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2017

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Clinical Value of Initial Host Molecular Signalling and Molecular Fingerprinting

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Clinical Value of Initial Host Molecular Signalling and Molecular Fingerprinting

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By due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at BMC I: I1345, Thursday 30th of March, 2017, 9.00 a.m.

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Organization LUND UNIVERSITY	Document name DOCTORAL DISSERTATION
	Date of issue 30th of March, 2017
Author(s) Nader Alaridah	Sponsoring organization

Title and subtitle
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Abstract

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In a second part of the thesis, we investigated the expression and function of GPCRs in a simple whole blood model from patients with pulmonary TB and in subjects with latent TB infection (LTBI). We found variations in GPCRs as pulmonary TB patients had significantly increased CXCR1 expression on blood cells compared to LTBI subjects and controls. These variations in receptor expression were linked to disease progression and affected the immune response against Mycobacterium tuberculosis (Mtb). As an airborne infection, tuberculosis (TB) has no boundaries and easily spreads by migration from one region to another. In this study, 93 patient Mtb-isolates, previously genotypically analysed by standard techniques, were re-analysed by whole genome sequencing (WGS). Compared to the standard genotyping, WGS had an overall high match in identifying cluster transmissions in this patient population. When comparing the different techniques individually, WGS and epidemiological data had the highest cluster similarity, while MIRU-VNTR had less cluster resolution. We can conclude that WGS is well suited for identifying transmission clusters in settings with low TB incidence.

Key words: TB, vaccine, G-protein-coupled-receptors, AECs, active tuberculosis, LTBI, Genotype, WGS		
Classification system and/or index terms (if any)		
Supplementary bibliographical information		Language English
ISSN and key title 1652–8220		ISBN 978-91-7619-432-4
Recipient's notes	Number of pages 74	Price
	Security classification	

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Tuberculosis

Clinical Value of Initial Host Molecular Signalling and Molecular Fingerprinting

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This dissertation is dedicated to my beloved family to whom I owe each moment of my life and without whom none of my success would be possible. Mom and Dad: thank you for encouraging me to go on every adventure, especially this one

"If you think research is expensive, try disease!"

Mary Woodard Lasker

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     Mycobacteria bypass mucosal NF-kB signalling to induce an epithelial anti-inflammatory IL-22 and IL-10 response.
     PLoS One. 2014 Jan 28;9(1): e86466. doi: 10.1371/journal.pone.0086466.

II. **Alaridah N**, Winqvist N, Håkansson G, Tenland E, Rönnholm A, Sturegård E, Björkman P, Godaly G.
     Impaired CXCR1-dependent oxidative defence in active tuberculosis patients.
     Tuberculosis (Edinb). 2015 Dec;95(6):744-50. doi: 10.1016/j.tube.2015.07.008.

III. **Alaridah N**, Lutay N, Tenland E, Rönnholm A, Hallgren O, Puthia M, Westergren-Thorsson G, Godaly G.
     Mycobacteria Manipulate G-Protein-Coupled Receptors to Increase Mucosal Rac1 Expression in the Lungs.
     J Innate Immun. 2016 Dec 24. doi: 10.1159/000453454.

IV. **Nader Alaridah**, Niclas Winqvist, Erik Sturegård, Jeanette Tångrot, Patrik Medstrand, Gabriela Godaly
     Transmission dynamics studies of tuberculosis isolates with whole genome sequencing in a low incidence country
     Manuscript

Other Related Papers by The Author

Mohanty S, Jagannathan L, Ganguli G, Padhi A, Roy D, **Alaridah N**, Saha P, Nongthomba U, Godaly G, Gopal RK, Banerjee S, Sonawane A.
A mycobacterial phosphoribosyltransferase promotes bacillary survival by inhibiting oxidative stress and autophagy pathways in macrophages and zebrafish.
J Biol Chem. 2015 May 22;290(21):13321-43. doi: 10.1074/jbc.M114.598482.

Tenland E, Håkansson G, **Alaridah N**, Lutay N, Rönnholm A, Hallgren O, Westergren-Thorsson G, Godaly G.
Innate Immune Responses after Airway Epithelial Stimulation with Mycobacterium bovis Bacille-Calmette Guérin.
PLoS One. 2016 Oct 10;11(10): e0164431. doi: 10.1371/journal.pone.0164431.

Abbrevations

AECs	Airway Epithelial Cells
AIDS	Acquired Immune Deficiency Syndrome
BALT	Bronchus-Associated Lymphoid Tissue
BCG	Bacillus Calmette–Guérin
cAMP	cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
CLRs	C‑type Lectin Receptors
CR	Complement Receptors
CREB	cAMP Response Element-Binding
DAG	Diacylglycerol
DAMPs	Damage-Associated Molecular Patterns
DC-SIGN	DC-Specific Intercellular-adhesion molecule-3 Grabbing Non-integrin
ECL1	One Extracellular Loop
ELISA	Enzyme-Linked ImmunoSpot Assay
ELISPOT	Enzyme-Linked ImmunoSpot
EMB	ethambutol
ERK-1	Extra-cellular Regulated Kinases 1
FADD	Fas-Associated Death
Fc	Fragment crystallizable
FDA	Food and Drug Administration
GAGs	Glycos-AminoGlycans
GALT	Gut-Associated Lymphoid Tissue
GPCR	G-Protein-Coupled Receptor
gp130	glycoprotein 130 dimer
GSK3	Glycogen Synthase Kinase 3
GTPases	Guanosine-5'-TriPhosphate hydrolysis enzymes
HBD	Hormone-Binding Domain
HIV	Human Immunodeficiency Virus
H_2O_2	Hydrogen Peroxide
ICL1-3	three Intracellular Loops
IFN-γ	InterFeron Gamma
IGRA	InterFeron Gamma Release Assay
I-kB kinase D	inhibitory IkappaB kinase D
IKK	I-kB kinase
IL-1	Interleukin 1
IL-6	Interleukin 6

IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-22	Interleukin 22
INH	IsoNicotinylHydrazide
IP3	Inositol-1,4,5-trisPhosphate
IRAK-4	Interleukin 1 Receptor Associated Kinase 4
IRF3	Interferon Regulatory Factor3
IS	InSertion elements
JAK	Janus kinase
JNK	c-Jun N-terminal Kinases
LAM	LipoArabinoMannan
LPS	Lipopolysaccharide
LTBI	Latent TB
MAL	MyD88 Adaptor Like
MAPK	Mitogen-Activated Protein Kinases
MBL	Mannose-Binding Lectin
MDR-TB	MultiDrug-Resistant Tuberculosis
MIRUs	Mycobacterial Interspersed Repetitive Units
MIRU-VNTR	MIRU-Variable Number of Tandem Repeat
PTR	Polymorphic Tandem Repeats
Mtb	Mycobacterium tuberculosis
MTC	Mycobacterium Tuberculosis Complex
MyD88	Myeloid Differentiation factor88
NAAT	Nucleic Acid Amplification Test
NEMO/IKKγ	NF-$\varkappa$B essential modifier / inhibitor $\varkappa$B kinase gamma
NADPH	Nicotinamide Adenine Dinucleotide PHosphate
NAG–NAM	N-Acetyl Glucosamine–N-Acetyl Muramic acid
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural Killer cells
NLRs	Nucleotide-binding and oligomerisation domain-Like Receptors
NO$-_3$	Nitrate
NOI	Nitrogen Oxygen Intermediates
NOS2	Nitric oxide synthase
O2−	superoxide anion
ONOO–	peroxynitrite
PAMP	Pathogen Associated Molecular Patterns
PE/PPE	proline-glutamic / proline-proline-glutamic acid
PGRS	Polymorphic GC-rich Repetitive Sequences
PH	Pleckstrin Homology
PHOX	phagocytic oxidase
PIM	Phosphatidyl-myo-Inositol Mannoside

PIP2	PI bisphosphate
PI3 kinase	PhosphatidylInositol-3-kinase
PI3Kg	PhosphatidylInositol-3-kinase g
PKC	Protein Kinase C
PLC	Phospholipase C
PMNs	polymorphonuclear leukocytes
PPAR	Peroxisome Proliferator-Activated Receptor
PRR	Pattern Recognition Receptors
PTK	Protein Tyrosine Kinases
PZA	PyraZinAmide
RD1	Region of Difference 1
RFLP	Restriction Fragment Length Polymorphism
RGS	Regulator of G Protein Signalling
RIF	RIFampin
RLRs	Retinoic acid inducible gene RIG‑I-Like Receptors
RNI	Reactive Nitrogen Intermediates
ROI	Reactive Oxygen Intermediates
SARM	Sterile-alpha and ARmadillo Motif-containing protein
SM	StreptoMycin
SNP	Single Nucleotide Polymorphisms
SOCS	suppressor of cytokine signaling proteins
SOG r	Somatostatin, Opioid and Galanin receptors
Sp-A	Surfactant protein A
Sp-D	Surfactant Protein D
STAT	Signal Transducer and Activator of Transcription
TAB1	TAK1 Binding protein 1
TAB2	TAK1 Binding protein 2
TAK1	TGF-α-Activated kinase 1
TB	Tuberculosis
TBK1	Tank-binding kinase 1
TDM	Trehalose DiMycolates
TH1	T helper 1
TH17	T helper 17
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain containing Adaptor Protein
TLRs	Toll Like Receptors
TMM	Trehalose MonoMycolates
TNF	Tumor Necrosis Factor
TRAF6	TNF Receptor-Associated Factor 6
TRAM	TRIF-Related Adaptor Molecule
Tregs	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β

TST	Tuberculin skin tests-
T7SS	Type seven Secretion System
WGS	Whole-Genome Sequencing
WHO	World Health Organization
XDR TB	Extensively Drug Resistant TB
7TM	seven-transmembrane
μ δ ϰ OR	μ δ ϰ Opioid Receptor

Abstract

Mycobacterium tuberculosis (Mtb) is principally a pulmonary pathogen infecting one-third of the world's population and causing two million deaths annually. The only approved tuberculosis vaccine today is the *Mycobacterium bovis* bacilli Calmette-Guérin (BCG). BCG vaccine is used as a benchmark to compare the immunogenicity of new vaccines, but not much is known about its mechanisms to induce protection. We investigated the initial events of mycobacterial activation of airway epithelial cells (AECs) through the signalling pathways of toll like receptors (TLRs) and the G-protein coupled receptors (GPCR; CXCR1, CXCR2). Our data indicate that mycobacteria attenuate epithelial pro-inflammatory production by supressing NF-κB activation, thereby supporting the production of the anti-inflammatory cytokines IL-22 and IL-10. BCG infection of AECs also resulted in epithelial actin redistribution that involved the MAPK signalling pathway. This study demonstrated that BCG infection of AECs manipulated the GPCRs to suppress epithelial signalling pathways. Future vaccine strategies could thus be improved by targeting GPCRs.

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General Background

Historical overview

Tuberculosis (TB) has plagued humankind worldwide for thousands of years [1]. It has been called by numerous names including "consumption" (because of the severe weight loss and the way the disease appeared to "consume" the patient), "phthisis pulmonaris" and also "the white plague" (because of the extreme pallor seen among those infected) [2]. An English writer narrated tuberculosis as "The captain among these men of death" at a time when tuberculosis case rates in London reached 1000 per 100 000 population each year [3]. During the 1800s, TB was the major endemic disease in Sweden and in major parts of Europe and 25% of all deaths were due to tuberculosis [4]. At this time, a majority of the Swedish population lived under poor housing conditions where different generations lived together with limited living space available.

Tuberculosis resulted not only in disease and death but also in social and economic challenges. TB patients suffered from stigma, social branding and were treated as outcasts while the economic situation of the core family deteriorated as the key provider was no longer able to earn money. It was not until the 19th century, with the help of Villemin's experiments, the founder of germ theory for tuberculosis disease, that the medical community began to accept that tuberculosis was indeed a contagious disease, transmitted by an etiological agent. In 1882, the Prussian physician Robert Koch utilized a new staining method and applied it to the sputum of tuberculosis patients, and by that discovering the causal agent of the disease: *Mycobacterium tuberculosis*, or Koch's bacillus [5]. In 1895, Wilhelm Roentgen discovered the X-ray, which allowed physicians to diagnose and track the progression of the TB disease. The modern TB history started with the powerful and optimistic speech of Robert Koch in a Nobel lecture on December 12, 1905:

> *"The struggle [against tuberculosis] has caught hold along the whole line and enthusiasm for the lofty aim runs so high that a slackening is no longer to be feared. If the work goes on in this powerful way, then the victory must be won."*

The first genuine successful tuberculosis immunization was developed from attenuated bovine-strain tuberculosis by Albert Calmette and Camille Guérin in 1906. It was called Bacille Calmette Guérin (BCG) and generated high hopes for TB prevention. However, the first administration was only after World War I [6]. Later

the same century, sanatoriums (containment) became favourable and the first effective antibiotic therapy was introduced in the form of an oral streptomycin as mycobactericidal drug [7].

Today, it is often said that TB is an ancient disease, but not a disease of the past. With timely diagnosis and correct treatment, most people who develop TB disease can be cured. However, in the context of war, catastrophes around the world, the HIV/AIDS pandemic, the new migration patterns, not to mention the rise of drug-resistant TB, TB this disease has revealed new challenges for the international community in the form of huge disease burden in developing countries [8]. Moreover, it is also re-emerging in several industrialized countries [9]. In 2015, WHO revealed that the global TB burden is higher than previously estimated. Global progress depends on major advances in TB prevention and care, and the world needs to move much faster to prevent, detect and treat TB to achieve "End TB Strategy" target goals in the next 15 years [10].

Tuberculosis Epidemiology (Local and global)

Tuberculosis is a major public health concern. About one third of the world's population is infected with tuberculosis (TB) bacteria (TB Latency). TB constitutes a major problem in high-income nations as well [11]. In Sweden, the estimate for TB incidence and mortality rate were 9.2 (7.9–11) and 0.32 (0.23–0.42) per 100 000 population (respectively) in 2015 [10]. However, only a small proportion of those infected will become sick with TB. People with weakened immune systems have a much greater risk of falling ill from TB [12].
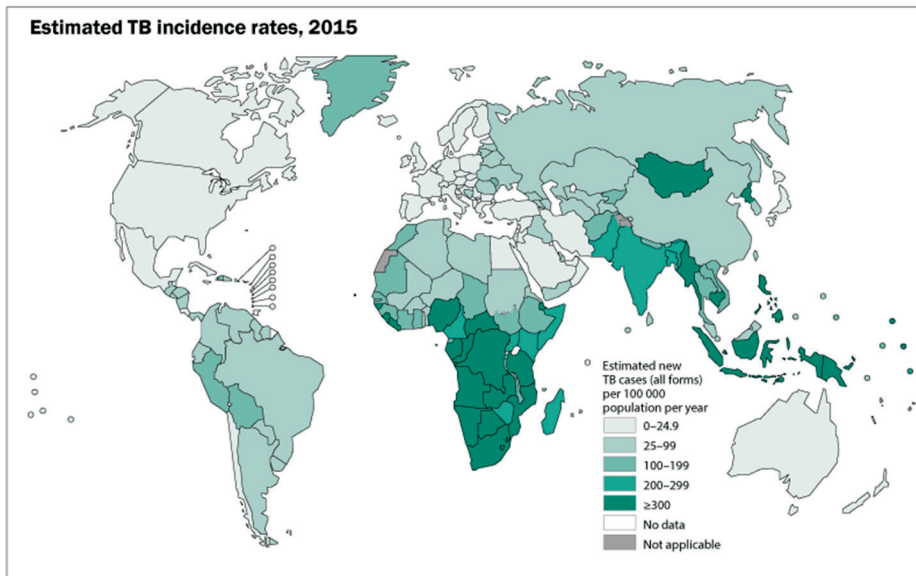
Estimated TB incidence rates, 2015

Estimated new
TB cases (all forms)
per 100 000
population per year

- 0–24.9
- 25–99
- 100–199
- 200–299
- ≥300
- No data
- Not applicable

Figure 1. Globally, nearly 10.4 million new TB cases and approximately 1.4 million TB-related deaths were reprted in 2015. Incidence varies, in Sweden, the annual incidence is 7.9–11 per 100,000 population, but in some countries in sub-Saharan Africa and Asia, the incidence rate is several hundred per 100,000. Reprinted from : WHO Global Tuberculosis report 2016, with persmission.

Despite the fact that much of the burden is concentrated in high-burden countries, in 2015 (Fig. 1), an estimated 10.4 million new TB cases were seen worldwide, of which 5.9 million (56%) were men, 3.5 million (34%) women and 1.0 million (10%) children. HIV infected patients accounted for 1.2 million (11%) of all new TB cases. TB resulted in approximately 1.4 million TB deaths in 2015, and an additional 0.4 million deaths among people living with HIV. Furthermore, the crisis of TB drug resistance detection and treatment continues. In 2015, only 125 000 (20%) of an estimated 580 000 TB infected patients, newly eligible for Multidrug-resistant (MDR), or extensively drug resistance (XDR) TB treatment, were enrolled [10]. The number of TB deaths decreased by 22% between 2000 and 2015, but once again TB remains a leading cause of infectious disease morbidity and mortality worldwide [13]. Even though the rate of decline in TB incidence was 1.5% from 2014 to 2015, a further 4–5% annual decline by 2020 is needed to reach the first milestones of the End TB Strategy. Global progress will depend on major advances in TB prevention and care especially in high burden settings [10].

Etiology

The genus *Mycobacterium* is mainly known for its two major pathogenic species, *M. tuberculosis* and *M. leprae.* These are the causative agents of two of the world's oldest diseases; tuberculosis and leprosy.

Domain: Bacteria; Phylum: Actinobacteria; Class: Actinobacteria; Order: Actinomycetales; family: Mycobacteriaceae; Genus: Mycobacterium

Tuberculosis is a communicable infectious disease and is caused by *Mycobacterium tuberculosis* complex (MTC), a genetically related group of Mycobacterium species that can cause tuberculosis in humans or other living things. It includes *M. tuberculosis* (Mtb), *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium caprae*, *Mycobacterium pinnipedii*, *Mycobacterium suricatte*, *Mycobacterium mungi*, *Mycobacterium dassie*, *Mycobacterium oryx* and *Mycobacterium canetti* [14,15] .

M. bovis was responsible for about 6% of all human tuberculosis deaths in Europe before the introduction of milk pasteurisation. Subsequent attenuation of a laboratory strain of *M. bovis* led to the development of the BCG vaccine in 1921 [6].

Species: The Mycobacterium tuberculosis complex (MTC) consists of Mycobacterium africanum, Mycobacterium bovis, Mycobacterium canettii, Mycobacterium microti, Mycobacterium tuberculosis (Mtb).

M. tuberculosis, a pathogen of the human respiratory system, is the most well-known member of the *Mycobacterium* species. Mtb is a slow-growing, obligate aerobe, facultative intracellular bacterium, with a non–spore-forming, non-motile, curved intracellular rod measuring 0.2-0.5 micrometer by 2-4 micrometer. Compared with the cell walls of other bacteria, its lipid-rich cell wall is relatively impermeable to basic dyes unless combined with phenol. Thus, *M. tuberculosis* is neither gram positive nor gram negative. It also retains red basic fuchsin dye after acid rinsing (acid-fast stain) [14,16,17].

M. tuberculosis divides every 18–24 h which is extremely slow compared with other bacteria, e.g. *Escherichia coli* divides every 20 minutes [16]. The slow replication rate together with its ability to persist in a latent state is the reason why *M. tuberculosis* infection require long duration drug therapy.
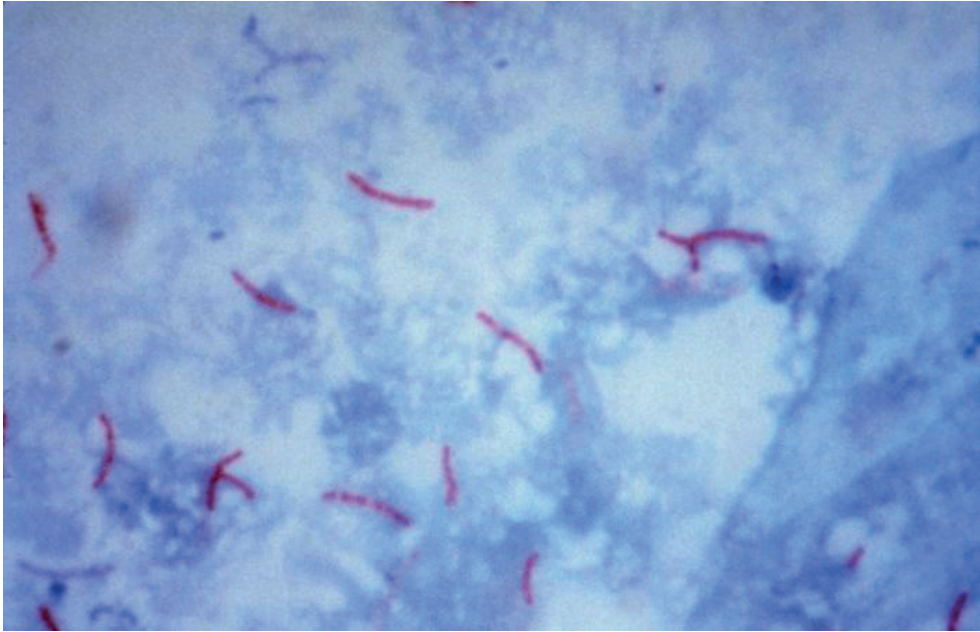
Figure 2. High-power micrograph of acid-fast bacilli in the sputum of a patient with tuberculosis, shown by Ziehl-Neelsen staining (×1000).

The cell envelope of mycobacteria

The cell wall structure of *M. tuberculosis* deserves special attention due to its uniqueness among prokaryotes, as well as being a major determinant of virulence for the bacterium. The mycobacterial cell wall is a complex structure that is required for cell growth, resistance to antibiotics and virulence [18,19]. It consists of an inner layer and an outer layer that surrounds the plasma membrane. The inner compartment is composed of three distinct macromolecules — peptidoglycans (PG), arabinogalactans (AG) and mycolic acids (MA) — covalently linked together to form a complex known as the MA-AG-PG complex. The MA-AG-PG complex extends from the plasma membrane outward in layers, starting with PG and ending with MAs which are surrounded by the non-covalently linked outer layer, the capsule, of proteins polysaccharides and associated lipids. The peptidoglycan layer surrounds the plasma membrane and comprises long polymers of the repeating disaccharide N-acetyl glucosamine–N-acetyl muramic acid (NAG–NAM) that are linked via peptide bridges [20,21] (Fig. 3). Most of the arabinan is ligated with long-carbon-chain mycolic acids, which form the characteristic thick waxy lipid coat of mycobacteria and are major contributors to the impermeability of the cell wall and to virulence. Mycolic acids can

be found in the cell walls of *Mycobacterium* spp. and *Corynebacterium* spp. and make up 50% of the dry weight of the mycobacterial cell envelope. They are composed of unique alpha-branched lipids where these strong hydrophobic molecules affect the permeability properties at the cell surface. The mycolic acids are thought to be a significant determinant of virulence in Mtb. It is thought that they prevent attack of the mycobacteria by cationic proteins, lysozymes, and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum. These mycolic acids are esterified to glycerol and trehalose where trehalose can contain one or two molecules of mycolic acids forming trehalose dimycolates (TDM) (Cord Factor) and trehalose monomycolates (TMM). Both compounds are present in the cell wall envelope and interact with other complex lipids and lipoglycans [21,22].
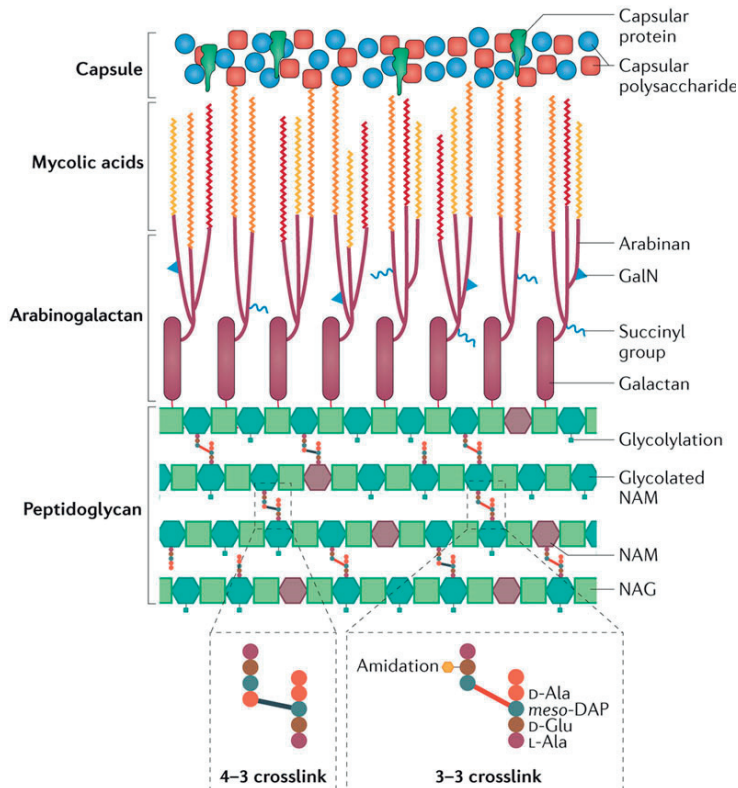
Figure 3. Mycobacterium tuberculosis cell wall : It is cosisted of three apperent macromolecules - peptidoglycan, arabinogalactan and mycolic acids- which are surrounded by a an outer capsule of proteins and polysaccharides. Moreover, mycobacterial peptidoglycan is heavily crosslinked. Reprinted from "How sisters grow apart: mycobacterial growth and division", Karen J. Kieser et al 2014, Nature Reviews Microbiology, with permission .

