic-di- Guanosine monophosphate - Adenosine monophosphate

CDN - cyclic dinucleotides

CFP - culture filtrate protein

cGAS - Cyclic GMP–AMP synthase

CLR - C-type lectin receptors

CNS - Central Nervous System

co-IP - Co-immunoprecipitation

CREB - cyclic adenosine monophosphate (c-AMP) response element-binding protein

CSP - cytosolic DNA surveillance pathway

CTL - cytolytic T lymphocytes

DAMP - danger associated molecular patterns

DC - Dendritic cell

DC-SIGN - DC-specific intercellular adhesion molecule-3-grabbing non-integrin

DOTS - Directly observed treatment short course

ELISA - enzyme-linked immunosorbent assay

EPTB - extra-pulmonary TB

ER - endoplasmic reticulum

ERK - Extracellular signal-regulated kinase

ESAT-6 - early secreted antigenic target of 6 kDa

ESX - ESAT-6 secretion system 1

ETM - ethambutol

H3K4me3 - Trimethylation of Histone H3 at Lysine 4

HbA1c - glycosylated Haemoglobin type A1c

HC - Healthy control

HEK - Human Embryonic Kidney cells

HHC - Household contact

HIV - human immunodeficiency virus

HPV - human papillomavirus

IFN - Interferons

IFNGR1 - interferon gamma receptor 1

IGRA - interferon-gamma release assay

IKK - IkB kinases

IL - Interleukin

INH - isoniazid

IRAK - IL-1R-associated kinase

IRF - interferon regulatory factor

JNK - c-Jun N-terminal kinase

LAM - Lipoarabinomannan

LC 3 - microtubule-associated-protein-1 light chain 3

LDL - low-density lipoprotein

LM - Lipomannan

LPS - Lipopolysaccharide

LRR - leucine- rich repeat

LTBI - latent TB infection

M.tb - Mycobacterium tuberculosis

MAL - MyD88 adapter like protein

MAMP - Microbe-associated Molecular Pattern

ManLAM - Mannosylated Lipoarabinomannan

MAPK - mitogen-activated protein kinases

MB21D1 - Mab21 domain-containing protein 1

MD2 - Myeloid Differentiation Protein 2

MDA5 - melanoma differentiation-associated protein 5

MDR - multi drug-resistant

MHC - major histocompatibility complex

MyD88 - myeloid differentiation 88

NAA - nucleic acid amplification

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NK - natural killer cells

NLR - NOD-like receptors

NLRC4 - NLR family CARD domain-containing protein 4

NLRP3 - NLR family pyrin domain containing 3

NO - nitric oxide

NOD2 - nucleotide-binding oligomerization domain 2

PAMP - pathogen-associated molecular patterns

PBMC - Peripheral blood mononuclear cells

PDIM - phthiocerol dimycocerosate

PGN - peptidoglycan

PI - Phosphatidyl inositol

PNG - polymorphonuclear granulocytes

PRAT4A - protein associated with TLR4

PRR - pattern recognition receptors

PTB - pulmonary TB

PZA - pyrazinamide

RD1 - region of difference 1

RIF - rifampicin

RIG I - retinoic acid-inducible gene I

RIP1 - receptor-interacting protein 1

RLR - RIG I like receptors

ROS - reactive oxygen species

RR - rifampicin resistant

RSV - Respiratory Syncytial Virus

SARS-CoV-2 - severe acute respiratory syndrome coronavirus 2

SNP - Single nucleotide polymorphism

STING - Stimulator of interferon genes

TAK - TGF-β activating kinase

TANK - TRAF Family Member Associated NFKB Activator

TB - Tuberculosis

TBK1 - TANK-binding kinase 1

Teff - effector T cells

Tfh - T follicular helper cells

TGF-β - transforming growth factor-β

Th - T helper cells

TIR - Toll/IL-1 receptor

TIRAP - TIR domain containing adaptor protein

TLR - Toll-like receptor

TNF - tumour necrosis factor

TRAF 6 - TNF receptor-associated factor 6

TRAM - TRIF-related adaptor molecule

Treg - regulatory T cells

TRIF - TIR domain-containing adaptor inducing interferon-β

TST - tuberculin skin test

Unc93B1 - Protein unc-93 homolog B1

WHO - World Health Organization

XDR - extensively drug-resistant

ZN - Ziehl–Neelsen

1. Introduction

1.1 Epidemiology

Tuberculosis (TB) is caused by Mycobacterium tuberculosis (*M.tb*), an acid fast, facultative intracellular bacterium first discovered by Robert Koch in 1882 (Sakula 1982). According to the 2021 Global Tuberculosis report TB is the second leading cause of death worldwide with an estimated range of 9.9 million new TB cases registered globally (Figure 1). 1.3 million deaths occurred in 2020, which is an increase from 1.2 million in 2019 potentially due to the impact of COVID-19 pandemic (World Health Organization 2021). India alone accounted for 38% of global TB deaths. India with 2.6 million cases in 2020 is one among the eight countries (China, Indonesia, Philippines, Pakistan, Nigeria, Bangladesh, and South Africa) accounting for 26% of the new cases constituting a total of two thirds of the global incident cases. 8% of the new TB cases are human immunodeficiency virus (HIV) positive worldwide. In 2020 about 0.21 million (16%) people died of HIV-associated TB.

About 157,842 people worldwide developed drug-resistant (isoniazid-resistant TB, rifampicin resistant (RR)-TB, multi drug-resistant (MDR-TB), pre-extensively drug-resistant TB (pre-XDR-TB) and extensively drug-resistant (XDR-TB)) TB in 2020. MDR-TB is defined as disease caused by TB bacilli that are resistant to at least isoniazid and rifampicin, the two generally most powerful anti-TB drugs. Pre-XDR-TB is resistant to rifampicin and any fluoroquinolone (a class of second-line anti-TB drug). XDR-TB is TB resistant to rifampicin, any fluoroquinolone, and to at least one of the drugs bedaquiline and linezolid. About 16% of MDR-TB/RR-TB cases had developed Pre-XDR-TB/XDR-TB TB in 2020.

M.tb usually infects the lung (85%) causing pulmonary TB (PTB). Mycobacteria invade many other organs during the primary infection called extra-pulmonary TB (EPTB). The extra-pulmonary involvement can be seen in >50% of patients with HIV coinfection and only in 10 - 20% without coinfection (Lin et al. 2009; Sterling et al. 2010; Guler et al. 2015). The most frequent sites of EPTB include parenchyma, such as the lymph nodes, pleura, abdomen, genitourinary tract, gastrointestinal tract, skin, joints and bones, or meninges. The diagnosis of EPTB is more difficult than that of PTB (Lin et al. 2009).

About one-third of the world's population has latent TB infection (LTBI) (Dye et al. 1999), while 5 - 10% progress to active TB during the first two years of infection due to complex environmental, genetic, and immunological interactions that are currently incompletely defined. The major risk factors for developing active disease include socio-demographic, behavioral, and co-morbidity conditions (Narasimhan et al. 2013). The risk factors for developing recurrent TB are poor medication adherence to anti-TB drugs, drug sensitivity to exogenous reinfection and a patient's immune status. According to WHO, among individuals with LTBI, people living with human immunodeficiency virus (HIV) have a 26-fold higher risk of getting TB than those without HIV.
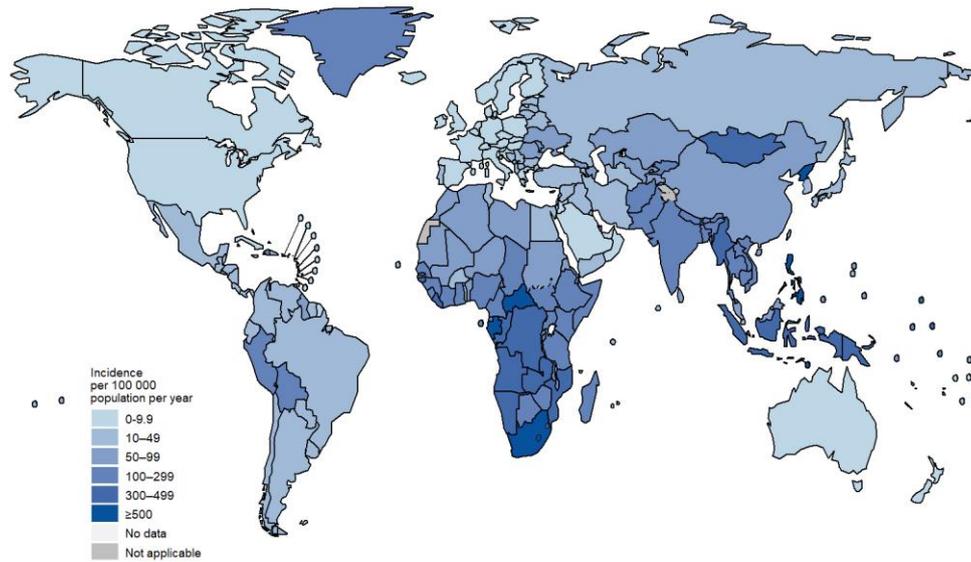
Figure 1. Global Estimated TB incidence rates, 2020 Global Estimated TB incidence rates in 2020 shown in shades of blue (Map and the data was adapted from WHO, Global tuberculosis report 2021)

1.2 Diagnosis

Patients with clinical symptoms such as non-resolving cough, hemoptysis, fevers, night sweats and weight loss should undergo chest radiography. If imaging suggests lesions in the lungs or airways, three sputum specimens should be submitted to identify acid-fast bacilli (AFB) in mycobacterial culture smear for confirmation of TB as the specificity of chest radiography is as low as 23 – 45% although the sensitivity is 90 – 100% (Harris et al. 2019). The primary diagnosis of PTB is by isolation of *M.tb* either from sputum culture, bronchoalveolar lavage, pleural fluid or tissue (pleural biopsy or lung biopsy) in developing countries (Pai et al. 2016). Sputum smear microscopy is performed by Ziehl-Neelsen (ZN) stain technique. The sample is labeled as "smear positive" or "smear negative", depending on the presence or absence of AFB. Specificity and sensitivity of the test ranges between 25.3 - 81.6%, and 83.4 - 99%, respectively (Bhalla et al. 2013; Deng et al. 2021). Culture remains the gold standard to detect TB, it also allows the diagnosis of drug resistance, including molecular analyses of emerging mutations. The sensitivity of the test is higher compared to smear because of the limit of detection is 100 bacilli/ml. However, the growth in a conventional egg-based medium takes anywhere from 4 to 8 weeks with an additional 4 weeks for drug sensitivity-testing by the conventional proportion method. Thus, it takes a median of 70 days to diagnose a case of MDR-TB by conventional culture methods (Shah et al. 2011).

Additional diagnostic tools include nucleic acid amplification (NAA) testing to detect the presence of TB DNA, as well as common mutations associated with RR along the rpoB gene (Xpert test: MTB/RIF or MTB/RIF Ultra endorsed by WHO). The Xpert assays have demonstrated 89% sensitivity and 99% specificity at diagnosing pulmonary TB in adults as well as EPTB compared to culture (Steingart et al. 2014). However, the Xpert diagnosis is a challenge in low-income countries and developing countries due to limited financial resources. In addition, a tuberculin skin test (TST) or interferon-gamma release assay (IGRA) can be performed to detect LTBI. However, sensitivity of these tests is poor (Dheda et al. 2016). Accurate and timely

diagnosis of active TB and identification of high-risk individuals is key to achieve the goal eradication of TB.

1.3   Treatment

TB is a treatable and curable disease. Drug-susceptible TB disease is treated with a standard 6 month course of 4 antimicrobial drugs which comprise an intensive phase with 2 months treatment consisting of rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (ETM) followed by a continuation phase with 4 months treatment of RIF and INH (Nahid et al. 2016). The "Directly observed treatment short course (DOTS)" is widely implemented by TB control programs. Here the antimicrobial drugs are provided with information, supervision, and support to the patient by a health worker or trained volunteer. In patients with positive smear test the test is repeated at 2nd and 5th month of the treatment. A successful completion of the treatment is defined by negative sputum smear during the follow-up.

Drug-resistant TB treatment is more complex, yet also feasible. The treatment course ranging from 9 to 24 months depending on the type of resistance and second line of drugs will be given to the patients which are often toxic (Nahid et al. 2019). Isoniazid therapy is given to LTBI and high-risk individuals ranging from 3 to 12 months (6month is effective) as a preventive treatment (Egsmose et al. 1965; Akolo et al. 2010). Identification of high-risk individuals and new preventive therapies for specific target groups of co-morbid conditions and the drug resistance will help to improve the prophylaxis of TB (Ai et al. 2016).

1.4   BCG vaccination

The "Bacille Calmette Guerin (BCG)" vaccine was developed by Albert Calmette and Camille Guérin from 1908 to 1921 by cultivating a virulent *M. bovis* strain for 230 serial passages on glycerinated bile potato medium until it lost its virulence properties and used as a vaccine for TB (Calmette 1931). Unlike *M.tb*, BCG has no 'region of difference 1' (RD1), which encodes the genes for two small secreted proteins, EsxA and EsxB (Behr et al. 1999). Nevertheless, both BCG and *M.tb* are similarly complex in their structure, stimulating various classes of pattern recognition receptors (PRRs) (Netea and van Crevel 2014). Since 1974 BCG vaccination has been included in the WHO Expanded Program on Immunization with about 350 million doses of procurement annually (Cernuschi et al. 2018). This makes it the most widely used vaccine in humans currently. Meta-analysis of literature shows that in children, BCG is >80% effective against severe forms of TB, including TB-meningitis and miliary TB (Colditz et al. 1995; Trunz et al. 2006). However, the efficacy of BCG in adults against PTB is ranging from only 0 to 80% (Colditz et al. 1994; Brewer 2000).

Several hypotheses have been put up potentially explaining the variable protective immunity of BCG including human and mycobacterial genetics, differences among the vaccine strains used in clinical studies, exposure of trial populations to environmental mycobacteria, coinfections with viruses and/or parasites, geographical location, nutrition, differences in trial methods, and variations among clinical *M.tb* strains (Fine 1995; Behr 2001; Moliva et al. 2017). All these factors mentioned may contribute to the heterogeneity of vaccine efficacy. Understanding the host immunity needed to confer adequate protection and analyzing the roadblocks of sustainable protective immunity following BCG vaccination is key in developing successful vaccines. The following are the vaccines (Table 1) that are developed either to prevent TB or to protect from active disease, which are currently undergoing clinical trials (Hatherill et al. 2019).

Table 1 Vaccine candidates in "pipeline" at different stages of clinical trials

Vaccine name	Type of vaccine	Composition	Function
M72/AS01E	Subunit vaccine	Fusion protein Mtb32A & Mtb39A, AS01E adjuvant	prevent pulmonary TB in adults already infected with *M.tb* (Ottenhoff 2020).
H4:IC31	Protein subunit vaccine	H4 antigen, IC31 adjuvant	Trigger multifunctional CD4+ T cell responses in previously BCG-vaccinated healthy individuals (Norrby et al. 2017)
ID93 + GLA/SE	Subunit vaccine	Fusion Rv1813, Rv2608, Rv3619, Rv3620 with GLA-SE adjuvant	functional humoral and Th1 type cellular response (Coler et al. 2018)
GamTBvac	Subunit vaccine	Ag85A & ESAT6-CFP10 fusion with dextran-binding domain immobilized on dextran mixed with adjuvant DEAE-dextran core, with CpG oligodeoxynucleotides	induce both humoral and cellular immune responses (Tkachuk et al. 2017)
MTBVAC	Live attenuated	Attenuated *M.tb* clinical isolate with ESAT6 & CFP10 & independent stable genetic deletions of phoP & fadD26 genes	induce durable antigen-specific Th1 cytokine-expressing CD4 cell responses in infants (Tameris et al. 2019)
VPM1002	Live recombinant BCG	Recombinant BCG vaccine with listeriolysin O encoding gene	prevent infection in newborn and protect form active disease from recurrence in adults by modifying the T-cell responses (Nieuwenhuizen et al. 2017)
Ad5Ag85A	Recombinant live vaccine	Adenovirus serotype 5 expressing Ag85A	Trigger polyfunctional T cell responses and improve local lung immunity (Smaill and Xing 2014)
TB/FLU-04L	Recombinant live vaccine	Attenuated replication-deficient influenza virus vector expressing	significantly improve the protective efficacy of BCG (Li et al. 2020)
M. vaccae	Attenuated vaccine	Heat-killed M. vaccae	Reduces pathological damage and improve immunity in drug sensitive cases (Li et al. 2020)
DAR-901	Attenuated vaccine	Agar-grown SRL172 by scalable, broth-grown manufacturing technique	effective BCG booster and trigger polyfunctional effector memory CD4+ T cell responses (Masonou et al. 2019)

1.4.1 Cross protection of BCG vaccination - Trained immunity

Innate immune responses following BCG vaccination have adaptive characteristics that are able to contribute to protection to subsequent unrelated infections, this effect recently has been termed "trained immunity" (Netea et al. 2016). Studies have shown that BCG vaccination confers protection against viral infections and non-TB infections. In children BCG vaccination is effective against Type 1 Diabetes by decreasing the HbA1C levels (Allen et al. 1999), multiple sclerosis by decreasing the active lesions in the Central Nervous System (CNS) (Ristori et al. 1999), and in cancer immunotherapy (Morra et al. 2017). BCG vaccination also leads to diminished SARS-CoV-2 incidence and death rates (Curtis et al. 2020; Netea et al. 2020). However, there are contradicting studies, with some of them failing to show evidence of protection of BCG against SARS-CoV-2 (Lindestam Arlehamn et al. 2020).

The potential mechanism of trained immunity is that following BCG vaccination monocytes exhibit epigenetic modification of H3K4me3 (histone tri methylation at lysine 4) associated with the promoters of the pro-inflammatory cytokine genes through the nucleotide-binding oligomerization domain 2 (NOD2) receptor (Kleinnijenhuis et al. 2012). These epigenetic modifications upregulate the expression of pattern recognition receptors (PPRs). Consequently, when these trained monocytes are exposed to a secondary infection, the pathogen is recognized by PPRs, leading to increased cytokine production (Figure 2).
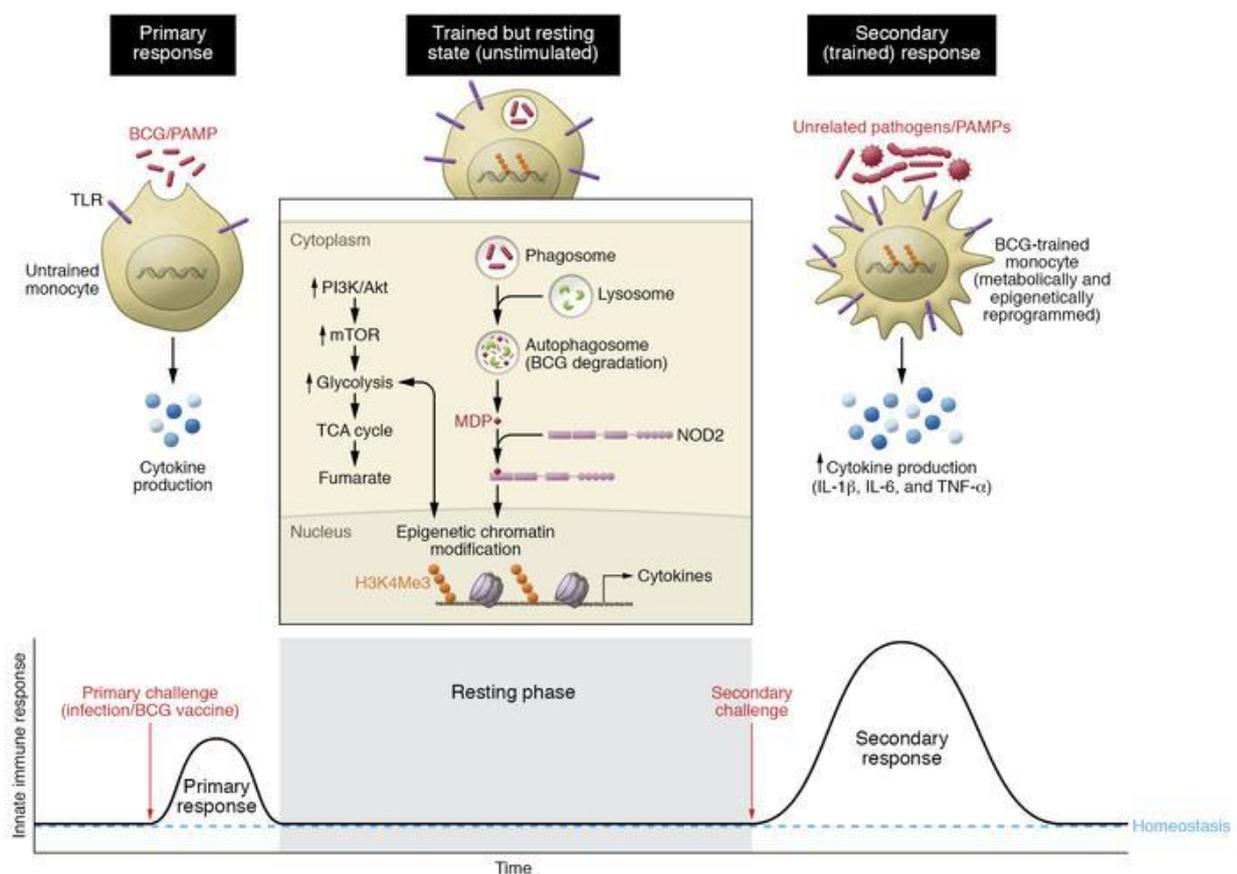
Figure 2. Epigenetic programming of monocytes and trained immunity BCG vaccination activates the innate immune system and induces epigenetic changes (Histone methylation H3K4Me3) in monocytes. This chromatin rearrangement induces a "trained" state in the cell that alter immune readiness to enhance the effectiveness of the innate immune response when exposed to a non-specific pathogen, inducing the secretion of pro-inflammatory cytokines,

such as tumour necrosis factor (TNF), Interleukin (IL)-1β, and IL-6 (Figure is adapted from (Singh et al. 2021)).