The outer compartment on the other hand consists of both lipids and proteins (Fig. 3). The lipid-linked polysaccharides associated with the outer cell wall consists of lipoarabinomannan (LAM), lipomannan, phthiocerol-containing lipids such as phthiocerol dimycocerosate, dimycolyl trehalose (cord factor), sulfolipids specific to M. tuberculosis, and the phosphatidylinositol mannosides [23].

The virulence determinants are divided into the several categories based on their function, molecular features and cellular localization [24]:

- Lipid and fatty acid metabolism
- Cell envelope proteins: including cell wall proteins, lipoproteins and secretion systems including the type seven secretion system (T7SS). The *M. tuberculosis* contains a total of five T7SS, also called ESX, that show similarity in gene content and gene order. Two members of T7SS; ESX-1 and ESX-5, have been shown to be involved in virulence. ESAT6 and CFP10 are located in a segment called Region of Difference 1 (RD1) and are absent in *M. microtti* and in the BCG vaccine IGRA [25].
- Proteins inhibiting antimicrobial effectors of the host, including those involved in responses to oxidative and nitrosative stresses, phagosome arresting and inhibition of apoptosis.
- Protein kinases, proteases, including metalloproteases.
- Metal-transporter proteins, divided into importer and exporters.
- Gene expression regulators, including two component systems, sigma factors and other transcriptional regulators.
- Proteins of unknown function, including the Pro-Glu (PE) or Pro-Pro-Glu (PPE) families [26].

The proliferation of bacterial cells can be divided into two stages: elongation of the mother cell and division of the elongated mother cell into two daughter cells. However, mycobacteria do not adhere to the 'one size fits all' rule, instead, they grow and divide in an asymmetric manner, which produces daughter cells of unequal sizes. This trait might have been selected for, as cells of different sizes might have distinct survival advantages in the highly variable host environment [21].

Genome structure

The *M. tuberculosis* consist of one circular chromosome where the genome comprises of 4,411,529 base pairs and contains around 4,000 genes. It has a very high guanine and cytosine content that is reflected in the biased amino-acid content of the proteins. *M. tuberculosis* differs radically from other bacteria in that a very large portion of its coding capacity is devoted to the production of enzymes involved in

lipogenesis, lipolysis and to two new families of glycine-rich proteins. These proteins contain a repetitive structure that may represent a source of antigenic variation [27], several of which carry highly repetitive motifs, polymorphic GC-rich repetitive sequences (PGRS) and major polymorphic tandem repeats (MPTR) [24].

Pathogenesis

Clinical TB disease is complex and shaped by the host–pathogen relationship. Intracellular Mtb bacilli are known to reside within phagosomal compartments [28] in a broad range of human cell types, especially those of the myeloid lineage such as macrophages and dendritic cell [1,29].

Pulmonary TB is by far the most common form of tuberculosis where the *M. tuberculosis* (viable bacilli) is typically inhaled into the body through the mouth or nose, passes through the airways and reaches the alveolar space in the lungs. Innate immune responses involving epithelia cells, alveolar macrophages, Natural killer (NK) cells and granulocytes begin to combat the infection; in some persons, the bacilli are cleared, whereas in others, infection is established [30-32]. Infected macrophages recruit additional macrophages, other immune cells from neighbouring blood vessels and adaptive immune response is initiated by being engaged through antigen-presenting cells (i.e. by poly-functional T cells, T_{H1}-type response, non-classical T cells, balanced activating and regulatory T cell responses and Mtb specific antibodies from B cells) [33,34]. The infection may also be cleared at this stage. However most exposed individuals will enter the latency phase, which may persist for life. In this phase, the bacteria are contained inside a dynamic and organized structures called granulomas [30], which are a pathological hallmark of TB. Mycobacteria use granuloma cells to expand, disseminate and colonize nascent granulomas and regional lymph nodes (Gohn's complex) which leads to both lymphatic and haematogenous dissemination [35], with seeding of multiple organs, which may eventually give rise to extra-pulmonary disease or dissemination. Pulmonary lesions evolve from cellular granulomas which are composed of lymphocytes, macrophages, foamy macrophages and neutrophils to necrotizing granulomas. The necrotic core, which is also known as the caseum, owing to its cheese-like appearance, is the result of host cell and bacterial lysis [36].

As the granuloma matures, it often develops several layers of fibroblasts (known as a fibrous cuff), which function to wall off the infection allowing the bacterium to remain in a clinically inactive state. However, the bacteria may ultimately start replicating and escape the immune control, resulting in clinically active tuberculosis [37]. Nevertheless, the stage of infection is determined by the ability of the host innate and adaptive immune systems to eradicate or control *M. tuberculosis* [33].

Therefore, latent and active tuberculosis do not represent two separate and distinct states, but exists as a continuum of host–pathogen interactions [38].

Clinical aspects

Transmission

TB is considered an airborne infectious disease although *M. tuberculosis* complex organisms can be spread through unpasteurised milk, direct inoculation and other means. The airborne transmission is succeeded through aerosolisation of *M. tuberculosis*. Transmission rate is faster during coughing, speech and singing, all considered effective aerosol-generating activities that are inhaled into the alveoli of a new host [39].

The underlying pathophysiology of TB is the "10/3/1 formula", i.e. 10 people exposed yields 3 people with latent TB (LTBI) and 1 person with active disease [40]. In other words, although the majority has been exposed to TB, through innate or adaptive immune function, they are able to clear the invading bacilli and withhold a sterilising immunity [41].

Diagnosis

TB is also considered a multi-systemic disease with several presentations and manifestations and is one of the top 10 causes of death worldwide [10]. *M. tuberculosis* usually attack the lungs, yielding pulmonary TB, but as TB bacteria can attack any part of the body such as the kidney, spine, and brain it may also yield extra-pulmonary TB. As previously mentioned, not everyone infected with TB bacteria becomes ill. Consequently, two TB-related conditions exist; latent TB infection (LTBI) and TB disease [30]. If not treated properly, TB disease can be fatal. People who have latent TB infection do not feel sick, do not have any symptoms, and cannot spread TB to others [30].

History
TB risk groups comprises young adults (more frequently males), people in developing countries, health care workers, and patients with impaired immune systems, such as HIV patients or regular smokers. Classic clinical features associated with active pulmonary TB are coughing, weight loss/anorexia, fever, night sweats, haemoptysis (coughing blood), dyspnea (chest pain) and malaise/fatigue. Furthermore, symptoms

of TB disease may vary and arise in other parts of the body depending on the area affected [37].

Physical Examination

As earlier mentioned, physical examination findings associated with TB depend on the organs involved. Patients with pulmonary TB experience abnormal breath sounds, especially over the upper lobes or involved areas. Crepitation, rales or bronchial breath signs may be noted, indicating lung consolidation. Clinical symptoms, chest radiography, and sputum smear examination are the mainstays of rapid diagnosis [3]. Ultimate diagnosis rests on identification of *M. tuberculosis* in culture or demonstration of a definite clinical response to therapy. In most reported series, sputum smear examination is positive in 50-75% of patients with tuberculosis [42]. Smears are more often positive in patients with cavitary disease and less often positive in patients with HIV infection. Co-infected HIV and TB patients often rather present manifestations of primary tuberculosis. Mtb may cause symptoms and signs of disease (TB disease), but may also results in no clinical evidence of the disease, i.e. LTBI.

Imaging & Laboratory Investigations

Imaging

Chest X-ray (CXR), Ultra-Sonography USG, Computed tomography (CT) chest - Multi-detector CT (MDCT), MRI, Positron emission tomography-CT (PET) are among other various imaging modalities that can be used for TB diagnosis [43], for treatment evaluation- response and to control residual activity. This technique can also be used for detection of disease complications/sequelae. However, the majority of pulmonary tuberculosis infections are clinically and radiographically unapparent [44].

Smear microscopy

Three specimens from each patient with suspected TB should be examined microscopically for Acid Fast Bacilli AFB (classically Ziehl-Neelsen) or mycobacteria can be demonstrated by yellow fluorescence after staining with auramin. Both liquid and solid mycobacterial cultures should be performed for every specimen, and recovered isolates should be identified according to standard criteria (local protocols) [13]. A nucleic acid amplification test (NAAT) is recommended in AFB-positive patients and in AFB-negative patients with high suspicion of pulmonary TB. Rapid molecular drug susceptibility testing for rifampin and, optionally, for isoniazid is recommended in AFB-positive or patients with a positive NAAT who are at risk for drug-resistant tuberculosis [13]. From each patient with confirmed TB, an isolate should be genotyped for epidemiological public health control reasons.

Tuberculin skin tests (TSTs)

TSTs use an intradermal injection of a standardised bacterial purified protein derivative into the volar surface of the forearm (Mantoux method) to produce a transient wheal that is measured after 48–96 h. This test is simple, cheap and there is no need for phlebotomy. TST detects cell-mediated immunity to Mtb through a delayed-type hypersensitivity reaction. Sensitivity and specificity are dependent on the number of millimetres [45,46]:

≥ 5 mm: Patients with HIV co-infection, close contacts of patients with infectious disease, and those with fibrotic lesions on a chest radiograph

≥10 mm: Other high-risk patients, including infants and children under the age of four, health care workers, recent immigrants from countries with a high prevalence of tuberculosis, malnutrition, prolonged immunosuppressive therapy) and patients with chronic medical conditions and malignancies

≥15 mm: healthy people; at low risk for developing tuberculosis

Nevertheless, there are some TST usage limitation: the need for a return visit to have the test read, inter- and intra-reader variability in interpretation, false-positive results due to the cross-reactivity resulting from exposure to environmental mycobacteria or previous BCG vaccination [13].

Also, persons who are not likely to be infected with *Mycobacterium tuberculosis* should generally not be skin tested because the predicted value of a skin test in low-risk populations is poor [47].

Interferon-gamma release assays (IGRAs)

IGRAs require a blood sample to be taken from the patients. IGRAs are in vitro, T cell–based immune assays that measure interferon gamma (IFN-γ) release by sensitized T cells after exposure to secreted proteins specific to only *M. tuberculosis* and not *M. bovis* (the vaccine strain Bacillus Calmette–Guérin BCG) [46]. Interferon-gamma released by cells that recognise these antigens is then assayed in the supernatant after incubation (QuantiFERON-TB Gold, Cellestis Inc.) or by counting the number of interferon-gamma producing cells in an enzyme-linked immunospot assay (ELISA) (ELISPOT) [48]. The need for phlebotomy, test availability and the high cost of the test are also among the disadvantages [49].

Tuberculosis culture

Culture for AFB is the most specific test for TB and allows direct identification and determination of susceptibility of the causative organism. Routine culture uses a

nonselective egg medium (Lowenstein-Jensen or Middlebrook 7H10) (Fig. 4). However, obtaining the test results is slow because of the 22-hour doubling time of *M. tuberculosis* [50]. Radiometric broth culture (BACTEC radiometric system) can be used to significantly reduce the time (10-14 days) for mycobacterial recovery. All isolates are notified to public health authorities, used for susceptibility and genotyping. A community based, low-cost, sensitive, user-friendly, high-throughput, and same-day point-of-care screening (triaging) test for tuberculosis is clearly needed [1].

Figure. 4-Colonies of Mycobacterium tuberculosis on Löwenstein-Jensen medium. Two media are used to grow MTB, Middlebrook's medium which is an agar-based medium and Lowenstein-Jensen medium which is an egg based medium.

Treatment success and challenges

The course of TB treatment depends on whether the individual is in the latent or active stage, and on his or her probability of risk. Treatment of TB usually involves a drug cocktail, or a mixture of multiple drugs, with an intensive initial 2-month phase followed by a slower 4- to 6-month continuation phase the main anti-tuberculosis drugs used in the chemotherapy of TB are: isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and either ethambutol (EMB) or streptomycin (SM) [51,52]. Isoniazid preventive therapy IPT is the recommended treatment for LTBI but the regimen's main drawback is the duration of therapy [49].

Drug-resistance

The treatment of multidrug-resistant tuberculosis is based on expert opinion and requires the creation of combination drug regimens chosen from five hierarchical groups of first-line and second-line drugs. Multidrug-resistant tuberculosis (MDR TB) is TB that is resistant to at least two of the best anti-TB drugs, isoniazid and rifampicin. These drugs are considered first-line drugs and are used to treat all persons with TB disease [53]. Extensively drug resistant TB (XDR TB) is a relatively rare type of MDR TB. XDR TB is defined as TB which is resistant to isoniazid and rifampin, plus resistant to any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin). Because XDR TB is resistant to first-line and second-line drugs, patients are left with less effective treatment options, and these cases has often worse treatment outcomes. Bedaquiline and Delamanid were approved by the Food and Drug Administration (FDA) at the end of 2012 for the treatment of adults with multidrug-resistant pulmonary tuberculosis for whom an effective treatment regimen is not otherwise available [54]. Two strategies may facilitate Mtb clearance: enhancement of anti-Mtb immune responses with the aim of eradicating the pathogen and a targeted decrease in inflammation (organ-saving strategy) [40].

Prevention

Mycobacterium bovis Bacillus Calmette–Guérin (BCG), an attenuated vaccine derived from *M. bovis*, is the only licensed vaccine against tuberculosis (TB) [55]. BCG vaccine was prepared at the Pasteur Institute, France in 1921 consisting of a live attenuated strain of *M. bovis*, a closely related subspecies of Mtb. By repeating sub-culturing (231 passages) of the virulent strain on ox bile glycerine–potato media every 3 weeks over 13 years, they produced an attenuated strain, which, by the year 1919, was shown to be avirulent in guinea pigs, cows, horses, hamsters, mice and rabbits [56]. This attenuation promoted genetic deletions from *M. bovis* and resulted in 16 genomic regions of differentiation (RD1–RD16) as compared to the Mtb genome [57]. Despite its protection against TB in children especially TB meningitis and the disseminated TB form (Miliary TB), its protective value in adults against pulmonary TB is questionable, with efficacies ranging between 0 to 80 % (average 50 %) have been reported from multiple clinical trials performed during the 20th century [58]. The reason for such variable protection may include BCG strain variation, the host genetic variability amongst and different ages of the vaccinated individuals, administration routes, high incidence vs low incidence settings, the dose of vaccine, interference by environmental, mycobacterial and helminthic infection, and patient nutritional status [59]. BCG vaccine could not be replaced by another vaccine today as this is the only vaccine that needs only one single dose, thereby eliciting a long-lasting immunity. This vaccine is also cheap, feasible to produce, stable and safe [60].

However, the manipulation of BCG vaccine to improve efficacy should be carefully considered, as it can bring in both favourable and unfavourable effects.

In spite of the current knowledge of molecular biology, immunology and cell biology, infectious diseases such as TB and HIV/AIDS are still challenges for the scientific community.

Clinical Value of
Initial Host Molecular Signalling

Innate and adaptive immune response in TB

The host response against tubercle bacilli is comprised of a balanced interaction of innate and adaptive immune responses [40]. The innate immune system provides the initial immune response to Mtb and is comprised of physical barriers to the environment (i.e., skin, mucosa), antimicrobial peptides and proteins, cells (i.e., epithelium, mucosa, natural killer (NK) cells, dendritic cells, neutrophils, macrophages, and monocytes), and soluble factors (i.e., cytokines, chemokines, complement) [61,62]. These are followed by inductive immune responses to inhaled antigens within the respiratory tract which occur mainly in the bronchus-associated lymphoid tissue (BALT) by induction of T- and B-cell responses. The specialized epithelium overlying the lymphoid aggregates consists of M cells heavily infiltrated with lymphocytes and significant dendritic cell populations directly below the epithelium [63]. Minimal inflammation occurs in the bronchial mucosa accounting to T_{regs} that inhibit T-cell activation and expansion. Instead, antigen is carried by local macrophages to the regional lymph nodes, where respiratory effector immune responses originate [64]. Balance of protection and pathology defines the chronic nature of intracellular *M. tuberculosis* infection. Although it is evident from human disease and from experimental mouse models that CD4+ and CD8+ T cells [29] in addition to IL-12, IFN-γ and TNF are all fundamental in the control of *M. tuberculosis* infection [65,66], there remains an incomplete understanding of the host factors that determine why some individuals are protected from *M. tuberculosis* infection while others go on to develop disease Balance of protection and pathology defines the chronic nature of intracellular bacterial infection.

The role of epithelial cells and respiratory mucosa in TB

The innate host defence of the conducting airway depends on the multiple barriers created by layers of mucus and the tight adhesions between epithelial cells. The first surface that immobile bacterium will encounter after inhalation into lungs would most typically be epithelia. Several groups have shown that *M. tuberculosis* invade, survive, manipulate and replicates within alveolar epithelial cell [67,68]. Furthermore, airway epithelial cells (AECs) are now recognized as active participants of the immune response against *M. tuberculosis* [68] and are critical during the progression to

active disease [69,70]. Furthermore, AECs facilitate a protective environment for Mtb replication where it could gain enhanced virulence by modifying envelope structure and gene expression [71,72].

Epithelial cells express pattern recognition receptors (PPRs), such as the Toll like receptors (TLRs) that interact with mycobacteria [73]. AECs also interact with other cells of the innate immune system, such as granulocytes, monocytes, macrophages, and innate lymphoid cells, to mount an effective defence against the invading pathogen as well as to activate the following specific immunity.

The extent of epithelial cytokine secretion may lead to tissue damage and breakdown of extracellular matrix, thus favouring bacterial persistence and facilitating mycobacterial transmission [74,75]. However, perturbed defence in immune-compromised patients can tilt this balance leading to active disease [76]. It has also been observed that mycobacteria manipulate epithelial production of the cytokine CXCL8 through the inhibitory G-protein-coupled receptor (GPCR) kinase 2 [77].

Traditionally, the adaptive immune responses, particularly central memory CD4+ and CD8+ T cells, are considered to be important for long-term immunity [41,78,79]. However, emerging evidence indicates that the cells of the innate immune system are equipped with "epigenetic memory" where genes encoding specific host defence molecules increase the response upon re-stimulation [80,81]. Moreover, mucosal vaccination with an attenuated *M. tuberculosis* strain induced a strong innate immune response, followed by a robust central memory answer [82].

Inductive immune responses to inhaled antigens within the respiratory tract occur mainly in the bronchus-associated lymphoid tissue (BALT). BALT comprised of lymphoid aggregates and function to provide protection against inhaled microbes by induction of T- and B-cell responses. The specialized epithelium overlying the lymphoid aggregates consists of M cells infiltrated with lymphocytes and significant dendritic cell populations directly below the epithelium. the main result of induction of immune responses in BALT is the production of secretory IgA. Pools of lymphocytes are present within the lung interstitium, made up of 10–20% T cells [63,83]. Macrophages are present on both the air side of the lung and airways as well as in the mucosa. Communication occurs between the gastrointestinal and respiratory mucosae through cell trafficking. Antigen-reactive T and B cells from the gut-associated lymphoid tissue (GALT), amongst Peyer's patches, can populate the bronchial mucosa. This common mucosal immune system feature has been exploited to develop oral vaccines against respiratory microbes [84].

The molecular repertoire for innate recognition of *Mycobacterium tuberculosis*

A major challenge in host–pathogen interaction in tuberculosis is to define the mechanisms used by *M. tuberculosis* for its survival inside the host. The innate immune response comprises several different cell types, has its own receptor system to recognize the presence of pathogens, and is a key to the initiation of an adaptive immune response in the host [85]. Therefore, pathogen recognition is considered as the most critical step behind eliciting adequate immune response during an infection [86]. After Mtb-containing aerosols is deposited into the pulmonary alveoli, the bacteria bind to phagocytic receptors and enter resident alveolar macrophages, dendritic cells, and alveolar epithelial cells [68,87,88]. Cellular receptors involved in the endocytosis of *M. tuberculosis*, such as the immunoglobulin receptors (Fc), complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18), mannose-binding receptors such as the soluble mannose-binding lectin (MBL), surfactant protein A and D (Sp-A, Sp-D), DC-specific intercellular-adhesionmolecule-3 grabbing non-integrin (DC-SIGN; CD209), Dectin-1 and the macrophage mannose receptor (MMRc), are capable of transducing intracellular signals [85,89]. However, these receptors do not appear to be a major contributor of *M. tuberculosis*-induced cytokine production [87].

Besides expressing phagocytic receptors, both classical and non-classical (including alveolar epithelial cells) immune cells express pattern recognition receptors (PRR) [73]. There are several families of PRRs that recognize conserved pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) such as the cytosolic nucleotide-binding oligomerization domain-like (NOD-like) receptors that sense bacterial products, the C‑type lectin receptors (CLRs) that binds β‑glucans, the retinoic acid inducible gene RIG‑I-like receptors (RLRs) that binds nucleic acid and scavenger receptors bind modified low-density lipoprotein [90][91,92]. Amongst the innate immune PRRs, Toll-like receptors (TLRs) are the most studied [93].

The TLRs are crucial for sensing microbial or endogenous products released from bacteria and viruses and in order to trigger the innate immune responses [94]. TLR signalling triggers a cascade of events in innate immune cells that includes change in chemokine and cytokine production, altered chemokine receptor expression, and modify signalling through G protein-coupled receptors (GPCRs). One of the mechanisms by which TLR signalling could modify GPCR signalling is by altering the expression of regulator of G protein signalling (RGS) proteins culminating in activation of mitogen-activated protein kinases (MAPK), PI3 kinase and NF-κB to initiate innate cytokine secretion and anti-microbial intermediates (e.g. radical oxygen species and nitric oxide) [95-97].

Immunobiology of TLRs

TLRs are expressed on the cell surface or intracellularly in many cell types, especially in cells of the innate immune system. TLRs can be divided into two sub groups such as transmembrane (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) and intracellular (TLR3, TLR7, TLR8, and TLR9) [98]. Mammalian TLRs represent a structurally conserved family of membrane receptors, which share common structural elements, containing an ecto-domain that contains leucine-rich repeats, a transmembrane region and a cytoplasmic Toll/IL-1-Receptor (TIR) domain [99].
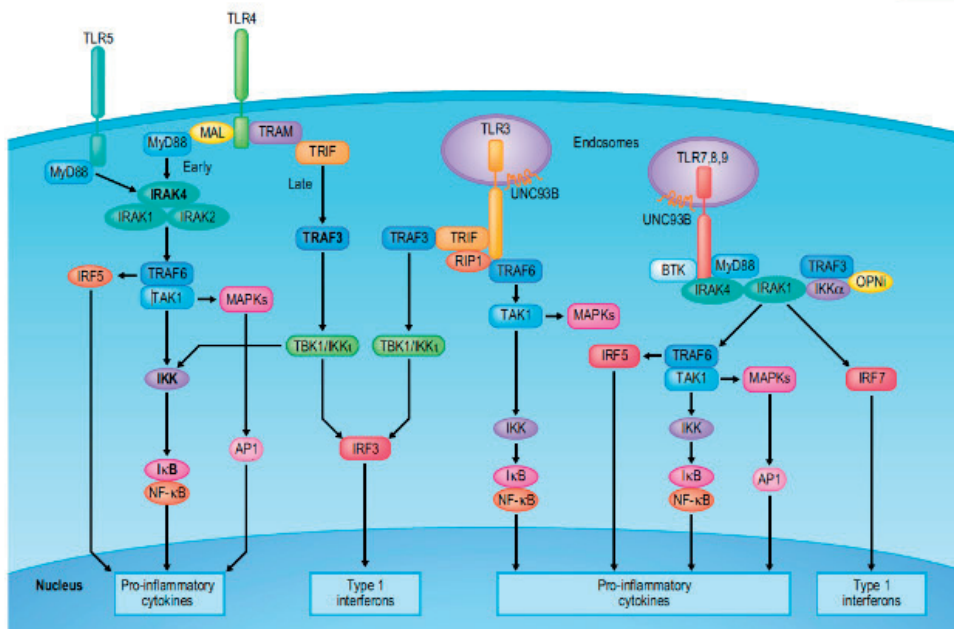

Figure 5. MyD88 dependent and independent TLR signaling pathways. MyD88 recruits interleukin 1 receptor associated kinase 4 (IRAK4) which phosphorylates IRAK1, 2 that in turn activate the TNF Receptor-Associated Factor TRAF6 and TAK-1. Subsequently, activation of the IKK complex and MAP kinases lead to activation of NFkB and AP1 transcription factors,respectively.The transcription factor IRF5 is also activated downstream of TRAF6. The TLR4 signaling utilizes four adaptor proteins.The adaptors MAL and MyD88 lead to activation of signaling cascade through the IRAKs. Subsequently, TLR4 is internalized and a signaling cascade dependent upon the adaptors TRAM and TRIF is activated. TLR3 activates a TRIF-dependent pathway leading to production of pro-inflammatory cytokines and IFN- β b. TLR7, 8, 9 are MyD88 dependent and activate the transcription factors NFkB, IRF5, AP1, and IRF7, resulting in production of pro-inflammatory cytokines and type 1 interferons Reprinted from "Clinical Immunology, principles and practise", Robert R. Rich, 2013, Elsevier, with persmission.