1.5   *M.tb* recognition particles

Bacteria are ingested by resident alveolar macrophages through recognition of conserved molecular structures called pathogen-associated molecular patterns (PAMPs) present on/in the bacilli by pattern recognition receptors (PRRs). These bacterial components not only serve as PAMPs that activate the host's immune system but also function as bacterial effectors that modulate the host response for its survival within the host. The major components of the mycobacterial cell wall are depicted in Figure 3, the biosynthesis of these components was reviewed in (Kaur et al. 2009; Mishra et al. 2011). The mycobacterial cell wall consists of lipomannan (LM), lipoarabinomannan (LAM) and its mannosylated form (ManLAM), lipoproteins, phthiocerol dimycocerosate (PDIM), and mycolic acids. The majority of these cell wall components (LM, LAM and ManLam) are formed by the Phosphatidyl inositol (PI) backbone with an addition of mannoses and arabinoses to PI. Sugar residues are present on the PI backbone and these compounds are either embedded in the plasma membrane or on the outer membrane by their lipid moiety (Pitarque et al. 2008). PDIM is a surface exposed bioactive lipid including several polyketide synthases, and a specific transporter, MmpL7 (Cox et al. 1999; Jain and Cox 2005). Mycolic acids are the major component of the mycobacterial cell wall with long chain fatty acids. They ultimately become the "cord factor" when conjugated to a trehalose sugar residue, the major cell surface lipid of *M.tb*. This whole complex lipid coat protects *M.tb* from host defence as well as presents a range of ligands for the PRRs to recognize. Besides these cell wall proteins, there is an embedded secretion system ESAT-6 secretion system 1 (ESX) that facilitates the secretion of early secreted antigenic target of 6 kDa (ESAT-6), which helps in phagosomal rupture (de Jonge et al. 2007) to release a variety of secreted PAMPs along with the nucleic acids to expose them to the cytoplasmic receptors.
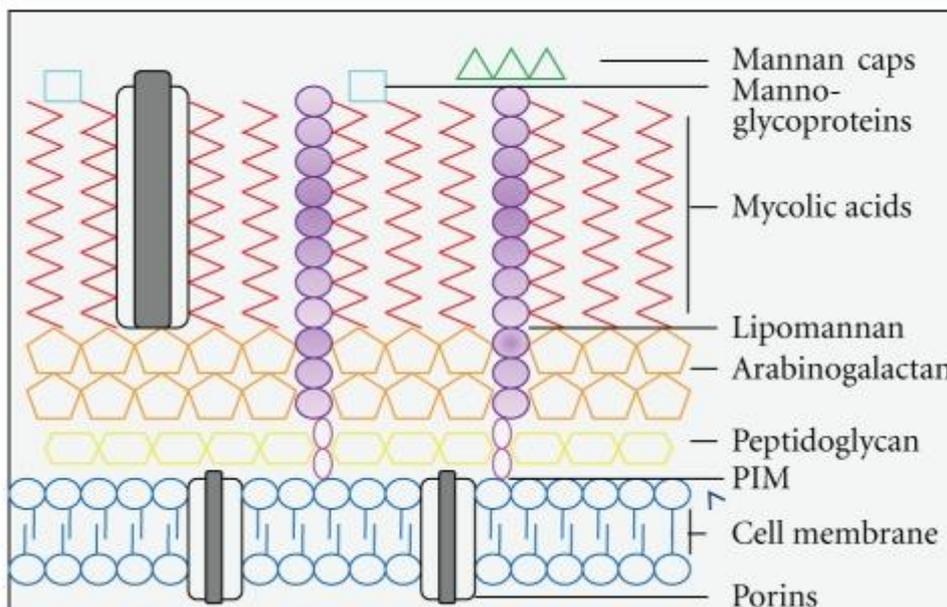

Figure 3. Mycobacterium tuberculosis cell wall structure This figure represents the major components of the mycobacterial cell wall. The outer layer consists of mycolic acids, glycolipids like (mannose-capped) lipomannan, and mannoglycoproteins. The inner layer is

composed of peptidoglycan, which is covalently linked to an arabinogalactan layer (the figure is adapted from (Kleinnijenhuis et al. 2011)).

1.6   Pathogenesis

M.tb is spread via small airborne droplets (droplet nuclei), which can remain airborne for minutes to hours (Frieden et al. 2003). The bacilli are then trapped by the mucus-secreting goblet cells situated in the upper parts of the airways. The cilia on the surface of the cells push the mucus along with the entrapped bacilli upward to remove the infection in most of the individuals exposed to TB (Torrelles and Schlesinger 2017). Inhalation of *M.tb* bacilli activates innate immune responses from pulmonary alveolar macrophages and dendritic cells (DCs) that contribute to host immunity. In the early phase of infection, *M.tb*, internalized by phagocytic immune cells, replicates intracellularly, and the bacteria-loaded immune cells can efficiently cross the alveolar barrier and transmits to various other extra-pulmonary sites (Teitelbaum et al. 1999; Bermudez et al. 2002). The intracellular replication and simultaneous dissemination of the pathogen occur prior to the development of the adaptive immune responses. This shows the unique feature of *M.tb* to establish a protected niche where they can avoid elimination by the immune system and persist forever (Chackerian et al. 2002; Hingley-Wilson et al. 2003). If infection persists, immune cell recruitment ultimately leads to the formation of large clusters of immune cells termed granuloma, which are considered as the hallmark of TB infection. A study by Davis and Ramakrishnan (Davis and Ramakrishnan 2009), shows that the mycobacteria replicate freely in macrophages and direct the recruitment of uninfected macrophages to the site of infection using the ESX-1/RD-1 virulence factors. Initial granuloma stages are mainly characterized by poorly organized structures consisting of macrophages, monocytes, and neutrophils. Macrophages within the granuloma develop into specialized cell types, such as epithelioid macrophages, foamy macrophages, and multi-nucleated giant cells that form after fusion of plasma membranes of multiple macrophages (Puissegur et al. 2007). Dendritic cells uptake and engulf the bacteria and migrate to the lymph nodes and *M.tb* can modulate the migration and delay in development of adaptive immunity which is required to stop bacterial proliferation.

In order to initiate an adaptive immune response, antigen-presenting dendritic cells migrate to the local lung-draining lymph node and drive naïve T-cell differentiation. When antigen-specific T-cells enter the site of infection, the granuloma stratifies and well-organized structures build up. At this stage, infected macrophages in the core of the granuloma are enclosed by uninfected and foamy macrophages which are surrounded by varying fractions of T- and B-lymphocytes (Ulrichs et al. 2004). A fibrous cuff may separate macrophages from lymphocytes, while the center of the granuloma necrotizes and generates the liquefied core termed 'caseum'. In the periphery of necrotic granulomas, secondary lymphoid structures comprising of myeloid, T- and B-cells appear (Ulrichs et al. 2004; Joosten et al. 2016) (Figure 4). In case of active disease, a functional granuloma disintegration occurs, leading to the release of viable, extracellular mycobacteria into the airways resulting in coughing and sneezing of the patient, which ultimately leads to transmission of the disease.
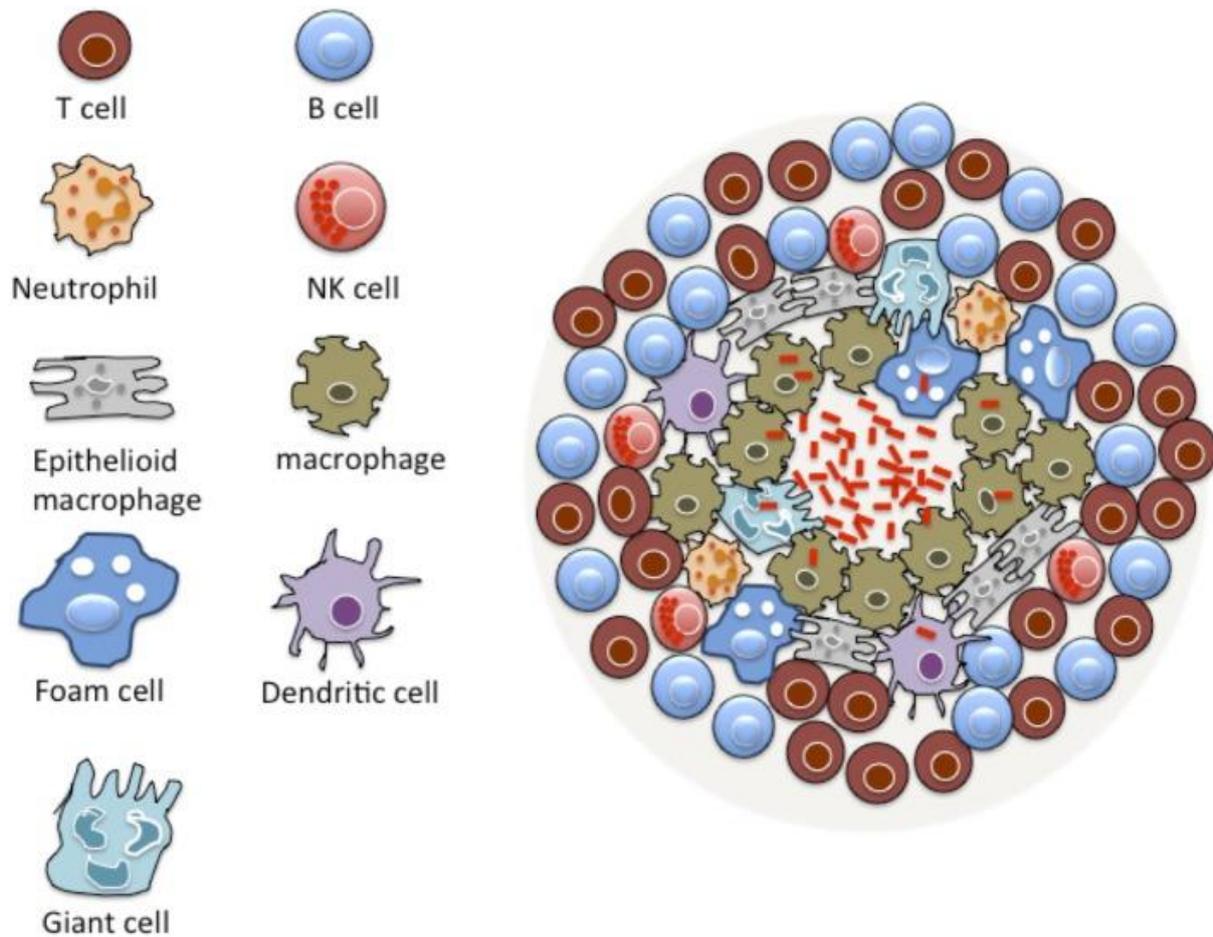
Figure 4. Structure of TB granuloma Bacilli containing macrophages at the center are sur-rounded by specialized macrophages, such as epithelioid cells, multi-nucleated giant cells, and foamy macrophages. Numerous other cells such as neutrophils, dendritic cells, natural killer (NK) cells, fibroblasts B and T cells constitutes granuloma formation. (The figure is adapted from (Ndlovu and Marakalala 2016).

Alveolar macrophages engulf *M.tb* bacilli and initiate their elimination through different mech-anisms. These include acidification of the phagosome, phagosome fusion with the lysosomes through production of nitric oxide (NO) / reactive oxygen species (ROS) and autophagy (van Crevel et al. 2002). Autophagy is a cellular process through which intracellular pathogens and cytoplasmic components are entrapped in a double-membrane-bound autophagosome. In-creased acidification in the phagosome then finally leads to mycobacterial killing (Gutierrez et al. 2004). Interferon γ (IFN-γ) also enhances the translocation of microtubule-associated-pro-tein-1 light chain 3 (LC3) an autophagy marker to form autophagosome and reduces the sur-vival of intracellular virulent *M.tb* H37Rv (Shi and Kehrl 2010). Innate immune cells can also eliminate the bacilli through apoptosis: During this process DCs engulf and degrade the bacilli to release *M.tb* products containing apoptotic bodies into the cytosol and to cross-presenting them to (cluster of differentiation 8) CD8+ T cells. This process, via MHC class I leads to a release of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β). These cytokines help in suppression of excessive inflammation and minimize the tissue damage caused by release of intracellular contents and digestive enzymes to the extracellular space (Lee et al. 2009). However, *M.tb* employs virulence strategies (ESX-1 secretion system) to evade host immunity and disseminates through inhibiting phagosome maturation and restrict lysosome fusion by altering acidic pH inhibiting autophagy (Houben et al. 2012).

The adaptive immunity develops 2 - 8 weeks post infection. Control of *M.tb* is mainly a team-work by T cells, macrophages, and dendritic cells (Cooper 2009). *M.tb*-containing antigen presenting cells (APCs) present the antigens to CD4+ T cells through MHC class II and various PRRs leading to the activation of different Th cell subsets (Mosmann and Sad 1996): Th1 cells produce IL-2 for T-cell activation, IL-12, IFN-γ, and TNF for macrophage activation and enhance microbicidal activity (Akira et al. 2006). Th17 cells produce IL-17 and IL-22, which activate polymorphonuclear granulocytes (PNGs) contributing to protective immunity (Khader et al. 2007). Th2 and regulatory T cells (Treg) normalize Th1 cytokines via IL-4, TGF-β and IL-10 also involving exhaustion of T cells to suppress Th1 protective immunity leading to granuloma caseation and active disease. CD8+ T cells produce IFN-γ and TNF, which activate macrophages besides being cytolytic T lymphocytes (CTL) secreting perforin and granulysin (Stenger et al. 1998), which lyse host cells and directly attack bacilli. These effector T cells (Teff) are succeeded by memory T cells and produce multiple cytokines, notably IL2, IFN-γ, and TNF (Figure 5) (Sallusto et al. 2004). An optimal Th1/Th2/Th17 immune response establishment is more critical for host protection and long term control *M.tb* infection by limiting tissue damage (Ndlovu and Marakalala 2016).
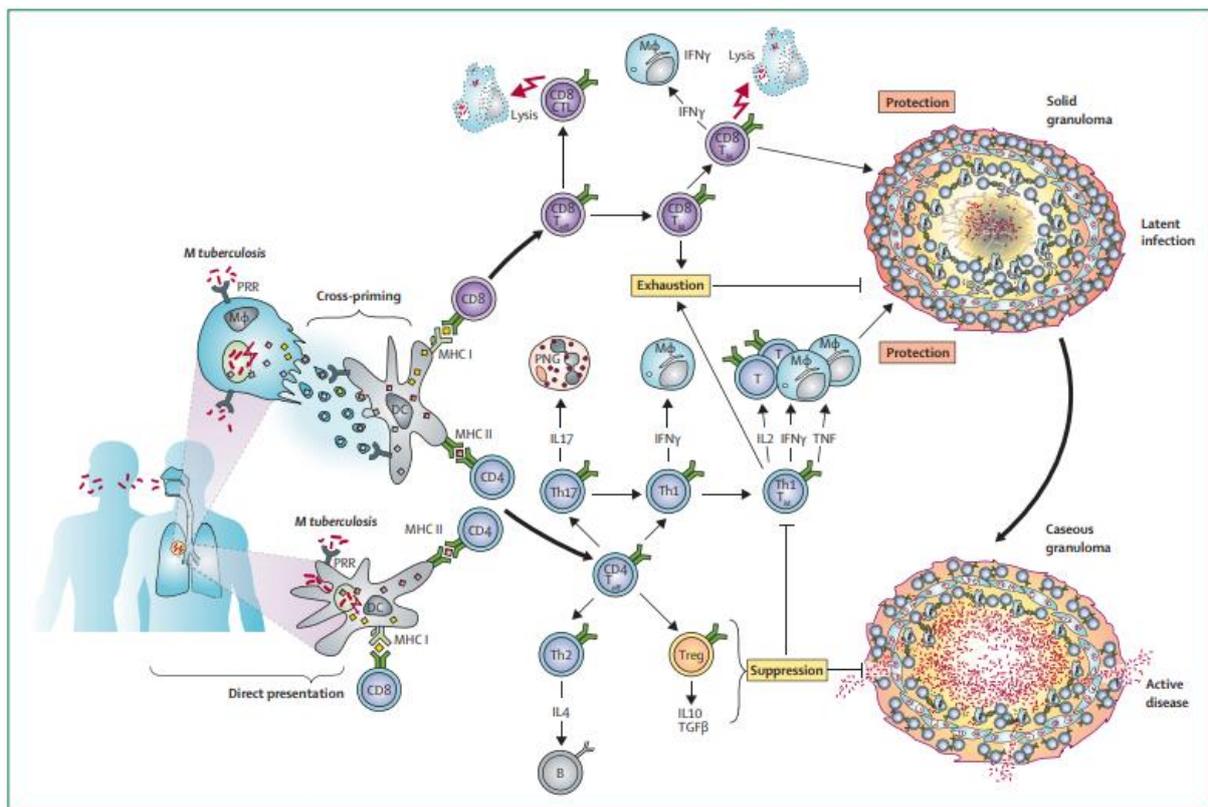

Figure 5. Chronological events in TB Pathogenesis The series of events during the pathogenesis of TB described above. Polarization of T helper subsets to induce several pro- and anti-inflammatory cytokines leading to protective immunity, granuloma formation or T-cell exhaustion and active disease.(Kaufmann et al. 2010)

1.7   Innate immunity to TB

Innate immunity is the first line of defence and is considered to be more crucial than the adaptive immunity in immediate elimination of the pathogen and to bring back the host's tissue homeostasis. Adaptive immunity develops and retains high antigenic specificity through T and B cell receptors. Innate immunity maintains specificity through PRRs by recognizing specific PAMPs that are expressed by a large variety of microbes and contributes to sustained immunity (Janeway 1989). Inhalation of TB bacilli activates PRRs, such as toll-like receptors (TLRs), NOD-like receptors (NLRs), complement receptors, C-type lectin receptors (CLRs) such as dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and mannose receptors expressed on immune cells. These trigger a variety of mechanisms such as phagosome maturation, DC maturation/migration - characterized by increase in expression of co-stimulatory molecules (CD40, CD80, CD83, and CD86), oxidative stress, cell death induction, production of proinflammatory cytokines, and eventually establish the adaptive immune response (Turvey and Broide 2010).

Various PRRs at distinct locations get activated simultaneously to induce host immune responses aimed at controlling *M.tb* growth and course of TB. The signalling of distinct PRRs appears to be redundant in some cases (Holscher et al. 2008), however, there may be unique regulatory adjustments performed by specific PRRs, which needs to be well characterized to develop better therapeutics (Dube et al. 2021). PRR signalling and the host's immunity are influenced by several factors such as polymorphisms and epigenetic modifications, which need to be thoroughly investigated to identify high-risk individuals and in order to potentially develop an individualized disease prophylaxis. Among the PRRs, TLRs are one of the first components of immune system to encounter pathogens and they serve as the link between innate and adaptive immunity by regulating the immune cells and cytokines (Duan et al. 2022).

1.8   Toll like receptor discovery

Christiane Nüsslein-Volhard was the first to discover the Toll protein in fruit flies in 1985 (Anderson et al. 1985; Anderson et al. 1985). The key function of Toll in drosophila is to maintain dorsoventral polarity in the fly. The mutated gene product of Toll was found to cause ventralization. A decade later Jules Hoffmann discovered that Toll was not only responsible for dorsoventral polarity but also has a role in the immune defense in Drosophila (Lemaitre et al. 1996). Without Toll, flies did not survive fungal infections. Interestingly, Toll activation triggered the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) cascade, which mounted the defense against fungi. Spätzle, a protein that induces ventralization by binding to Toll, elicits an NF-κB cascade (Gonzalez-Crespo and Levine 1994). Charles Janeway first reported on similar receptors but failed to understand their nature (Janeway 1992). Ruslan Medzhitov and Charles Janeway were the first to report the cloning of a mammalian homologue, a Toll-like receptor (now called TLR4) (Medzhitov et al. 1997). Its ligand remained unknown. However, by constructing a constitutively active mutant, Medzhitov et al could determine that TLR induces NF-κB activation in a similar way as ligation of IL-1 receptor and similarly to Drosophila Toll. The discovery of TLR4 as a receptor for LPS (Poltorak et al. 1998), the active component in endotoxin from Gram-negative bacteria led to the understanding of TLRs that constitute a family of PRRs, which recognize PAMPs, danger associated molecular patterns (DAMPs) and Microbe-associated Molecular Pattern (MAMPs). In 2011, Jules Hoffmann and Bruce Beutler were awarded the Nobel prize for discoveries on how Toll (in flies) and TLRs (in mammals) activate innate immunity (Wagner 2012).

So far, there have been 13 TLRs described in mammals (Tabeta et al. 2004). TLR11, 12 & 13 are only expressed in mice (Seki and Brenner 2008). In humans TLR11 exists as pseudogene, and TLR12 and -13 are completely absent (Yarovinsky 2014). TLRs are type I transmembrane

proteins, the cytoplasmic portions of TLRs (C-terminal domain) are similar to that of interlukin-1 (IL-1) receptor family, named Toll/IL-1 receptor (TIR) domain. These are required for activation of mitogen-activated protein kinases (MAPK) and subsequent downstream signaling pathways (Gao et al. 2017). The extracellular portions of both receptors are distinct from each other: IL-1 consists of an Ig-like domain while TLRs possess 20-27 leucine- rich repeats (LRRs). Each TLR contains a unique set of LRR repeats that recognize specific PAMPs of various pathogens including bacteria and viruses. TLRs distinguish themselves with ligand specificity, signal transduction pathways and subcellular localization (Singh et al. 2014). TLRs are expressed not only on immune cells (such as macrophages, NK cells, DCs, monocytes, neutrophils, T and B lymphocytes) but also on epithelial cells, endothelial cells and fibroblasts (Delneste et al. 2007).

1.9   Toll like receptor signaling pathway

TLRs are localized either on the plasma membrane (TLR1, -2, -4, -5 and -6) or on intracellular compartments (TLR3, -4, -7, -8, and -9) (Akira et al. 2006). Upon recognition of specific molecular patterns on pathogens TLRs initiate signaling via the TIR domain to recruit the adaptors myeloid differentiation (MyD) 88, TIR domain-containing adaptor inducing interferon-β (TRIF), TIR domain containing adaptor protein (TIRAP), or TRIF-related adaptor molecule (TRAM) as depicted in Figure 6. All TLRs except TLR3 activate NF-κB through MyD88, which then recruits the downstream signaling molecules to form the myddosome (Medzhitov et al. 1998). MyD88 then interacts with IL-1R-associated kinase (IRAK) family (IRAK1–4) (Cao et al. 1996). IRAK1 disassociate from the complex and further activates the E3 ubiquitin ligase - TNF receptor-associated factor 6 (TRAF-6) (Deng et al. 2000), which then recruits and activates transforming growth factor-β (TGF-β) activating kinase (TAK1) (Wang et al. 2001). The TAK1 complex phosphorylates IkB kinases (IKK), and ultimately activates and translocates NF-κB to the nucleus. TAK1 also activates MAPKs such as MKK4/7 and MKK3/6 to induce JNK and p38 respectively, and the IKK complex also activates MKK1 and MKK2 to induce ERK1/2. All these MAPKs leads to activation of cyclic adenosine monophosphate (c-AMP) response element-binding protein (CREB), and activator protein 1 (AP1) to cooperate with NF-κB for induction of pro-inflammatory cytokines (O'Neill 2002). TLRs -3 and -4 also activate an alternative (TRIF/TRAM) pathway to recruit TRAF6 and -3. TRAF6 further recruits the kinase receptor-interacting protein 1 (RIP1) to activate the TAK1 and IKK complex leading to the activation of MAPKs and NF-κB. While TRAF3-dependent activation of the TANK-binding kinase 1 (TBK1) and IKKε (originally IKKi) phosphorylates and activates interferon regulatory factor (IRF) 3 and IRF7 (by TLRs 7, 8 and 9) (Fitzgerald et al. 2003), which is activated by IRAK1 and IKKα. Activation of IRF3 and IRF7 leads to the induction of Type I IFN (Figure 6) (Oshiumi et al. 2003).

So far TLR1/2, TLR3, TLR4, TLR2/6, TLR7/8 and TLR9 have been proposed as key receptors in recognition of *M.tb* and are involved in host defense and inflammation through the production of inflammatory cytokines including IL-1, IL-6, TNF, IL-12, CD4+T cell responses and CD8+T cell responses (Pahari et al. 2017). However, deficiencies of adapter proteins along with polymorphisms greatly impair the conformational changes of TLR proteins and subsequent signalling (Akar-Ghibril 2022). Endosomal TLRs play a crucial role in recognizing danger signals (dsRNA, ssRNA and hypomethylated dsDNA) as the *M.tb* proteins sequestered in the endosome either clears the bacilli or develop the active disease based on the immune response elicited by type I IFNs (α/β). Endosomal TLRs also help in regulating the internalized phagocytic material and TLR themselves; DC migration; antibody production and memory formation (Nguyen et al. 2020).
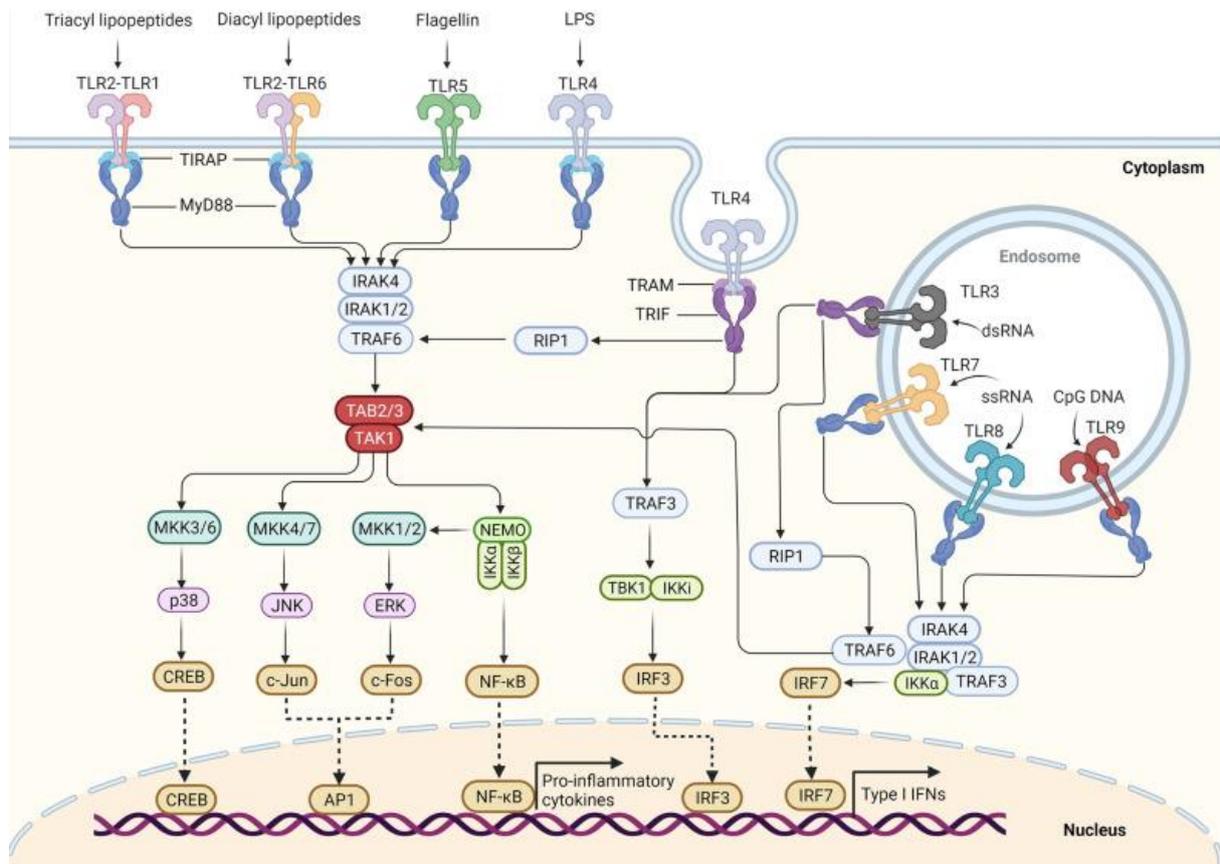
Figure 6. Toll like receptor pathway Upon recognition of specific PAMPs, TLR signalling is initiated by dimerization of receptors, leading to the engagement of TIR domains with TIRAP and MyD88 (or directly interact with MyD88) or with TRAM and TRIF (or directly interact with TRIF) ultimately activating NF-κB and IRFs to induce proinflammatory cytokines and type I IFNs respectively. (Adapted from (Duan et al. 2022))

1.10 Toll like receptor 4

The TLR4 gene is located on chromosome 9 and is expressed on a variety of human cells, such as monocytes, mast cells, neutrophils, dendritic cells, T cells and endothelial cells. Lipopolysaccharide (LPS) from Gram-negative bacteria is the key ligand of TLR4. Various ligands of *M.tb* such as heat shock proteins 60/65, 38-kDa antigen, secretory protein (Rv0335c), lipomannan (LM), have been proposed to also activate TLR4, however, there is no strong evidence (Jung et al. 2006; Hossain and Norazmi 2013; Sharma et al. 2021). TLR4 is synthetized in the endoplasmic reticulum where glycoprotein 96 (gp96) helps in folding of the receptor and protein associated with TLR4 (PRAT4A) helps in its maturation (glycosylation) (Takahashi et al. 2007; Yang et al. 2007). TLR4 is a unique transmembrane receptor which is expressed both, on cell surface, as well as on endosomal membranes. It utilizes MyD88-, and TRIF-dependent pathways respectively. It has been established that CD14 controls the LPS-induced TLR4 endocytosis along with Myeloid Differentiation Protein 2 (MD2) (Tan et al. 2015). However, in the presence of *M.tb* ligands, the TLR4 endocytosis process is not clear (Thada et al. 2021). Unstimulated cells also detect TLR4 in the endoplasmic reticulum (ER), Golgi apparatus and the plasma membrane (Latz et al. 2002).

TLR4 at the plasma membrane interacts with TIR domain containing adapter protein (TIRAP) (also called MyD88 adapter like protein (MAL). The TLR4-TIRAP-MyD88-IRAK forms the so-

called "myddosome" to induce the nuclear translocation of NF-κB (Lin et al. 2010). This ultimately leads to the expression of pro-inflammatory cytokines, such as TNF, IL-6, cyclooxygenase 2, and type III interferons (IFNλ1/2), the latter is required for epithelial barrier integrity, and is crucial for host defense (Kawai and Akira 2011; Odendall et al. 2017). In addition, this pathway also induces the production of the anti-inflammatory cytokine IL-10 to supress inflammation (Chanteux et al. 2007). The TRIF-dependent signaling pathway of TLR4 at the endosome interacts with TRAM and activates TRAF3 to ultimately recruit IRF3 to induce expression of genes encoding type I IFN helps in DC maturation. TRIF also induces NF-κB via recruitment and activation of TRAF6 to induce cytokine production and can also trigger necrotic cell death (Sato et al. 2003).

There are several lines of evidence that TLR4 recognize *M.tb* cell surface lipid antigens and participates in host's protective immunity, however, the overall TLR4 role in TB pathogenesis is still unclear. (i) TLR4 activation induces autophagy through TRIF dependent pathway (Xu et al. 2007);(ii) TLR4 induces activation of canonical and non-canonical nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain-containing 3 (NLRP3) inflammasome pathway through (MyD88) adaptor molecule to produce IL-1β, and IL18 for an effective TB control strategy (Kelley et al. 2019) upon *M.tb* antigen recognition.

Integrity of TLR signaling components is essential for protective immunity. Changes in confirmation of these molecules resulting from single nucleotide polymorphism (SNPs) are often associated with susceptibility to various infectious diseases. A recent meta-analysis of TLR4 SNPs rs4986790, rs10759932 indicated increased risk of PTB, while rs4986791 and rs11536889 SNPs did not confer risk for PTB (Muheremu et al. 2022).

1.11   Toll like receptor 8

The TLR8 gene is present on the X-chromosome and influences the immune system by its overexpression to viral infections in women (Conti and Younes 2020). TLR8 resides on immune cells such as monocytes, macrophages, neutrophils, myeloid dendritic cells, T reg cells and epithelial cells. It has been reported that a chaperone protein, Protein unc-93 homolog B1 (Unc93B1), regulates the stability and transportation of endosomal TLRs (TLR3, -7, -8 and -9) (Pelka et al. 2018).TLR-Unc93B1 complexes move to the endosomal compartment and release Unc93B1 to induce ligand binding dimerization of TLRs (Miyake et al. 2021).

TLR8 recognizes ssRNA AU- and GU-rich sequences, whereas TLR7 is activated by GU-rich sequences only (Zhang et al. 2016). TLR8 is able to distinguish self and non-self RNA by nucleoside modifications (Kariko et al. 2005). TLR8 also serves as vita-PAMP receptor that can recognize molecular structures from viable microbes and drives T follicular helper (Tfh) cell differentiation (Ugolini et al. 2018).TLR8 also senses imidazoquinoline resiquimod R848 (Jurk et al. 2002). Activation of TLR8 promotes CD4+ T cell proliferation to induce Th differentiation and reverse the suppression of Treg cells through the TLR8-MyD88-IRAK4 signaling pathway and induce the production of TNF, IL-6 and IL-12 (Peng et al. 2005). TLR8 activation has been shown to induce production of ROS in neutrophils (Makni-Maalej et al. 2015). TLR8 also plays a crucial role in autoimmune diseases (Duan et al. 2022). Since TLR8 is not functional in mice, the role of TLR8 in tumor progression has been studied by replacing the exon 3 of mouse TLR8 with the human version, and it was found to activate IFN-γ and TNF positive CD4+ T cells and effector T cells. *M.tb* tRNA activates TLR8 to induce IL-18, IFN-γ and IL-12p70 (Keegan et al. 2018). TLR8-humanized mice permitted higher bacillary load, however ESAT-6 along with TLR8 agonist vaccine adjuvant restored enhanced memory T cell formation and Th1 humoral response (Tang et al. 2017). Davila et al., first reported that TLR8 rs3764879G/C, rs3788935G/A, rs3761624G/A, rs3764880G/A SNPs associated with PTB in males in Russian and Indonesian populations (Davila et al. 2008). rs3764880G/A (A1G or

M1V) SNP changes the start codon resulting in truncated TLR8 protein and correlated to gain of function SNP (Oh et al. 2008).

1.12   Role of TLR dimerization in signal transduction

Ligand-induced multimerization is a key event of TLR activation followed by recruitment of adaptor proteins to their intracellular TIR domains which also contain TIR domains. The TIR-TIR interactions are critical to form myddosome for initiating downstream signalling (O'Neill and Bowie 2007). The activated TLR complex is typically forms "m" shape, the C-terminal regions of the two TLRs are positioned in close proximity to promote dimerization and initiate signalling. Endosomal TLR ligand interaction is a complex process involving recognition of degradation products of nucleic acids at two distinct ligand-binding sites and cleavage of the Z-loop followed by dimerization and activation of TLR (Asami and Shimizu 2021). Although most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or -6 to recognize tri-acylated and diacylated lipopeptides respectively confirmed by crystallization (Jin et al. 2007; Kang et al. 2009). TLRs also depend on other co-receptors for full ligand sensitivity, as in TLR4's recognition of LPS, requiring MD-2 (Park et al. 2009). TLRs show remarkable versatility of the ligand recognition mechanisms, which is essential for defence against diverse microbial infections. SNPs in TLR dimer complexes may disrupt confirmation leading to differences in dimerization and signaling cascade activation.

1.13   Cytosolic receptors: cGAS/STING

Cyclic GMP-AMP synthase (cGAS) (also known as Mab21 domain-containing protein 1—MB21D1) is a member of the nucleotidyl transferase (NTases) family, and it acts as a sensor for cytosolic dsDNA via the endogenous second messenger cGAMP in multiple cell types including macrophages (Wu et al. 2013). cGAS interaction with DNA precedes the synthesis of cyclic-di-GMP-AMP (c-diGAMP) from ATP and GTP, which then binds STING that dimerizes and translocates to the Golgi from the ER (Ishikawa and Barber 2008; Saitoh et al. 2009). This leads to the availability of STING carboxyl terminus to subsequent recruitment and phosphorylation of TBK1 and IRF3 (Tanaka and Chen 2012; Liu et al. 2015) leading to the induction of IFNβ production (Sun et al. 2013; Wu et al. 2013; Zhang et al. 2018) (Figure 7). STING also activates NF-κB, which functions together with IRF3 to turn on the transcription of type I IFNs and other cytokines (Burdette and Vance 2013). Cytosolic DNA is also vital for autophagy induction (Watson et al. 2012). Thus, it is important to understand the role of cytosolic DNA surveillance pathway (CSP) during mycobacterial infection either protective or detrimental to the host (Figure 7). cGAS knockout human and mouse macrophages were shown to block the cytokine production and induction of autophagy (Sun et al. 2013; Collins et al. 2015; Wassermann et al. 2015) through an ESX-1 dependent manner (Watson et al. 2015). cGAS cannot discriminate pathogenic or self-DNA. Hence, it is considered as a double-edged sword: When foreign DNA invade the host's cell cytoplasm, the cGAS pathway induces high levels of IFN to resist infection. However, if cGAS encounters self-DNA and damaged mitochondrial DNA (mtDNA) in the cytoplasm (Hopfner and Hornung 2020) it will lead to serious autoimmune diseases (An et al. 2017).

STING is a downstream adaptor protein and acts as a PRR itself and senses bacterial cyclic dinucleotides (Burdette et al. 2011) independently of cGAS. There are many reports suggesting that the variation in the STING gene could lead to conformational change near the c-terminal region where the ligand-binding packet lies (Huang et al. 2012; Shu et al. 2012; Yin et al. 2012), which eventually could lead to differences in the binding efficiencies of STING with the respective ligands. Hence, it is important to study the effect of these SNPs in different infectious

and autoimmune diseases in order to understand the underlying mechanism. STING HAQ haplotype (R71H-G230A-R293Q) occurs in high frequency in human populations and alters the conformation ligand binding region impairing recognition of cyclic dinucleotides (Yi et al. 2013). Hence, it is important to understand the role of SNPs influencing the mechanism of cGAS/STING during the course of disease and whether structural differences in cGAS also modulate the interaction of cGAS/STING.
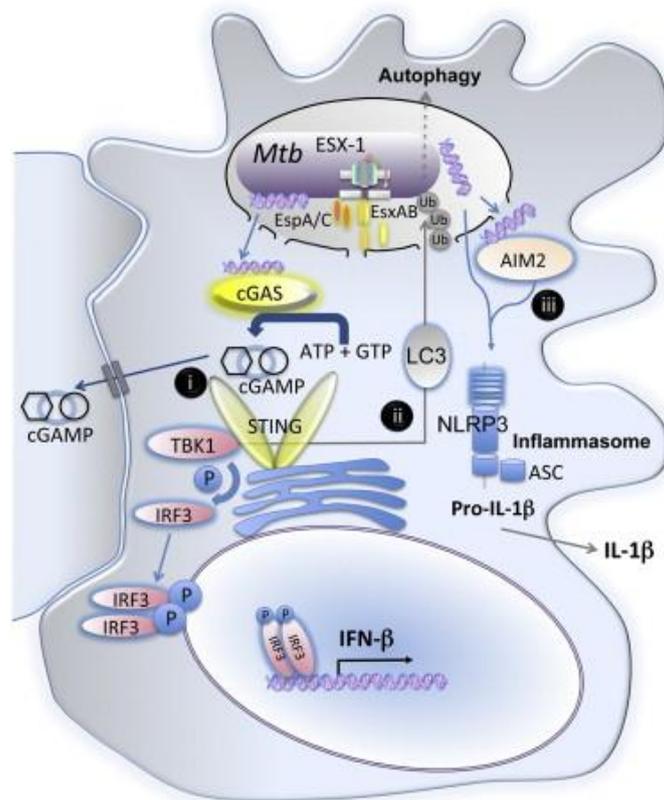

Figure 7. cGAS/STING pathway Infected macrophages with *M.tb* virulence type VII secretion system secrete ESAT-6 along EspA and EspC inducing phagosome membrane rupture and release of its DNA into the cytoplasm to trigger the immune system (i), activating cGAS-STING pathway to induce TBK-1-IRF-3-IFN-β signaling axis. (ii) TBK-1 also activates autophagy via recruitment of LC3-II marker. (iii) NLRP3/AIM-2 inflammasome axis can also be activated by dsDNA contributes to release of mature IL-1β. (the figure is adapted from (Majlessi and Brosch 2015).

2. Objectives and Aims

TB is a complex disease and PRR activation is a coordinated process between different cells and at different stages of infection. Therefore, it is important to study the function of each PRR in the pathogenesis of TB with an emphasis on their genetic modifications to better understand the disease, which is important for eventually developing novel intervention strategies or for an effective vaccine. Studies have shown that TLRs are able to interact and form heterodimers that could enhance, inhibit, or modulate immune responses. In this study we hypothesize that TLR4 and -8 interact at the endosome to maintain the Th1 and type I IFN balance and that the potential genetic modifications within these receptors may disrupt the balance leading to modulation of the immune responses and thus changes in disease progression.

Specific objectives:

1. To study the frequency and potential involvement in susceptibility to and course of TB of functional SNPs in TLR4 rs4986790 A/G (aa code D299G) and rs4986791 C/T (aa code T399I); TLR8 rs3764880 A/G (aa code M1V); cGAS rs610913 C/A (aa code P261H) and rs311686 A/G (aa code K625E 8.98 kb upstream in the cis-regulatory region); and STING rs78233829 G/C (aa code G230A), rs1131769 A/G (aa code H232R) and rs7380824 C/T (aa code R293Q) in an Indian TB cohort, a healthy control cohort and a second German healthy control cohort, and to study the potential impact of the SNPs on potential confirmational changes in ligand binding sites.

2. To prove *M.tb* RNA and TLR8 ligand (R848) recognition potentially by forming TLR4/8 heterodimers by overexpression studies in human embryonic kidney (HEK) system, confocal microscopy, Co-immunoprecipitation (co-IP) analysis, mass spectrometry and in silico modeling.

3. To measure TLR4-, and TLR8- genotype-specific cytokine induction either alone or in combination with TLR8 ligand stimulated peripheral blood mononuclear cells (PBMCs).

3. Original Articles

3.1 Interaction of TLR4 and TLR8 in the Innate Immune Response against Mycobacterium Tuberculosis

Article

Interaction of TLR4 and TLR8 in the Innate Immune Response against Mycobacterium Tuberculosis

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Abstract: The interaction and crosstalk of Toll-like receptors (TLRs) is an established pathway in which the innate immune system recognises and fights pathogens. In a single nucleotide polymorphisms (SNP) analysis of an Indian cohort, we found evidence for both TLR4-399T and TRL8-1A conveying increased susceptibility towards tuberculosis (TB) in an interdependent manner, even though there is no established TLR4 ligand present in *Mycobacterium tuberculosis* (Mtb), which is the causative pathogen of TB. Docking studies revealed that TLR4 and TLR8 can build a heterodimer, allowing interaction with TLR8 ligands. The conformational change of TLR4-399T might impair this interaction. With immunoprecipitation and mass spectrometry, we precipitated TLR4 with TLR8-targeted antibodies, indicating heterodimerisation. Confocal microscopy confirmed a high co-localisation frequency of TLR4 and TLR8 that further increased upon TLR8 stimulation. The heterodimerisation of TLR4 and TLR8 led to an induction of IL12p40, NF-κB, and IRF3. TLR4-399T in interaction with TLR8 induced an increased NF-κB response as compared to TLR4-399C, which was potentially caused by an alteration of subsequent immunological pathways involving type I IFNs. In summary, we present evidence that the heterodimerisation of TLR4 and TLR8 at the endosome is involved in Mtb recognition via TLR8 ligands, such as microbial RNA, which induces a Th1 response. These findings may lead to novel targets for therapeutic interventions and vaccine development regarding TB.