The broad cellular expression of TLRs and their diverse agonists allow detection of a wide variety of pathogens despite the existence of a limited number of TLRs. Engagement of TLRs by these pathogen-specific ligands, an early event in the interaction of Mtb with its host cell, activate cytosolic TIR domain and associated adaptors. Myeloid differentiation factor 88 (MyD88), TIR-related adaptor protein inducing interferon (TRIF), TRIF-related adaptor molecule (TRAM), TIR domain containing adaptor protein (TIRAP) or MyD88 adaptor like (MAL), and Sterile-alpha and Armadillo motif-containing protein (SARM) are the five major important adaptors of TLR activation [94].

Upon TLR activation, MyD88 recruits interleukin 1 receptor associated kinase 4 (IRAK4) which phosphorylates IRAK1 that in turn activate the TNF Receptor-Associated Factor 6 (TRAF6). Both proteins leave the receptor complex and interact with TGF- α -activated kinase 1 (TAK1) and two TAK1 binding proteins TAB1 and TAB2. TAK1 becomes phosphorylated and activates the I-kB kinase (IKK) complex comprising IKKα, IKKβ, and NEMO/IKKγ, and Mitogen-activated protein kinase (MAPK) leading to the activation of NF-κB and c-Jun N-terminal kinases (JNK) signalling pathways respectively for triggering the expression of a wide variety of cytokines [100]. TAK1 is also capable of phosphorylating MKK6 and 7 which leads to the activation of p38 and JNK.

MAL or TIRAP is the second adaptor that transmits signal from TLR4 and TLR2 by facilitating transcription factor activation like NF-κB, JNK and the extra-cellular regulated kinases 1(ERK-1) [101]. TRIF is the third adaptor for TLR4 and TLR3 that activates Interferon regulatory factor3 (IRF3) by activating the kinases TBK1 and the I-kB kinase D. Fourth adaptor is TRAM which is one of the interacting partners of TRIF. TRAM is involved in transmitting the TLR4 signalling resulting in MyD88-independent interferon-γ production. The fifth adaptor is SARM, which mainly interact with TRIF, thereby negatively regulating the NF-κB and IRF3 activation.

A MyD88-independent TLR4 signalling pathway involves TRAM and TRIF and leads to activation of type 1 IFNs. Thus, individual TLRs may utilize different signalling systems that characterize their specific functions. Therefore, the modulation of TLR protein function may hold great potential in combating active, latent and multidrug-resistant *M. tuberculosis* infection in humans [94].

TLR stimulation through PI3K kinase activates further kinases downstream the signalling pathway [102]. Activation of Akt or p38 inactivates the glycogen synthase kinase 3 (GSK3) that is found further down the signalling pathway [103]. GSK3 is constitutively active in resting cells leading to the pro-inflammatory NF-κB transcription, but phosphorylation of GSK3 by p38/Akt switches the transcriptional activity to cAMP response element-binding protein (CREB) [104]. TLR activation

can either lead to a pro-inflammatory cytokine response by activation of NF-κB pathway or an anti-inflammatory CREB-related cytokine response.

M. tuberculosis and TLRs interaction

TLR2 expression increases upon mycobacterial infection of alveolar epithelium and blocking of TLR2 decreases cytokine responsiveness [105]. Accumulated data indicate that Mtb expresses ligands that bind to TLRs family, especially TLR2, TLR4 and TLR9 [106-108]. However other TLR1 or TLR6 has been documented to be involved in Mtb infection [107]. The 19-kDa lipoprotein was the first Mtb ligand shown to interact specifically with TLR2 to induce TNFα and nitric oxide production from both murine and human macrophages [87]. Other mycobacterial lipoproteins, lipomannan and phosphatidyl-myo-inositol mannoside (PIM) also interact with TLR2 to initiate cellular activation[109] and the non-methylated CpG motifs of mycobacterial DNA an activator of the TLR9-pathway, also induces rapid antimycobacterial responses in macrophages, in a phospholipase D-dependent manner [87]. TLRs signalling results in secretion of inflammatory cytokines such as IL-1, IL-6, IL-10, IL-12, TNFα, chemokines such as IL-8 and reactive oxygen and nitrogen intermediates (ROI, NOI). Previous studies indicate that signalling through TLRs is important for the phagocytosis of bacteria, as TLR-mediated MyD88-dependent activation of p38 is required for phagosome maturation [110,111].

Intracellular pathogens such as *Listeria monocytogenes* manipulate TLRs through the MAPK pathway to avoid phagosome maturation, autophagy and cytoskeletal changes [112-114]. TLRs play also a role in inhibition of reactive oxygen and nitrogen intermediates, in addition to stimulation of the cytokine network. The p53 pathway acts in synergy with the p38 MAPK pathway to mediate cell cycle arrest, cellular senescence and apoptosis [115], MyD88 binds Fas-associated death domain (FADD) and activates apoptosis of infected cells through the caspase cascade. TLR2 and TLR4 are also known to modulate the activation of peroxisome proliferator-activated receptor (PPAR)c that mycobacteria utilize to affect the NF-κB activation [116,117]. Activation of Akt or p38 inactivates the glycogen synthase kinase 3 (GSK3) that is found further down the signalling pathway [103]. GSK3 is constitutively active in resting cells leading to the pro-inflammatory transcription, but p38/Akt phosphorylation of GSK3 switches the transcriptional activity to cAMP response element-binding protein (CREB) [104]. TLR activation can thus either lead to a pro-inflammatory cytokine response by activation

Chemokines as mediators of defence in TB

Chemokines constitute the largest family of cytokines, consisting of approximately 50 endogenous chemokine ligands (6–14 kDa) in humans and mice. Chemokine receptors constitute the largest branch of the γ subfamily of rhodopsin seven-transmembrane receptors [118]. Chemokines are a family of small proteins secreted by a great variety of cell types. Their name (derived from chemoattractant cytokines) is due to their ability to control the migratory patterns and positioning of all immune cells and also critical for the generation of primary and secondary adaptive cellular and humoral immune responses [119]. Correct mononuclear cellular recruitment and localization are essential to ensure control of bacterial growth without the development of diffuse and damaging granulocytic inflammation. Nonetheless, their functions include growth regulation and haematopoiesis, tissue and cell development, modulation of specific immune recognition and participation in several pathophysiologic situations [120,121].

Chemokines can be classified as inflammatory or homeostatic. Some of these molecules are considered to be pro-inflammatory as their expression, which is very low or absent increases when cells are damaged. These chemokines, such as CCL2 and CCL3, can be induced during an immune response to promote cells of the immune system to the site of infection, whereas others are considered homeostatic and are involved in controlling the migration of cells during normal processes of tissue maintenance or development to guarantee constant recirculation of leucocytes during physiology. The 'extracellular nonconventional receptors' named glycosaminoglycans (GAGs) are extracellular molecules that bind chemokines and stabilize their concentration gradient, determining therefore the most effective chemotaxis in tissues [122]. Cytokines have pleotropic effects and their receptors are redundant (i.e. they may have more than one receptor and their receptors often share subunits).

Immunobiology of chemokines

Chemokines are structurally related, most chemokines contain at least four cysteines that form two disulfide bonds, one between the first and the third and one between the second and the fourth cysteine (C) residues [123]. Four groups are defined on the basis of the presence or absence of non-conserved amino acids between those cysteines: CC (adjacent cysteine residues), CXC (separated cysteine residues by single amino acid residue), CX3C (separated by three amino acid residues) and C (where Lymphotactin is the only known chemokine that contains only two cysteines [120].

A nomenclature that tries to standardize the names of chemokines withhold the following codes: CCLn, CXCLn, CX3CLn or XCLn, where 'n' is a correlative number that individualizes each molecule, and 'L' is an abbreviation of 'ligand' which

is used to distinguish CKs from their receptors (CCRn, CXCRn, CX3CRn and XCRn) which use 'R' (an abbreviation of 'receptor'. A subfamily of CXC chemokines are further classified according to the presence of the tripeptide motif glutamate–leucine–arginine (ELR) motif near the N terminal of the molecule (designated ELR+) are specific for leukocytes (such as CXCL8), whereas ELR− chemokines (such as CXCL13) attract lymphocytes [124]. Some chemokine receptors pair monogamously with their chemokine ligand. However, most are promiscuous, but restricted to one chemokine group. The binding of the chemokine to the receptor activates signalling cascades, most signalling depends on coupling through Bordetella pertussis toxin-sensitive G proteins. CXCR1 and CXCR2 couple most commonly through $G\alpha i2$, but also through $G\alpha 14$, $G\alpha 15$, and $G\alpha 16$, but not $G\alpha q$ or $G\alpha 11$ that culminate in the rearrangement, change of shape, and cell movement of actin [125].

CXCL8, CXCR1 and CXCR2 in tuberculosis disease

The chemokine CXCL8 (IL-8) is secreted early in the infectious disease process and acts as a strong neutrophil chemoattractant, but is also chemotactic for monocytes and lymphocytes [126,127]. In TB patients, CXCL8 is found abundantly in sputum, lymph nodes and plasma [128,129] . CXCL8 binds to the two GPCRs, CXCR1 and CXCR2, which are primarily expressed by neutrophils, but also by peripheral blood mononuclear cells, alveolar macrophages, TH cells, alveolar and bronchial epithelial cells, and vascular cells [77,130-132]. Neutrophils are an essential component of the innate immune system and are the first group of cells that migrate to sites of infection. However, sustained neutrophil response in severe TB have also been associated with delayed Mtb clearance and disease severity [133,134]. The imbalance between anti-inflammatory and pro-inflammatory factors can lead to dysregulated inflammation in TB is thought to be partly due to the accumulation of early influx of neutrophils and NK cells whom both express the receptor CXCR2 [135-137]. Between the two CXCL8 receptors, CXCR2 has gained particular interest in the pathology of several pulmonary diseases, such as ischemia/reperfusion injury, chronic obstructive pulmonary disease and pulmonary fibrosis [138]. In addition, lung epithelial cells can directly sense Mtb and produce chemokines, resulting in a potentiation of immune cell recruitment. CXCL8 and CXCL5 are produced by alveolar epithelial cells and by human bronchial epithelial cells and can increase neutrophil recruitment. Recent reports have shown increased neutrophils in broncho-alveolar lavages from patients with cavitary TB in comparison to non-cavitary TB patients, further associated with decreased CXCL10 and IL-6, which may indicate the failure of adaptive immunity at this stage of disease [139]. Therefore, understanding the functions and interactions between cytokines and chemokines is therefore critical to our attempts to limit TB.

IL-22

IL-22 is primarily produced by T cells as well as γδ T cells, natural killer (NK) cells, and innate lymphoid cells following exposure to innate or infectious stimuli. The major functions of IL-22 are the regeneration and survival of the intestinal, airway, and external epithelium, as well as stimulating the secretion of antimicrobial peptides such as lipocalin and β-defensin [140]. In the context of TB, IL-22 is expressed at higher levels than IL-17 at the site of infection and within granulomas from TB patients. IL-22 has also been shown to inhibit intracellular growth of *M. tuberculosis* in human monocyte-derived macrophages. Current data suggest a protective role for IL-22 in TB disease progression, possibly via antimicrobial peptide production, cellular function, and promotion of epithelial repair [141].

IL-10

The major function of IL-10 is to serve as an anti-inflammatory and immunosuppressive cytokine. Furthermore, IL-10 strongly inhibits the production of IL-1, IL-6, IL-8, IL-12, TNF, and other immune and inflammatory cytokines. IL-10 can be produced by T-cell subsets including T_{REG}, T_{H1} and T_{H17} cells, macrophages, some dendritic cell subsets, myeloid-derived suppressor cells, B cells, and neutrophils [142]. It inhibits macrophage antigen presentation and decreases expression of MHC class II, adhesion molecules, and the co-stimulatory molecules CD80 (B7.1) as well as CD86 (B7) [143]. IL-10 signals through the IL-10R, which comprises IL-10R1 and IL-10R2. IL-10R1 is induced on hematopoietic cells, while IL-10R2 is expressed constitutively on most tissues and immune cells. In the context of TB, was initially identified as a regulatory "cytokine synthesis inhibitory factor" polymorphisms in the IL-10 gene, are significantly associated with an increased TB risk [144].

IL-6

IL-6 has a wide array of biological actions on both lymphoid and non-lymphoid cells. It is important in host defence and in inflammatory responses. It is considered to possess both pro-inflammatory and anti-inflammatory qualities. IL-6 serves as a major inducer of fever and the synthesis of acute-phase proteins in the liver, such as fibrinogen, serum amyloid A, haptoglobin, C-reactive protein [145]. This cytokine is also involved in the essential cellular processes of differentiation, proliferation, and apoptosis. IL-6 signals through soluble and membrane-bound IL-6R of which the glycoprotein 130 dimer (gp130) is an essential component. Downstream signalling is mediated by a phosphorylation cascade involving JAK, mitogen activated protein kinase (MAPK), and STAT pathways [146]. The pluripotency of IL-6 warrants regulation and this is mediated by suppressor of cytokine signalling (SOCS), which inhibits STAT signalling [147]. IL-6 also play a role in potentiating immunity during early *M. tuberculosis* infection [146].

Cytokine-induced host-protective cellular mechanism (Respiratory burst)

Innate immune system increase their oxygen consumption through the activity of an NADPH-oxidase that generates superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) The release of reactive oxygen intermediates such as O_2 and H_2O_2 is an important component of the innate immune bactericidal machinery [148]. Activation of a membrane-bound NADPH oxidase by stimulation with IFNγ or IgG initiates an oxidative burst that generates ROI, these oxygen metabolites give rise to yet other reactive oxygen species ($\bullet O_2^-$, H_2O_2, OH^-, and $\bullet OH$ radical) that are strongly anti-microbial, but may also cause damage by destructing surrounding tissue and inducing apoptosis in other immune reactive cells [149]. Human neutrophils, that abundantly express myeloperoxidase, but also in blood monocytes, the activity of reactive oxygen intermediates (ROI) is further augmented by the formation of hypochlorous acid. Oxidation of bacterial lipids and proteins results in bacterial killing. The importance of ROIs in antibacterial defence is underlined by recurrent infections in patients whose phagocytes fail to generate an oxidative burst. Professional phagocytes deliver NO to the phagolysosome harbouring bacteria while consuming O_2 and L-arginine. NO is further oxidized to NO_2^- and NO_3^-. Nitrification and/or oxidation then functions by inactivating bacterial molecules needed for bacterial growth [150]. The formation of reactive nitrogen intermediates (RNI) $\bullet$NO is catalyzed by NOS2, which is promoted by both immunological stimuli such as IFNγ and TNF, and microbial products such as LPSs, lipoteichoic acid, and mycobacterial lipids. RNIs exert their bactericidal activity by destroying iron-/sulfur-containing reactive centres of bacterial enzymes, and by synergizing with ROIs to form highly reactive peroxynitrite ($ONOO^-$). A central role for NOS2 in protection against intracellular bacteria is well established in murine models of infection [151].

G-protein–coupled receptors (GPCRs)

In 2012, The Nobel Prize in Chemistry was awarded to Brian K. Kobilka and Robert J. Lefkowitz for studies of G-protein–coupled receptors (GPCRs). It all started with noticing the remarkable similarities to the one for rhodopsin, known then as a light-sensing receptor of the eye and concluded that there likely exists an entire protein family of such receptors with similar structure and function confirmed the hypothesis about the common folding of all GPCRs [118]. The name GPCR refer to a common mode of receptor signalling via heterotrimeric GTP-binding proteins on the inside of the cell. Because their polypeptide chain passes seven times through the plasma membrane, the GPCRs are also called seven-transmembrane (7TM) receptors. Human cells are constantly communicating between each other and over long distances in the body. This requires a molecular scaffold and a means for transmission of information across the plasma membrane. The seven-transmembrane spanning GPCRs are critically involved in transmitting extracellular stimuli including

light, hormones and neurotransmitters into specific cellular responses. Today we recognize that GPCR play a vital role in various diseases and that understanding its role could provide a rational basis for the design of novel therapies in various clinical conditions. GPCRs are also pharmacologically important because they are the targets of about 30% of commercially available drugs [152].

GPCRs structure

GPCRs comprise seven transmembrane α-helices (7TMH) connected by three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3). The extracellular (EC) region, which is responsible for ligand binding, also includes the N-terminus that can range from relatively short sequences in rhodopsin-like receptors to large extracellular domains in other classes of GPCRs, e.g. the hormone-binding domain (HBD) in adhesion receptors. The intracellular (IC) region interacts with G proteins, arrestins and other downstream effectors. The ligand binding triggers a slight change in GPCR conformation that is propagated through the whole protein, ultimately causing alterations at the receptor's cytoplasmic surface that permit binding to its cognate G protein [153]. The GPCR superfamily is divided into five main families: glutamate (former class C), rhodopsin (former class A), adhesion (part of former class B), frizzled/taste2 (former class F), and secretin (part of former class B). The rhodopsin family can be further divided into four subfamilies: α, β, γ and δ. the γ subfamily consists of three main branches: somatostatin, opioid and galanin receptors (SOG) receptors (including crystallized μOR, δOR, ϰOR and nociceptin opioid receptors), MCH receptors, and chemokine receptors [118].

The molecular framework of G-protein–coupled receptors (GPCRs)

The cascade reaction on the inside of the cell starts with nucleotide exchange where the heterotrimeric G protein dissociates into subunits (Gα, Gβ and Gγ;) [154]. Gα binds to and stimulates enzymes such as adenylate cyclase. This produces the cyclic nucleotide cAMP, which diffuses easily and serves as a 'second messenger' [155]. Other proteins may interact with Gβ and Gγ to further modulate the signal. This in turn activates diverse G protein-dependent effectors, including phospholipases A2, C (subtypes b2 and b3) and D, phosphatidylinositol-3-kinase(PI3Kγ), protein tyrosine kinases (PTK) and phosphatases, low molecular weight GTPases, and mitogen-activated protein kinases (Fig.6).
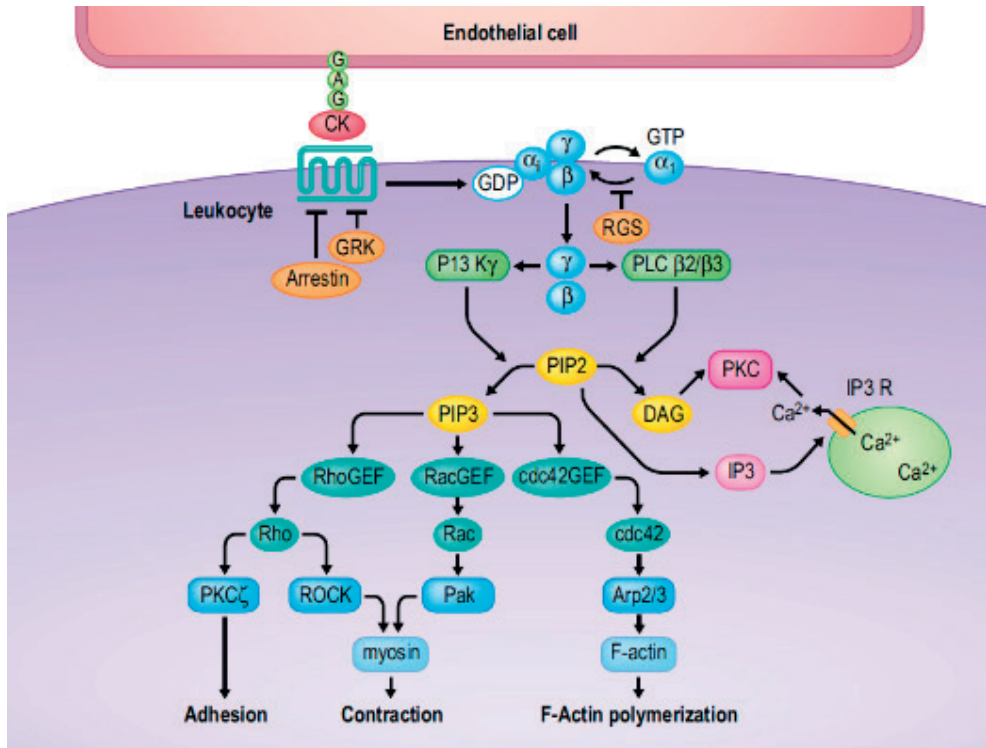
Figure 6. Chemokine signal transduction in chemotaxis. two main pathways induced by most chemokines.The PI3Kγ pathway is particularly important for cell migration. Chemokines are able to activate other pathways as well, including non-Gi-type G proteins, protein tyrosine kinases, and MAP kinases. Reprinted from "Clinical Immunology, principles and Practise", Robert R Rich et al, 2013, Elsevier, with permission

Cytosolic and calcium-independent PLA2 catalyse formation of arachidonic acid from membrane phospholipids and enhance chemokine activation of human monocyte chemotaxis [118]. PLC hydrolyzes PI bisphosphate (PIP2) to form 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) (Fig.6). IP3 induces Ca^{+2} release from intracellular stores, which acts with DAG to activate protein kinase C (PKC) [125]. The PI3Kg phosphorylates PIP2 to form PIP3, which recruits proteins containing pleckstrin homology (PH) or PHOX (PX) domains to lamellipodium, thereby converting shallow extracellular chemokine gradients to steep intracellular effector gradients. Four PH domain-containing targets—Akt, and GEFs for Rac, Rho, and Cdc42—modulate distinct phases of cell movement in various model systems. Rho regulates cell adhesion and chemotaxis, and myosin contraction [156] (Fig 6). Rac and Cdc42 control lamellipodia and filipodia formation, respectively. Downstream targets of Rac include Pak1, which also regulates myosin contraction and respiratory burst [157,158].

To trigger GPCR signal transduction in AECs, intracellular bacteria such as *Shigella*, are known to engage GTPases in actin polymerization [159,160]. These low-molecular- weight proteins belong to the Ras GTPase superfamily and include Rab and Rho/Rac, with the ability to act as molecular switches by coupling extracellular signals to different cellular responses, cytoskeletal integrity, intracellular vesicular transport, and trafficking of proteins [161]. Inhibition of Rac1 was recently shown to repeal tumour protein p53 suppression of STAT and NF-κB, and where Rho was essential in the establishment and maintenance of tight junctions [162].

Tuberculosis Molecular Fingerprinting

Molecular Epidemiology of tuberculosis: role of whole genome sequencing

The molecular epidemiology field emerged from the integration of molecular biology, statistics, clinical medicine and epidemiology that studies the role of genetic and environmental risk factors at the molecular/cellular or biochemical level in disease aetiology and transmission among populations [163]. Despite the fact that much is known about the epidemiology of TB, some key questions have confounded classical epidemiologists for long. Researchers still struggle to find the rates of active transmission by differentiating disease due to high/low burden settings and recent/previous infection. The determination of whether recurrent tuberculosis is attributable to exogenous reinfection or reactivation, is another question that may be answered through molecular epidemiology studies. As is the question to whether all *M. tuberculosis* strains exert similar epidemiologic characteristics in populations. Furthermore, an understanding of transmission dynamics on group-specific levels, or cluster-levels, may well identify extensive transmission or outbreaks from what appear to be sporadic, epidemiologically unrelated cases. Several molecular epidemiologic methods have facilitated studies that address most of these very questions [164].

Given the plethora of molecular tools available, it is critical to choose an appropriate method to address the transmission dynamics, outbreaks, or phylogenetics. In general terms, the key in choosing the adequate molecular approach for studying TB transmission is the observed rate of polymorphism, i.e. the stability of the biomarker, and the genetic diversity of strains in the population. Indeed, the rate of change of a biomarker must be adequate to distinguish non-epidemiologically related strains and yet be sufficient to reliably link related cases. This requirement, coupled with the general background of TB prevalence, should be taken into consideration when choosing molecular epidemiologic methods or in evaluating data.

The completion of the first human genome drafts was just the start of the modern DNA sequencing era which was further applied on microbes [165,166]. The TB research community welcomed the genomic era in 1998 with the publication of the complete annotated genome of the *M. tuberculosis* laboratory strain H37Rv [27]. The *M. tuberculosis* complex genome is highly conserved in relation to other bacterial pathogens. It has a very high guanine and cytosine content and carries highly repetitive motifs [57,167]. While the members of the *M. tuberculosis* complex display diverse phenotypic characteristics and host ranges, they represent an example of

interspecies genetic homogeneity, with an estimated rate of synonymous, or functional, nucleotide polymorphisms of 0.01% to 0.03% and no significant evidence for horizontal genetic transfer between genomes, unlike most bacterial pathogens [168].

For over 20 years, investigators have been discovering and utilizing genetic elements of the Mtb genome as molecular genotype markers [163,169]. The *M. tuberculosis* genotyping methodologies include utilizing insert elements, as in Insertion element (IS6110), the direct repeat locus-based spacer oligonucleotide typing (spoligotyping) and mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) typing [170-173].

The advent of novel DNA sequencing technologies has revolutionized the field of tuberculosis by generating wealth of data, massively parallel sequencing and designing novel methods to exploit this information. Many mycobacteriologists fully embraced the revolution, understanding that the generation of high numbers of genomes from a single species, strain or clonal group would allow to reconstruct the history of a bacterium in time and space, to trace its movements and relevant evolutionary events, and to understand the success of specific strains. This genomic epidemiology approach has been applied to mycobacterium tuberculosis [174,175]. Comparative genomics and whole-genome sequencing (WGS) have paved the way for high-resolution analysis of the genome and become a common technique for investigation of the pathogenic bacteria, and have been used to address various aspects of tuberculosis [176-178]. Sequencing technologies are advancing at a rapid pace. With time, these advances hold promise to overcome current technical limitations. Moreover, as the cost of reagents and instruments continues to decrease, the opportunities for applying sequencing on the front line of the tuberculosis fight will only expand.