Keywords: TLR4; TLR8; tuberculosis; SNP analysis; heterodimerisation

1. Introduction

The recognition of potentially pathogenic microorganisms followed by an inflammatory response of the host is regulated by the immediate reaction of the innate immune

system [1]. Activation of this evolutionary older system is also crucial for an efficient function of the second arm of the immune response present only in vertebrates, the acquired immune system. Antigen-presenting cells (APCs) are activated and migrate to the lymph nodes, where they bridge the innate and adaptive immune systems by presenting antigens, leading to the generation of an efficient antibody response [2]. Pattern Recognition Receptors (PRRs), which have been identified and structurally characterised over the last 20 years, play a major role in mounting an effective innate immune response by recognising the presence of pathogens via Pathogen-Associated Molecular Patterns (PAMPs) [3]. Toll-like receptors (TLRs) are one important subgroup of PRRs mainly present on APCs such as alveolar macrophages and dendritic cells (DCs) [4]. Several TLRs located in the cell surface membrane have the main function of recognising bacterial cell wall compounds, internalising the microbe, and activating a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated inflammatory response. Others are expressed within the endosomal membrane and act to recognise microbial nucleic acids, inducing type I interferons (IFNs) [5]. TLRs act as dimers, and while most receptors organise as homodimers, some have been structurally analysed as functional heterodimers [6]. For example, the plasma membrane located TLR2 can form a heterodimer either with TLR1 or -6, resulting in a change in its ligand-binding capacity and a more specific response to Gram-positive bacteria [7].

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Mtb), and both the cell wall composition and the immune response elicited by Mtb within the host are unique. Certain cell wall compounds of Mtb have been described to interact with PRRs located mainly on the outer cell membrane, particularly TLR2/1 [8,9]. Furthermore, TLR4 has been suggested to recognise mycobacterial lipoarabinomannan (LAM) and lipomannan (LM), although it has remained unclear how a ligand so distinct from the typical TLR4-ligand lipopolysaccharide (LPS) can interact with TLR4 [10]. TLR4 can be localised either on the cell surface or the endosomal membrane. For the interaction of TLR4 with LPS, MD-2 and CD14 are required [11,12]; however, for other ligands, this might not be the case. Furthermore, MD-2 and CD14 promote the LPS-induced endocytosis of TLR4 [13].

Interestingly, the endosomal localisation of TLR4 changes the utilisation of adapter molecules, leading to a differentiated inflammatory response. TLR4 activated within the endosome does not recruit its standard adaptor protein myeloid differentiation primary response 88 (MyD88) but instead, it recruits TIR-domain-containing adapter-inducing interferon-β (TRIF), which activates the transcription factor IFN regulatory factor (IRF)-3 and thereby changes the immune response from NF-κB-dependent cytokines to type I IFNs [14,15]. Generally, TLR4 activation promotes a T helper (Th)1 response by activating APCs and promoting DC maturation, inducing interleukin (IL)-12, tumour necrosis factor (TNF)α, IFNγ, major histocompatibility complex class (MHC)-II, CD80, and 86, and NO, as well as enhancing phagocytosis and inhibiting IL-10 production [16–20]. Mtb is known to strongly inhibit the expression of TLR4 and both its adaptor proteins TRIF and MyD88 [21,22]. Furthermore, mycobacteria evade PRR recognition of the cell wall by persisting intracellularly in macrophageal phagosomes. Therefore, once Mtb is internalised, intracellular PRRs must assume immunosurveillance. Recently, evidence has accumulated that mycobacterial nucleoside recognition both in the endosome (e.g., by TLR8) and within the cytoplasm is important for an effective immune response [23–25]. TLR8 is an endosomal receptor recognising uridine-rich and short ssRNA mainly expressed in macrophages and myeloid DCs [26,27]. Activation leads to an induction of NF-κB via MyD88, resulting in the production of IL-12, TNFα, and IFNγ, as well as the induction of type I IFNs through IRF5 and IRF7 [28]. Thus, a Th1 response is promoted. Similarly to TLR4, mycobacteria have developed mechanisms to impair the function of endosomal PRRs by inhibiting endosomal acidification [29,30].

Evidence for both TLR4 and TLR8 being involved in TB pathogenesis comes from clinical trials assessing the frequency of single nucleotide polymorphisms (SNPs) in the TLR4 and TLR8 genes in TB patients compared to healthy controls. Increased suscepti-

bility toward TB in individuals carrying TLR4 SNPs has been described for the variants Asp299Gly (rs4986790) and Thr399Ile (rs4986791) [31]. For TLR8, susceptibility has been associated with the less functional variant of TLR8 Met1Val (rs3764880) [23,32]. In this study, we confirm the evidence for TLR4 playing a role in TB immunity and hypothesise that the endosomal cooperation of TLR4 and TLR8 by forming a heterodimer modulates the immune response to Mtb.

2. Results

2.1. Cohort Characteristics and Genotyping

We analysed a TB cohort from an unmatched case-control study that was previously described for genetic susceptibility (Table S1, [23,24,33,34]). The cohort consisted of 346 TB patients and 301 controls. TB patients were either diagnosed with pulmonary (224 patients, PTB) or extrapulmonary TB (121 patients, EPTB). Additionally, there were 95 relapse cases. There were significantly more females among the patients (58.9%) than controls (50.9%) and TB patients, on average, were younger (25.5 years) than controls (32.9 years). Among the relapses, 49.5% were female, and the mean age was 30.6 years. More controls than patients had received Bacillus Calmette–Guérin (BCG) vaccine in their childhood (82.9% and 48.8% respectively). Relapse cases had the lowest mean body mass index (BMI, 16.49), followed by pulmonary (16.6) and extrapulmonary cases (19.9), and controls (24.3). Relapse cases also had the lowest portion of BCG positives (36.92%) in comparison to primary TB cases (48.8%) and controls (82.9%).

Regarding SNP distribution, TLR4-Thr399Ile (cytosine (C)>thymine (T)) and TLR4-Asp299Gly (adenine (A)>guanine (G)) were not fully linked (cosegregation in only 73%), unlike among Caucasians (Table S2). TLR4-399T was more frequent among TB patients than controls, and there was evidence that it conveyed susceptibility towards TB (OR = 1.97 [1.15–3.37]; p = 0.013; Table 1, for full model Table S3). There was no evidence for effect modulation by BCG vaccination (p = 0.392). There was also no evidence for a different distribution of TLR4-A299G alleles between TB patients and controls (OR = 0.72 [0.49–1.07], p = 0.101), nor for an impact of allele distribution on the site of manifestation (PTB or EPTB) for TLR4-C399T (OR = 0.85 (0.51–1.41), p = 0.523) or TLR4-A299G (OR = 1.24 (0.74–2.05), p = 0.413). As we previously reported [23], TLR8-1A was associated with a susceptibility towards being a TB case (OR = 1.68 (1.08–3.63); p = 0.022; Table 1), with weak evidence for an interaction between BCG and TLR8-Met1Val (A > G) (p = 0.071, Table S4). Interestingly, the susceptibility conveyed by TLR8-1A was only seen in individuals carrying the TLR4-399T allele (OR= 1.97 (1.15–3.37), p = 0.013); among homozygote TLR4-399C individuals, no impact of TLR8-1A on TB disease was observed (OR = 1.19 (0.52–2.72); p = 0.681, Table 1). Notably, there was no evidence for the statistical interaction of the two SNPs in primary TB (p = 0.363) or relapse (p = 0.750) cases.

Table 1. Allele distributions of single nucleotide polymorphisms (SNPs) in the Indian cohort among controls and primary TB cases.

TLR SNPs (Nucleotide Change)	Alleles	N	Allele Frequency [N(%)] Controls Primary TB		OR [95% CI] *	*p*-Value
TLR4-Asp299Gly (A > G)	G	533	72 (27.48)	100 (33.22)	0.72 [0.49–1.07]	0.101
TLR4-Thr399Ile (C > T)	T	552	68 (23.37)	105 (31.44)	1.57 [1.04–2.36]	0.027
TLR8-Met1Val (A > G)	A	556	139 (47.60)	199 (58.70)	1.68 [1.08–2.63]	0.022
TLR8-1, when TLR4-399CT/T	A	395	34 (50.00)	59 (56.19)	1.97 [1.15–3.37]	0.013
TLR8-1, when TLR4-399CC	A	155	105 (47.09)	137 (59.83)	1.19 [0.52–2.72]	0.681

* Odds Ratios (ORs) based on Likelihood Ratio Tests (LRTs) adjusted for gender and age, as well as BCG status in case of TLR8.

Regarding relapses, we did not observe a significantly different distribution between primary TB patients and relapse cases regarding TLR4-A299G (OR = 0.80 (0.49–1.32), p = 0.381) or TLR4-C399T (OR = 1.36 (0.81–2.28), p = 0.245, Table 2). However, there was also strong evidence for an increased risk for being a relapse case associated with TLR8-1A (OR = 1.99 (1.03–3.82); p = 0.006, Table S5). Regarding a potential interaction of TLR4 and TLR8,

the same pattern as above was observed, namely that TLR8-1A conveyed susceptibility to TB depending on TLR4-399T although with only weak evidence (OR = 2.90 (0.87–9.59), *p* = 0.069; Table 2). Again, there was no evidence for formal statistical interaction (*p* = 0.750). Nevertheless, this observation led us to suspect that there might be an interaction on the molecular level between TLR4 and TLR8, and we further investigated this in different in silico and in vitro systems.

Table 2. Allele distributions of SNPs in the Indian cohort among primary TB and relapse cases.

TLR SNPs	Alleles	N	Allele Frequency [N(%)] Primary TB Relapses		OR [95% CI] *	*p*-Value
TLR4-Asp299Gly	G	383	100 (33.22)	36 (39.13)	0.80 [0.49–1.32]	0.381
TLR4-Thr399Ile	T	376	105 (31.44)	33 (38.82)	1.36 [0.81–2.28]	0.242
TLR8-Met1Val	A	355	140 (58.70)	68 (72.34)	1.99 [1.03–3.82]	0.035
TLR8-1, when TLR4-399CT/T	A	111	59 (56.19)	25 (75.76)	2.90 [0.87–9.59]	0.069
TLR8-1, when TLR4-399CC	A	231	137 (59.82)	37 (71.15)	1.62 [0.69–3.81]	0 265

* ORs based on LRTs adjusted for gender and age, as well as BCG status in case of TLR8.

2.2. Docking Outcome

To evaluate the possible structural implications of an amino acid residue change in TLR4 at position 399, we performed in silico analysis. Furthermore, we performed molecular docking studies to investigate the idea of interaction between TLR4 and TLR8. The docking outcome revealed that the TLR4-399C molecule (threonine at position 399) could undergo heterodimerisation with TLR8 in presence of the agonistic ligand R848 (Figure 1). Threonine-399 and serine-400 residues of TLR4 could link to the TLR8 molecule by hydrogen bonds of 2.12 and 2.24 angstroms, respectively. The major non-ligand residues from TLR8 involved in the hydrophobic contacts were Tyr353, Gly351, Val378, Ser352, Ile349, and Tyr348. However, the structure of the TLR4-399T molecule (with isoleucine at position 399) did not show this phenomenon. This demonstrated that the ability of TLR4 to form a heterodimer with TLR8 was lost by changing the residue TLR4 residue threonine-399 to isoleucine-399. This change might lead to conformational rearrangements in the protein structure that could alter the ligand-binding capacity and thereby prevent the R848-facilitated formation of a heterodimer with TLR8.
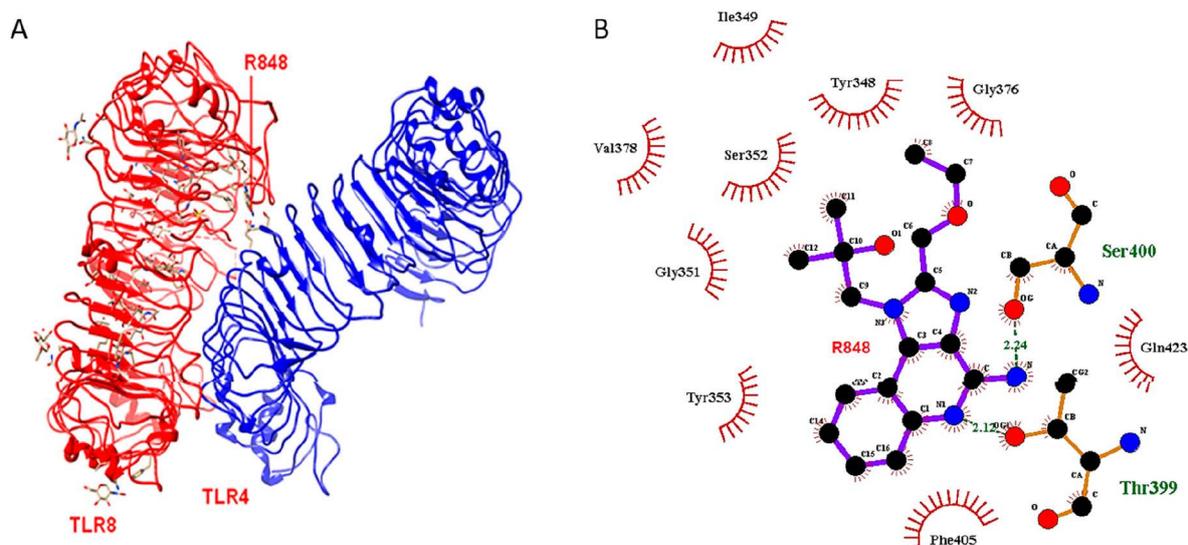

Figure 1. Molecular Docking: (**A**) Toll-like receptor (TLR)4-TLR8 heterodimer mediated by agonistic ligand R848 of TLR8. Both are wild types. (**B**) Ligand plot showing TLR4 variant (Threonin-399, i.e., 399C) interacting with agonistic ligand R848 and assisting in heterodimerisation.

2.3. Mass Spectrometry

To further explore the potential interaction of TLR4 and TLR8, we conducted mass spectrometry analysis on co-immunoprecipitation (IP) to find evidence for heterodimer formation. With IP, we pulled down with anti-human influenza hemagglutinin (HA)-TLR8 in human embryonic kidney (HEK)293 cells that were either transfected with TLR4- and with or without TLR8-HA and analysed the samples for the presence of TLR4, TLR8 and other proteins. As expected, we saw a significant difference in TLR8 intensity between samples transfected with or without TLR8-HA. Furthermore, we identified protein unc-93 homolog B1 (UNC93B1) in the proteomic analysis as statistically significantly enriched when co-immunoprecipitating with TLR8-HA in HEK cells (Figure 2), as expected and reported by others [35]. The TLR4 receptor was identified in five out of six TLR8-HA+TLR4 co-transfected cell lysate immunoprecipitations, with two to five unique peptides, but not in any control sample. However, due to fluctuating LFQ intensities for TLR4, co-IP with TLR8-HA, and high variance in the whole dataset of the experiments, the enrichment of TLR4 in TLR8-HA co-transfected cells immunoprecipitated with anti-HA did not reach statistical significance after Benjamini–Hochberg correction (TLR4 log2 fold change = 4.27 and $p > 0.05$ after B.H. adjustment; before $p = 0.001$). The successful identification of a TLR4 peptide in TLR4/8 co-IP samples by higher-energy collisional dissociation spectrum is shown in Figure S1. Altogether, the data indicate that TLR8 can interact with TLR4 in HEK cells overexpressing both receptors.
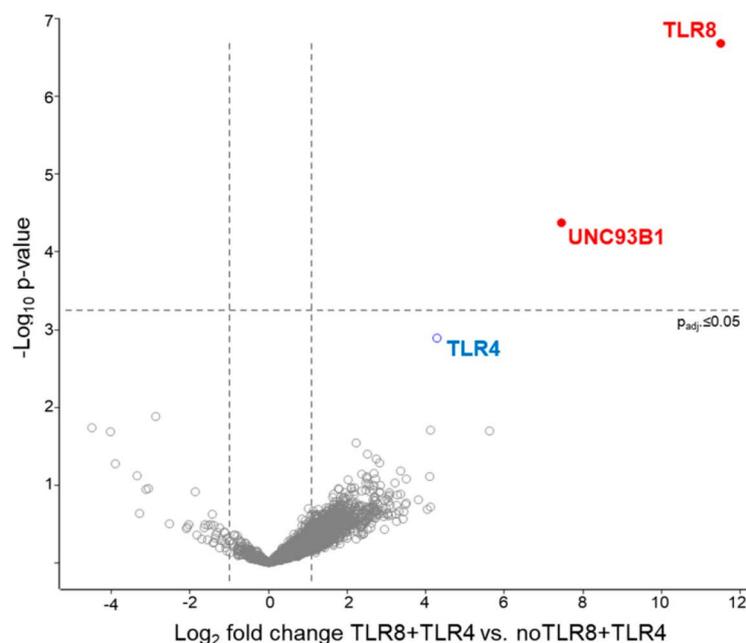

Figure 2. Interaction of TLR8 with TLR4 in HEK cells. Volcano plot of the label-free quantitative MS data plotting the logarithmic difference in protein levels in the HA-immunoprecipitated fraction of TLR8-HA and TLR4 expressing HEK cells and cells expressing TLR4 alone versus the negative logarithmic p values of the t-test performed of six experiments per group. The dotted lines indicate significance thresholds (fold change ≥ 2 and p-value $_{\text{(Benjamini-Hochberg adj.)}} \leq 0.05$). In red (filled circles), statistically significant differentially abundant proteins, in blue (open circle) TLR4, in grey (open circles), proteins with no statistically significant abundance.

2.4. Co-Immunoprecipitation

For further investigation of the potential interaction of TLR4 and TLR8, we performed co-IP experiments with TLR4 variants. HEK293XL/hTLR8-HA+UNC93B1 cells were transiently transfected with TLR4–mCherry–myc 399C or 399T followed by stimulation with LPS, R848, LPS+R848, and Mtb RNA, or left untreated for 2 h. First, the lysates were

immunoprecipitated with anti-HA antibody to pull down TLR8 and then immunoblotted with either anti-TLR4 or anti-HA (for indirect blotting of TLR8) antibody (Figure 3). As expected, without transfection for TLR8, neither TLR4 nor TLR8 were identified in the precipitates. Interestingly, TLR4 was identified in all cells transfected with both TLR4 and TLR8, even in unstimulated cells. The successful blot of TLR4 after precipitation for TLR8 indicated an interaction of the two TLRs. Of note, adding MD2/CD14 to the transfection abolished this effect (Figure S2). In hTLR8HA+UNC93B1 cells co-transfected with TLR4-399C (the variant that we have identified to be able to interact with TLR8), R848 stimulation resulted in higher TLR4 band intensities as compared to other stimulants (Figure 3B). Adding LPS to R848 decreased TLR4-399C band intensity. Comparing the genotypes of TLR4 T399C by the quantification of band intensities, 399T-transfected cells exhibited less TLR4 band intensities upon stimulation with R848 and Mtb RNA, although both input and unstimulated cells showed higher band intensities for 399T than 399C (Figure 4).
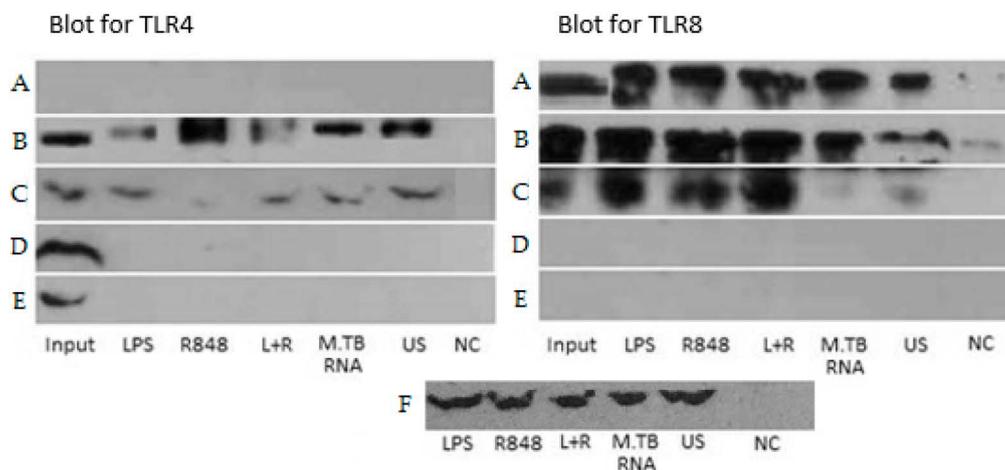

Figure 3. Co-immunoprecipitation. HEK 293 cells transfected as indicated per line, followed by stimulation as indicated per column (2 h for lipopolysaccharide (LPS) (10 ng/mL), R848 (2 µg/mL), LPS and R848 (L+R), 16 h for MTB RNA (1 µg/mL), unstimulated (US) and negative control (NC)). After 2 h, immunoprecipitation procedure was started. The left panel shows immunoprecipitation and -blot with anti-HA antibody (≈110 kDa), indirectly precipitating for TLR8. The right panel shows immunoprecipitation with anti-HA-antibody, followed by immunoblot with anti-TLR4 antibody (100 kDa). (**A**) hTLR8HA+UNC93B1, (**B**) hTLR8HA+UNC93B1+TLR4 399C-mCherry-myc, (**C**) hTLR8HA+UNC93B1+TLR4 399T-mCherry-myc, (**D**) TLR4 399C-mCherry-myc, (**E**) TLR4 399T-mCherry-myc, (**F**) hTLR8HA+UNC93B1 native cells blot with loading control—anti-GAPDH antibody (≈37 kDa). TLR8 was pulled down by anti-HA antibodies and identified in the immunoblot. When HEK cells were co-transfected with both TLR4 and TLR8, TLR4 could be identified in lysates precipitated for HA/TLR8, indicating heterodimerisation.