Current genotyping methods

IS6110-based Restriction Fragment Length Polymorphism (RFLP)

Insertion elements (IS) are small mobile genetic elements, usually less than 2.5 kb in size, that are widely distributed in most bacterial genomes [179]. IS elements are commonly defined as carrying only the genetic information needed for their transposition and regulation, unlike transposons, which can also carry genes that encode phenotypic markers (e.g., antibiotic resistance). IS6110-based RFLP genotyping detects variations generated by the insertion element IS6110. Strains can

differ in both the number of copies of IS6110 and the positions of IS6110 in the bacterial DNA [180].

IS6110-based RFLP is performed by using an internationally standardized protocol: purification of DNA from a culture of *M. tuberculosis* followed by the addition of a restriction enzyme that cuts the DNA at specific sequences into hundreds of different fragments. The fragments are separated by size on an agarose gel and transferred to a membrane. A probe is then used to detect fragments containing IS6110, and the image is captured on film. Each copy of IS6110 produces one band. Isolates from infected TB patients with epidemiologically unrelated strains have different RFLP patterns, whereas those from patients with epidemiologically linked strains generally have identical RFLP patterns. Nevertheless, there are limitations inherent to IS6110-based RFLP analysis concerning the interpretation: a) Strains with fewer than six IS6110 insertion sites have a limited degree of polymorphism, b) IS6110-based RFLP requires sub-culturing of the isolates for several weeks to obtain sufficient DNA [181].

PCR based mycobacterial interspersed repetitive units (MIRUs), variable numbers of tandem repeats (VNTRs)

The genome of *M. tuberculosis* contains many mycobacterial interspersed repeat units (MIRUs), some containing identical repeat units and others containing repeats that vary slightly in sequence and length [182]. This method relies on PCR amplification of multiple loci using primers specific for the flanking regions of each repeat locus and on the determination of the sizes of the amplicons, which reflect the numbers of the targeted MIRU-VNTR copies [163].

PCR based Spacer oligonucleotide typing (spoligotyping)

Spoligotyping is a hybridization assay that detects variability in the direct repeat (DR) region in the DNA of *M. tuberculosis*. The DR region consists of 10 to 50 copies of a 36-bp direct repeat, which are separated from one another by spacers that have different sequences (the standard spoligotyping assay uses 43). Different *M. tuberculosis* strains differ in terms of the presence or absence of specific spacers, the pattern of spacers in a strain can be used for genotyping [170].

Single nucleotide polymorphisms(SNPs) based Whole Genome Sequencing (WGS)

There have been major advances in sequencing technology, DNA sample preparation, genome assembly, and data analysis is naming a few [183]. A number of

sequencing techniques have been introduced making bacterial genome sequencing significantly cheaper and easier. However, the workflow of bacterial sequencing sample preparation such as DNA sequencing, sequence assembly, bioinformatics analysis in order to identify polymorphic sequences with potential relevance to disease pathogenesis, immunity, transmission and evolution- remains generally unchanged [184-186].

The potential of WGS is sometimes described as the early days of PCR, i.e. only imagination will set the primary limitation to its use. Using the current WGS technologies, only short reads (40–250 bp) are generated, this approach does not typically capture long repeat regions. In *M. tuberculosis*, in particular, insertion elements and PE–PPE genes (which constitute a highly repetitive class of antigenic genes) are often excluded [187]. Short reads are also likely to under-sample genome rearrangements. Paired-end sequencing and jumping libraries can help to mitigate these issues. Advances in genome assembly from short reads can also generate longer contiguous sequences [188].

Each sequencing technology has specific strengths and weaknesses, making the selection of the appropriate technique important to achieve the desired experimental results. Illumina being the most widely used platform in the field and may be used as an example [189]. The HiSeq (Illumina Inc., San Diego, CA, USA) machines utilizes a "sequencing by synthesis" technique, where individual DNA molecules are attached to the surface of flow cells and isothermal "bridging" amplification is used to amplify the signal. The DNA molecule is then sequenced using reversible fluorophore-labelled nucleotides, which are optically read from each flow cell. While these have high accuracies, and produce large amounts of raw data, the individual read lengths tend to be shorter, which can be problematic for genomes with large repeats. However, it allows for template amounts as low as 50 ng, which can be useful for organisms that are difficult to culture [190]. While raw sequence data is useful, it is significantly more valuable after assembly into contiguous DNA sequences (contigs). Two strategies for assembly exist, and sequences can be assembled either de novo or assembled against a reference sequence. Sequences that do not meet quality guidelines are automatically trimmed out [191].

In addition to rapidly determining genotype/phenotype association, WGS has allowed the retrospective differentiation between relapse and reinfection, the reconstruction of transmission chains, and the prediction of super-spreaders, drug resistance mutations, to study bacterial evolution and to find potentially undiagnosed cases [192-196]. However, the abundance of sequence information makes bioinformatics the bottleneck in utilization of sequences in clinical samples [197].

Insights into the transmission of tuberculosis

It is confirmed that variability in *M. tuberculosis* isolate genotype between different patients denotes epidemiologically unrelated tuberculosis, whereas identical genotypes of isolates from different patients imply that they were infected by a common source. Furthermore, clustered cases of tuberculosis defined as those in which the isolates have identical or closely related genotypes, have usually been transmitted recently. In contrast, cases in which the isolates have different genotypes generally represent infection acquired in the distant past [198,199]. However, there are two precautions to bear in mind with this concept. First, the genotyping data must be interpreted together with the epidemiologic information, which is usually obtained by interviewing patients, preferably when tuberculosis is diagnosed. Second, accurate identification of clustered cases requires the evaluation of a large percentage of tuberculosis cases in the population over a long period. The ability to identify and trace back individual clones of Mtb may provide control strategies to tuberculosis pathogenesis, transmissibility dynamics and novel disease therapeutics [200-202].

Aims and conclusion

Paper I

Aims

- Investigate mycobacteria induced epithelial signalling pathways from the activation of TLRs.
- Analyse Alveolar epithelial cell's cytokine secretion upon Mtb infection

Conclusion

Our data indicate that mycobacteria suppress epithelial pro-inflammatory production by suppressing NF-κB activation thereby shifting the infection towards an anti-inflammatory state. This balance between the host immune response and the pathogen could determine the outcome of infection.

Paper II

Aims

- Investigate CXCR1 and CXCR2 expression in blood of patients with pulmonary TB, LTBI subjects and close contacts.
- Investigate the capacity to phagocytose in whole blood in pulmonary TB patients, LTBI subjects and close controls

Conclusion

We found that variations in receptor expression are linked to disease progression and affect the immune response against Mtb. Expression pattern of GPCRs could provide an important platform for tuberculosis diagnostics.

Paper III

Aims

- Investigate initial events of mucosal BCG interaction using primary airway epithelial cell (AECs) and a murine model.
- Analyse the impact of G-protein coupled receptors (GPCRs) CXCR1 and CXCR2 in mucosal BCG interaction

Conclusion

This study demonstrated that BCG infection of AECs manipulated the GPCRs to suppress epithelial signalling pathways which led to GPCR-dependent Rac1 upregulation, resulting in actin redistribution.

Paper IV

Aims

- Investigate whether whole genome sequencing (WGS) is well suited for identifying transmission clusters in low TB incidence settings.
- Compare traditional genotyping methods and WGS in their discriminatory power to distinguish between related transmission chains.

Conclusion

WGS has provided new information on the evolution of mycobacterium tuberculosis during outbreaks and provided better resolution of transmission than standard genotyping methods.

Future directions

One of the main 'stop TB' strategies by WHO is to develop a simple, rapid and cheap test for TB diagnosis. We plan to investigate the expression pattern of CXCR1 and CXCR2 in pregnant women and children with pulmonary TB, LTBI subjects and close contacts as well as utilizing extracted genomic material from TB subjects to look for mutations that can affects the host susceptibility to *M. tuberculosis* infection, tuberculosis disease immune-pathogenesis and the outcome of *M. tuberculosis* exposure and/or infection. On the other hand, we plan to continue with the data generated from *M. tuberculosis* high throughput next generation whole genome sequencing of clinical isolates and extend the scope of the analysis to include: drug resistance mutations, relapse and reinfection, and transmission dynamics on the national level in Sweden. The WGS approach might become routine surveillance/outbreak investigation which can direct public health action without additional epidemiological evidence.

Acknowledgment

Most of all, I thank the Almighty God for giving me a blessed career.

Associate professor **Gabriela Godaly**, my supervisor and friend. Thank you for being a genuine and kind hearted scientist. I am grateful for all the trust and confidence that put in me. Your patience and dedication have been an inspiration and motivation throughout my PhD study. This thesis is dedicated to you and in all my future career I will always looks back and cherish our scientific as well as friendly conversations. I will also take with me your love for making the deadlines at the very last second ;)

Professor **Patrik Medstrand**, my co-supervisor, I thank you for all the challenging scientific discussions. Thank you for giving me your time, time you should've spent with family during last Christmas holidays, to help finalize our last manuscript.

I have been lucky to have two colleagues sitting with me in the same office: **Erik Tenland** and **Anna Rönholm**. Erik, you are a true friend who made me feel quite at home here in Sweden. Sharing scientific, political and personal discussions made my cold, Swedish winter days pass more easily. Anna, what a pleasure it has been to work with you. I thank you for all kind good mornings and talks about all matters in life.

Devoted to science, you are the role model for all the ambitious scientists in the research world, professor **Catharina Svanborg**. Seeing you every day working early and staying late before leaving is a guidance for hard work. Thank you everyone at Catharina's lab- **Manoj**, **Therese**, **Aftab**, **Daniel**, **Anna**, **Suzan**, **Ines**, **Nina Caterina** and **Waleed**. We shared lab secrets and international dinners, and you always welcome new members to the group with a great smile.

I thank you **Niclas winqvist**, **Per Björkman**, **Erik Stårigård** and **Sten Skogmar** for welcoming me and introducing me to the world of Tuberculosis in Malmö clinic and the TB think tank. It has been a real privilege to collaborate with you and to share in house knowledge.

If you have one true friend you have more than your share. Thank you **Enas Sheik-khalil** for all the help I received from you.

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Paper I

Mycobacteria Bypass Mucosal NF-kB Signalling to Induce an Epithelial Anti-Inflammatory IL-22 and IL-10 Response

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Abstract

The mechanisms by which mycobacteria subvert the inflammatory defence to establish chronic infection remain an unresolved question in the pathogenesis of tuberculosis. Using primary epithelial cells, we have analysed mycobacteria induced epithelial signalling pathways from activation of TLRs to cytokine secretion. *Mycobacterium bovis* bacilli Calmette-Guerin induced phosphorylation of glycogen synthase kinase (GSK)3 by PI3K–Akt in the signalling pathway downstream of TLR2 and TLR4. Mycobacteria did not supress NF-κB by activating the peroxisome proliferator-activated receptor γ. Instead the pro-inflammatory NF-κB was bypassed by mycobacteria induced GSK3 inhibition that promoted the anti-inflammatory transcription factor CREB. Mycobacterial infection did not thus induce mucosal pro-inflammatory response as measured by TNFα and IFNγ secretion, but led to an anti-inflammatory IL-10 and IL-22 production. Apart from CREB, MAP3Ks p38 and ERK1/2 activated the transcription factor AP-1 leading to IL-6 production. Interestingly, blocking of TLR4 before infection decreased epithelial IL-6 secretion, but increased the CREB-activated IL-10 production. Our data indicate that mycobacteria supress epithelial pro-inflammatory production by supressing NF-κB activation thereby shifting the infection towards an anti-inflammatory state. This balance between the host immune response and the pathogen could determine the outcome of infection.

Introduction

Successful pathogen *Mycobacterium tuberculosis* (*M. tuberculosis*) use intricate strategy to evade the immune response. This pathogen invades the epithelial cells that cover the alveolar space of the lung and modulate or fine-tune the immune responses to produce a selective cytokine response [1–5]. The first phagocytes to be attracted to the infectious foci are the neutrophils [1,2,6,7], followed by monocytes, and these leukocytes cooperate in the elimination of mycobacteria [8]. The extent of epithelial cytokine secretion may lead to tissue damage and breakdown of extracellular matrix, thus favouring bacterial persistence and facilitating mycobacterial transmission [9,10]. However, perturbed defence in immune-compromised patients can tilt this balance leading to active disease [11]. These initial innate events, depending on the magnitude of the host immune responses, could thus determine the outcome of mycobacterial infection.

Epithelial cells express molecular pattern associated receptors, such as the Toll like receptors (TLRs) that interact with mycobacteria [12]. TLR2 expression increases upon mycobacterial infection of alveolar epithelium and blocking of TLR2 decreases cytokine responsiveness [4]. Mycobacteria express multiple ligands that bind to members of the TLR family, especially TLR2 and TLR4. Mycobacterial products, such as

lipoarabinomannan (LAM) and the cell wall-associated and secreted 19-kDa glycolipoprotein, activate TLR signalling [4,6,13,14]. TLR2 and TLR4 are also known to modulate the activation of peroxisome proliferator-activated receptor (PPAR)γ [15] that mycobacteria utilize to affect the NF-κB activation [16,17]. Ligand binding to TLR initiates a signalling cascade through a MyD88-dependent and/or a MyD88-independent gene expression [18]. The MyD88-dependent activation leads to a pro-inflammatory cytokine response by the IRAK-NF-κB pathway, but also to chromatin remodelling by the MAPK kinases that regulates extracellular signal-regulated kinase 1/2 (ERK1/2), p38 proteins and c-Jun N-terminal kinase (JNK) [18]. The cytosolic domains of several TLRs bear also a conserved YxxM PI3K binding motif and phosphorylation of Akt, a downstream kinase activated by PI3K, is detected upon TLR stimulation [19]. Activation of Akt or p38 inactivates the glycogen synthase kinase 3 (GSK3) that is found further down the signalling pathway [20]. GSK3 is constitutively active in resting cells leading to the pro-inflammatory NF-κB transcription, but p38/Akt phosphorylation of GSK3 switches the transcriptional activity to cAMP response element-binding protein (CREB) [21]. TLR activation can thus either lead to a pro-inflammatory cytokine response by activation

of NF-κB pathway, or an anti-inflammatory CREB-related cytokine response.

The initial events of mycobacterial infections are not clear. The first surface that the immobile bacterium will encounter after inhalation into the lungs would most likely be epithelial. Several groups have demonstrated that *M. tuberculosis* invades and survives within human type II alveolar epithelial cells [1,22,23]. Previous research revealed that the epithelia remain unresponsive to the infection until the third day, when the cells secreted a distinct pattern of cytokines [4,5]. There are conflicting reports regarding the activation of NF-κB by pathogenic mycobacteria. In the present study, we have analysed mycobacteria induced epithelial signalling pathways from activation of TLRs to cytokine secretion. Our data indicate that mycobacteria avoid epithelial pro-inflammatory production by bypassing NF-κB activation thus balancing the infection towards an anti-inflammatory state.

Materials and Methods

Ethical Statement

The Swedish Research Ethical Committee in Lund (FEK 413/2008) approved the isolation of the bronchial material for primary cell cultures. Bronchial material for primary cell cultures was obtained from lung explant from healthy donors with irreversible brain damage and with no history of lung disease. Lungs were to be used for transplantation but could instead be included in this study as no matched recipients were available at that moment. Written consent was obtained from their closest relatives.

Bacterial strains and growth conditions

Mycobacterium bovis bacillus Calmette-Guerin (BCG) Montreal strain containing the pSMT1 shuttle plasmid was prepared as previously described [24]. Briefly, the mycobacteria were grown in Middlebrook 7H9 culture medium, supplemented with 10% ADC enrichment (Becton Dickinson, Oxford, UK) and hygromycin (50 mg/l; Roche, Lewes, UK), the culture was washed twice with sterile PBS, and re-suspended in media again and then dispensed into vials. Glycerol was added to a final concentration of 25% and the vials were frozen at -80°C. Prior to each experiment, a vial was defrosted, added to 9 ml of 7H9/ADC/hygromycin medium, and incubated with shaking for 72 h at 37°C. Mycobacteria were then centrifuged for 10 minutes at $3000\times g$, washed twice with sterile PBS, and re-suspended in 2 ml of sterile PBS.

Cell Culture

Bronchial tissue was dissected from lungs and kept in Dulbecco's Modified Eagle Medium supplemented with gentamicin, penicillin, streptomycin, Fungizone and 10% fetal calf serum (FCS) (all from Gibco, Paisley, UK) until further isolation. After removing intraluminal mucus and surrounding tissue, bronchi were digested in 0.1% Protease (Sigma St. Louis, MO) prepared in Minimum Essential Medium Eagle Spinner Modification (Sigma-Aldrich) supplemented with gentamicin, penicillin, streptomycin and Fungizone for 24 hours. Bronchial epithelial cells (HBEC) were recovered by repeated intraluminar rinsing with Dulbecco's Modified Eagle Medium supplemented with gentamicin, penicillin, streptomycin, Fungizone and 10% FCS. Cells were filtered through a 100 μm strainer (Falcon, Becton Dickinson) and seeded in cell culture flasks coated with 1% Collagen-1 (PureCol, Inamed Biomaterial, Freemont, CA) in Bronchial Epithelial Cell Growth Medium (Clonetics). The following day cells were thoroughly washed with a medium change every other day. Experiments were performed in passage 3 and 4.

Infection and treatments of the cells

For the infection experiments, primary cells were grown in 6-well plates (2.0×10^5 cells/well; Fisher Scientific, UK), infected with BCG (one bacterium per cell; 1 MOI) or phenol purified LPS (1 ng/ml; Sigma-Aldrich), lipoarabinomannan (LAM, 1 μg/ml, Lionex GmbH) or 19-kDa glycolipoprotein (1 μg/ml, Lionex GmbH) at 37°C for up to three days. For the blocking experiments, monoclonal mouse anti-human TLR2 or monoclonal mouse anti-human TLR4 antibodies (R&D Systems) 10 μg/ml were added to the epithelial cells 30 minutes before the addition of bacteria.

For cytokine analysis, the samples were collected after 0, 6, 24, 48 and 72 hours and for western blot analysis, the cells were detached by versene (140 mM NaCl, 2.4 mM KCl, 8 mM Na$_2$HPO$_4$, 1.6 mM KH$_2$PO$_4$, 0.5 mM EDTA, pH 7.2) and washed with PBS.

To investigate whether epithelial cells survive mycobacterial infection, we analysed cell viability by trypan blue exclusion assay according to manufactures instructions (Sigma Aldrich, Germany). For analysis of bacterial survival within the epithelial cells, infected epithelial cells were lysed in 300 μl of sterile distilled water for 15 minutes. 100 μl of the suspension was plated on Middlebrook 7H10 supplemented with 10% OADC Enrichment (Becton Dickinson, Oxford, UK) and grown for 3 weeks.

Western Blot

The primary cells were washed with PBS containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml PepstatinA, 5 μg/ml Leupeptin (Sigma-Aldrich) and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and lysed with modified Mammalian Protein Extraction Reagent (M-PER) solution (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 50 mM ZnCl, 1% NP-40, 0.1% deoxycholate, 0.1% SDS; Pierce) containing phosphatase (1:10) and the complete protease inhibitor cocktail (1:25). The cells were then placed on a shaker for 5 minutes, collected and centrifuged at 10,000×g for 5 minutes. Protein samples were used immediately for western blot analysis or stored at -80°C.

Protein levels were measured in cells treated with BCG and cells blocked for TLR2 or TLR4 with the NanoDrop^{TM} 8000 Spectrophotometer using the Pierce 660 nm assay (Thermo Scientific). Medium alone, LPS, LAM and 19 kDa were used as controls. Protein samples were mixed with PBS, 4× NuPAGE LDS sample buffer (Life Technologies) and 1 M DTT and incubated at 90°C for 10 minutes followed by centrifugation at 218×g for 5 minutes. Equal amounts of protein (10 μg/well) were loaded on a NuPAGE 4%–12% Bis-Tris Gel (Life Technologies) and separated by sodium dodecyl sulfate-PAGE. A molecular weight marker (Novex® Sharp Prestained; Life Technologies) was loaded onto each gel for protein band identification. After separation, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Healthcare Amersham). The membrane was then blocked with either 5% dry-milk (Santa Cruz Biotechnology, Santa Cruz, CA) or with 5% bovine serum albumin (BSA; Santa Cruz Biotechnology) for 1 hour on a shaker at room temperature. Membranes were then incubated on a shaker overnight at 4°C with rabbit anti-human p-GSK-3α/β (1:500; AF1590, R&D systems, Denmark), GSK-3α/β (1:250; AF2157, R&D systems), p-CREB (1:1000; #9198 Cell Signalling Technology, Inc., Danvers, MA), IκBα (1:1000; #4812 Cell Signalling Technology), ERK1/2 (0.1 μg/mL, AF1018 R&D systems), PPARγ (1:1000; NBP1-61399 Novus Biologicals), GAPDH (1:500; sc-25778 Santa Cruz Biotechnology) or mouse anti-human NF-κB p65 (1:200; sc-8008 Santa Cruz Biotechnol-

ogy, Heidelberg, Germany), or β-actin (1:10.000; Sigma-Aldrich) primary antibody. Incubation was followed by washing 3×5 minutes with Tris-buffered saline (TBS)-Tween 20 and 1×5 minutes TBS. The membrane was then incubated with goat-anti-rabbit IgG HRP (1:2000; Santa Cruz Biotechnology) IgG secondary antibody or with rabbit anti-mouse IgG$_1$ HRP (1:4000; Dako) secondary antibody for 2 hours on a shaker at room temperature followed by washing with TBS-Tween 20 and TBS. The housekeeping protein GAPDH and β-actin were used to confirm equal loading on the wells. The membrane was developed using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK) and GelDoc equipment (Bio-Rad Laboratories). Blot intensity was quantified using ImageJ software 28 and normalized against GAPDH or β-actin. If required, membranes were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), blocked and re-probed with new antibodies.

Phospho-kinase array

Protein phosphorylation was examined with the Proteome Human Phospho-Kinase Array Kit (Proteome Prolifer Array, R&D Systems, Abingdon, Oxford, UK), which is a membrane based sandwich immunoassay. The assay was performed according to the manufacturers' instructions. Briefly, total cell extracts were prepared from stimulated near-confluent cultures of normal human primary epithelial cells grown in 6-well plates. Untreated cells were used as control. The cell extracts containing 500 µg of total protein were incubated with the Human Phospho-Kinase Array. The proteins present in a lysate sample were captured by discrete antibodies printed in duplicate across the nitrocellulose membranes. The array was washed 3× with 1X Wash Buffer for 10 minutes on a rocking platform shaker to remove unbound proteins. Washing was followed by incubation with a cocktail of biotinylated detection antibodies (monoclonal anti-human of phosphorylated Akt (S473), Akt (T308), AMPK alpha1 (T174), AMPK alpha2 (T172), beta-Catenin, Chk-2 (T68), c-Jun (S63), CREB (S133), EGF R (Y1086), eNOS (S1177), ERK1/2 (T202/Y204, T185/Y187), FAK (Y397), Fgr (Y412), Fyn (Y420), GSK-3 alpha/beta (S21/S9), Hck (Y411), HSP27 (S78/S82), HSP60, JNK pan (T183/Y185 T221/Y223), Lck (Y394), Lyn (Y397), MSK1/2 (S376/S360), p27 (T198), p38 alpha (T180/Y182), p53 (S15), p53 (S392), p53 (S46), p70 S6 Kinase (T421/S424), PDGF R beta (Y751), PLC gamma-1 (Y783), PRAS40 (T246), Pyk2 (Y402), RSK1/2/3 (S380/S386/S377), Src (Y419), STAT2 (Y689), STAT3 (S727), STAT3 (Y705), STAT5a (Y694), STAT5a/b (Y694/Y699), STAT5b (Y699), STAT6 (Y641), TOR (S2448), WNK-1 (T60), Yes (Y426) and subsequent application of streptavidin-HRP conjugate. The signals were detected with the ECL Plus Western Blotting Detection System (GE Healthcare). Developed signals were analyzed using ImageJ 1.45s analysis software.

Immunofluorescence microscopy

Expression of p-CREB and NF-κB in primary cells was detected by immunofluorescence staining. After blocking and infection for 72 hours the cells were fixed with 3.7% formaldehyde and then permeabilized in a mixture of PBS, 0.25% Triton X-100 and 5% fetal calf serum (FCS) for 30 minutes shaking at room temperature. Specimens were then incubated for 2 hours shaking at room temperature with PBS, 5% FCS, and the primary anti-rabbit p-CREB-1 (Ser133) or anti-mouse NF-κB p65 antibodies (1:50; Santa Cruz Biotechnology). The cells were washed two times with PBS at 400×g for 5 minutes and then incubated with goat anti-rabbit or rabbit anti-mouse secondary antibody (1:100; Invitrogen)

in PBS and 5% FCS for 1 hour (shaking in dark) in room temperature. After additional washing the cells was stained with 1 µg/ml of 4′, 6-diamidino-2-phenylindole (DAPI) dissolved in PBS for 5 minutes in dark and then washed again with PBS. Finally the slides were mounted in fluoromount Aqueous Mounting Medium (Sigma Aldrich, F4680). The slides were examined with an inverted Nikon microscope (Nikon Diaphot 300) equipped with a 100 W mercury lamp (Osram, Berlin, Germany) and Ploempac with the filter set for fluorescein isothiocyanate and BioRad MRC 1024, controlled via LaserSharp (version 5.2 for PC/Windows) and further examined with the LSM 510 DUO confocal equipment with LSM software version 4.2 SP1 (Carl Zeiss, Jena, Germany). Sections incubated without primary or secondary antibody were used as negative controls to verify the lack of auto-fluorescence and unspecific secondary antibody staining.