As a control, we performed the same experiment with hTLR7FLAG instead of hTLR8HA, and no TLR4 was found after IP for FLAG (Figure S3). In order to check different species, we repeated the experiment with Rhesus and C. atys TLR4 FLAG-tagged along with HEK293XL/hTLR8-HA+UNC93B1 with the result of successful identification of TLR4 in the co-IP, which is similar to hTLR4 (Figure S4). Altogether, data from co-IP supported the data from modelling, indicating that TLR4 and TLR8 interact at the endosomal level, particularly in cells co-transfected with TLR4-399C and stimulated with R848.
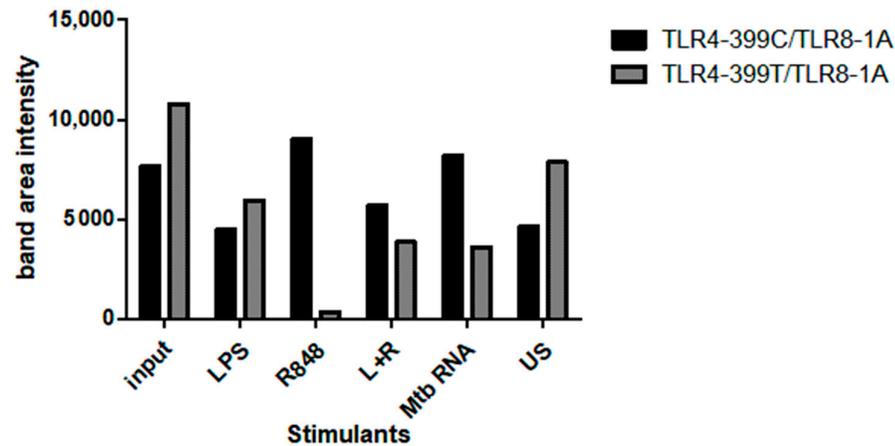
Figure 4. Co-immunoprecipitation quantification. HEK 293 cells transfected with hTLR8HA+ UNC93B1+TLR4 399C-mCherry-myc compared with hTLR8HA+UNC93B1+TLR4 399T-mCherry-myc were stimulated with LPS, R848, LPS and R848 (L+R), *Mycobacterium tuberculosis* (Mtb) RNA and unstimulated (US), followed by immunoprecipitation with anti-HA-antibody and immunoblot with anti-TLR4 antibody: In cells stimulated with R8484 and Mtb RNA, the band intensity is higher in hTLR8HA+UNC93B1+TLR4 399C-mCherry-myc transfected cells compared with hTLR8HA+UNC93B1+TLR4 399T-mCherry-myc.

2.5. Co-Localisation

HEK293/hTLR8-HA+UNC93B1 cells were transiently transfected with TLR4-mCherry-myc 399C, as well as the accessory proteins gp96, PRAT4A, CD14, and MD2, and stimulated with LPS, R848, LPS+R848, or left untreated for 2 h. Furthermore, ssRNA40 was used for stimulation, as it produced less cell stress due to easier transfection (already complexed with transfection agent) and higher stability, resulting in clearer results comparted to MTB-RNA/LyoVec. As expected, TLR4 was identified both at the outer cell membrane and the endosome, whereas TLR8 was only seen at the latter. Microscopy showed that within the endosome, TLR4 and TLR8 co-localised in all cells transfected with hTLR8HA+ UNC93B1+TLR4-mCherry-myc 399C, irrespective of the stimulant (Figure 5A–E). The number of co-localising endosomes increased in cells stimulated with LPS ($p < 0.003$) or R848 ($p < 0.001$) (Figure 6). For the combination of LPS and R848, an additive effect for co-localisation could be observed ($p < 0.001$). Additionally, cells were treated with dynasore

seen (Figure 5F). Of note, no difference in the results reported was observed for transfection without MD2 and CD14 (data not shown).
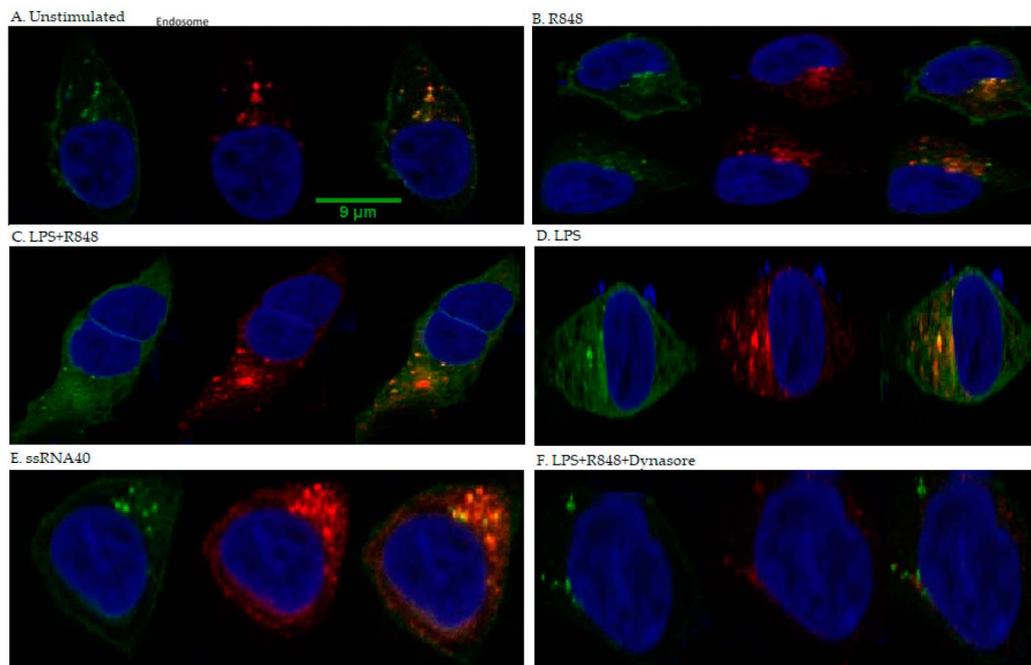
Figure 5. Confocal microscopy. Fluorescence microscopy of HEK293 cells stably transfected with TLR8-HA and transiently transfected with fluorescently tagged TLR4-mcherry along with accessory proteins gp96, PRAT4A, CD14, and MD2. Cells were (**A**) unstimulated or treated with (**B**) R848, (**C**) LPS+R848, (**D**) LPS, (**E**) ssRNA40, and (**F**) LPS+R848+Dynasore for 2 h and stained with an anti-HA Alexa 647-conjugated antibody for TLR8 and Dapi for nuclei. In the false-coloured merged image, double co-localisation of TLR4 (green) and TLR8 (red) in endosomes appears as areas of yellow (arrowhead). Scale bar 9 μm. (**F**) Inhibition of dynamin-dependent endocytosis blocked TLR4-TLR8-triggered downstream pathways by Dynasore.
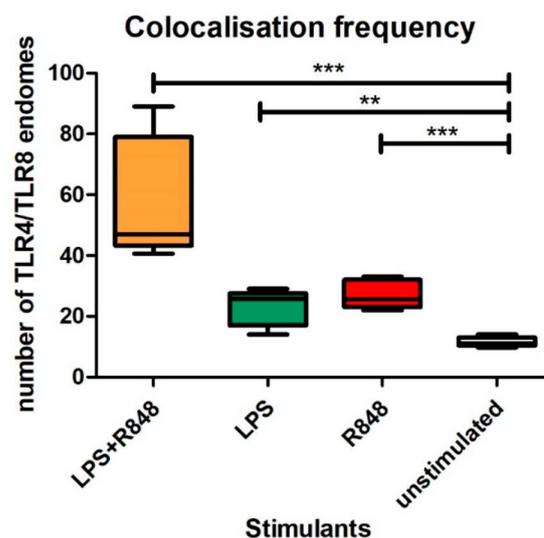

Figure 6. Co-localisation frequency. HEK293 cells stably transfected with TLR8-HA and transiently transfected with fluorescently tagged TLR4-mcherry along with accessory proteins gp96, PRAT4A, CD14 and MD2. Cells were unstimulated or treated with R848 (2 μg/mL), LPS (10 ng/mL), and LPS+R848 for 2 h. Co-localisation was observed under Leica SP5 SMD confocal microscope. The co-localisation frequency increased in cells stimulated with LPS+R848 as compared to LPS, R848, and unstimulated ($p < 0.009$, $p < 0.01$, and $p < 0.006$ respectively). ** $p \leq 0.01$, *** $p \leq 0.001$.

2.6. Functional Studies

We further explored the functional impact of the interaction of TLR4 and TLR8 using experiments with TLR-transfected HEK293-cells, including the different SNPs of interest. First, the different variants of TLR4 were transiently transfected along with MD2 and assessed for LPS responsiveness (Figure 7A). The 'wild-type' variant of TLR4-299A-399C showed the highest NF-κB induction as compared to other variants with a significant difference in comparison to 399-T ($p < 0.01$) but not 299-G ($p < 0.218$). Next, we co-transfected TLR4-variants with TLR8-1A (Figure 7B). NF-κB induction upon LPS stimulation was not detected due to a lack of MD2. Upon stimulation with R848, TLR8-1A co-transfected with TLR4-399C showed a significantly reduced NF-κB induction compared to TLR4-399T ($p < 0.007$). Adding TLR4-399C to TLR8-1A did significantly reduce NF-κB responsiveness ($p < 0.012$), while TLR4-399T failed to do so ($p < 0.148$). Adding MD2 and CD14 to the transfection of TLR4-399C and TLR8 increased LPS and decreased R848 responsiveness (Figure S5). Stimulation with LPS+R848 and mycobacterial RNA showed similar patterns (Figure S6). Of note, as a control, we transfected HEK blue cells with TLR7 and the TLR4 variants of interest and did not observe any differences upon adding TLR4 to TLR7.

To further support the interaction of TLR4 and TLR8 at the endosome, we used CLI-095, which specifically inhibits TLR4 signalling [36]. CLI-095-treated human monocyte-derived macrophages (THP cell line) showed a decreased NF-κB-response upon LPS stimulation ($p < 0.0001$) and increased NF-κB response in the presence of TLR8 ligands R848 ($p < 0.001$) and Mtb RNA ($p < 0.01$; Figure 7C). Furthermore, we blocked endosomal signalling pathways in THP cells with siRNA for MyD88, TRIF-related adaptor molecule (TRAM), or directly TLR4 (Figure 7D). Upon stimulation with TLR8-specific ligands, the NF-κB response was diminished when MyD88, TRAM, and TLR4 were silenced, which was not the case for TLR2-specific stimulation with PAM$_3$CSK$_4$. Blocking TLR8 signalling by either completely blocking the endosome through treatment with bafilomycin or siTLR8 abolished NF-κB induction, while LPS-stimulated cells did not show any difference (Figure S7). Altogether, experiments with HEK cells and THP cells indicated that the interaction of TLR4 with TLR8 diminishes NF-κB responsiveness upon TLR8 stimulation, which could be partly reversed by blocking TLR4 signalling and completely inhibited by TLR8-specific or total endosomal blockage.

Next, we analysed peripheral blood mononuclear cells (PBMCs) from healthy controls with TLR8-1A, which differed in TLR4-C399T (Figure 7E). Regarding TNFα, individuals with TLR4-399T exhibited higher levels of TNF release upon stimulation with LPS ($p < 0.001$), R848 ($p < 0.014$), or the combination of LPS and R848 ($p < 0.013$). This effect was even more pronounced when looking at IL12p40, with a remarkable difference in induction upon stimulation with both LPS and R848 ($p < 0.002$) (Figure 7F).

Finally, in order to assess different signalling pathways altered by an interaction of TLR4 and TLR8, we performed Western blotting of IRF3 from supernatants of HEK293 cells transiently transfected with different combinations of TLR4 and TLR8 variants and stimulated with TLR4- and TLR8-specific ligands (Figure 8). Transfection with TLR4, even unstimulated, led to high IRF3 expression, which was strongly increased with TLR8 co-transfection, implying that TLR4 together with TLR8 strongly activates type I IFNs, potentially even by spontaneous heterodimerisation in HEK cells. Cells double transfected with TLR4-399C and TLR8 showed higher band intensities than mono-transfected cells (Figure 9A) or cells transfected with 399T and TLR8 (Figure 9C). With TLR4-399T and TLR8, the differences were less pronounced and only in unstimulated and LPS-stimulated cells IRF3 band intensity was higher in double transfected cells (Figure 9B). Altogether, this would support our hypothesis that with TLR4-399C, the heterodimerisation is more likely than with TLR4-399T.
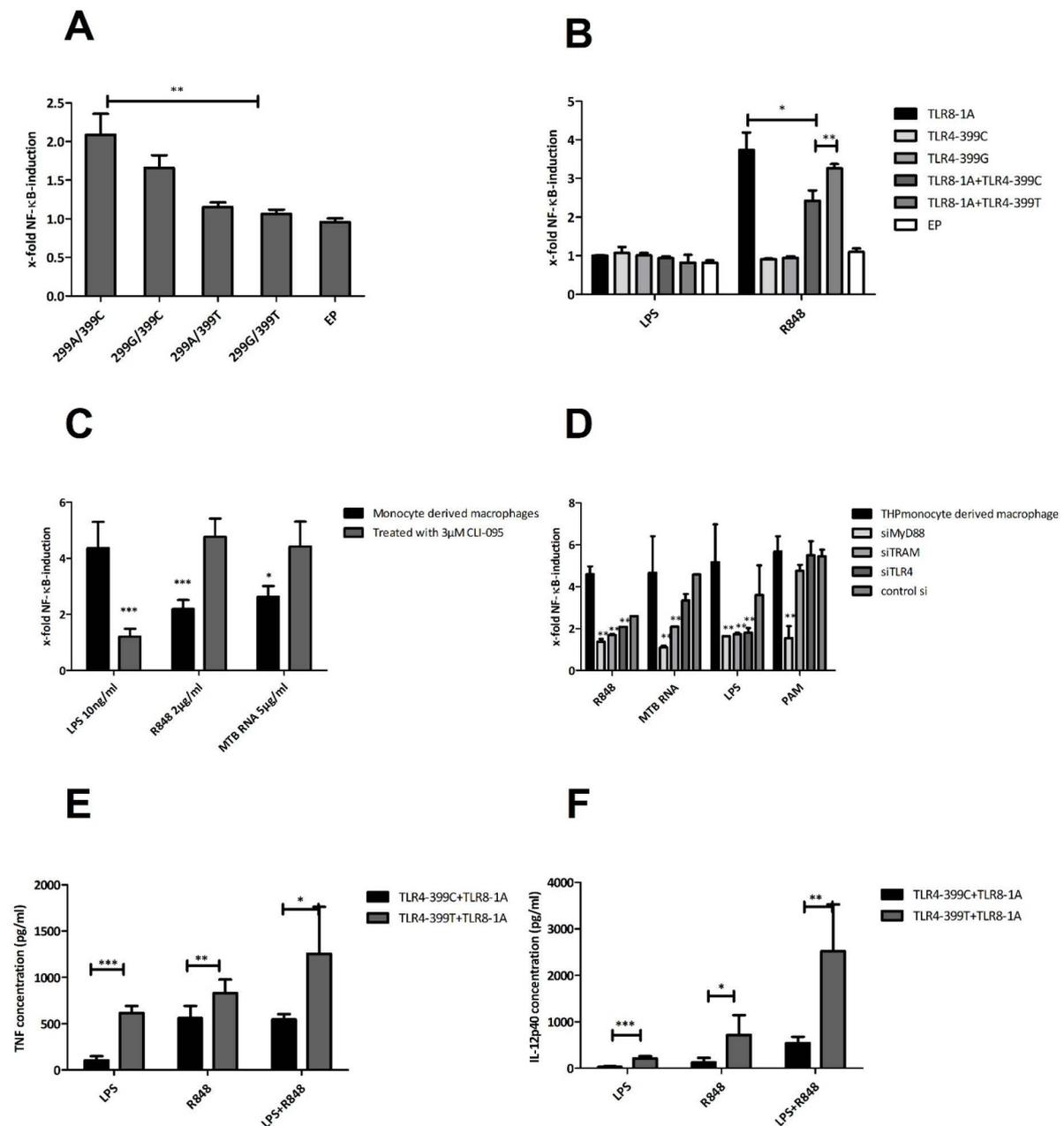
Figure 7. Functional studies within different cell lines to analyse the impact of the SNP TLR4-C399T on the interaction with TLR8. Used cells: (**A**,**B**): HEK293 blue null 1 cells, transiently transfected with TLRs or empty plasmid (EP) as indicated. (**C**,**D**): THP monocyte-derived macrophages. (**E**,**F**): Peripheral blood mononuclear cells (PBMCs) isolated from healthy homozygous volunteers that differed in their status of TLR4-399. Stimulation took place with LPS (100 ng/mL if not otherwise specified), R848 (2 μg/mL if not otherwise specified), Mtb-RNA (5 μg/mL) complexed with Lyovec or PAM$_3$CSK$_4$ as indicated for 16 h. NF-κB activation was measured by secreted embryonic alkaline phosphatase (SEAP) reporter gene assay, TNFα and IL12-p40 were measured by enzyme-linked immunosorbent assay (ELISA). (**A**) LPS responsiveness of different TLR4 SNPs: Transfection with variants human TLR4 along with human MD2. NF-κB fold induction was significantly raised in cells transfected with TLR4-299A-399C/MD2 compared to TLR4-299G-399C/MD2 ($p < 0.01$) and TLR4-299G-399T/MD2 ($p < 0.007$). (**B**) Co-transfection of TLR8 with TLR4-variants. Without accessory proteins, LPS

stimulation was insignificant. TLR8 was stimulated successfully with R848. When adding TLR4-399C, NF-κB levels were significantly lower ($p < 0.012$). The difference between TLR8-1A+TLR3-399C and TLR8-1A-399T was also significant ($p < 0.007$). TLR8-1A and TLR8-1A+TLR4-399T did not show a significantly different response ($p < 0.147$). (**C**) Inhibition of TLR4 signalling with CLI-095. THP monocyte-derived macrophages were stimulated with or without 3 μM CLI-095 (LPS at 10 ng/mL). NF-κB response in the presence of TLR8 ligand R848 ($p < 0.001$), Mtb RNA ($p < 0.01$), and LPS ($p < 0.001$) decreased (**D**) TLR signalling adaptor protein inhibition with siRNA. THP monocyte-derived macrophages were with or without silencing MyD88, TRAM, or TLR4 (LPS at 10 ng/mL, R848 at 5 mg/mL). TLR8 ligand stimulation significantly decreased in presence of siMyD88 and siTRAM ($p < 0.05$). (**E**) TNFα and (**F**) IL-12p40-levels of PBMCs. Individuals with TLR4-399T showed more tumour necrosis factor (TNF)α upon stimulation with LPS ($p < 0.001$), R848 ($p < 0.014$), and LPS+R848 ($p < 0.032$) in comparison to individuals with TLR4-399C. Regarding IL-12p40, there were significantly higher concentrations in individuals with TLR4-399T with LPS ($p < 0.001$), R848 ($p = 0.016$), and LPS+R848 ($p < 0.002$) than 399C. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.
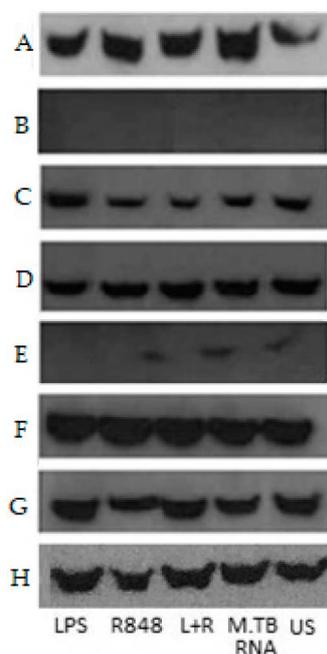

Figure 8. IRF3 Western blotting. (**A**) native HEK293 cells (**B**) HEK293 cells transfected with TLR4-399C/MD2 (**C**) HEK293 cells transfected with TLR4-399C (**D**) HEK293 cells transfected with TLR4-399T (**E**) HEK293 cells transfected with TLR8-1A (**F**) HEK293 cells transfected with TLR4-399C+TLR8-1A (**G**) HEK293 cells transfected with TLR4-399T +TLR8-1A were stimulated with LPS, R8484, LPS+R848 for 2 h and Mtb RNA (16h) or left untreated (US). (**H**) Native HEK293 cells blot with loading control–anti GAPDH antibody (≈37 kDa). IRF3 (≈55 kDa) could be identified in all double-transfected cell lysates.
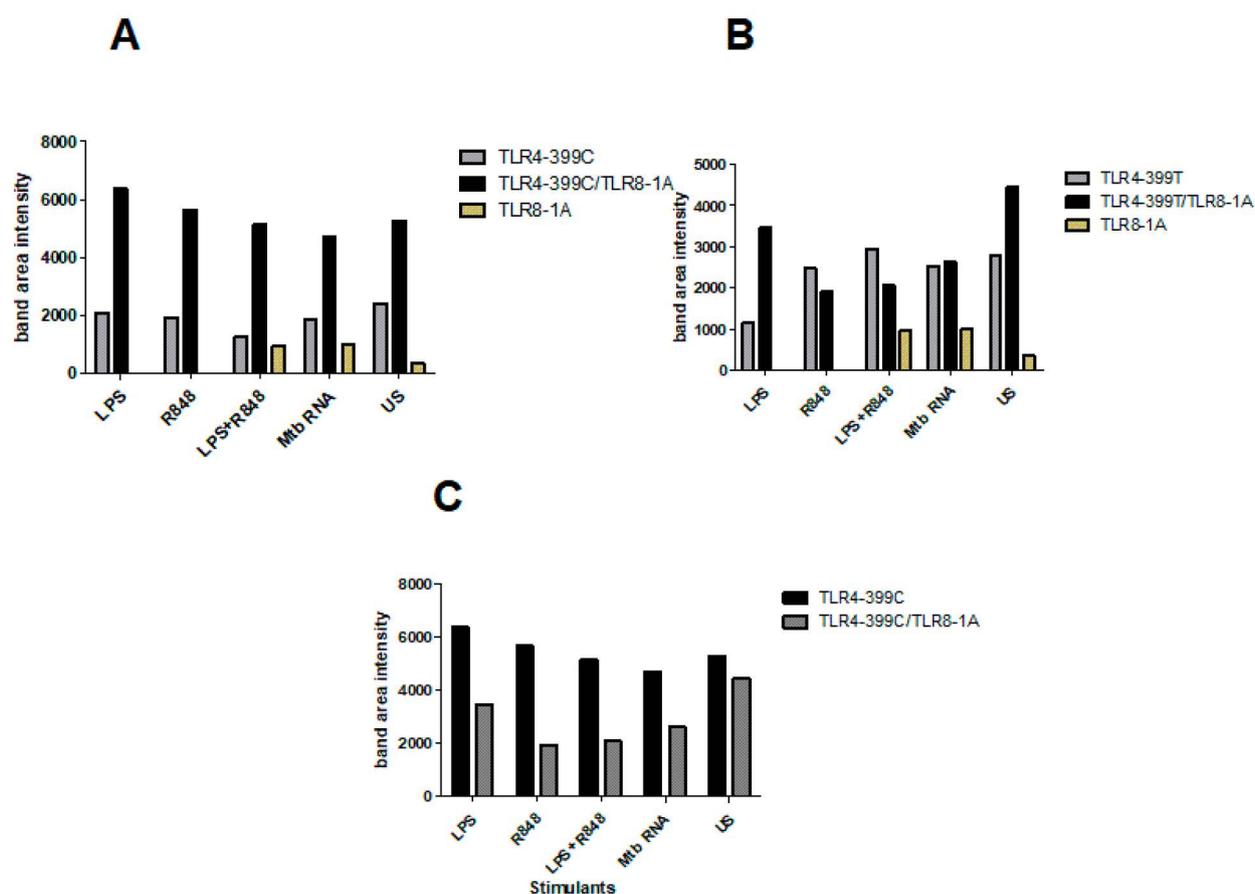
Figure 9. IRF3 Western blot quantification in HEK293 cells: (**A**) cells transfected with TLR4-399C compared with TLR4-399C+TLR8-1A cells. IRF3 band intensity is higher in TLR4-399C+TLR8-1A double transfected cells under LPS, R8484, LPS+R848, Mtb RNA stimulation and unstimulated (US) cells than in cells transfected with TLR4-399C (**B**) in cells transfected with TLR4-399T compared with TLR4-399T+TLR8-1A cells, upon LPS or in unstimulated cells, band intensity is higher in double-transfected cells, whereas with R848 with or without LPS, it was slightly less (**C**) cells transfected with TLR4-399C+TLR8-1A compared with TLR4-399T+TLR8-1A cells. IRF3 band intensity is higher in TLR4-399C+TLR8-1A cells, irrespective of stimulation.