ELISA

IL-6 (D6050), TNFα (DTA00C), IFNγ (DIF50), IL-10 (D1000B) and IL-22 (D2200) secretion by the infected cells were quantified in supernatants by Human Quantikine ELISA Kits (R&D Systems, Oxon, UK) according to manufactures instructions. NF-κB (EK1111) and AP-1 (c-Jun, EK1041) were quantified with nuclear extraction kits containing ELISA-kit according to manufacturers instructions (Affymetrix Panomics, UK).

Statistics

The statistical program used was SigmaStat, version 3.5, for Windows XP. The statistical difference between two groups was investigated by Mann-Whitney test. Multiple comparisons were done by one-way Analysis of Variance followed by Bonferroni test or Dunnett's test (***P≤0.001, ** P<0.01, *P<0.05, ns = non significant).

Results

Mycobacteria supress NF-κB and c-Jun

We used a low infection dose of 1:1 (bacterium:cell) [25,26] and analysed alveolar nuclear extracts for NF-κB and c-Jun by ELISA. BCG at low MOI was shown to invade and survives in alveolar epithelial cells three days after infection without affecting epithelial viability (Figures S1 and S2). The TLR4 agonist LPS was used as a control. Infection of primary epithelial cells did not induce NF-κB activation during the three days of infection (Figure 1a). However, mycobacterial infection induced an early activation of c-Jun proteins that was suppressed two days after infection (Figure 1b). LPS induced an early NF-κB activation that was significantly higher than medium control and BCG up to 48 hours after addition to primary epithelial cells. Interestingly, BCG induced significantly higher c-Jun protein activation at 6 hours than LPS (p = 0.0177). We could confirm that BCG at low MOI invades and survives in primary epithelial cells [5,27] three days after infection (Figure S3).

Mycobacteria inactivates GSK3αβ signalling pathways

GSK3 consist of the isoforms α and β. The un-phosphorylated form of GSK3 promotes NF-κB activation, while phosphorylation of GSK3 by p38 and Akt promotes CREB anti-inflammatory activation. To investigate mycobacteria induced signalling pathways in primary epithelial cells, we analysed the GSK3αβ-pathway by Phospho-kinase array (Figure 1c–e). LAM and 19 kDa were used as controls for mycobacterial virulence factors and are known to signal through TLR2 [13,14], while LPS is a known TLR4 ligand. In the beginning of infection, live mycobacteria, induced
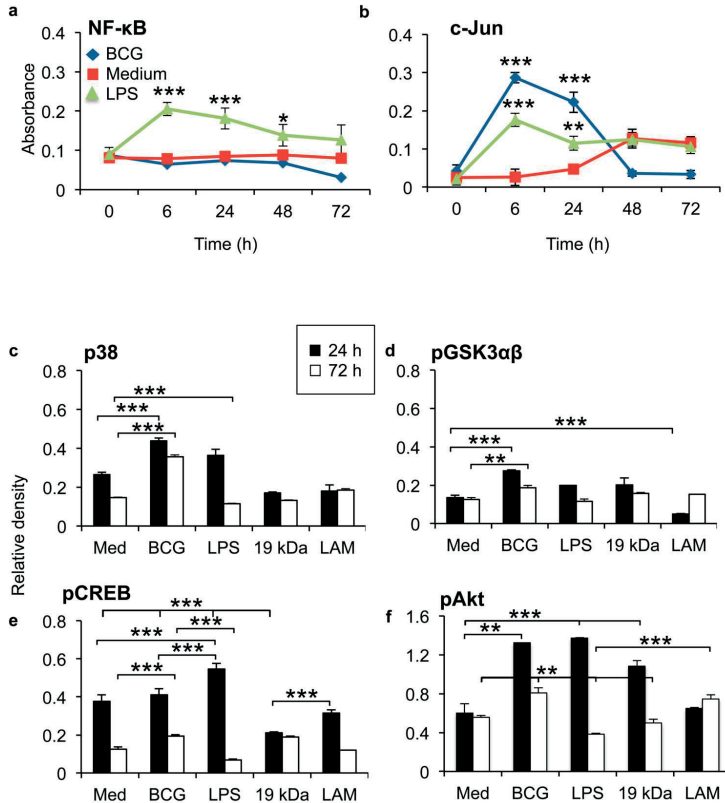
Figure 1. Mycobacteria bypass epithelial NF-κB signalling. (a) Infection of primary epithelial cells did not induce NF-κB activation quantified by ELISA, but an early activation of c-Jun proteins in epithelial cells was observed. (b) Epithelial GSK3αβ-pathway was analysed by Phospho-kinase array upon mycobacteria infection. In the beginning of infection, live mycobacteria, the virulence factors LAM and 19 kDa, and the TLR4 agonist LPS, induced comparable induction of p38, pAkt and pGSK3αβ. During the first 24 h, LPS induced higher increase of pCREB protein levels than mycobacteria (p = 0.0017). Third day of infection, mycobacteria significantly increased epithelial pCREB compared to medium control (p = 0.0357) or LPS (p = 0.0089). Epithelial stimulation with LAM induced an increase in pGSK3αβ and pAkt phosphorylation (p = 0.001 respectively p = 0.0196) during the later stages of infection compared to the early time-point. Generally, mycobacteria induced a more persistent increase of the investigated transcription factors three days after infection in primary epithelial cell than the controls LPS, 19 kDa and LAM. Data are presented as mean ± SEM of three separate experiments; **p<0.01 and ***p<0.001.
doi:10.1371/journal.pone.0086466.g001

higher induction of p38 and pGSK3αβ than the virulence factor 19 kDa and the TLR4 agonist LPS (Figure 1c–d). Interestingly, mycobacterial virulence factor LAM significantly down-regulated pGSK3αβ after 24 hours of stimulation (Figure 1d). Mycobacteria and LPS induced higher increase of Akt than LAM and 19 kDa, but mycobacteria induced less pCREB protein levels during the first 24 hours, compared to LPS (p = 0.0017) or medium control (not significant) (Figure 1e–f). Three days after infection there was a significant increase of pCREB in mycobacteria infected epithelium compared to medium control (p = 0.0357) and LPS (p = 0.0089) (Figure 1e). Generally, mycobacteria induced a more persistent increase of the investigated transcription factors three days after infection in primary epithelial cell than the controls LPS, 19 kDa and LAM (Figure 1c–f). Interestingly, epithelial stimulation with LAM induced a late increase in pGSK3αβ and

pAkt phosphorylation (p = 0.001respectively p = 0.0196) during the later stages of infection compared to the early time-point.

Mycobacteria bypass NF-κB activation, but activated ERK1/2 and cFos

To investigate mycobacteria induced epithelial signalling pathways further we analysed several molecules in TLR-signalling pathway by Western blotting. By comparing infected cells with un-infected cells during the investigated time-points, we could confirm that mycobacterial infection did not induce higher NF-κB- or IκB-activation (Figure 2a,b) than medium control during infection. Variations in suppression were observed during the time of infection. Mycobacteria supressed epithelial IκB and pGSK3αβ proteins at the beginning of infection (Figure 2b; p = 0.002 and Figure 2d; p = 0.0148 respectively), while the pGSK3αβ and
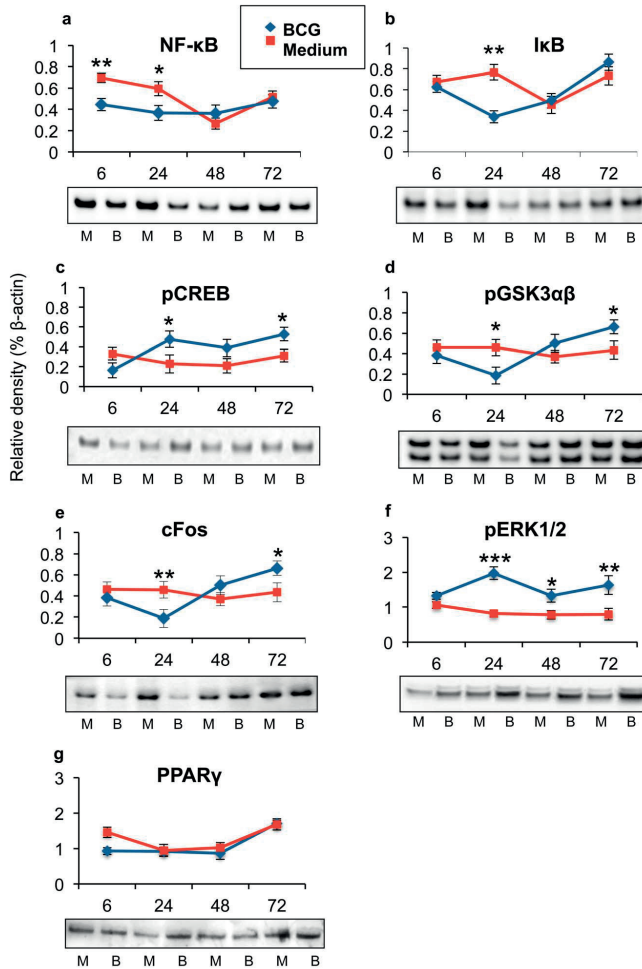
Figure 2. Mycobacteria modulate epithelial signalling pathways. Several molecules in the TLR-signalling pathway were analysed by Western blotting upon mycobacterial infection. (a–b) We could confirm that mycobacterial infection did not induce NF-κB- or IκB-activation. Mycobacterial suppression of primary epithelial (b) (p = 0.002) IκB and (d) (p = 0.0148) pGSK3αβ proteins were mostly pronounced at 24 hours of infection. The phosphorylated forms of (c) (p = 0.0163) CREB and (d) (p = 0.0248) GSK3αβ proteins reached highest levels 72 hours after infection. (e) Mycobacterial infection increased the Fos family of AP-1 proteins, as c-Fos protein levels significantly increased 72 hours after infection (p = 0.0038). (f) Mycobacteria induced two peaks of pERK1/2 protein levels, after 24 hours (p<0.001) and after 72 hours (p = 0.0034) of infection. (g) Epithelial cells express PPARγ protein, but mycobacterial infection did not significantly increase epithelial PPARγ amount. Data are presented as mean ± SEM of three experiments; *p<0.05, **p<0.01 and ***p<0.001.
doi:10.1371/journal.pone.0086466.g002

pCREB proteins reached highest levels 72 hours after infection (Figure 2c,d; p = 0.0163 and p = 0.0248 respectively). Mycobacteria affected both GSK3 isoforms similarly. Interestingly, mycobacterial infection increased the Fos family of AP-1 proteins, as c-Fos protein levels significantly increased 72 hours after infection (Figure 2e; p = 0.0038). Mycobacteria induced two peaks of pERK1/2 protein levels, after 24 hours (p<0.001) and after 72 hours (p = 0.0034) of infection (Figure 2f). Mycobacteria were

previously reported to induce PPARγ in order to modulate NF-κB responses [16,17], but we could not observe that BCG significantly affected epithelial PPARγ protein concentration compared to medium control (Figure 2g). The actin loading controls are shown in the Figure S3.

Mycobacterial infection controls epithelial cytokine production

Generally, the pro-inflammatory cytokines, such as IFNγ and TNFα, orchestrate innate and adaptive host immune responses, while anti-inflammatory cytokines, such as IL-10 and IL-22, confine the inflammation and postpone the generation of adaptive immunity [28]. Mycobacterial control of induced transcriptional factors was analysed as epithelial cytokine secretion from six hours up to three days after infection. Infection induced a significant IL-6 and IL-10 secretion that peaked at 72 hours (Figure 3a,b). In contrast, mycobacterial infection induced an early significant IL-22 secretion from primary epithelial cells that ended 24 hours after infection (Figure 3c). Mycobacterial infection did not induced epithelial TNFα or IFNγ secretion during the studied time interval (data not shown).

Mycobacteria regulate TLR-induced inflammatory response

TLR-induced CREB activation is important for IL-10 production [21]. To determine the impact of TLR2 and TLR4 on mycobacteria induced pro- and anti-inflammatory cytokine production, the receptors were blocked prior to mycobacterial three-day infection of the primary epithelial cells (Figure 4). Antibody blocking of TLR2 or TLR4 before infection decreased epithelial IL-6 secretion ($p = 0.0011$ and $p = 0.0047$ respectively) (Figure 4a). The blocking of TLR2 or TLR4 did not affect alveolar survival during infection (Figure S1b). LPS induced a significantly higher IL-6 response than BCG ($p = 0.0063$), while 19 kDa induced a lower response compared to live mycobacteria ($p = 0.0029$). Mycobacteria induced higher production of the anti-inflammatory IL-10 production than LPS ($p = 0.0032$) in human primary epithelial cells (Figure 4b). Blocking of TLR4 prior to infection increased IL-10 secretion compared to unblocked infection ($p = 0.0399$). Blocking with TLR2 or addition of 19-kDa to the epithelial cells did not induce a significant change in epithelial IL-10 production compared to mycobacteria.

Mycobacteria regulates CREB through TLRs

Mycobacterial infection was previously shown to increase epithelial TLR2 and TLR4 [4]. The impact of TLR2 and TLR4 were analysed by immuno-fluorescence staining of pCREB and NF-κB expression in primary cells (Figure 5a). Mycobacterial infection increased nuclear pCREB protein levels compared to unstimulated cells, while the expression of NF-κB did not increase. Blocking of TLR4 before mycobacterial infection resulted in a granular cytoplasmic pCREB distribution, similar to pCREB aggregation in 19 kDa-stimulated cells. TLR2 blocking and LAM treatment induced similar pCREB distribution as live mycobacteria. Epithelial treatment with mycobacterial virulence factor 19-kDa resulted in a granular cytoplasmic pCREB distribution, while LAM treatment induced similar pCREB distribution as live mycobacteria (Figure 5a). Further confirming our results, detection of epithelial pCREB by confocal immuno-fluorescent microscopy revealed that mycobacterial infection significantly increased pCREB expression ($p<0.001$), but NF-κB expression was not affected (Figure 5b). Blocking of TLR2 or TLR4 before mycobacterial infection increased pCREB expression even further ($p = 0.0187$ and $p<0.001$ respectively) compared to unstimulated cells, but NF-κB expression was not affected (Figure 5b).
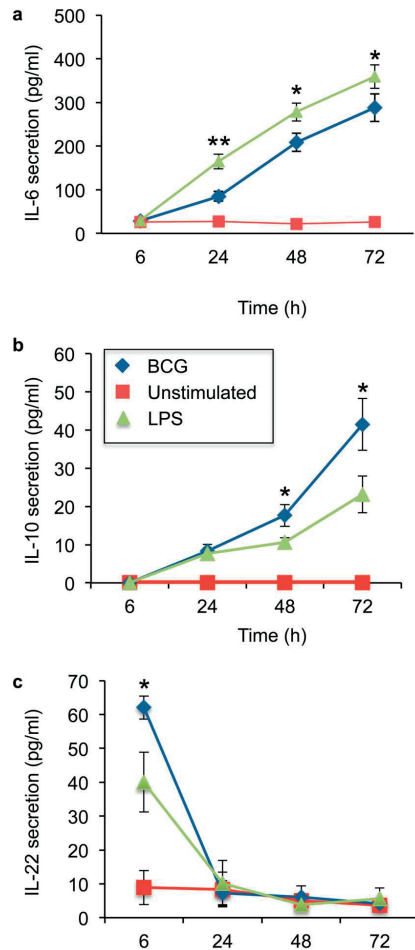
Figure 3. Controlled epithelial cytokine secretion. Mycobacterial control of induced transcriptional factors was analysed as epithelial cytokine secretion from six hours up to three days after infection. Infection induced a significant (a) IL-6 and (b) IL-10 secretion that peaked at 72 hours ($p = 0.0425$ and $p = 0.0186$ compared to LPS). (c) Mycobacterial infection of primary epithelial cells induced an early significant IL-22 secretion ($p = 0.0463$ compared to LPS) that ended 24 hours after infection. Data are presented as mean ± SEM of four separate experiments; *$p<0.05$ and **$p<0.01$.
doi:10.1371/journal.pone.0086466.g003

TLRs are involved in mycobacterial regulation of mucosal inflammation

To further determine the impact of TLR2 and TLR4 on mycobacteria induced cytokine production, the receptors were blocked prior to mycobacterial infection and the impact of modulated epithelial signalling was studied by Western blotting three days after infection. Blocking of TLR2 ($p = 0.0063$) or TLR4 ($p = 0.0047$) prior to infection or stimulation with 19 kDa
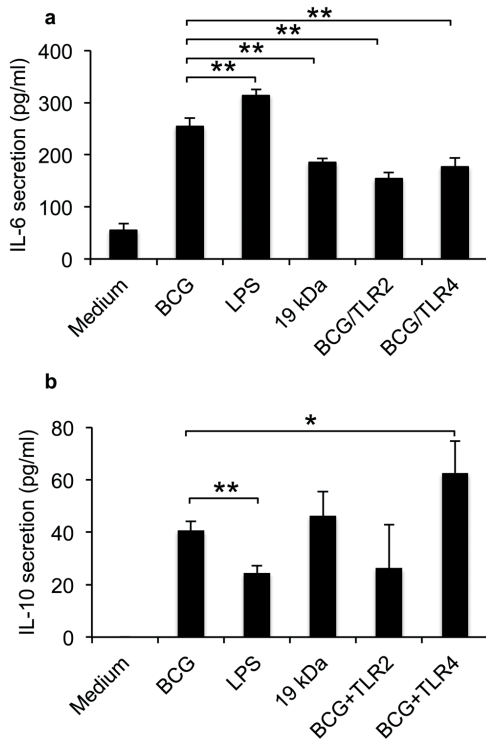
Figure 4. Mycobacterial regulation of TLR-induced cytokines.
To determine the impact of TLR2 and TLR4 on mycobacteria induced pro- and anti-inflammatory cytokine production, the receptors were blocked prior to mycobacterial infection of the primary epithelial cells. (a) Blocking of TLR2 or TLR4 before infection decreased epithelial IL-6 secretion (p = 0.0011 and p = 0.0047 respectively) after three days. LPS induced a significantly higher IL-6 response than BCG (p = 0.0063), while 19 kDa induced a lower response compared to live mycobacteria (p = 0.0029). (b) Mycobacteria induced higher production of the anti-inflammatory IL-10 production than LPS (p = 0.0032) in human primary epithelial cells. Blocking of TLR4 prior to infection increased IL-10 secretion compared to unblocked infection (p = 0.0399). Blocking with TLR2 or addition of 19-kDa to the epithelial cells did not induce a significant change on epithelial IL-10 production compared to mycobacteria. Data are presented as mean ± SEM of three separate experiments; *p<0.05 and **p<0.01.
doi:10.1371/journal.pone.0086466.g004

significantly increased epithelial pCREB production (p = 0.0163) (Figure 6a). Blocking of TLRs or 19 kDa stimulation of epithelial cells had a non-significant impact on pGSK3βα expression (Figure 6b). Blocking of TLR2 or TLR4 before mycobacterial infection of primary epithelial cells non-significantly restored the NF-κB values to background levels (Figure 6c). The GADPH loading controls are shown in the Figure S4.

Discussion

Functional NF-κB activation is essential for the maintenance of physiological immune homeostasis and protective host defence.

We found that *Mycobacterium bovis* bacilli Calmette-Guerin bypassed NF-κB activation during the first days of infection. BCG is equipped with several genes coding for invasin/adhesin-like proteins [27,29–33] and the mycobacterial adhesion heparin-binding haemaglutinin [34] is believed to be involved in invasion of human alveolar epithelial cells [35]. Activated NF-κB was recently shown to be essential for mycobacterial elimination, since blocking of this pathway prevented bacterial killing and allowed the bacteria to grow in macrophages [36]. To date, reported data regarding the activation of NF-κB by pathogenic mycobacteria are conflicting. *M. tuberculosis* was shown to supress NF-κB pathway in some studies [37,38], induce a transient NF-κB activation in other studies and some studies observed activated NF-κB pathways under some conditions [39–42]. However, several bacteria are known to subvert the cell-intrinsic innate immunity by targeting NF-κB. *Salmonella*, *Shigella* and enteropathogenic *Escherichia coli* (EPEC) are known to supress the NF-κB pathway to counteract the host defences [43,44]. Recent genetic studies revealed that EPEC suppression of host NF-κB signalling and NF-κB dependent anti-inflammatory cytokine production requires NleE, a type III-secreted effector that has homologues in *Shigella* and certain *Salmonella* species [45–47]. Recently, genome-wide screens identified previously unidentified gene products for *M. tuberculosis* persistence [48], but whether mycobacteria possess similar elements are not known.

Innate recognition of mycobacteria involves the activation of TLR2 and TLR4. Signalling through TLR activates the adaptor protein MyD88 leading to NF-κB signalling and the activation of ERK1/2, p38 and JNK [18]. Besides of MyD88, activation of TLRs triggers also PI3K activation leading to subsequent Akt phosphorylation. Akt and p38 phosphorylate the glycogen synthase kinase 3 (GSK3), which switches the transcription from the pro-inflammatory NF-κB to the anti-inflammatory CREB activation [21]. We observed that mycobacteria induced the MyD88 stimulated p38, ERK1/2 and AP-1 signalling. Interestingly, mycobacterial infection induced an early activation of the c-Jun family of AP-1 proteins in primary epithelial cells, and a late activation of the AP-1 protein Fos. Mycobacterial activation of PPARγ is known to supress NF-κB in macrophages [16], but we could not observe mycobacteria-induced PPARγ activation in primary epithelial cells. GSK3 regulates the transcriptional activity of CREB and NF-κB by competing for the limited amount of CREB-binding protein (CBP) [21]. TLR activation could therefore either lead to a pro-inflammatory cytokine response by activation of NF-κB pathway, or an anti-inflammatory CREB-related cytokine response. In this study, mycobacterial infection induced increased GSK3 phosphorylation, switching thus the transcriptional activity from NF-κB to CREB. Indeed, epithelial cells responded early to mycobacterial infection by secreting IL-6 and the anti-inflammatory IL-22, while the anti-inflammatory IL-10 increased two days after infection. The cytokine IL-6 is transcribed by CREB, C/EBP, STAT3 and AP-1 [49,50], and can act as both pro- and anti-inflammatory in many chronic inflammatory diseases. IL-6 trans-signalling is critically involved in the maintenance of a disease state by promoting transition from acute to chronic inflammation [51]. In addition, IL-6 is required in the rapid expression of an initial protective IFNγ response during *M. tuberculosis* infection [52]. However, concomitant IFN production can tilt the anti-inflammatory qualities of IL-10 and IL-22 towards a pro-inflammatory state [53]. We could not observe epithelial IFNγ production, suggesting that the secreted IL-10 and IL-22 are produced to damper the inflammation. IL-10 modulates the anti-inflammatory mechanisms by targeting NF-κB thereby inhibiting cellular production of TNFα, which could be one of the
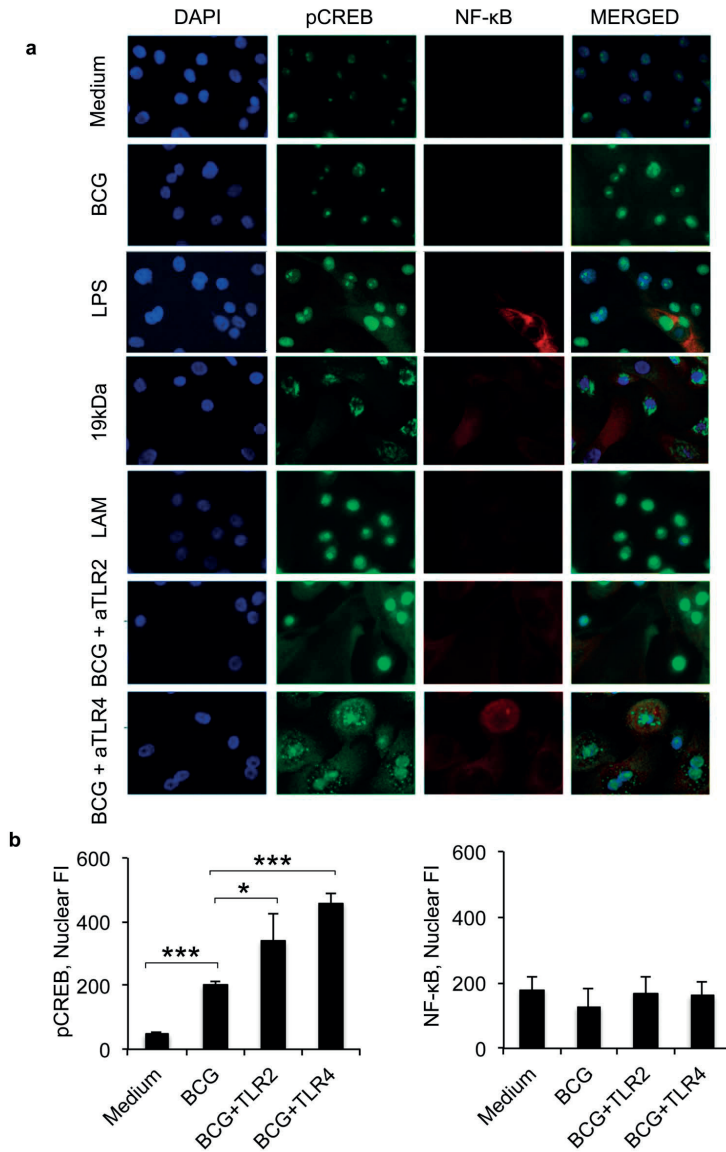
Figure 5. TLR4 blocking results in cytoplasmic CREB aggregation. Mycobacterial modulation of TLR signalling pathways was confirmed by immuno-fluorescence staining of pCREB and NF-κB expression in primary epithelial cells. (a) Mycobacterial infection increased nuclear pCREB protein levels compared to unstimulated cells, while the expression of NF-κB did not increase. Blocking of TLR4 before mycobacterial infection resulted in a granular cytoplasmic pCREB distribution, similar to pCREB aggregation in 19kDa-stimulated cells. TLR2 blocking and LAM treatment induced similar pCREB distribution as live mycobacteria. (b) The results were further analysed by LSM software. Mycobacterial infection increased significantly epithelial (p<0.001) pCREB expression as detected by confocal immuno-fluorescent microscopy, but NF-κB expression was not affected. Blocking of TLR2 or TLR4 before mycobacterial infection increased pCREB expression even further (p = 0.0187 and p 0.001 respectively) compared to unstimulated cells, but NF-κB expression was not affected. Original magnification × 300. Data are presented as representative images or mean ± SEM of three separate experiments; *p < 0.05 and ***p < 0.001.
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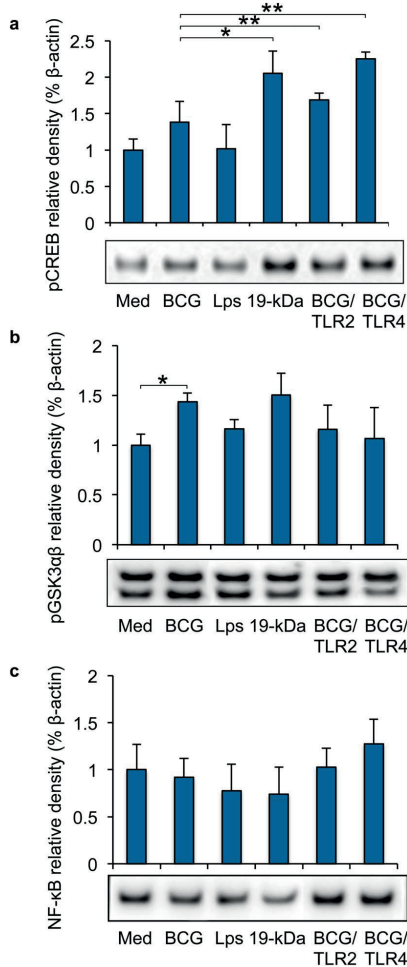
Figure 6. Mycobacteria control epithelial TLR responses. The impact of TLRs on mycobacterial modulated epithelial signalling was studied by Western blotting prior to infection and three days after infection. (a) Blocking of TLR2 (p = 0.0063) or TLR4 (p = 0.0047) prior to infection or stimulation with 19 kDa significantly increased epithelial pCREB production (p = 0.0163). (b) Blocking of TLRs or 19 kDa stimulation of epithelial cells had an non-significant impact on pGSK3βα expression. (c) Blocking of TLR2 or TLR4 before mycobacterial infection of primary epithelial cells non-significantly restored the NF-κB values to background levels. Data are presented as mean ± SEM of three separate experiments; *p<0.05 and **p<0.01.
doi:10.1371/journal.pone.0086466.g006

mechanisms of NF-κB suppression that we observed in our study [54,55]. Mycobacteria was previously reported to induce IL-10 secretion from neutrophils through the phosphorylation of p38 and Akt kinases [56]. Mycobacterial infection of $Il10^{-/-}$ mice show enhanced protection while showing no signs of aberrant host-mediated pathology, which perhaps reflects the slow disease

progression [57,58]. The role of IL-10 could be to limit mycobacterial clearance during the early immune response through the inhibition of IL-12p40 [59]. IL-22 is found in large amounts in pleura from TB patients [60] and this cytokine is primarily expressed by CD4^{+} T cells [61], but other leukocyte subsets also express this cytokine [62]. IL-22 acts through the IL-22 receptor complex expressed by epithelial cells and hepatocytes, where it promotes regeneration and protects against tissue damage [63,64], but accumulating evidence suggests that IL-22 can be either pathogenic or protective depending on host conditions [65]. Using the TB mouse model, a recent study showed that neutralization of IL-22 did not have any effect on the lung bacterial burden or granuloma formation [66]. The mycobacterial vaccine strain used in our study did not induce TNFα or IFNγ secretion. Interestingly, recent studies support the IL-17-CXCL13 pathway rather than the IFNγ pathway as a new strategy to improve mucosal vaccines against tuberculosis [67]. We are currently investigating if alveolar epithelia induce IL-17 or CXCL13 upon mycobacterial infection.

Blocking of epithelial TLR4 before mycobacterial infection decreased the pro-inflammatory IL-6 secretion, but increased the anti-inflammatory IL-10 secretion. TLR4 blocking prior to mycobacterial infection resulted in a granular cytoplasmic pCREB distribution similar to the 19-kDa stimulated cells. We could not find any explanation of the cytoplasmic granular accumulation, but granular accumulation of pERK in cytoplasm was shown to alternate downstream signalling in Parkinson's disease [68]. Normally, signals that induce NF-kB activity usually lead to IkB phosphorylation by the IkB kinase (IKK) complex, and subsequent multi-ubiquitination and degradation of this protein via proteasome, allowing NF-kB dimers' translocation to nucleus [69]. We observed that TLR4 blocking induced cytoplasmic accumulation of NF-κB as well, although no increased NF-κB translocation to epithelial nuclei was detected.

Mycobacteria cause persistent infections by minimizing the degree of overt pathology, allowing long-term association with the host. We have observed that mycobacterial infection of primary epithelial cells supress NF-kB activation by increasing the inhibitory GSK3, thereby supporting the production to the anti-inflammatory cytokines IL-22 and IL-10. Production of anti-inflammatory cytokines is known to impair antigen presentation, which confines the inflammation and postpones the generation of adaptive immunity resulting in antigen-specific anergy. These events could lead to an impaired innate immune response by which mycobacteria create a safe haven for chronic infection and transmission to new hosts.

Supporting Information

Figure S1   Intracellular viability of mycobacteria.
(TIF)

Figure S2   Epithelial viability visualized by trypan blue exclusion assay three days after infection, with or without blocking of TLR2 or TLR4.
(TIF)

Figure S3   Actin loading controls (Figure 2).
(TIF)

Figure S4   GADPH loading controls (Figure 6).
(TIF)

Author Contributions

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Paper II

IMMUNOLOGICAL ASPECTS

Impaired CXCR1-dependent oxidative defence in active tuberculosis patients

CrossMark

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ARTICLE INFO

SUMMARY

Much of the pronounced host inflammatory response that occurs in tuberculosis (TB) is related to failed immunity against the invading pathogen. The G-protein coupled receptors CXCR1 and CXCR2 are implicated in important signal transduction pathways in lung inflammatory responses. We investigated the expression and function of these receptors in a simple whole blood model from 24 patients with pulmonary TB and in subjects with latent TB infection (LTBI). Healthy controls were recruited from close contacts to the pulmonary index patients. We found that pulmonary TB patients had significantly increased CXCR1 expression on blood cells compared to LTBI subjects and controls ($p < 0.001$). In contrast, LTBI subjects had a significant increase in CXCR2 expression compared to pulmonary TB patients ($p < 0.001$) and controls ($p < 0.01$). Leukocyte function, measured as oxidative capacity, was decreased in pulmonary TB patients compared to LTBI and controls ($p < 0.001$) and correlated with the increased CXCR1 expression. Leukocyte recruitment, measured as the expression of microRNA-223 was increased in pulmonary TB patients compared to LTBI ($p < 0.05$). We found that variations in receptor expression are linked to disease progression and affect the immune response against *Mycobacterium tuberculosis* (Mtb).

1. Introduction

The reason why some individuals develop disease after exposure to *Mycobacterium tuberculosis* (Mtb) while others do not is not well understood. Predisposing factors include nutritional deficiencies that affect the immune system and comorbid medical conditions, such as cancer, diabetes and HIV infection, but various host genetic factors are also known [1—4]. The immune response has a strong influence, both for the establishment of infection, but also for subsequent pathogenesis and disease manifestations. In many exposed individuals Mtb is cleared by innate immunity before manifest infection and when that fails, infection is controlled by protective encapsulation in most cases [5,6]. The exaggerated immune response leading to tissue destruction, as observed in active TB, is thus seen as a failure of immunity to eliminate or control the infection.

Inhaled Mtb is phagocytosed by a number of cells in the alveoli, such as neutrophils, epithelial cells, alveolar macrophages and dendritic cells [7—10]. Collectively, these target cells initiate a first chemokine and cytokine cascade that attracts other leukocytes and lymphocytes to the site of infection. The chemokine CXCL8 (IL-8) is secreted early in the infectious disease process and acts as a strong neutrophil chemoattractant, but is also chemotactic for monocytes and lymphocytes [11,12]. Neutrophils are an essential component of the innate immune system and are the first group of cells that migrate to sites of infection. These leukocytes contribute to the control of Mtb in infected patients, but sustained neutrophil

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response in severe TB have also been associated with delayed Mtb clearance and disease severity [13,14]. The microRNA-223 was recently found to control the neutrophil-driven fatal TB inflammation by regulating the murine counterpart to human CXCL8 [14].

In TB patients, CXCL8 is found abundantly in sputum, lymph nodes and plasma [15–18]. CXCL8 binds to two G-protein coupled receptors, CXCR1 and CXCR2, which are primarily expressed by neutrophils, but also by peripheral blood mononuclear cells, alveolar macrophages, T$_H$ cells, alveolar and bronchial epithelial cells, and vascular cells [8,19–21]. Signalling through these receptors stimulates chemotaxis of myeloid cells, but they are also involved in mucus hypersecretion of epithelial cells and increased vascular permeability of endothelial cells [20,22]. Mtb adherence to cells is known to trigger signal transduction events involving the G-proteins in actin polymerization with the subsequent uptake of the bacteria [23–25]. Barrios-Payán recently showed that during latent infection, Mtb is present in the majority of endothelial and epithelial cells, but does not provoke an inflammatory response or granuloma formation [26]. Between the two CXCL8 receptors, CXCR2 has gained particular interest in the pathology of several pulmonary diseases, such as ischemia/reperfusion injury, chronic obstructive pulmonary disease and pulmonary fibrosis [27].

Using a simple whole blood model, we investigated the expression pattern of CXCR1 and CXCR2 in patients with pulmonary TB, LTBI subjects and close contacts. Additionally, the function of these receptors were analysed in the different study groups. We found significant differences in receptor expression in pulmonary TB patients and LTBI subjects. Furthermore, leukocyte capacity to destroy bacteria was impaired in pulmonary TB patients. The distinct chemokine receptor expression in active and latent TB patients could potentially have an impact on future therapies.

2. Materials & methods

2.1. Patient parameter description

We prospectively recruited active TB-cases and TB-exposed controls from the department of Infectious diseases, Skåne University Hospital, Malmö, Sweden (Figure 1). During a period of 35 months, from January 1st 2012 through November 2014, patients who met the inclusion criteria (age >18 years, ongoing treatment for active, microbiologically confirmed, smear positive pulmonary TB) were offered participation in connection with ordinary clinical assessment visit. Patients were excluded if they

Figure 1. Flow chart illustrating the complete recruitment process to the study.

participated in other studies or if they were HIV or hepatitis B and C positive. Two groups of controls exposed to Mtb were recruited from contact tracing around sputum smear positive TB patients at the same department. These groups were categorized as patients with either latent tuberculosis infection (LTBI) and TB-exposed, but not infected patients. For this categorization subjects exposed to TB through close contact with a patient with confirmed, smear positive, pulmonary TB were investigated with QuantiFERON®-TB Gold In-Tube (QFT-G; Cellestis Limited, Carnegie, Victoria, Australia). Asymptomatic individuals with positive QFT-Gs were categorized as LTBI and were offered Isoniazid preventive therapy (IPT). Subjects with LTBI were followed according to Swedish guidelines for contact tracing for contagious TB, i.e. 3–18 months from study inclusion to exclude signs of active TB. Subjects who were negative in QFT-G were categorized as TB-exposed, but not infected patients. Individuals with inconclusive QFT-G ($n = 4$) were not eligible for participation in the study. Among exposed controls with valid QFT-G result, 19 with LTBI and 25 without infection were considered to have had very close contact with a sputum smear positive contact and were offered inclusion. Study subjects originated from Sweden, Finland, Somalia, Afghanistan, Uganda, Chile, Bosnia, Iran, Thailand, Zimbabwe, Romania, Cameroon, Syria and Tanzania.

2.2. Sample collection

For the receptor studies, 2×4 ml blood sample was obtained from each participant in heparin containing blood collection tubes and in PAXgene Blood RNA Tubes (BD, Sweden), for the leukocyte receptor analysis, functionality assays and for the preparation of microRNA. Three additional aliquots were obtained at the same time for the QFT-G assay.

For the blocking experiments, blood was donated from healthy volunteers and treated with monoclonal mouse anti-human CXCR1 and/or monoclonal mouse anti-human CXCR2 antibodies (R&D Systems) 10 μg/ml for 30 min on ice; mouse IgG isotype control, R&D Systems, Denmark was used as a negative control. The cells were washed twice in PBS and neutrophil oxidative capacity was measured by Phagoburst (see below).

2.3. Flow cytometry analysis

CXCR1 and CXCR2 expression was investigated by FACS analysis. Heparinized blood (100 μL/antibody) was mixed with lysis buffer (BD, Sweden), incubated for 10 min at room temperature and washed twice in PBS-buffer. After centrifugation at $200 \times g$ the supernatant was discarded and monoclonal IgG-FITC labelled mouse anti-human CXCR1 or monoclonal mouse anti-human CXCR2 antibodies (10 μg/ml; R&D Systems, Denmark) were added. FITC-labelled monoclonal mouse immunoglobulin G (10 μg/ml; mouse IgG isotype control, R&D Systems, Denmark) was used as a negative control. The samples were incubated 35 min on ice, washed two times in PBS and analysed by flow cytometry (Accuri, Becton Dickinson, Oxford, UK). A total of 5000 cells were counted in each sample. Mean fluorescence intensities (MFI) were calculated from the negative control.

2.4. Oxidative burst assay

The oxidative capacity of leukocytes was measured by Phagoburst (Glycotope Biotechnology, Heidelberg, Germany) according to manufacturer's instructions. The experiments were set up using all three reagents supplemented by the manufacturer, i.e. the peptide fMLP, released from both gram− and gram+ bacteria, as low stimulus, the plant derived toxin PMA as high stimulus and *Escherichia coli* as specific stimulus. As we were more interested in
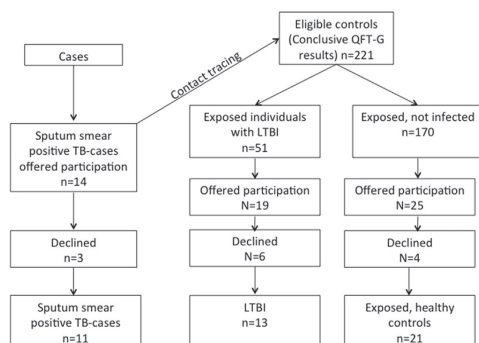
leukocytes function to eliminate bacteria, we choose to perform the oxidative burst analysis using *E. coli*. Briefly, whole blood from patients and controls (100 μL) was incubated with opsonised *E. coli* for 10 min at 37 °C. Dihydrorhodamine-123 solution was added (20 μL) and the samples were incubated 10 min at 37 °C. Subsequently, the blood was lysed and fixed by the addition of 2 ml of lysis solution. After 20 min incubation in 37 °C, samples were washed once and finally incubated for 10 min with 200 μl of DNA staining solution. The respiratory burst was measured by flow cytometry (Accuri, Becton Dickinson, Oxford, UK). The mean fluorescence intensity (MFI) of the background (washing solution) was calculated and subtracted by an overlay technique from the MFI of the sample. The stability of the fluorescence signal was checked and adjusted using FluoroSpheres® (Dako, Copenhagen, Denmark).

2.5. miRNA isolation and quantification

Venous blood from participants was collected directly into PAXgene blood RNA tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland) containing a reagent that lyses blood cells and immediately stabilize intercellular RNA to preserve the gene expression profile. PAXgene Blood miRNA Kit (PreAnalytiX GmbH, Hombrechtikon, Switzerland) was used for miRNA extraction according to the manufacturer's protocol and subjected to on-column DNase I treatment with RNase-free Dnase (Qiagen). RNA quantity and quality were determined using a Thermo Scientific NanoDrop 2000c. Total RNA (50 ng) was reverse transcribed to cDNA and qRT-PCR was performed to quantify mature miRNA-223 expression using the TaqMan miRNA Assay (Applied Biosystems) according to manufacturer's instructions. Samples were run in duplicates in three different experiments. A template controls were carried out in each PCR. Ct is defined as the fractional cycle number at which the fluorescence exceeds the defined threshold. miRNA-103 was chosen as a reference for data analysis [28] and all fold changes were calculated using the ΔCt method [29].

2.6. Statistics

Prism 6f for Mac OS X was used for statistical analysis. The statistical difference between two groups was investigated by Mann—Whitney *U* test. Multiple comparisons were done by one-way Analysis of Variance followed by Bonferroni or Dunnett's post-tests (***$p \leq 0.001$, **$p < 0.01$, *$p < 0.05$, ns = non significant).

3. Results

3.1. Study population and patient characteristics

During the study period 14 cases of pulmonary TB were identified at the study site. Of these, eleven pulmonary TB patients were assigned into group 1 (active) (Figure 1 and Table 1). Patients were included 0—7 months after initiation of treatment. In total 225 persons were identified as exposed to Mtb and subjected to LTBI investigation. Out of 225 contacts, 51 (22.7%) were defined as having LTBI, 4 (1.8%) contacts had inconclusive QFT-G results, and the remaining 170 (75.6%) contacts were QFT-G negative. Ten contacts declined and 34 were included, of which 13 with LTBI were enrolled into group 2 (LTBI). 21 healthy individuals in the patient's immediate vicinity were recruited as close contacts with the active patient and were enrolled into group 3 (controls).

There was no difference in age ($p < 0.3223$) or gender ($p < 0.3161$) between the groups (Table 1).

Table 1
Patient demographic data.

	Active	LTBI	Controls
Patient (n)	11	13	21
Age (year)			
Median	36	39	38
Range	18—79	19—77	18—60
Gender (male/female)	(8/3)	(9/4)	(8/13)
Clinical			
Sputum smear positive (%)	73	0	0
Culture-positive (%)	100	0	0
IGRA- positive	NA	13	0
Co-morbidity			
Diabetes	2	1	0
Rheumatoid arthritis	0	0	1
Asthma	1	0	0
IBD	0	0	1

3.2. CXCR expression linked to TB stages

Regulation of receptor expression in response to Mtb has been described before [30,31]. Mtb has been reported both to down-regulate and to up-regulate the chemokine receptor CCR5 on leukocytes. To investigate CXCR1 and CXCR2 expression during pulmonary TB and LTBI we adopted a simple whole blood model for flow cytometer analysis. TB patients had a significant increase in CXCR1 expression ($p < 0.001$) compared to LTBI subjects and controls (Figure 2 and Supplemented Figure 1). For CXCR2 expression, the LTBI subjects had a significant increase compared to TB patients ($p < 0.001$) and controls ($p < 0.01$).

3.3. miRNA-223 is expressed in active TB

miRNA-223 was recently identified as regulator of neutrophil recruitment to the lung during TB [14]. We investigated blood miRNA-223 profiles in TB patients, LTBI subjects and close contacts (Figure 3). Using the human TaqMan miRNA-223 assay, we identified significant upregulation of miRNA-223 in TB patients compared with LTBI cases (*$p < 0.05$).

3.4. Impaired oxidative burst in active TB patients

CXCR1 has been previously implicated in bacterial killing [32], while CXCR2 is known to be involved in the release of fibrotic markers. We investigated the capacity to induce oxidative killing in whole blood in pulmonary TB patients, LTBI subjects and close controls (Figure 4). Oxidative burst was significantly impaired in pulmonary TB patients compared to LTBI and controls (Figure 4A; $p < 0.001$). We found a strong correlation between increased CXCR1 expression and decreased oxidative burst in pulmonary TB patients (Figure 4B).

To investigate the connection between CXCR1 expression and oxidative killing even further, we blocked CXCR1 or CXCR2 in healthy volunteers before performing the oxidative burst assay (Figure 4C). Blocking of CXCR1 decreased the capacity to induce oxidative burst compared to CXCR2 blocking ($p < 0.001$).

4. Discussion

As an essential component of the innate immune defence, leukocytes migrate from the blood into inflammatory tissue. Inadequate innate immune response could lead to destruction of organ structure, a main characteristic of acute tuberculosis. We found an increased expression of CXCR1 in active pulmonary TB patients, and an increased CXCR2 receptor expression in LTBI subjects. Latency is considered today as a state of persistent mycobacteria-specific T-cell
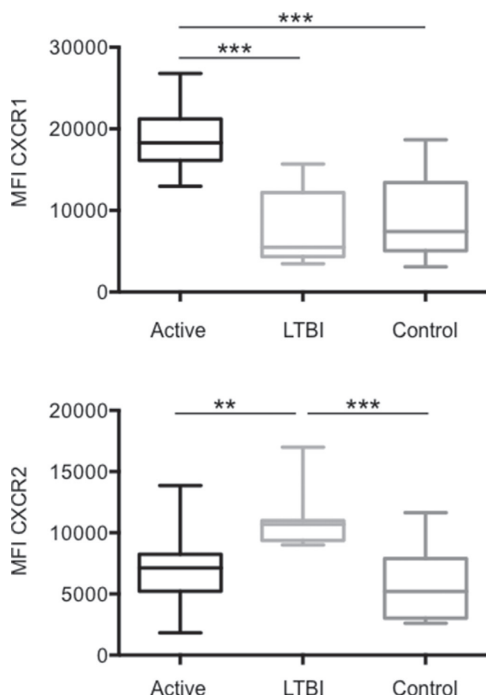
Figure 2. CXCR expression linked to TB pathology. The expression of CXCR1 and CXCR2 was investigated by monoclonal antibodies in whole blood from pulmonary TB patients (active, $n = 11$), LTBI subjects ($n = 13$) and close contacts ($n = 21$). (A) CXCR1 was significantly increased in active TB patients ($p < 0.001$) compared to LTBI subjects and controls. (B) LTBI subjects had increased CXCR2 expression compared to active TB patients ($p < 0.001$) and controls ($p < 0.01$). The mean fluorescence intensities (MFI) were calculated from the negative control. Data are presented as min/max with horizontal bar representing mean (ANOVA/Bonferroni post-test and Mann–Whitney U test); $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Figure 3. Increased miRNA-223 expression in active TB patients. qRT-PCR assay of miR-223 in peripheral blood from pulmonary TB patients (active, $n = 7$), LTBI subjects ($n = 7$) and close contacts ($n = 7$). miRNA-223 is significantly up-regulated in whole blood of active TB patients compared with LTBI participants and uninfected controls ($^*p < 0.05$). Data are presented as Relative miR-223 expression (ΔCt); ANOVA with Bonferroni test.

responses in the absence of clinical evidence for TB [33,34]. Like active TB, which can manifest on a continuum of severity, there is a spectrum of latent infection. Pro-inflammatory cytokines, such as IFNγ and TNFα, are produced in the lung during latent TB infection and both of these cytokines can regulate CXCR2 expression [35,36]. Generally, the cellular expression of these receptors is dependent on ligand-induced receptor internalization, where CXCR1 is internalized at higher chemokine concentrations than CXCR2, yet recycles back to the membrane faster [37,38]. However, pulmonary TB patients entered our study after an average treatment for four months, when the chemokine cascades that can affect the recycling of CXCR1/2 are back to normal [39]. The subjects in our control group have been in close contact with the index patient for a long time, but avoided infection. However, the statistically significant difference in receptor expression between the groups cannot tell us about biological significance, as measured in terms of a biological outcome. From this perspective, we are currently investigating whether a genetic factor could be predisposing the exaggerated CXCR1 expression in pulmonary TB patients. Interaction of host cells with the pathogen or parts of the pathogen can also induce increased receptor expression [31]. A previous study reported that both CXCR1 and CXCR2 were down-

regulated on neutrophils isolated from pulmonary TB patients compared to healthy volunteers [40]. Another study identified an increased CXCR2 expression on isolated mononuclear cells from two TB patients [19]. Our results differ, but could be explained with the controls used, as we compare our results with data obtained from TB exposed close contacts. Moreover, in both previous studies the leukocytes were isolated from blood before measuring receptor expression. Since isolation procedure activates cells [41], we chose to perform the analysis in whole blood.

G protein-coupled receptors (GPCRs), such as CXCR1 and CXCR2, are important regulators of pulmonary diseases [42]. CXCR2 is of particular interest since several studies identified this receptor in the pathology of a wide diversity of chronic pulmonary diseases and modulation of CXCR2 function is considered as a possible therapeutic strategy. Consistent with the observation in chronic pulmonary diseases we observed an increased CXCR2 expression in LTBI subjects compared to the pulmonary TB patients and the uninfected controls. CXCR2 is proposed to be involved in mucus hypersecretion and neutrophil recruitment in chronic obstructive pulmonary disease (COPD) [22,43]. Increased receptor expression is found in injured areas in the lungs of COPD patients, which goes along with the presence of tissue neutrophils during severe exacerbations of COPD [43,44]. Interestingly, COPD patients have an increased risk of developing active TB compared to general population [45] and LTBI increases the risk of COPD [46]. The role of CXCR1 and CXCR2 in these progressions is not known, but could be of great interest.