3. Discussion

In this paper, we argue for an interaction of TLR4 and TLR8 as a heterodimer, which has functional importance for TB immunity. We came to this conclusion on the basis of (1) finding TLR4 in co-immunoprecipitated lysates of transfected HEK-cells for TLR8, particularly after R848 stimulation, (2) confirming this result with mass spectrometry, (3) seeing co-localisation with confocal microscopy, which increased upon stimulation, (4) finding a significantly enhanced susceptibility towards TB among individuals with TLR4-399T and TLR8-1A, the latter depending on the first, (5) finding evidence in modelling that TLR4-399C can form a heterodimer with TLR8 in the presence of a TLR8-ligand R848, while TLR4-399T might not, and finally (6) seeing in co-IP that with TLR4-399C, R848 stimulation induced a higher TLR4 band intensity than with TLR4-399T. Regarding the functional impact of this interaction, we found that (1) TLR4-399C showed higher NF-κB levels after LPS stimulation compared to TLR4-399T in HEK-cells, (2) in combination with TLR4, TLR8 transfected HEK cells secreted less NF-κB with TLR4-399C, but not with TLR4-399T, (3) blockage of TLR4 in monocyte-derived macrophages led to higher levels of TLR8-induced NF-κB, (4) in PBMCs, TRL4-399T led to more TNFα and IL12p40, and (5) IRF3 seems to be

enhanced spontaneously upon co-transfection of TLR4 and TLR8, possibly more so with TLR4-399C.

TLR4 has been suggested to be a main receptor involved in TB immunity by recognising mycobacterial antigens upon which a MyD88- and TRIF-dependent Th1 answer is fostered, although no clear explanation for this has been provided [10]. TLR4 involvement has been supported by mechanisms that Mtb has evolved to avoid the host immune system involving TLR4: mycobacterial anti-inflammatory proteins such as phosphatidylinositol mannosides, LM, and LAM that specifically inhibit TLR4-induced pathways or lead to TLR4-triggered immunosuppression [21,37,38]. Furthermore, Mtb is known to block the acidification and maturation of phagosomes, thereby generally inhibiting host immune receptors that require a low pH to function properly.

TLR4 polymorphisms are differently distributed around the world, and they are attributed to evolutionary pressure from infectious diseases and the migration of mankind over time. TLR4-299G without linkage with TLR4-399T can be found among African populations and is reported to be protective against malaria [39]. However, in Europe, this allele is linked with TLR4-399T [40]. We found that among the Indian population, TLR4-399T can occur as a single non-linked mutation, next to the TLR4-299G/-399T haplotype. TLR4-399T has already been associated with increased TB susceptibility [41,42]. Reduced LPS responsiveness is a known functional implication of this SNP, as we saw in HEKs. However, in THPs and PBMCs, we also surprisingly found hyperresponsiveness. These conflicting data have been reported in the literature, and recently, a mouse model with the human SNPs TLR4-299 and TLR4-399 confirmed that both SNPs contribute to cell hyporesponsiveness [43].

TLR8-1A, by being less functional than TLR-1G, is also associated with TB susceptibility [32]. What we report here, and to our knowledge for the first time, is the direct interaction at the endosomal level of TLR4 and TLR8. Our experiments show that synergy through the simultaneous stimulation of both receptors leads to higher levels of IL-12, and others have shown increased IL-12 in monocyte-derived DCs [29] and a higher expression of antigen-presenting, co-stimulatory molecules on matured DCs [30]. Crosstalk between TLRs to modulate the immune response is an established concept; for instance, studies show that the co-activation of both TLR3 and TLR8 is necessary to achieve a strong IL12p70 answer [27]. It is also known that the co-stimulation of TLR8 and -2 induces a shift towards a Th17-immunity [44]. Another example is the endosomal heterodimerisation of TLR4 and -6 in the presence of the co-receptor CD36 in responses to oxidised LDL during atherogenesis, independent of MD-2 and CD14 [45]. This signalling induced both MyD88- and TRIF-dependent genes. Similarly, in our study, we could show that the heterodimerisation of TLR4 and TLR8 activated both NF-κB- and IRF3-linked pathways.

Co-IP showed that the co-transfection of TLR4 and TLR8 lead even in unstimulated cells to the ability to precipitate TLR4 through TLR8, potentially indicating spontaneous heterodimerisation even without stimulation. Mass spectrometry of the lysed precipitates revealed that UNC93B1, a chaperone required for TLR8 endosomal trafficking, was identified alongside TLR8 [35]. For TLR4, UNC93B1 is not required. From our data, we cannot conclude whether UNC93B1 was merely pulled down alongside TLR8 homodimers or promoted heterodimerisation with TLR4, but this might be a focus of further research.

In confocal studies, we saw co-localisation upon co-transfection with an increase of co-localisation frequency even if only one receptor was stimulated. However, in contrast to co-IP, co-localisation frequency even further increased with double simulation, which was possibly due to the different read-outs and the close proximity of the receptors, not being able to distinguish between co-localisation, homo- and heterodimerisation upon stimulation.

The formation of a heterodimer in co-IP studies and confocal microscopy was observed without MD2 and CD14, although it is the established concept that TLR4/MD2/CD14/LPS is necessary for TLR4 internalisation [46]. This might be due to the experimental set-up, as, in HEK-cells, we delivered TLR4 by transfection directly to the endosome. Interestingly,

with MD2 and CD14 along the transfection for TLR4 and TLR8, no TLR4 could not be identified after the precipitation of TLR8 in co-IP-studies, and R848-induced levels of NF-κB in co-transfected HEK-cells decreased with the addition of MD2 and CD14, arguing for an inhibitory effect on the heterodimerisation of TLR4 and TLR8 by the accessory proteins, which is potentially due to the promotion of the homodimerisation of TLR4. In line with this, adding LPS to R848 in co-transfected cells decreased the intensity of the TLR4 band, which is possibly due to the formation of a homodimer of TLR4, thereby decreasing the chances of interaction with TLR8. Inhibiting the interaction of TLR4 and TLR8 with CLI-095 in THPs reversely led to an increase of TLR8-induced NF-κB-levels. In contrast to that, with siRNA, a slight decrease of the NF-κB-signal upon stimulation could be observed, which is possibly due to more cell stress due to the necessary double transfection, as well as, potentially, a less complete inhibition by siRNA compared to CLI-095.

Furthermore, in HEK cells, we could see that mere co-transfection of TLR4 and TLR8 led to an expression of IRF3, which activated the type I IFN axis. This might explain how TLR4 could negatively regulate NF-κB induction by TLR8 activation, namely by shifting the balance from the NF-κB towards the type I IFN pathway. This might also explain why the blockage of TLR4 enhanced NF-κB induction by TLR8 activation.

The most pronounced difference between the TLR4 variants in co-IP was that TLR4-399C, the variant identified as more prone towards heterodimer formation, when undergoing co-transfection with TLR8, showed increased TLR4 band intensity after TLR8 stimulation with R848 compared to both LPS and unstimulated cells. In contrast to that, with TLR4-399T, R848-stimulated precipitation of TLR4 was decreased as compared to after LPS-stimulated and in unstimulated cells, further supporting the notion that the interaction of TLR4 and TLR8 is impaired with the nucleotide change from C to T. For Mtb RNA, the same trend was observed, although to a lesser extent. This might be because stimulation with RNA requires another transfection medium, thereby increasing cell stress, potentially resulting in reduced reactivity. Another reason might be that modelling actually identified R848 as the ligand promoting heterodimerisation, resulting in higher band intensities in our experiments.

In functional studies, we could show that the combination of TLR4-399T and TLR8-1A led to increased NF-κB, TNFα, and IL12p40 levels in PBMCs and THPs upon stimulation with TLR8 ligands in comparison to TLR4-399C, although both TLR4-399T and TLR8-1A individually are each the less functional variants of the SNP. Based on modelling data, with TLR4-399C, heterodimerisation is more likely to occur, possibly leading to more activation of IRF3, thus potentially leading to more type I IFNs but less direct activation of the NF-κB axis. With TLR4-399T, this effect is hindered, thereby producing more NF-κB. Keeping this rationale in mind, we propose that both loss-of-function alleles of TLR8 and -4 convey susceptibility towards TB by altering the balance of the NF-κB and type I IFN axes, possibly more pronouncedly reducing the latter, and that this interaction plays a crucial role in a successful host response against Mtb.

Eliminating TB is a set goal by the WHO by 2030 [47]. In order to achieve this goal, novel intervention strategies are needed, which will be based on a complete understanding of the pathophysiology. Furthermore, individual risk stratification will be important to improve prevention strategies. Therefore, the interaction of TLR4 and TLR8, by offering new treatment targets and understanding individual progression risk, might contribute to eliminating TB in the future. This is particularly needed in the face of increasing incidence of multi-drug resistant TB.

Implications for the importance of TLR4 and TLR8 interaction might be found beyond TB. Endosomal TLRs recognising RNA such as TLR8 play an important role in viral diseases. Regarding the current SARS-CoV-2 outbreak, i.e., it has been suggested that SARS-Cov-2 contains more RNA sequences recognisable by TLR7/8 than SARS-CoV-1, and by that potentially causing more frequently a hyperinflammatory syndrome [48]. Similarly, there have been studies claiming an important role of TLR4 in SARS-CoV-2, as in silico studies identified TLR4 as very likely to respond to spike proteins of SARS-

CoV-2 [49]. Interestingly, TLR4 is also associated with cardiometabolic comorbidities such as obesity and hypertension, which are known risk factors for severe COVID with hyperinflammation [50]. Furthermore, TLR4-deficient mice were less susceptible to acute respiratory distress syndrome (ARDS) upon inhalation trauma [51].

Taken together, our data suggest that TLR4 and TLR8 form a heterodimer changing the immune response towards a Th1 balance. Mutations leading to a loss of function of this specific pathway seem to convey susceptibility towards TB. Thus, the interaction of TLR4 and TLR8 might open up new targets for vaccines or therapeutic drugs. Finally, genetic risk stratification may lead to better prevention strategies of individuals at increased risk.

4. Materials and Methods

4.1. Study Subjects

The cohort of TB patients and controls in Hyderabad has been described before [34]. In brief, the cohort consisted of 346 TB patients with either PTB, EPTB or a relapse, and 301 Controls (HC) including healthy household contacts (HHC). Patients, who attended Free Chest TB Clinic with directly observed treatment surveillance (DOTS) at Mahavir Hospital and Research Centre, Hyderabad, were confirmed with the sputum microscopy for acid-fast bacilli, culture, and chest X-ray or histopathology as per the guidelines of the Revised National Tuberculosis Control Program (RNTCP). Patients with diabetes, hypertension, HIV, and other comorbidities were excluded from the study. Informed consent was obtained from all subjects. The study was approved by the institutional ethics committee of Bhagwan Mahavir Medical Research Centre (BMMRC), Hyderabad, and Charité Medical University Berlin. The German cohort consisted of 853 volunteers, as described earlier [52]. All studies followed the ethical principles of the declaration of Helsinki.

4.2. SNP Analysis

Genomic DNA was extracted from whole blood of TB patients and healthy volunteers using a DNA Blood mini kit (Qiagen GmbH, Hilden, Germany) or from buccal swabs using a DNA kit (Qiagen) according to the manufacturer's protocol. Quantity of DNA was confirmed by NanoDrop and DNA was stored at $-20\,^{\circ}$C. Functionally relevant SNPs were analysed using Light Cycler Assays (Roche) based on the differentiation of fluorescence signals due to nucleic acid differences and the respective melting curves. Primers used are found in Table S6.

4.3. Modelling and Molecular Docking

Homology model of the human TLR4 with threonine at 399 was determined, using the crystal structure of TLR4 (PDB ID: 4G8A) as a template with MODELLER [53]. The structure 4G8A had isoleucine (I) in position 399. Refinement and quality estimation of the model was carried out using Swiss PDB viewer [54] and SAVES server (https://servicesn.mbi.ucla.edu/SAVES/). The structure of TLR8 (PDB ID: 3W3M) with resiquimod (R848) ligand was obtained from the PDB database (www.rcsb.org). Molecular docking was implemented using PatchDock [55] and FireDock [56]. In this process, transformations of docking elements obtained from PatchDock were given as an input to FireDock. Firedock initially performs coarse refinement followed by refinements and energy-based rankings. Next, it implements chain optimisation to reduce steric clashes [56]. The generated model of TLR4 having threonine (position 399) and the structure TLR8 was used as inputs during docking. Similarly, in another study, the structure of TLR4 (I399) was considered to find out if it formed a dimer with TLR8 in the presence of the ligand R848. The interacting residues were visualised with LigPlot v2.

4.4. In Vitro Experiments

4.4.1. Stimulants and Reagents

The stimulants LPS, R848, ssRNA40, and PAM$_3$CSK$_4$ and the antagonists bafilomycin, polymyxin B (PMB), dynasore, and CLI-095 were purchased from Invivogen (Toulouse, France). The concentration of above stimulants and antagonists were standardised as LPS (10 ng/mL), R848 (2 µg/mL), ssRNA40 (5 µg/mL), PAM$_3$CSK$_4$ (2 µg/mL), bafilomycin (1 µM), PMB (10 µg/mL), Dynasore (50 µM), and CLI-095 (3 µM). Mycobacterial RNA was extracted from gamma-irradiated Mycobacterium tuberculosis H37Rv (BEI Resources, NR-14819) with InnuPrep RNA Mini Kit (Analytik Jena, Germany). Purity was confirmed by Scandrop analysis (Analytik Jena). A 260 nm/280 nm extinction quotient of 1.9-2.0 was considered pure. For transfection, if not otherwise specified, LyoVec (Invivogen) was used in a 3 µg/100 µL dilution according to protocol.

4.4.2. Mutagenesis

hTLR8-pUno3 and hTLR4-pUno3 plasmid were purchased from Invivogen and hTLR4 mcherry-myc. All these plasmids were mutated with QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Genes, Frankfurt am Main, Germany) according to user's manual using the primers designed with primerX software (Table S7). Maxi Prep of mutated and original plasmid was performed with Plasmid Maxi Kit (Qiagen, Hilden, Germany). Successful mutation was confirmed by Value Read sequencing (Eurofins, Ebersberg, Germany), using the primer 5′CTGTAGTCGACGATTGCTGC3′ for TLR8 and 5′AGGTAAATGAGGTTTCTGAGTGA3′ for TLR4 designed with Primer3 software.

4.4.3. Cell Line Experiments

THP NF-κB (Invivogen) cells were harvested in RPMI 1640 + 10% FCS + 100 µg/mL blasticidin. Cells were counted and plated on 96-well plates with 1×10^5 cells/well in 150 µL of RPMI 1640 + 10% FCS Medium. The cells were differentiated to macrophages by using PMA (50 ng/mL) 3 h prior to transient transfection. Cells were treated with or without bafilomycin, PMB, dynasore, CL-095 1 h prior to stimulation of R848, LPS, or Mtb RNA/Lyovec for 18–24 h. Then, 20 µL of supernatant were transferred to QUANTI-Blue detection medium (Invivogen) for SEAP estimation at 620 nm absorbance, corresponding with NF-κB-activity.

Hek Blue Null 1 (Invivogen) cells were harvested in Dulbecco's Modified Eagle's Medium (DMEM) + 10% fetal calf serum (FCS) + 100 µg/mL zeocin. 1.5×10^5 cells were distributed in T25-flasks and cultured overnight before transient transfection. After 48 h, cells were counted and plated on 96-well plates with 5×10^4 cells/well in 160 µL of Hek Blue Detection Medium (Invivogen). For stimulation, LPS, R848, a combination of LPS/R848 or mycobacterial RNA/LyoVec was added, making up to 200 µL well volume. After 16 h (unless specified) of stimulation, SEAP levels were measured at 620 nm absorbance each value was normalised to the respective negative control (PBS). HEK 293XL hTLR8 HA cells overexpressing UNC93B1-mCitrine cells were cultured in 24-well plates prior to transient transfection [35].

Note: We have confirmed that there was no LPS contamination in the TLR8 ligands by treating THP monocyte-derived macrophages with PMB specifically blocking LPS stimulation by binding to lipid A of LPS (Figure S8).

4.4.4. Transient Transfection

For Co-IP and confocal microscopy, HEK 293XL hTLR8 HA cells were used. HEK 293XL hTLR8 HA transiently transfected using Extreme Gene 9 (Roche) in a 1:3 ratio according to protocol for 24 h. The following plasmids were used for transient transfection: hTLR4 mCherry-myc and its variant forms, with and without the accessory proteins gp96, PRAT4A, CD14, and MD2 as indicated, and empty plasmid (pUno3). All the plasmid combinations were attained to a final concentration of 3 µg. Regarding the additional set of experiments with monkey TLR4 plasmids, pEF1a_rhesus TLR4 N-FLAG IRES DsRed

Express2 and pEF1a_sooty mangabey TLR4 N-FLAG IRES DsRed Express2 (provided by Prof. Dr. Sauter, Ulm) were performed as described above. For functional studies with HEK Blue Null 1 cells, transient transfection as described above was performed with and without MD2 and CD14 as indicated.

THP NF-κB: The cells were transfected using the Amaxa Nucleofector (Amaxa, Cologne, Germany) according to the manufacturer's protocol (Cell Line Nucleofector Kit V, Program T-08) with 2 μg DNA/10^6 cells, psiRNA TLR8, psiRNA TLR4, psiRNA MyD88 or psiRNA Ticam2 (plasmid-based siRNA designed by Invivogen).

4.4.5. Immunofluorescence Staining of TLR8HA/Confocal Microscopy

First, 1×10^5 HEK 293XL hTLR8-HA UNC93B1-mCitrine cells/well were seeded in imaging dishes (Ibidi), which was followed by transfection including accessory proteins gp96, PRAT4A, CD14, and MD2 for 48 h and stimulation with various ligands for 2 h. TLR8-HA was stained with anti-HA antibody (Sigma Aldrich, Munich, Germany) at a dilution of 1:200 in PBS containing 1% (*w/v*) bovine serum albumin (BSA) for 1 h at room temperature. Specimens were washed three times with PBS and incubated with anti-rabbit Alexa647 antibody diluted 1:2000 in PBS containing 1% (*w/v*) BSA for 30 min at room temperature. TLR4 plasmid has mCherry fluroprobe. Then, cells were imaged on a Leica SP5 AOBS with SMD confocal microscope, with a 63×, NA 1.20 water-immersion objective, at a lateral resolution of 120 nm. Cell Profiler and Fiji software were used to analyse the co-alocalisation, which was defined as a spatial overlap of fluorescent TLR4- and TLR8-labels indicated by yellow dots [57].

4.4.6. Co-Immunoprecipitation

Per condition, 5 million cells were lysed in 250 μL NP40 lysis buffer for 30–60 min on ice. Lysates were collected by centrifuging at 4000× *g* for 5 min at 4 °C. Then, 30 μL of lysate was saved as a control, and the rest of the lysate was used for IP. IP was performed with HA agarose beads/anti-FLAG-M2 affinity gel (Sigma Aldrich, Munich, Germany) according to the manual. Per IP, 50 μL of the 1:1 suspension of the anti-HA agarose was used, and IP was performed for 2 h at 4 °C shaking. After the last wash, 30 μL 2× Lämmli was added. The lysate collected before IP (Input) served as a positive control, for negative control (NC) lysis buffer without antibody was used.

4.4.7. Western Blot Procedure

Cell lysates were separated by SDS-PAGE and blotted. Membranes were first exposed to Abs specific for anti-TLR4, anti-HA, anti-FLAG, anti-IRF3, and anti-GAPDH (Santa Cruz Biotechnology, Heidelberg, Germany) and subsequently incubated with secondary Abs. Proteins were detected using electrochemiluminescence (ECL) (32106 Pierce™ ECL Western Blotting Substrate). The band intensities were quantified using image J.

4.5. Mass Spectrometry (MS)

4.5.1. Sample Preparation

Eluted proteins from IPs (1% SDS in PBS) were reduced with 50 mM of Dithiothreitol (5 min, 95 °C) and diluted with 8 M Urea in 100 mM Tris/HCl pH = 8.0. Buffer exchange and protein digestion was done according to the filter-aided sample preparation protocol [58]. In brief, the reduced proteins were transferred to a 30 kDa Microcon filter unit (YM-30 filter units, Millipore) and centrifuged at 14.000× *g* for 20 min in all consecutive steps, and the flow-through discarded. For washing, 200 μL urea buffer (8 M Urea, 100 mM Tris HCL, pH 8.0) was added, and the centrifugation was repeated. Then, 100 μL of alkylation solution (0.1 M iodoacetamide in urea buffer) was added, and samples were incubated for 20 min in the dark. The alkylation solution was removed by centrifugation followed by two additional centrifugation steps with 200 μL 8 M urea buffer. Afterwards, samples were washed and centrifuged twice with 200 μL 50 mM ammonium bicarbonate buffer. Proteins were digested by the addition of 0.5 μg trypsin in 50 μL digestion buffer (50 mM

ammonium bicarbonate). Proteolytic cleavage was allowed for 16 h at 37 °C, and peptides eluted by centrifugation. To collect residual peptides, the centrifugation was repeated twice after the addition of 50 μL ammonium bicarbonate buffer (50 mM). Eluted peptides were dried in a SpeedVac (Thermo Fisher) and reconstituted by adding 20 μL of 0.3% formic acid in water.

4.5.2. Mass Spectrometric and Statistical Analysis

Tryptic peptides were analysed with a Dionex UHPLC (Thermo Scientific) coupled to an Orbitrap Fusion LC-MS/MS system (Thermo Scientific). Full mass spectrometry scans were acquired in the Orbitrap (m/z range 370–1570, quadrupole isolation) at a resolution of 120,000 (full width at half maximum) during a 60 min, non-linear gradient from 2 to 90% acetonitrile/0.1% formic acid. Peptides were fragmented by higher-energy collisional dissociation (HCD, 30% collision energy) and maximum 10 fragment ion spectra were acquired per cycle in the Orbitrap analyser at a resolution of 15,000 using quadrupole isolation (m/z window 1.6). The following conditions were used: spray voltage of 2.1 kV, heated capillary temperature of 275 °C, S-lens RF level of 60%, a maximum automatic gain control (AGC) value of 4×10^5 counts for MS1 with a maximum ion injection time of 50 ms and a maximum AGC value of 5×10^4 for MS2, with a maximum ion accumulation time of 45 ms. A dynamic mass exclusion time window of 5 s was set with a 10 ppm maximum mass window.

All raw files were searched against the human UniProt database (version 05.2016, reviewed sequences) with MaxQuant version 1.5.5.1 (Max Planck Institute of Biochemistry, Germany) [59]. The default parameters were used or set as follows: first search peptide tolerance: 20 ppm, main search peptide tolerance: 4.5 ppm (for MaxQuant); enzyme: trypsin, max. 2 missed cleavages; static modification: carbamidomethylation of cysteine residues; variable modifications: methionine oxidation; min. peptide length: 6, max. peptide mass: 7600 Da. Normalisation was omitted and Label-Free Quantification (LFQ) min. ratio count was set to 1 (unique and razor peptides). Peptide specific match (PSM) and protein false discovery rate was set to 0.01. Label-Free Quantification (LFQ) values of all samples were loaded into Perseus (version 1.5.5.0) [60]. Groups were created, with 6 samples per group: (a) TLR4 with and without stimulation, (b) TLR8+TLR4 with and without stimulation, (c) TLR8 with and without stimulation. The resulting matrix was reduced as proteins were identified as "possible contamination" or "only identified per site", while "reverse identified proteins" and "identified in less than 2 samples per group" were discarded. LFQ values were log2-transformed, and missing values imputed by default parameters. The negative logarithmic difference of the means of protein intensities of MS was plotted against the *p*-values from respective *t*-tests.

4.6. PBMC Experiments

PBMCs from individuals of the Indian cohort differing in their genotype of TLR8-M1V and TLR4-T399I were isolated with lymphocyte separation medium (LSM 1077 GE Healthcare) according to the user's manual, seeded at 3×10^5 cells per well on 96-well plates in RPMI 1640 + 10% FCS and left overnight at 370 in a humidified incubator with 5% CO2. Then, PBMCs were stimulated with LPS, R848, a combination of LPS and R848 or mycobacterial RNA/LyoVec. Cytokines were analysed in the supernatants by enzyme-linked immunoassays according to the respective standard manufacturer's recommendations. TNFα levels were determined after 4 h (BD Pharmingen: 551220, 554511), and IL-12p40 (BD Biosciences) levels were measured after 24 h post-stimulation.

4.7. Statistical Analysis

Statistical analysis was performed using STATA 16. Descriptive characteristics were obtained, and for the assessment of differences of basic characteristics, *t*-tests for continuous variables and chi-square for categorical data tests were used. For assessing the odds associated with a specific allele, genotypes were dichotomised, summarising minor alleles,

and a logistic regression model was used; 95% confidence intervals are given in square brackets. The main outcome was being a TB patient or a relapse, depending on the context. Gender and age were included a priori, as was BCG status whenever a TLR8-SNP was assessed, based on previous reports. Other variables were included according to the evidence for confounding based on comparison of crude and adjusted OR, using Wald's test. Final significance testing and tests for effect modification were based on likelihood ratio tests (LRTs). For analysis of functional, Prism (Version 5.01) was used, performing either Mann–Whitney U or T-tests, as appropriate. For analysis of mass spectrometry, Persus software was used.

Abbreviations

A	Adenine
APC	Antigen-presenting cell
ARDS	acute respiratory distress syndrome
BCG	Bacillus Calmette–Guérin
BMI	Body mass index
BSA	Bovine serum albumin
C	Cytosine
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme-linked immunosorbent assay

EPTB	Extrapulmonary TB
FCS	Fetal calf serum
G	Guanine
HA	Human influenza hemagglutinin
HC	Healthy control
HEK	Human embryonic kidney
HHC	Healthy household contact
IFN	Interferon
IL	Interleukin
IP	Immunoprecipitation
IRF	Interferon response factor
LAM	Lipoarabinomannan
LM	Lipomannan
LPS	Lipopolysaccharide
LRT	Likelihood ratio test
MHC	Major histocompatibility complex
Mtb	Mycobacerium tuberculosis
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen-associated molecular pattern
PMB	Polymyxin B
PRR	Pattern recognition receptor
PTB	Pulmonary TB
SEAP	Secreted embryonic alkaline phosphatase
T	Thymine
TB	Tuberculosis
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
WB	Western blotting

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3.2   A SNP upstream of the cyclic GMP-AMP synthase (cGAS) gene protects from relapse and extra-pulmonary TB and relates to BCG vaccination status in an Indian cohort

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## 4.	Discussion

Nucleic acids from pathogens play an important role as ligands as the immune system is designed to respond effectively against invading pathogens. PRRs recognizing nucleic acids can be divided as endosomal PRRs and cytosolic PRRs based on their location. Both classes of PRRs are essential to generate protective immune responses (Barrat et al. 2016). Endosomal PRRs are TLRs 3, -7, -8, and -9. While the cytoplasmic PRRs are retinoic acid-inducible gene I (RIG I) like receptors (RLRs), melanoma differentiation-associated protein 5 (MDA5), cGAS, STING and absent in melanoma 2 (AIM2).

Endosomal TLR activation by their ligands such as double-stranded (ds) RNA (TLR3), single-stranded (ss) RNA (TLR7 and -8), or unmethylated CpG containing ssDNA (TLR9) requires cleavage of their ectodomains by cathepsins for further signal transduction (Fukui et al. 2018). This mechanism is in place to distinguish self- and non-self-nucleic acids. However the outcome of endosomal TLR activation (uncontrolled activation may be detrimental to the host in chronic infections, autoimmune diseases and cancer) may depend on TLR expression, distribution, proteolysis in the endosomal compartments, dose and time of interaction with the receptor and genetic variations of TLRs modulate the downstream signaling through conformational changes (Andon et al. 2022).

M.tb RNA activates endosomal TLR3 and triggers IL-10 production which downregulates IL-12 p40 production leading to disease activation (Bai et al. 2014). TLR7 & -8 are also activated by *M.tb* RNA, however the latter is present on monocyte/macrophages and triggers IL-18, IFNγ, IL12p70, and type I IFNs, which helps in effective elimination of bacteria (Bao et al. 2017; Keegan et al. 2018).

Apart from these resident endosomal TLRs, TLR4 also signals through the endosome via the TRIF/TRAM pathway. TLR4 at the endosome activates IRF3 to induce type I IFNs as well as NF-κB to induce inflammatory cytokines. Both IRF3 and NF-κB activation regulate each other based on the type of infection, however, the underlying mechanism is not completely elucidated. Endosomal TLR4 activation upon *M.tb* challenge leads to DC maturation, induces Th polarization, and helps in bacterial elimination (Sepehri et al. 2019).

In the cytoplasm MDA5 and RIG1 can detect dsRNAs. cGAS and STING identifies dsDNAs and cyclic dinucleotides (CDN) leading to type I IFN responses (Burdette et al. 2011; Sun et al. 2013). Another cytosolic sensor is AIM2 also detecting dsDNA by forming the inflammasome complex with apoptosis-associated speck-like protein (ASC) and caspase-1. This complex eliminates the pathogen by secreting pro-inflammatory cytokines IL-1 and IL-18 and induce pyroptosis, a form of inflammatory cell death (Latz et al. 2013). The AIM2 inflammasome fails to distinguish between microbial and cellular dsDNAs and promotes the development of autoimmune disorders (Kumari et al. 2020). *M.tb* DNA activates endosomal TLR9 (through CpG sites), AIM2 and cGAS/STING to promote bacterial clearance.

In this study we focussed on endosomal TLRs:

(i) TLR4, as *M.tb* doesn't contain LPS or other PAMPs interacting with TLR4 and is not clear how *M.tb* activates TLR4. We hypothesize that TLR4 gets endocytosed to form a heterodimer with TLR8 to recognize the TLR8 ligands such as ss RNA and its analogues.

(ii) TLR8, as bacterial RNA has been shown to stimulate TLR8 and

(iii) Cytosolic receptors: cGAS/STING for association studies with tuberculosis.

4.1 SNP Association studies

4.1.1 TLR4 SNP analysis

In this study we compared haplotypes of TLR4 399C/T and TLR4 299A/G among Indian and German healthy cohort and found that the SNPs were not in complete linkage disequilibrium in the Indian cohort (cosegregation is only 73%, D' = 0.687), unlike Caucasians (cosegregation is 98%, D' = 0.999). Similar to that of previous studies, where it was reported D' = 0.8 in healthy Indian population (Najmi et al. 2010) and D' = 0.82 in Chinese population (Wu et al. 2015). The difference in the prevalence of TLR4 haplotypes across different ethnicities may be due to local infectious pressure and the migration of populations (Ferwerda et al. 2007; Plantinga et al. 2012).

We found TLR4 399T was significantly associated with disease susceptibility in TB patients compared to controls (OR=1.57 [1.04-2.36]; p<0.027), however this effect of the SNP is independent of the BCG vaccination status. We failed to observe any impact of allele distribution neither on the site of manifestation (PTB or EPTB) nor on the reactivation status (among relapse cases). As evident from cosegregation analyses, we did not find any association with TLR4 299A/G SNP between TB patients and controls unlike TLR4 399C/T SNP.

In line with this study, in a Saudi population (Fouad et al. 2019) 399T was found to be associated with risk in PTB and EPTB in a Kashmiri population (Wani et al. 2021). Similar results were obtained in a Chinese population with PTB (Wu et al. 2015) contradicting a Southern Chinese study, where 299 G was associated with increased risk of TB (Chen et al. 2022). In a Brazilian study 399T was associated with TST conversion and development of active disease (Cubillos-Angulo et al. 2019). In an Iranian population 399T along with 299G SNPs were associated with susceptibility to PTB (Jafari et al. 2016). The homozygous state of these mutations (299 GG, 399 TT) was significantly associated with severity of PTB in Asian Indian populations (Najmi et al. 2010). Contradicting to these studies in a Mexican population, these two SNPs were associated with decreased risk of active TB (Ortega et al. 2020). Two meta-analysis studies performed in 2015, found that TLR4 399T mutation is associated with disease susceptibility in an African subpopulation (Zhao et al. 2015) and in Asian subpopulations (Schurz et al. 2015). A current meta-analysis shows TLR2 299G mutation to be a risk factor for developing PTB (Muheremu et al. 2022). However, in Gambian (Newport et al. 2004), Mexican (Rosas-Taraco et al. 2007), Colombian (Sanchez et al. 2012), South Indian (Selvaraj et al. 2010) and in South-eastern Chinese population (Xue et al. 2010), there was no association of TLR4 SNPs with TB susceptibility. These two SNPs were found to be associated with various diseases (reviewed in (Schroder and Schumann 2005; Mukherjee et al. 2019).

Both TLR4 299A/G and 399C/T mutations are present in the extracellular domain of the receptor between the LRR10 and -14 regions. These SNPs were predicted to affect a ligand-binding region and MD2 binding region, respectively (White et al. 2003). However, there were no conformational changes of these SNPs observed compared to that of the wildtype (Ohto et al. 2012) in line with our own TLR4 modelling.

4.1.2 TLR8 SNP analysis

We have previously shown that TLR8-1A was associated with susceptibility towards being a TB case (OR=1.68 [1.08-3.63]; p<0.022), with weak evidence for an interaction between BCG and TLR8-1A (p<0.071). In addition, we found that there was strong evidence for an increased risk for being a relapse case associated with TLR8-1A (OR=1.99 [1.03-3.82]; p<0.006). Little

is known about individual genetic risk factors that could influence TB reactivation and to our knowledge no clear correlation with a SNP of PRRs has been found in terms of containing the disease as we show here. The replacement of A nucleotide with G increases the flexibility of the protein and probably improves the receptor's ability to adapt to side-chain rearrangement and dimerization (Ugolini et al. 2018). Previous reports in line with this study showed TLR8 1A to be associated with susceptibility to PTB in males in Russian, Indonesian populations, Turkish male children, South African population, Kazakhstan population and Moldavian population (Davila et al. 2008; Dalgic et al. 2011; Yerezhepov et al. 2014; Salie et al. 2015; Varzari et al. 2019). However, in Pakistani and Chinese Han population TLR8 1A associated with reduced risk of getting disease (Bukhari et al. 2015; Wang et al. 2018). Kobayashi et al., and Chimusa et al., did not report any association of this SNP with TB susceptibility (Kobayashi et al. 2012; Chimusa et al. 2014).

It has been shown that TLR7/8 recognizes viable bacteria and induces MyD88-dependent signalling (Gidon et al. 2017). TLR8 knockdown in a THP-1 cell line led to an increase in apoptosis upon BCG infection (Tang et al. 2016). TLR8 also activates neutrophil leading to the production of IL-6, TNF, and IL-23, promoting Th17 differentiation (Tamassia et al. 2019). The fate of *M.tb* RNA in host cells has been recently reviewed (Burkert and Schumann 2020).

4.1.3 Gene-gene interaction

Interestingly, the susceptibility conveyed by TLR8-1A was seen in individuals carrying the TLR4-399T allele (OR= 1.97 [1.15-3.37], p<0.013); among homozygote TLR4-399C individuals, no impact of TLR8-1A on TB disease was observed. In relapse cases, the same pattern as above was observed, however with only weak evidence (OR=2.90 [0.87-9.59], p<0.069). It has been previously shown that gene-gene interactions of TLR4 and IFNGR1 with NOS2A were associated with TB susceptibility (Azad et al. 2012). Nevertheless, this observation led us to suspect that there might be an interaction on the molecular level between TLR4 and -8, and we further investigated this in different in-vitro systems.

4.1.4 cGAS/STING SNP analysis

Intracellular bacteria (Such as *M.tb*) can activate cGAS indirectly by inducing cellular stress by releasing mitochondrial DNA (mtDNA) into the cytoplasm leading to autophagic cell death (Wiens and Ernst 2016). It was reported that cGAS/STING/TBK1/IRF3 activates bone marrow derived dendritic cells (BMDCs) upon *M.bovis* infection to induce type I IFN production, which was necessary for T cell proliferation, IL-10-, IL-6- and IL-12p70-induction (Li et al. 2019). Previously it was argued that type I IFN promotes intracellular *M.tb* growth but helps in regulating Th1 cell differentiation. cGAS/STING upon dsDNA recognition initiates antimicrobial and inflammatory signaling pathways producing type I interferon (IFN-I) (Stanley et al. 2007; Sun et al. 2013). NLRP3/AIM2 inflammasome activation by cytosolic DNA leads to the production of IL-1β and IL-18 (Dorhoi et al. 2012; Kupz et al. 2016). These pathways partially counteract each other in restricting or developing disease depending on the host immune status (Wassermann et al. 2015; Labzin et al. 2016).

We found a significantly different distribution of allelic frequencies of rs311686_G, STING230_C and STING293_T among Indian and German healthy controls. As explained earlier the importance of this shift over time may be due to the selective pressure for local infections over time. cGAS SNPs were in moderate Linkage Disequilibrium, whereas STING SNPs were strongly linked and hence we investigated STING 230 SNP for further analysis.

4.1.5 cGAS SNP analysis

We found several lines of evidence pointing towards protectivity of the G-Allele for rs311686 SNP: In primary TB patients and in HHC (household contact) the G-Allele frequency was higher than in the HC (unrelated healthy control). HHC were either related or living in the surrounding areas of patients, thus comparison of HHC and TB can largely rule out confounding factors. An interesting aspect is that in the unvaccinated sub cohort, primary TB patients had lower G-allele frequency compared to HHC which argues for a potential protective effect originating from the mutation, which might be masked by BCG. Strikingly, comparing PTB and EPTB patients, the frequency of the G-allele is lower in EPTB, and even lower in relapse cases. From our results, we can speculate on an impact for more contained disease modulated by the G-Allele, which helps against dissemination as well as developing a relapse, in combination with an immunological memory. However, BCG does not have the ESX-1 locus, which helps in translocation of bacteria into cytosol, activation of the cGAS/STING pathway induced by the vaccination seems unlikely. However, we see an interaction with the SNP in the unvaccinated group, which would argue that it rather acts upon re-infection, when a memory was already built, and might regulate the efficacy of reactivation. Dissemination, at least to the lymph node, would be necessary to activate central memory T cells but also supports spreading of *M.tb*, thus a possible explanation of stronger impact of the SNP on EPTB could be proposed. As reported in a study, where a low-virulence, ESX-1 engineered with recombinant BCG induced the cGas/STING/TBK1/IRF-3/type I interferon axis and AIM2 and NLRP3 inflammasome, released higher proportions of CD8+ T cell effectors specific to BCG and polyfunctional CD4+ Th1 cells specific to ESX-1 locus (Groschel et al. 2017). In another study it was reported that the loss of the functional variant in the NLRC4/IL18 axis was unable to effectively contain mycobacteria within granulomas, leading to eventual extra-pulmonary dissemination (Souza De Lima et al. 2020). We did not find any structural conformational changes of cGAS regarding rs311686 SNP, which is located upstream of the cGAS gene and most likely affects the transcription rates of the gene. cGAS is regulated at cellular level by the threshold of DNA sensing as well as by the posttranslational modifications (Ablasser and Chen 2019) for its activation, further in vitro studies in aid of these regulations by rs311686 SNP may help in understanding different activation pattern of the cGAS promoter.

We did not find any significant differences with respect to rs610913 SNP between TB and controls. However, structure analysis showed a change from Proline(P) to histidine(H) at position 261. This mutation results in loss of Helix ($p < 0.04$) as well as loss of glycosylation ($p < 0.04$), which might cause changes during DNA-induced oligomerization of cGAS. However, the H variant has a larger volume in the second cleft implying larger binding sites and better bonding capacity. A study reported that the rs610913 AA slows down telomere shortening in the Polycyclic aromatic hydrocarbons (PAHs) exposure group, which are the main carcinogenic components in coke oven emissions (COEs) (Duan et al. 2020). A recent study proposed that rs610913 alters cGAS-mediated DNA sensing and viral infection based on the molecular modeling hinting towards an additional binding site for a potential cellular cofactor in cGAS dimers (Kazmierski et al. 2022). However, upon DNA challenge, reconstituted THP-1 and PBMCs from donors homozygously expressing rs610913 exhibited only a trend of reduced type I IFN response as compared to wildtype, suggesting that cGAS wild type and P261H similarly sense viral infections in vivo. Similar to our study, there is no association of rs610913 with that of HPV infection in Chinese population (Xiao et al. 2016) also there was no association of this SNP with that of metastatic colorectal cancer (Wang et al. 2022).