Effective bactericidal activity and cooperation with macrophages could theoretically eliminate Mtb at an early stage, but failure of killing could result in continuous neutrophil trafficking to the site of infection. MicroRNAs are important regulators of immune responses and could be used as future therapeutic targets. The expression of microRNA-223 is mostly found in neutrophils where it controls their activation [47]. We found increased microRNA-223 expression in the blood of active TB patients compared to the latent TB patients or index controls. Dorhoi et al. identified miRNA-223 in blood and lung parenchyma of tuberculosis patients and during experimental mouse models of the disease [14]. Deletion of microRNA-223 led to decreased neutrophil infiltration and subsequent exacerbated lung inflammation. Sustained neutrophil recruitment at the site of disease is thus likely to contribute to on-going inflammation, especially in the context of
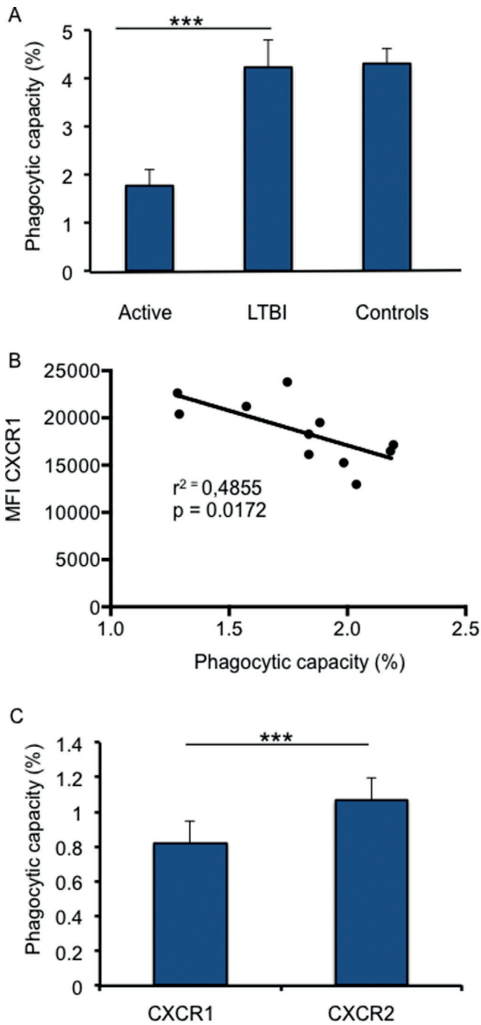
Figure 4. Impaired phagocytosis in pulmonary TB patients. Intracellular killing was investigated with opsonized *Escherichia coli* by oxidative burst in whole blood from pulmonary TB patients (active, $n = 11$), LTBI subjects ($n = 13$) and close contacts ($n = 21$). (A) Pulmonary TB patients showed decreased killing of bacteria showed as a reduced production of oxidative radicals ($p < 0.001$) compared to LTBI subjects and controls. (B) Correlation of CXCR1 expression in pulmonary TB patients (active, $n = 11$) with bacterial-killing capacity. Bacterial killing capacity is shown as percentage of negative control and the MFI for CXCR1 expression was calculated from the negative control. (C) Blocking of CXCR1 or CXCR2 by monoclonal antibodies in healthy volunteers ($n = 8$) before performing the assay for bacterial killing. Blocking of CXCR1 decreased the phagocytic capacity compared to CXCR2 blocking. Un-blocked cells were used as control ($p < 0.001$). Data were analyzed by regression curve after one-way ANOVA/Bonferroni post-test and Mann–Whitney U test.

immunological dysfunction. The study is considered to reveal an essential role for a single miRNA in tuberculosis and the potential of miRNAs for diagnosis and therapy of tuberculosis is currently considered [48,49].

Despite evidence that CXCR1 and CXCR2 signal through similar G proteins, there are marked differences in the activation of signalling cascades, which may identify disparate physiological roles under inflammatory conditions. CXCR1 is the functionally important receptor involved in neutrophil degranulation [50–53], while CXCR2 activation is coupled to the release of elastase, markers of fibrosis, and the matrix metalloproteinase 9 (MMP9) [54]. CXCR2 signalling was previously shown to lead to NADPH-oxidase (NOX)-dependent generation of ROS and cell death [55]. In contrast, recently the reactive oxygen species generated via the phagocyte NADPH oxidase 2 (NOX2) complex were shown to contribute to the resolution of inflammation [56]. We observed decreased intracellular superoxide production despite increased CXCR1 expression in active TB patients. Decreased CXCR1 induced superoxide production can be achieved by the mechanisms of desentization [57,58]. Leukocyte deactivation is also found in septic patients and function as a negative predictor of survival [59,60]. Furthermore, since phagocytosis is known to down-regulate the expression of CXCR1 and CXCR2 [61], our data suggests that a non-functional CXCR1 could explain the impaired phagocytosis observed in active TB patients. Blocking of CXCR1 in healthy volunteers resulted in decreased intracellular superoxide production, supporting our hypothesis on impaired CXCR1 function. A cleaved CXCR1 was recently observed to disable bacterial killing in patients with chronic pulmonary diseases [32]. Our next step will be to investigate the cause behind the impaired receptor function.

The global burden of TB remains high, but the determinants of susceptibility to severe infection and latent disease remain poorly understood. Tissue damage during TB arises primarily from the immune response and not as a direct consequence of the bacterial infection. In terms of rapid diagnosis, sputum microscopy will only identify approximately 50% of patients with active pulmonary TB, while IGRA cannot distinguish active TB from symptomatic patients with latent infection. We identified distinct chemokine receptor expression in pulmonary TB patients and LTBI subjects, and an impact of these receptors on immune defence. This finding could open up a new area of future therapies.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2015.07.008.

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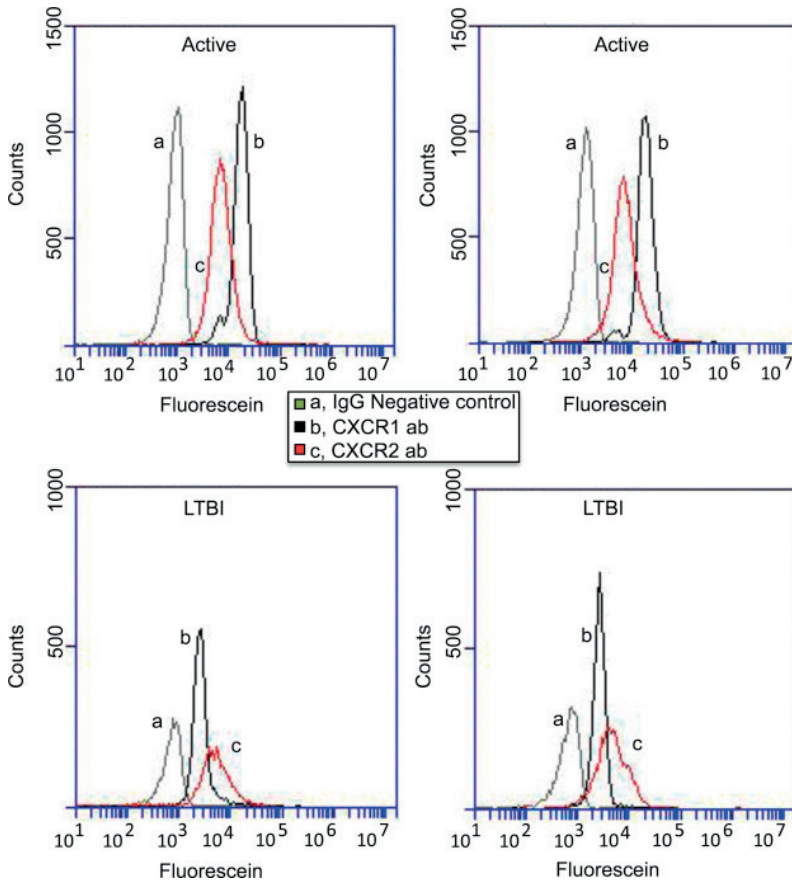
Supplementary Figure 1. CXCR expression in pulmonary TB patients and latent objects as determined by flow cytometry analysis. The x axis indicates fluorescence intensity measured on log10 scale, and the y axis indicates event counts per channel on a linear sca...

Nader Alaridah, Niclas Winqvist, Gisela Håkansson, Erik Tenland, Anna Rönnholm, Erik Sturegård, Per Björkman, Gabriela Godaly

Impaired CXCR1-dependent oxidative defence in active tuberculosis patients

Paper III

Mycobacteria Manipulate G-Protein-Coupled Receptors to Increase Mucosal Rac1 Expression in the Lungs

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Abstract

Mycobacterium bovis bacille Calmette-Guérin (BCG) is currently the only approved vaccine against tuberculosis (TB). BCG mimics *M. tuberculosis* (Mtb) in its persistence in the body and is used as a benchmark to compare new vaccine candidates. BCG was originally designed for mucosal vaccination, but comprehensive knowledge about its interaction with epithelium is currently lacking. We used primary airway epithelial cells (AECs) and a murine model to investigate the initial events of mucosal BCG interactions. Furthermore, we analysed the impact of the G-protein-coupled receptors (GPCRs), CXCR1 and CXCR2, in this process, as these receptors were previously shown to be important during TB infection. BCG infection of AECs induced GPCR-dependent Rac1 up-regulation, resulting in actin redistribution. The altered distribution of the actin cytoskeleton involved the MAPK signalling pathway. Blocking of the CXCR1 or CXCR2 prior to infection decreased Rac1 expression, and increased epithelial transcriptional activity and epithelial cytokine production. BCG infection did not result in epithelial cell death as measured by p53 phosphorylation and annexin. This study demonstrated that BCG infection of AECs manipulated the GPCRs to suppress epithelial signalling pathways. Future vaccine strategies could thus be improved by targeting GPCRs.

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Introduction

It has been proposed that mucosal delivery of the bacille Calmette-Guérin (BCG) vaccine provides superior protection against tuberculosis (TB), a disease that kills 1.9 million people each year [1, 2]. Traditionally, the adaptive immune responses, particularly central memory CD4+ and CD8+ cells, are considered to be important for long-term immunity [3–6]. However, emerging evidence indicates that the cells of the innate immune system are equipped with "epigenetic memory" where genes encoding specific host defence molecules increase the response

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upon re-stimulation [4–7]. Recent studies further demonstrated that airway epithelial cells (AECs) harbour *Mycobacterium tuberculosis* (Mtb) and are critical during the progression to active disease [8, 9]. These cells could thus be important for both host defence and vaccine development, but a more comprehensive knowledge of direct interaction between AECs and mycobacteria is currently lacking [10].

BCG mimics Mtb in its ability to persist in the body, and is similar to the tubercle bacillus in its physiological, molecular, and metabolic responses [11–14]. This invasive bacterium actively induces its own uptake by macropinocytosis to enter normally non-phagocytic cells such as AECs [15]. Until now, Toll-like receptor (TLR)2, TLR4 and C-type lectins have been implicated in mycobacteria-induced epithelial signalling and uptake [16, 17]. It has also been observed that mycobacteria manipulate epithelial production of the cytokine CXCL8 through the inhibitory G-protein-coupled receptor (GPCR) kinase 2 [18]. To trigger GPCR signal transduction in AECs, intracellular bacteria, such as *Shigella*, are known to engage GTPases in actin polymerization [19–21]. These low-molecular-weight proteins belong to the Ras GTPase superfamily and include Rab and Rho/Rac, with the ability to act as molecular switches by coupling extracellular signals to different cellular responses, cytoskeletal integrity, intracellular vesicular transport, and trafficking of proteins [22]. Inhibition of Rac1 was recently shown to repeal tumour protein p53 suppression of STAT and NF-κB, and Rho is essential in the establishment and maintenance of tight junctions [23]. Previous studies indicate that signalling through TLRs is important for the phagocytosis of bacteria, as TLR-mediated MyD88-dependent activation of p38 is required for phagosome maturation [24, 25]. Intracellular pathogens such as *Listeria monocytogenes* manipulate TLRs through the MAPK pathway to avoid phagosome maturation and autophagy [26, 27]. The p53 pathway acts in synergy with the p38 MAPK pathway to mediate cell cycle arrest, cellular senescence and apoptosis [28].

The innate host defence of the conducting airway depends on the multiple barriers created by layers of mucus and the tight adhesions between epithelial cells. In the human lung, AECs are able to harbour Mtb and are critical during progression to active disease [29]. Recently, mucosal vaccination with an attenuated Mtb strain induced a strong innate immune response, followed by a robust central memory answer [30]. AECs facilitate a protective environment for Mtb replication where it could gain enhanced virulence by modifying envelope structure and gene expression [31]. AECs also interact with other cells of the innate immune system, such as granulocytes, monocytes, macrophages, and innate lymphoid cells, to mount an effective defence against the invading pathogen as well as to activate the following specific immunity. AECs are now recognized as active participants of the immune response against Mtb [32]. In this study, we analysed mycobacteria-induced epithelial signalling pathways and the contribution of GPCRs to further elucidate these responses. We could conclude that BCG induced Rac1 up-regulation, resulting in long-term actin cytoskeleton distribution. Inhibition of GPCRs decreased BCG-induced Rac1 expression but increased AEC transcriptional activity and epithelial cytokine production.

Material and Methods

Ethical Statement

The Swedish Research Ethical Committee in Lund (FEK 413/2008) approved the isolation of the primary AECs. These were acquired from lung explants of healthy donors with irreversible brain damage and no history of lung disease; the lungs were intended for transplantation, but could instead be used in this study as no matched recipients were available at that moment. Written consent was obtained from the donors' closest relatives. The murine study was approved by the Animal Experiment Ethics Committee at the Lund District Court in Sweden (M7–15).

Bacterial Strain and Growth Condition

Mycobacterium bovis BCG Montreal strain containing the pSMT1 shuttle plasmid was prepared as previously described [33]. Briefly, the mycobacteria were grown in Middlebrook 7H9 broth, supplemented with 10% ADC enrichment (Becton Dickinson, Oxford, UK) and hygromycin (50 mg/L; Roche, Lewes, UK), the culture was washed twice with sterile PBS, re-suspended in broth, and then dispensed into vials. Glycerol was added to a final concentration of 25% and the vials were frozen at −80°C. Prior to each experiment, a vial was defrosted, added to 9 mL of 7H9/ADC/hygromycin medium, and incubated with shaking for 72 h at 37°C. Mycobacteria were then centrifuged for 10 min at 3,000 g, washed twice with PBS, and re-suspended in 10 mL of PBS.

Cell Culture

Bronchial tissue was dissected from lungs and kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin, penicillin, streptomycin, Fungizone, and 10% FCS (all from Gibco, Paisley, UK) until further isolation. After removing intraluminal mucus and surrounding tissue, bronchi were digested in 0.1% protease (Sigma, St. Louis, MO, USA) prepared in a minimum essential medium Eagle, Spinner modification (Sigma-Aldrich) supplemented with gentamicin, penicillin, streptomycin, and Fungizone for 24 h. The cells were recovered by repeated intraluminal rinsing with DMEM supplemented as above. Cells were filtered through a 100-μm strainer (Falcon, Becton Dickinson) and seeded in cell-culture flasks coated with 1% collagen-1 (PureCol, Inamed Biomaterial, Freemont, CA, USA) in bronchial epithelial

cell growth medium (Clonetics). The following day, cells were thoroughly washed with a medium change every other day. Experiments were performed in passage 3 or 4.

Incubation of AECs with Mycobacteria

AECs were grown in 6-well plates (2.0×10^5 cells/well; Fisher Scientific, Loughborough, UK) for 2 days until confluent (80%). Before the experiment, the medium was changed and the cells were treated with monoclonal mouse anti-human CXCR1 and/or monoclonal mouse anti-human CXCR2 antibodies (10 μg/mL; R&D Systems) and/or Rac1 inhibitor (50 μM; Merck Millipore) for 30 min on ice, followed by infection with BCG. The cells were infected with a multiplicity of infection (MOI) of 1:1 at 37°C for up to 3 days. As a control, we used uninfected cells treated with CXCR1, CXCR2 antibodies, or the Rac1 inhibitor.

Western Blot

The primary cells were washed with PBS containing 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin A, and 5 μg/mL leupeptin (Sigma-Aldrich), and then lysed with modified mammalian protein extraction reagent solution (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 50 mM ZnCl, 1% NP-40, 0.1% deoxycholate, 0.1% SDS; Pierce) containing phosphatase and complete protease inhibitor cocktail (1:100). The cells were then placed on a shaker for 5 min, collected, and then centrifuged at 10,000 *g* for 5 min. Protein samples were used immediately for Western blot analysis or stored at −80°C. Protein levels were measured with a NanoDrop™ 8000 spectrophotometer using the Pierce 660 nm assay (Thermo Scientific). Medium alone and only BCG-infected cells were used as controls. Protein samples were mixed with PBS, 4× NuPAGE LDS sample buffer (Life Technologies), and 0.1 M DTT, and incubated at 90°C for 10 min followed by centrifugation at 218 *g* for 5 min. Equal amounts of protein (20 μg/well) were loaded on a NuPAGE 4–12% Bis-Tris Gel (Life Technologies) and separated by SDS-PAGE. A molecular weight marker (Novex® Sharp Prestained; Life Technologies) was loaded onto each gel for protein-band identification. After separation, the proteins were transferred to a PVDF membrane (GE Healthcare, Little Chalfont, UK). The membrane was then blocked with either 5% dry milk (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 5% BSA (Santa Cruz Biotechnology) for 1 h on a shaker at room temperature. Membranes were then incubated on a shaker overnight at 4°C with mouse monoclonal antibodies against human Rac1 (1:500; 23A8 Millipore), Rho A (1:500; 26c4; Santa Cruz Biotechnology), Rab4 (1:500; 46-K; Santa Cruz Biotechnology) and goat polyclonal against human actin (1:500; I-19; Santa Cruz Biotechnology). Bound primary antibodies were detected with goat-anti-mouse IgG horseradish peroxidase (HRP) or rabbit-anti-goat IgG HRP (1:5,000; Santa Cruz Biotechnology) using ECL (GE Healthcare) and GelDoc equipment (Bio-Rad Laboratories). The housekeeping protein GAPDH was used to confirm equal loading on the wells. Blot intensity was quantified using ImageJ v1.49, and normalized against GAPDH. If required, membranes were stripped with Restore Western Blot stripping buffer (Pierce, Rockford, IL, USA), blocked, and re-probed with new antibodies.

Phospho-Kinase Array

A proteome human phospho-kinase array kit (Proteome Profiler Array, R&D Systems), a membrane-based sandwich immunoassay, was used to measure protein phosphorylation. The assay was performed according to the manufacturer's instructions. Briefly, total cell extracts were prepared from stimulated near-confluent cultures of AECs grown in 6-well plates. Untreated cells were used as controls. The cell extracts containing 500 μg of total protein were incubated with the human phospho-kinase array. The proteins present in a lysate sample were captured by discrete antibodies printed in duplicate across the nitrocellulose membranes. The array was washed 3 times with 1× wash buffer for 10 min on a rocking platform shaker to remove unbound proteins. Washing was followed by incubation with a cocktail of biotinylated detection antibodies (monoclonal anti-human of phosphorylated PYK2 (Y402), MEK1/2 (S218/S222, S222/S226), JNK pan (T183/Y185, T221/Y223), p38, p53 (S15), p53 (S46), p53 (S392), STAT1 (Y701), STAT5b (Y699), STAT6 (Y641), and a subsequent addition of streptavidin-HRP conjugate. The signals were detected with the ECL Plus Western blotting detection system (GE Healthcare). Developed signals were analysed using ImageJ v1.49 analysis software.

Detection of Apoptosis and Necrosis

After incubation of AECs with BCG, the cells were detached by EDTA for about 5 min at room temperature, followed by the addition of a trypsin inhibitor, washed twice in PBS for 5 min (1,000 rpm), and stained with annexin V-Alexa Fluor®488 (1:100; Life Technologies Europe BV, Stockholm, Sweden) for 15 min in the dark on ice to detect early apoptosis and 7-aminoactinomycin D (1:100; BD Via-Probe, BD Pharmingen Biosciences, San Diego, CA, USA) for 15 min in the dark on ice to test for late apoptosis and necrosis. Mean fluorescence intensity was analysed by flow cytometry (Accuri, Becton Dickinson).

Murine BCG Infection

Male BALB/c mice, aged 8–10 weeks, were obtained from Jackson Laboratories (Bar Harbor, ME, USA). They were maintained in the animal facilities at the Department of Microbiology, Immunology, and Glycobiology, Lund University, Lund, Sweden. They were anaesthetized by isoflurane inhalation for 10–20 s. The mice were divided into 2 groups: BCG infected (*n* = 5) and uninfected (*n* = 3). For infections, BCG at 5×10^4 CFU in 10 μL PBS was given intranasally, and 10 μL PBS was given to the control mice. After 5 weeks, the mice were sacrificed by intraperitoneal administration of pentobarbitone (60 mg/mL, 0.05–0.1 mL/mice). The largest lobe was saved in paraformaldehyde (4%), and used for immunohistochemistry staining. Remaining lobes were homogenised and plated on 7H11 agar plates and incubated at 37°C for 4 weeks for CFU measurements.

Immunofluorescence Microscopy

The expression of actin and Rac in the sections of lung tissue from BCG-infected BALB/c mice was detected by immunofluorescence staining. The fixed tissue samples were dehydrated by overnight incubation in ethanol, followed by xylene, and placed in Histowax (Histolab Products, VästraFrölunda, Sweden), according to the manufacturer's recommendations. The samples were embedded in paraffin; sections (4–5 μm) were cut and placed on glass slides. Deparaffinization, rehydration, and antigen retrieval of the specimens were done, followed by blocking in 10% FCS with 1% BSA in TBS for 2 h at room temperature, followed by incubation overnight, shaking in a cold room with PBS with 1% BSA; the goat polyclonal anti-actin (sc-1616) (Santa Cruz Biotechnology) or
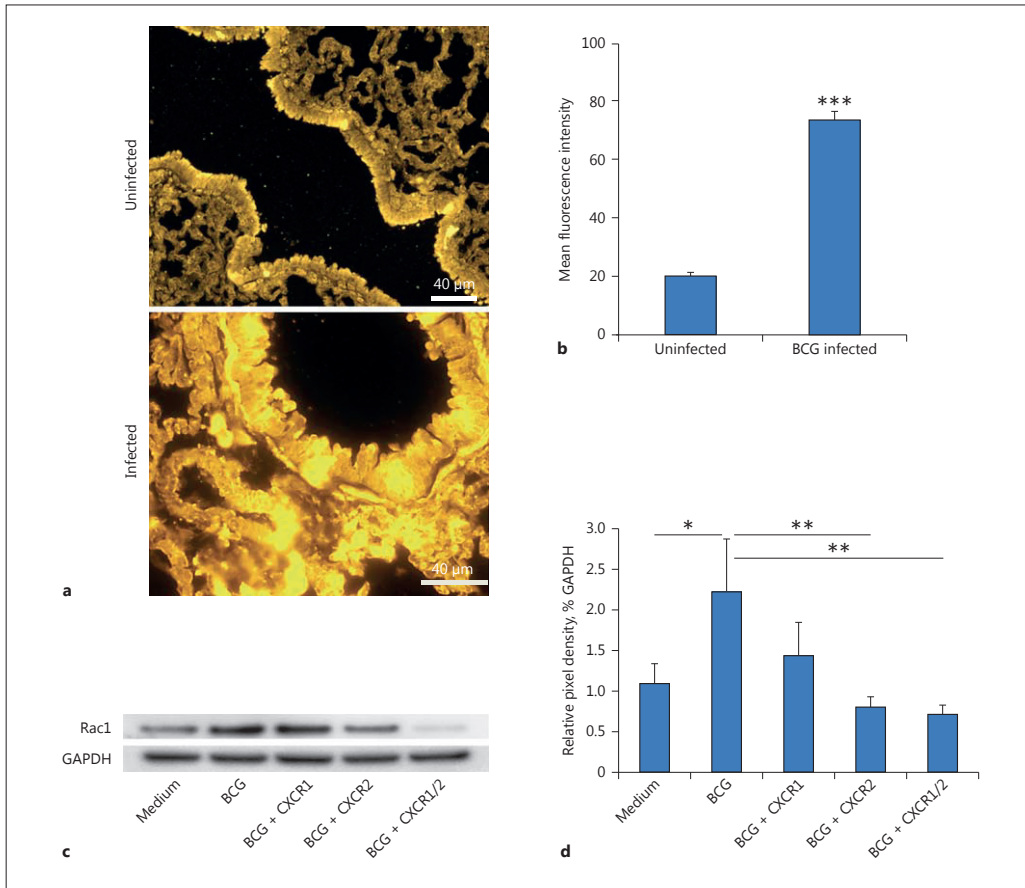
Fig. 1. Mycobacteria increase Rac1 expression. The impact of BCG infection, CXCR1 and CXCR2 on modulated epithelial Rac1 expression was studied in a murine model and by Western blot. **a, b** Mycobacterial infection significantly altered Rac1 expression in vivo as detected by immunofluorescence microscopy compared to uninfected mice. **c, d** BCG infection increased epithelial Rac1 expression, but CXCR1/2 blocking prior to infection decreased Rac1. Data are presented as representative images or as mean ± SEM of 3 separate experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

mouse monoclonal antibody against Rac1clone 23A8 (Millipore) antibodies were used. As a control, we used an isotype antibody by replacing the primary antibody with antibody diluent (blocking buffer). The specimens were washed twice with PBS plus 0.0025% Triton X-100 for 5 min, and then incubated with fluorophore-conjugated rabbit anti-goat or goat anti-mouse secondary antibody (1: 2,000; Invitrogen) in PBS with 1% BSA for 2 h in the dark. Slides were then examined by fluorescence microscopy (AX60, Olympus Optical).