4.1.6 STING SNP analysis

As mentioned earlier the STING SNPS G230A, H232R & R293Q were in complete linkage disequilibrium. Hence, we analysed G230A further. We did not find any significant association of STING 230 with that of TB susceptibility or protection. However, there is a slightly more

frequent C allele in TB patients compared to HHC but this failed to reach statistical significance after adjustment. Similar to our study, in HPV infected Chinese populations, no association of G230A was observed (Xiao et al. 2016). A study reported that G230A substitution lies at the C-terminal domain in the lid portion of the c-di-GMP binding pocket and alters it's binding by making STING protein a more sensitive responder to lower concentrations of the ligand (Yi et al. 2013). It has been shown that murine STING R231A substitution (equivalent position to R232 in humans) caused a loss of responsiveness to bacterial exogenous CDNs but not to endogenous 2′3′ cGAMP. This could be because the R231H and G230A mutations lie within the CDN binding regions of STING and alter the binding capacity with different CDNs (Walker et al. 2020). However, the mechanism of STING SNPs in TB needs to be investigated in further studies.

Taken together, cGAS/STING plays an important role in TB pathogenesis, as it induces type I IFN not only in myeloid cells but also in T and B cells (Donovan et al. 2017). This could explain the cGAS rs311686 SNP association in dissemination and reactivation. However, it is important to study these SNPs in larger sample sizes and in different ethnic groups and functional analysis of these variations help in better understanding of disease progression.

4.2   Functional analysis of TLR4 & TLR8 potential SNP variations & evidence of TLR4/8 Heterodimer formation which is hindered byTLR4 399 SNP

4.2.1   LPS responsiveness

In HEK & PBMC against TLR4 wildtype & variant forms:

In our study overexpression of TLR4 wildtype and variant forms along with MD2 in HEK Blue Null 1 showed 'wildtype' TLR4-299A-399C to induce highest NF-κB induction with a significant difference in comparison to 399-T and double mutants 299G-399T ($p<0.01$), but not 299-G ($p<0.218$). We also checked the LPS responsiveness on TNF levels in PBMC of individuals differing in their TLR4 genotype. Surprisingly, we found higher TNF induction in double mutants and 399T homozygous bearing PBMCs compared to wildtype and 299G variant forms (Figure 8). We speculate that the less NF-κB activation in HEK cells could be because of MyD88 dependent pathway, over the time surface TLR4 gets endocytosed and signals through TRIF/TRAM pathway.

TNF levels in PBMC stimulated with LPS (10ng/ml)

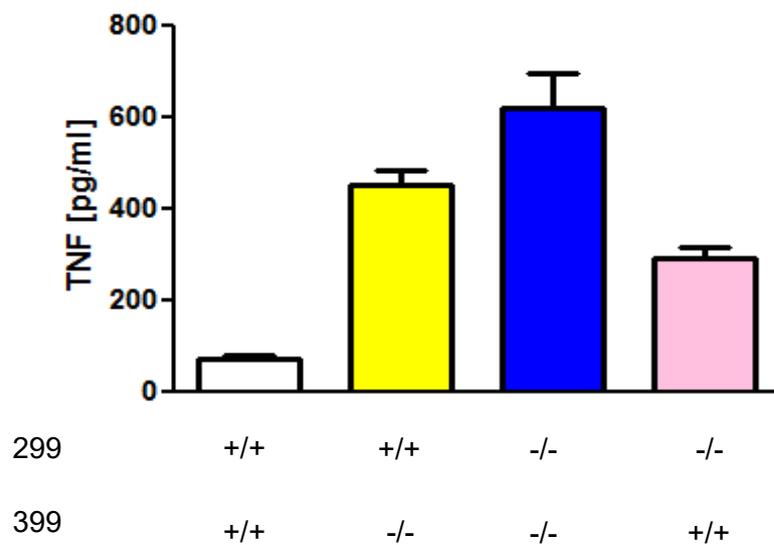| 299 | +/+ | +/+ | -/- | -/- |
| 399 | +/+ | -/- | -/- | +/+ |

Figure 8. LPS responsiveness TNF levels estimated by ELISA: PBMCs isolated from healthy volunteers that homozygously differed in their status of TLR4 wildtype (+/+) and variant forms (-/-).

It is well established that TLR4 is involved in TB pathogenesis, for example, TLR4 mutant C3H/HeJ mice producing lower TNF, IL-12p40 resulted in impaired elimination of mycobacteria compared to control C3H/HeN mice (Abel et al. 2002). In another study upon infection with highly virulent *Mtb* K-infection of TLR4 deficient mice, C3H/HeJ showed significantly increased neutrophils and production of IL-10 with an impaired Th1 response (Park et al. 2020). Similar to our study, the TLR4 variant forms i.e., 299, 399 and double mutants induced less NF-κB as compared to the wild-type in HEK cells and that the cytokine levels (TNF, IL-1β, IL-6 & IL-10) were high in PBMCs of double heterozygous carrier individuals. Moreover, monocytes from these individuals were shown to downregulate the genes involved in TRIF/TRAM pathway leading to sub-optimal immune responses to infection upon LPS challenge (Hold et al. 2014). In another study these human TLR4 SNPs were engineered to knock in murine strains expressing the D298G and N397I (corresponding to human 299 and 399 mutations respectively) homozygously. These have been associated with LPS-hyporesponsiveness, and differential susceptibility to various infections for example increased sensitivity to bacterial infection, resistance to influenza infection, and increased M1 phenotype but decreased M2 phenotype to RSV infection (Richard et al. 2021). Resistance to the latter two infections may be due to the fact that endocytosed TLR4 along with these viral PAMPs signals through TRIF/TRAM pathway similar to our study. There are several other lines of evidence that TLR4 plays a crucial role in TB pathogenesis reviewed in (Sepehri et al. 2019).

In contrast to our study, Ferwerda et al., analysed PBMCs from heterozygous 299AG individuals from Dogon ethnicity showing high TNF levels and no difference in IL-10 whereas double heterozygous individuals (299AG, 399CT) from Netherlands did not show any difference from that of the wildtype (Ferwerda et al. 2007). In another study 299G was shown to be LPS hyporesponsive against NF-κB activation compared to wildtype and 399T (Arbour et al. 2000) in THP-1 cells.

4.2.2 TLR4/8 interaction

To our knowledge, this is the first report on a potential TLR4/8 interaction. The individual TLR4 docking studies with that of 299 and 399 mutations did not show any conformational difference from that of wildtype and TLR8 docking studies reveals that TLR8-1G has higher ligand binding capacity as compared to that of the wildtype. The docking outcome of TLR4 and -8 together with R848 ligand revealed that the TLR4-399C (without MD2) could undergo heterodimerization with TLR8 in presence of the agonistic ligand R848. As discussed earlier, ligand induced TLR heterodimers might lead to conformational rearrangements in the protein structure that could alter the ligand-binding capacity and thereby enhance, inhibit, or modulate immune responses (Oosenbrug et al. 2017). We have validated this result with confocal microscopy and mass spectrometry.

We found the presence of TLR4 in co-immunoprecipitated lysates of HEK TLR8 cells transfected with TLR4 399C particularly after R848 stimulation. Although it is the established concept that TLR4/MD2/CD14/LPS is necessary for TLR4 internalization, adding MD2/CD14 to the transfection abolished this effect. This might be potentially due to the promotion of TLR4 homodimerization. In line with this study, endosomal heterodimerization of TLR4 and -6 in presence of the co-receptor CD36 in responses to oxidized LDL during atherogenesis, has been shown to also be independent of MD-2 and CD14. This signaling induced both, MyD88- and TRIF-dependent genes (Stewart et al. 2010). Similarly, in our study, we could show that heterodimerization of TLR4 and -8 activated both NF-κB- and IRF3-linked pathways.

We could see that there are synergistic effects of LPS and R848 in PBMCs by producing high TNF and IL-12p40. This is even more pronounced in individuals bearing TLR4 399T with TLR8 1A. *M.tb* RNA as a ligand also exhibited the same trend although to a lesser extent. This might be because stimulation with RNA requires another transfection medium, thereby increasing cell stress, potentially resulting in reduced reactivity. There are several studies displaying synergistic effects of various PRRs in recognition of *M.tb* and subsequent disease clearance or activation. For instance, in one study it was demonstrated that *M.tb* induced apoptosis is blocked in TLR2- and MyD88-deficient macrophages while in TLR4-deficient cells apoptosis was decreased but necrosis enhanced. However, upon treatment of wildtype cells with peptidoglycan (PGN) and LPS together apoptosis was induced upon *M.tb* infection through simultaneous activation of TLR2 and TLR4 pathways (Sanchez et al. 2010). Enhanced proinflammatory cytokine release was observed in cells stimulated with TLR4 plus TLR7/8 agonists through P38, JNK, c-Fos/AP1 pathway (Fischetti et al. 2017). Similarly, blocking p38 reduced the synergy of LPS and R848 on IL-12p70 release (Bohnenkamp et al. 2007). Co-activation of TLR8 and TLR3 was shown to induce a strong IL12p70 response (Ishii et al. 2014); with TLR2 inducing a shift towards a Th17-immunity (Bosl et al. 2018). In contrast, TLR8 sensing of pyrogenic bacteria and production of IL-12p70 has been shown to be blocked by activation of surface receptors such as TLR4 possibly due to the competition for myddosome components (Moen et al. 2019).

With TLR4-399T transfected TLR8 HEK cells upon R848-stimulation, precipitation of TLR4 was decreased in Co-IP, further supporting the notion that the interaction of TLR4 and TLR8 is impaired with the nucleotide change from TLR4 399C to 399T. Previously it was reported that TLR1/2 forms a dimer to recognize *M.tb* PAMPs and that the SNP in TLR1 248S greatly impaired the confirmation of TLR1 hindering the formation of dimer with TLR2 and this SNP is associated with PTB susceptibility (Dittrich et al. 2015). This is confirming that potential SNPs in ligand binding site could alter the dimerization leading to altered immune responses.

The shift from TLR4 399C to 399T induced higher NF-κB in HEK cells, increased TNF, and IL12p40 levels in PBMCs upon stimulation with TLR8 ligands in comparison to TLR4-399C in combination with TLR8 1A. We confirmed that the TLR4/8 heterodimer activate IRF3, thus potentially leading to more type I IFNs but less NF-κB. With TLR4-399T this effect is hindered,

thereby producing more NF-κB. Based on these observations we propose that both loss-of-function alleles of TLR8(1A) and TLR4(399T) convey susceptibility towards TB by altering the Th1 balance.

In conclusion, our study emphasizes the importance of innate immune receptors in recognition of *M.tb* nucleic acids inducing type I IFN which is important in regulating Th1 responses to restrict the bacterial growth. Besides, in the future, it is important to study the activation of bystander non infected host cell regulation by type I IFNs to understand dissemination, memory formation also the effect of potential SNPs of immune receptors in exacerbating protective immunity.

Zusammenfassung

Erkennung von M. tuberculosis durch angeborene Immunrezeptoren - Ein neuer Mechanismus der TB-Pathogenese, der durch genetische Analyse einer indischen Kohorte und In-vitro-Studien aufgedeckt wurde

Tuberkulose (TB) ist weltweit immer noch eine der führenden Todesursachen. Die Wirt-Pathogen Interaktion ist ein entscheidender Faktor, um den Schutz vor Infektionen einzuleiten und den Ausgang von Erkrankungen zu beeinflussen, und involviert komplexe Prozesse. *Mycobacterium tuberculosis (M.tb)* ist ein Erreger, der intrazellulär erfolgreich ist, indem er die angeborene Wirtsabwehr umgeht und in einer geschützten Nische überlebt. Erfolgreiche anti-TB Strategien werden dringend benötigt, und Ansätze, diesen Mechanismus zu überwinden, könnten zu neuen Interventionsstrategien führen. In dieser Studie wird untersucht, ob genetische Variationen endosomaler (Toll-like Rezeptor (TLR)-4 und -8) und zytosolischer (zyklische-GMP-AMP-Synthetase/Stimulator Interferon-induzierter Gene (cGAS/STING)) musterekennender Rezeptoren („PRRs") zur Krankheitssuszeptibilität beitragen. Wie bekannt ist, sind sowohl die endosomale als auch die zytosolische Erkennung intrazellulärer Pathogene wie *M.tb.* von entscheidender Wichtigkeit für die Aktivierung der angeborenen Immunität. Durch die Untersuchung von „Einzelnukleotidpolymorphismen (SNPs)" einer indischen TB-Kohorte und dem Vergleich ihrer Häufigkeit mit Kontrollgruppen wurde eine Mutation in TLR4 identifiziert, die die Aminosäure 399 (C/T) betrifft und in Indien häufig vorkommt, und das TB-Risiko beeinflusst. Dabei waren zwei hier untersuchte TLR4 Varianten (299 & 399) in der indischen Kohorte deutlich seltener miteinander gekoppelt (73%) als bei bisher untersuchten „kaukasischen Kontrollen" (98%). Diese Unterschiede in der Verteilung von genetischen Variationen könnten auf unterschiedlichen Selektionsdruck durch Infektionen und die Migration der Menschheit zu erklären sein. Die Varianten TLR4-399T und TLR8-1A waren mit einer erhöhten TB-Suszeptibilität assoziiert unabhängig voneinander, obwohl kein TLR4 Ligand in *M.tb.* bekannt ist. Auf der anderen Seite haben wir Varianten von cGAS/STING in einer indischen TB-Kohorte untersucht. Dieser Signalweg ist wichtig für die Wirtsabwehr nach der Internalisierung von *M.tb.* im Zytosol und der Freisetzung von mykobakterieller DNA, die die Produktion von Typ I Interferonen (IFN) induziert. Wir fanden, dass der rs311686 SNP, der dem cGAS-Gen vorgelagert ist, Individuen vor der TB Erkrankung schützt und dass diese Genvariation unterschiedlich verteilt ist in Patienten mit pulmonaler TB verglichen mit extrapulmonaler TB und „relapse-Fällen". Dieser SNP ist außerdem unterschiedlich verteilt wenn Patientengruppen nach ihrem Bacillus Calmette-Guerin (BCG)-Impfstatus unterschieden werden. Der rs610913 cGAS SNP scheint die Konformation von cGAS zu verändern, wie wir durch in silico modeling zeigen konnten.

Funktionelle Analyse von Wildtyp- und Variantenformen von TLR4 ergab, dass doppelt homozygote Varianten (299/399) und die 399 Variante dazu führten, dass sich größere Mengen Tumornekrosefaktor (TNF) in peripheren mononukleären Zellen (PBMCs) durch Lipopolysaccharid (LPS) induzieren ließen, was im Widerspruch zur Aktivierung von nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) in human embryonalen Nierenzellen (HEK)-steht, die die jeweiligen Rezeptorvarianten überexprimieren. Die Ursache für diese Diskrepanz könnte darin liegen, dass es neben dem NF-κB Signalweg in HEK-Zellen noch den TIR domain-containing adaptor inducing interferon-β/TRIF-related adaptor molecule (TRIF/TRAM)-Signalweg gibt, der Typ-I IFN induziert. Die Ausbildung von TLR-TLR Dimeren ist beschrieben, um so Pathogen besser erkennen und bekämpfen zu können. Wir zeigen hier erstmalig, dass eine Heterodimerisierung von TLR4 und -8 stattfindet, die durch die Bindung eines TLR8-Liganden (R848) induziert wird. In-silico modeling führte zu der Annahme, dass die TLR4-399T-Variante diese Interaktion unmöglich macht. Diese Ergebnisse wurden durch co-Immunopräzipitations- und massenspektometrische Analysen bestätigt: In HEK-Zellen, die mit TLR8-Liganden stimuliert wurden, konnten wir TLR4 mit an Agarose gekoppelten Antikörpern, die gegen TLR8 gerichtet waren, kopräzipitieren. Konfokale Mikroskopie bestätigte eine hohe Frequenz der Kolokalisierung von TLR4 und -8, die weiter zunahm, wenn

TLR8 stimuliert wurde. Kolokalisierung von TLR4 und -8 führte zu einer moderaten Aktivierung von NF-κB (in HEK-Zellen) und zu einer Induktion von TNF und Interleukin-12p40 (IL-12p40) (in PBMCs) gemeinsam mit der Aktivierung von interferon regulatory factor3 (IRF3). Die TLR4 Variante TLR4-399T konnte gemeinsam mit TLR8 mehr NF-κB aktivieren, was möglicherweise durch die Veränderung anderer Signalwege, die Typ I IFN beinhalten, bedingt ist. Insgesamt präsentieren wir hier Evidenz dafür, dass eine endosomale Heterodimerisierung von TLR4 und -8 in die *M.tb*-Erkennung über TLR8-Liganden, wie *M.tb*-RNA involviert ist, was zu einer optimalen Th1-Antwort führt. Zusammengefasst zeigen unsere Ergebnisse, dass die Erkennung der Nukleinsäuren von *M.tb.* wichtig für die Pathogenese der TB ist und dass dieser Mechanismus von zentraler Bedeutung für die Pathogenese dieser Erkrankung sein könnte. Eine Genotypisierung von Individuen bzgl. der hier genannten Gene könnte helfen, eine Risikostratifizierung durchzuführen. Dies könnte in der Zukunft dazu führen, die Prävention zu verbessern und mglw. neue Impf- und Behandlungsstrategien für die TB zu entwickeln.

Summary

Tuberculosis (TB) today still is one of the leading causes of death worldwide. Host-pathogen interaction is crucial for establishing protection against pathogens and involves complex processes. *Mycobacterium tuberculosis* (*M.tb)* is a successful intracellular pathogen and evades host immune system to establish a protected niche. There is a great need to develop successful anti-TB therapies, and to overcome this mechanism of *M.tb* potentially may lead to novel intervention strategies. In this study genetic contribution of endosomal (Toll-like receptor (TLR)-4 and -8) and cytosolic (Cyclic GMP–AMP synthase/Stimulator of interferon genes (cGAS/STING) "pattern recognition receptors (PRRs") towards disease susceptibility has been studied. It has been shown that both, endosomal and cytosolic recognition of microbial products is critical for the initiation of innate immune response against intracellular pathogens such as *M.tb*. In "single nucleotide polymorphism (SNP)" analyses of an Indian TB cohort, a TLR4 mutation affecting amino acid 399 (C/T), found frequently in India was shown to be associated with TB risk. The two mutations in TLR4 (299 & 399) analysed were not in complete linkage disequilibrium in our Indian cohort (73%) unlike Caucasians (98%). The difference in genotypic distribution among different ethnicities might be due to differences in local infection pressure during the migration of mankind. Furthermore, TLR4-399T and TLR8-1A conveyed increased susceptibility towards TB in an interdependent manner, even though there is no established TLR4 ligand present in *M.tb*. On the other hand, we analyzed cGAS/STING SNPs in an Indian TB-cohort. This pathway is an essential defence pathway within the cytosol after *M.tb* internalization and it's DNA release inducing the production of type I Interferons (IFNs). We found that the presence of rs311686 SNP upstream of cGAS provides protection from TB overall and is differently distributed in pulmonary TB patients as compared to patients with extra-pulmonary and particularly relapse cases. This SNP furthermore differs in distribution when comparing individuals with respect to Bacille Calmette-Guérin (BCG) vaccination status. Conformational changes of cGAS were found by in silico modelling with respect to rs610913 SNP.

Functional analysis of wildtype and variant forms of TLR4 revealed that the double homozygous variant form (299/399) and 399 variant forms to induce higher tumor necrosis factor (TNF) levels in peripheral blood mononuclear cells (PBMCs) stimulated with Lipopolysaccharide (LPS) as compared to the wildtype and 299 mutation alone, which is in contrast to nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) levels induced in human embryonic kidney (HEK) cells overexpressing the TLR variants. This could be because of the alternate pathway (TIR domain-containing adaptor inducing interferon-β/TRIF-related adaptor molecule (TRIF/TRAM)) which also induce type I IFNs in addition to NF-κB in HEK cells. TLR-TLR dimerization is an established pathway to recognize and fight pathogens in a better fashion. We describe here for the first time TLR4 and -8 heterodimer formation through TLR8 ligand (R848) interaction in in-sillico modeling and that the TLR4-399T variant disrupted this interaction with TLR8. This was confirmed by the co-immunoprecipitation and mass spectrometry analyses: Here we observed precipitated TLR4 with TLR8-targeted antibodies immobilized on agarose beads in TLR8 ligand stimulated HEK cells. Confocal microscopy confirmed a high co-localisation frequency of TLR4 and TLR8 that further increased upon TLR8 stimulation. This heterodimerization of TLR4 and TLR8 led to moderate activation NF-κB (in HEK cells) inducing TNF and Interleukin -12p40 (IL-12p40) (in PBMCs) along with the activation of interferon regulatory factor3 (IRF3) (in HEK cells). The variant form of TLR4-399T with TLR8 in contrast activated increased NF-κB, which was potentially caused by an alteration of subsequent immunological pathways involving type I IFNs. Taken together, we present evidence that the heterodimerization of TLR4 and TLR8 at the endosome is involved in *M.tb* recognition via TLR8 ligands, such as *M.tb* RNA, which induces optimal Th1 response. In summary, our findings implicate *M.tb* nucleic acid recognition in TB pathogenesis is an essential mechanism to understand the course of the disease. Genotyping for the genes investigated here could help in the future in TB risk stratification of individuals. This may ultimately help in prevention of disease and aid in developing new vaccination and treatment strategies.

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Publications & scientific contributions

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Shruthi Thada, Sanne Burkert, Saubashya Sur, Suman L. Gaddam, Ralf R. Schumann. Innate immune recognition of M. tb - Genetic analysis of an Indian cohort and in-vitro studies of toll-like receptor (TLR) variants. International Conference „Innate immunity of the lung" Berlin, Germany. September 15 – 16, 2016.

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In the context of this work, there are no conflicts of interest due to contributions from third parties.

Selbstständigkeitserklärung

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Berlin, den 21.02.2023   Shruthi Thada