For in vitro actin expression, AECs were seeded on glass slides (12 mm Ø), and allowed to attach for 1 day at 37°C in a 5% CO_2 atmosphere. Rac1 inhibitor was added for 30 min on ice before BCG infection. Bacteria were added to the AECs at MOI 1:1, and incubated for 72 h, followed by fixation in 4% paraformaldehyde. The glass slides were washed twice in PBS with 5% FCS, and blocked and mounted overnight with CytoPainter Phalloidin-iFluor 488 Reagent (Abcam) and anti-Mtb antibody (ab905; 1:200; Abcam).

Statistical Analysis
Prism 6f for Mac OS X was used for statistical analysis. The statistical difference between two groups was investigated by means of the nonparametric Mann-Whitney U test. Multiple comparisons were done by one-way analysis of variance followed by the Kruskal-Wallis test with the Bonferroni correction and the Dunnett post hoc test. Significance was accepted at $p < 0.05$, $p < 0.01$, or $p < 0.001$.

Results

Mycobacteria Utilize CXCR1 and CXCR2 to Induce Epithelial Rac1 Activation

Rac1, a member of the Ras superfamily of small GTPases, regulates the basal level of actin assembly and the reorganization of the actin cytoskeleton in response to GPCR stimulation [34]. BCG-induced mucosal Rac1 expression was analysed in vivo by immunohistochemistry staining of mycobacteria-infected mouse lung sections (Fig. 1a). BCG infection induced sustained mucosal Rac1 expression 5 weeks after infection ($p = 0.0001$; Fig. 1a). We confirmed that BCG infection of primary AECs increased Rac1, compared to uninfected cells ($p = 0.015$; Fig. 1c, d). Blocking of CXCR1 or CXCR2 decreased Rac1 production down to basal levels ($p = 0.092$, $p = 0.001$, and $p = 0.001$ for CXCR1, CXCR2, and CXCR1/2, respectively; Fig. 1c, d).

Mycobacteria Up-Regulate Actin Distribution

During inflammation, actin cytoskeletal changes regulate junctional integrity leading to disturbed barrier function [35]. Actin expression of BCG-infected AEC was studied by Western blot and immunofluorescence (Fig. 2a, b). BCG infection induced increased actin expression compared to uninfected cells ($p = 0.002$; Fig. 2a). Blockage of the CXCR2 decreased BCG-induced actin production ($p = 0.07$, $p = 0.001$, and $p = 0.006$ for CXCR1, CXCR2, and Rac1 inhibition respectively; Fig. 2a). With immunofluorescence, we observed that BCG infection induced epithelial actin redistribution (Fig. 2b; online suppl. Fig. 2; for all online supplementary material, see www.karger.com/doi/10.1159/000453454). To confirm that BCG infection regulates actin expression and distri-

bution in vivo, lung tissues from BCG-infected mice were stained for actin cytoskeletal changes (Fig. 2c, d). Compared to in the uninfected animals, BCG infection increased sustained actin up-regulation for 5 weeks after infection (Fig. 2d).

Mycobacteria Activate the Epithelial MAPK Pathway

Previous studies indicated that mycobacteria regulate the epithelial inflammatory response through GPCR kinases [18]. GPCR-mediated signalling is further known to activate the pathways of major kinases, including MAPK and JNK, new targets in drug discovery [36]. To investigate mycobacterial epithelial kinase activation, we used the human phospho-kinase array on primary AECs (Fig. 3). Mycobacterial infection led to increased epithelial phosphorylation of PYK2 ($p = 0.0045$; $p = 0.0003$), p38 kinase ($p = 0.0001$; $p = 0.0005$) and MEK1/2 (MAP2K1/2; $p = 0.0003$; $p = 0.0015$) compared to medium control at both 6 and 72 h after infection (Fig. 3). In contrast, JNK had decreased after 6 h, and increased after 72 h, compared to the control (Fig. 3). Actin distribution requires p38 and MEK1/2, and both of these molecules were activated by BCG infection of AECs (Fig. 3).

Mycobacterial Induction of p53 Does Not Affect Epithelial Survival

Intracellular bacteria trigger actin polymerization in AECs by engaging GTPases such as Rac and Rab [20, 37, 38]. Inhibition of Rac1 was recently shown to abolish tumour protein p53 suppression of the transcription factors STAT and NF-κB [23]. We investigated the mycobacterial influence on epithelial p53 with a phosphorylation assay (Fig. 4). BCG infection of AECs had increased the phosphorylation of p53 at the regulatory S319 and apoptotic S46 domains, at 6 and 72 h post infection, but not at the S15 domain, which activates transcription and cell survival. Rac1 inhibition prior to infection decreased p53 (S392 and S46), possibly suggesting that Rac1 is involved in transcription and cell survival (online suppl. Fig. 1).

To analyse if mycobacteria induced cellular death, we measured epithelial apoptosis and necrosis (data not shown). We found that mycobacteria did not affect epithelial survival.

BCG Manipulate Epithelial STAT Phosphorylation

Compared to our observation using BCG, pathogenic mycobacteria are known to suppress the MAPK and JAK/
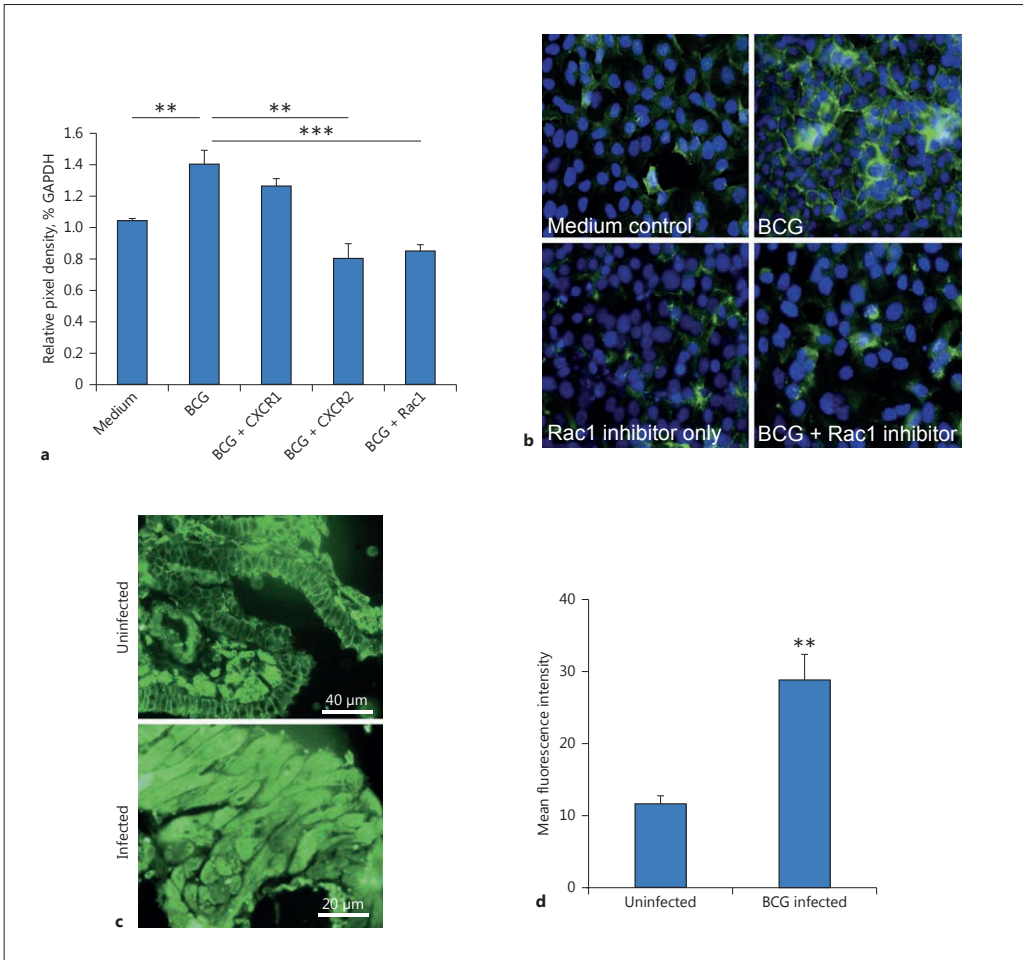
Fig. 2. Mycobacteria up-regulate actin distribution. **a**, **b** BCG-induced increased actin expression was detected in BCG-infected AECs. CXCR2 blocking or the addition of Rac1 inhibitor prior to infection decreased the actin expression. **c** In vivo, the infection increased actin distribution in the BCG-infected mice compared to the uninfected controls, visualised by immunofluorescence microscopy. **d** The images were further analysed by ImageJ software. Data are presented as representative images or as mean ± SEM of 3 separate experiments. ** $p < 0.01$; *** $p < 0.001$.

STAT signalling pathways that are crucial for many innate and adaptive immune responses [36]. Patients with disseminated BCG infection have been found to have dominant-negative mutations in *STAT1* that affect IFNγ signalling [39]. BCG infection in primary AECs reduced the phosphorylation of STAT1 (Y701), STAT5b (Y699), and STAT6 (Y641) 6 h after infection, and increased phosphorylation on STAT1 (Y701) and STAT5b (Y699) at 72 h after infection, but the STAT6 (Y641) levels were reduced (Fig. 4).

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Fig. 3. Mycobacteria activate the epithelial MAPK pathway. We used BCG to investigate epithelial kinase modulation using the human phospho-kinase array. Mycobacterial infection led to increased epithelial phosphorylation of PYK2, p38 kinase, and MEK1/2 (MAPK1/2) compared to medium control 6 and 72 h after infection. JNK had decreased after 6 h but increased after 72 h compared to the control. Data are presented as mean ± SEM of 3 experiments. *** $p < 0.001$.
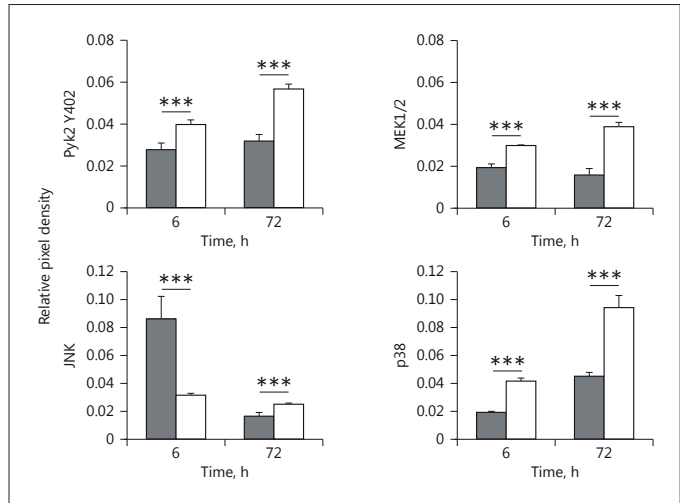
Fig. 4. Mycobacteria modulate epithelial p53 and STAT pathways. Mycobacterial influence on epithelial p53 and STAT was investigated with the phosphorylation assay. BCG infection of primary AECs increased the phosphorylation of p53 at the regulatory S319 and apoptotic S46 domains, 6 and 72 h after infection. In contrast, the phosphorylation at S15, which activates transcription and cell survival, was not induced by the infection. STAT1 (Y701), STAT5b (Y699), and STAT6 (Y641) were suppressed 6 h after infection, while BCG induced phosphorylation of STAT1 (Y701) and STAT5b (Y699) 72 h after infection. STAT6 (Y641) levels remained reduced during the study. Data are presented as mean ± SEM of 3 separate experiments. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.
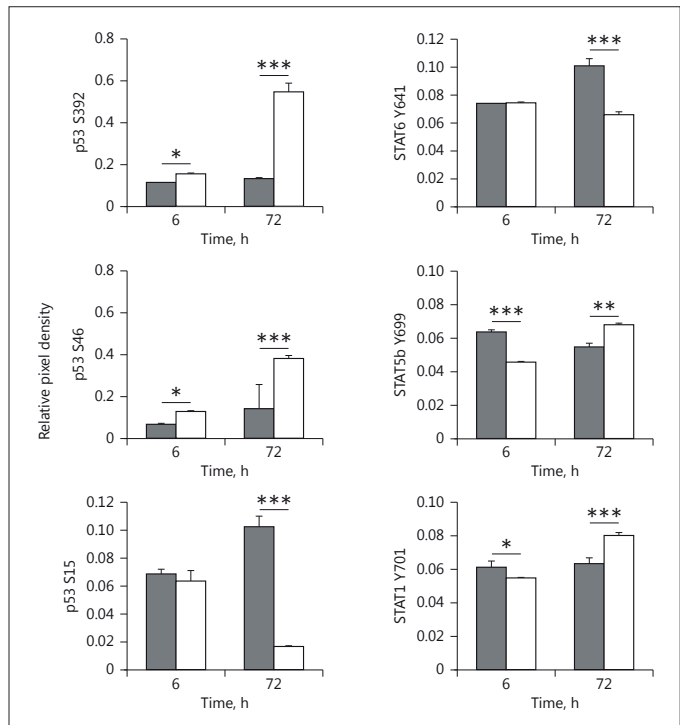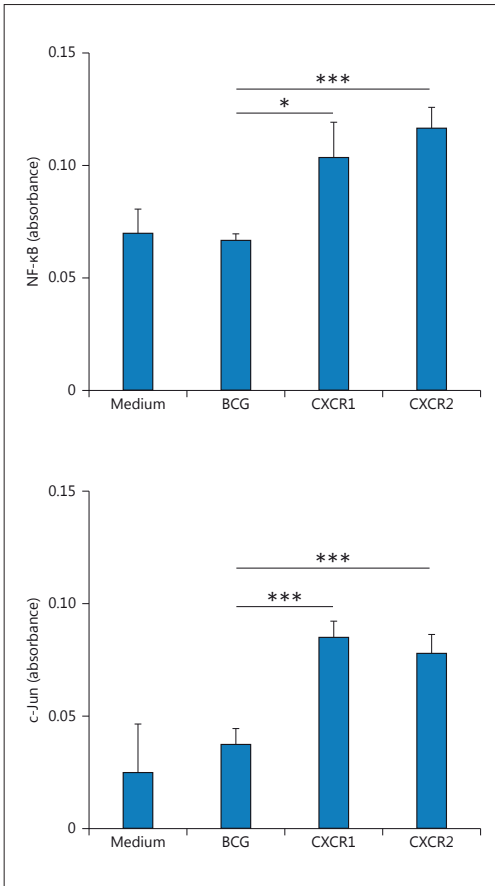
Fig. 5. Mycobacteria regulate NF-κB and c-Jun through CXCR1 and CXCR2. We determined the impact of CXCR1 and CXCR2 blockage on mycobacteria-induced NF-κB and c-Jun regulation. BCG infection did not affect epithelial NF-κB and AP-1 activation. However, the blockage of CXCR1 or CXCR2 prior to infection increased NF-κB and c-Jun protein levels compared to uninfected cells. Data are presented as mean ± SEM of 3 separate experiments. * $p < 0.05$; *** $p < 0.001$.
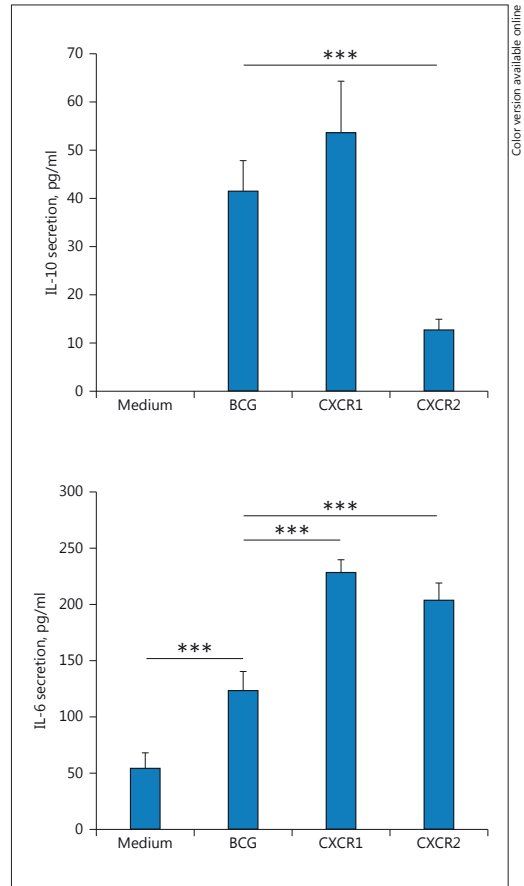
Fig. 6. Controlled epithelial cytokine secretion. Mycobacterial control of transcriptional factors was analysed as epithelial cytokine secretion. BCG infection of AECs induced significant IL-6 and IL-10 secretion. Blockage of CXCR1/2 prior to infection increased IL-6 secretion even further. IL-10 levels were decreased significantly by CXCR2 blockage, but not by CXCR1 antibodies. Data are presented as mean ± SEM of 3 separate experiments. *** $p < 0.001$.

Mycobacteria Regulate NF-κB and c-Jun through CXCR1 and CXCR2

The data on NF-κB activation by pathogenic mycobacteria is still conflicting. BCG was previously shown to bypass NF-κB activation, but activate the signalling through ERK1/2 and cFos instead [40]. We could confirm that BCG did not increase the activation of NF-κB or AP-1 (c-Jun). Interestingly, blocking the epithelial GPCRs CXCR1 and CXCR2 prior to mycobacterial infection increased the NF-κB and c-Jun levels compared to uninfected cells (Fig. 5).

Mycobacteria Control Epithelial Cytokine Secretion

Mycobacteria infection has been suggested to evoke the pro-inflammatory immune response in order to evade host immune responses. IL-6 bridges innate and adaptive host immune responses, while the anti-inflammatory IL-10 suppresses inflammation and postpones the generation of adaptive immunity. Mycobacterial engagement of CXCR1 and CXCR2 to control cellular transcriptional factors was analysed as epithelial cytokine secretion. Infection of AECs induced significant IL-6 and IL-10 secretion (Fig. 6). Blockage of CXCR1 or CXCR2 prior to infection increased IL-6 secretion even further ($p = 0.0001$; $p = 0.0004$). However, IL-10 levels were significantly decreased by CXCR2 blockage compared to BCG-infected cells ($p = 0.0001$), while CXCR1 antibodies did not affect BCG-stimulated epithelial IL-10 production (Fig. 6). The treatment of uninfected cells with CXCR1 or CXCR2 antibodies did not affect IL-10 and IL-6 secretion (data not shown).

Discussion

Reprogramming of AECs could be beneficial for the development of vaccines and treatment strategies [4–6]. Evidence is now accumulating that innate immunity can "remember" previous exposure to a microorganism and could contribute to host defence against infection and vaccine-induced immunity. We found that BCG infection of mucosal AECs induced epithelial signalling pathways leading to actin remodelling and selective cytokine secretion. Actin cytoskeletal changes regulate junctional integrity that leads to disturbed barrier function during inflammation [35]. Mtb invasion of AECs is both receptor- and actin-mediated [41]. BCG is also known to invade AECs [18]; in this study, we show that the infection resulted in Rac1 up-regulation followed by altered epithelial actin distribution. Actin distribution by BCG infection requires p38 that is activated by phosphorylation at the T180/Y182 sites. Recently, macropinocytosis, in contrast to phagocytosis, was shown to lead to rapid elimination of mycobacteria, suggesting that the cytoskeletal mobilization could be involved in the initiation of the adaptive immune response [42]. A previous study showed that Mtb infection increased the oxidative stress in AECs through the p38-ERK-NF-κB axis and led to the cytotoxicity of human lung epithelial cells [43, 44]. Mtb could thus facilitate its own dissemination by compromising the epithelial lining. Contrary to this finding, our results demonstrated that BCG stimulated the MAPK pathway by signalling through the PYK2-p38-MAPK2-JNK axis, pointing to a sustained epithelial barrier after BCG infection.

A wide variety of cellular processes are controlled by a complicated regulatory network consisting of positive and negative regulators. Additionally, post-translational modifications often affect points of regulation in a protein, which allows switching activities [45]. S15/S20 phosphorylation at p53 promotes the recruitment of transcriptional co-activators [46], while the phosphorylation of S46 is critical for p53-mediated induction of pro-apoptotic genes [47, 48]. Phosphorylation of C-terminal S392 in response to ultraviolet light activates specific DNA binding through the stabilization of the p53 tetramer [49]. S392 phosphorylation was recently shown to promote cell survival by stabilizing p53 and enhancing its transcriptional activity [50]. Interestingly, inhibition of Rac1 resulted in decreased p53 S392 and S46 phosphorylation, possibly affecting transcription and cell survival in BCG-infected AECs**.** Our results indicate that BCG infection of AECs stimulate cell survival, which was also confirmed by the negative result of the cell death assay.

Bacterial adherence to cells is also known to trigger signal transduction events involving the G-proteins in actin polymerization, with the subsequent uptake of the bacteria [21, 37, 51]. GPCRs, such as CXCR1 and CXCR2, are important regulators in pulmonary diseases [52]. Previous research revealed that mycobacteria decrease epithelial cytokine production by manipulating these receptors [18], and active TB patients were found to have increased CXCR1 expression [53]. CXCR2 is important in the pathology of a wide diversity of chronic pulmonary diseases, and the modulation of CXCR2 function is considered as a possible therapeutic strategy [54]. Interestingly, blocking of these GPCRs prior to mycobacterial infection repealed the mycobacteria-induced actin redistribution and suppression of epithelial NF-κB and c-Jun, further supporting the theory that mycobacteria utilize GPCRs to manipulate cellular signalling [23]. GPCRs are also required for the activation of STAT, and *STAT1* mutations were recently identified in patients with disseminated BCG infection [55, 56]. The effect of STAT is to increase the transcriptional activity of quiescent genes and the transcription of less active genes [56]. BCG induced the phosphorylation of the transcription supporting STAT1 and STAT5b, while the STAT6 levels were reduced. Blocking CXCR1/R2 increased epithelial NF-κB and c-Jun activation, and the secretion of IL-6

was increased. IL-6 is a multi-potent cytokine that acts in both pro- and anti-inflammatory ways. Exerting its pro-inflammatory qualities, IL-6 suppresses the development of regulatory T cells and favours the differentiation of effector T helper 17 cells during inflammation or infection [57]. Locally produced IL-6 could thus increase CD4+ T cell memory formation. Blocking CXCR2, but not CXCR1, decreased mycobacteria-induced IL-10 secretion. This cytokine modulates the anti-inflammatory mechanisms by targeting NF-κB [58, 59], which could have been the mechanism of suppressed transcriptional activity that we observed in our study. Impaired cellular activation and recruitment was previously related to increased IL-10 production and decreased CXCR2 expression during septic conditions [60, 61]. In our study, blocking of epithelial CXCR2 reduced BCG-induced epithelial IL-10 secretion, possibly suggesting that CXCR2 signalling is important for BCG-induced IL-10 production.

It has been proposed that AECs and innate immune cells drive respiratory diseases [62]. AECs express MHC-I/II [63], and the macropinocytosis of mycobacteria by AECs was recently shown to lead to rapid bacterial elimination [42]. The cytoskeletal mobilization due to macropinocytosis could thus be involved in antigen capture and presentation for incoming T cells [42]. More knowledge is needed on how mycobacteria manipulate cellular kinases and phosphatases, as these molecules constitute potential targets for future TB therapeutics [64]. We showed that the early event of mycobacterial AEC infection led to sustained manipulation of epithelial Rac1 and actin remodelling. Furthermore, our finding that mycobacteria utilized CXCR1 and CXCR2 to manipulate the inflammatory response clarifies the role of GPCRs in pulmonary disease, and possibly towards chronic disease [65]. Future vaccine strategies could thus be improved by targeting GPCRs.

Acknowledgements

Disclosure Statement

The authors have declared that no conflict of interests exists.

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Paper IV

Transmission dynamics studies of tuberculosis isolates with whole genome sequencing in a low incidence country

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ABSTRACT

As an airborne infection, tuberculosis (TB) has no boundaries and easily spreads by migration from one region to another. In Sweden, TB is mostly reported in migrant where this fragile group constitutes the major reservoir. In this study, we compared whole genome sequencing (WGS) with standard genotyping methods such as restriction length polymorphism (RFLP), mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) and spoligotyping, on 93 *Mycobacterium tuberculosis* isolates from 2004 to 2014 collected at Scania University Hospital in Southern Sweden. Genome-based transmissions were defined as isolated pairs separated by <12 single nucleotide polymorphisms (SNPs). Comparing all techniques, WGS confirmed all eighteen clusters, although the distribution differed between distinctive technique. WGS overlapped 14 of 20 RFLP determines transmission clusters, 17 of 18 epidemiological determined clusters, 7 of 8 MIRU-VNTR clusters, and 8 of 9 spoligotyped clusters. WGS and RFLP had the best ability to identify unclear outbreaks in the largest cluster, consisting of 11 homeless patients, where the isolates were separated by ≤ 9 SNPs. To summarize, WGS provided better resolution of transmission than standard genotyping methods. We can conclude that is well suited for identifying transmission clusters in settings with low TB incidence.

INTRODUCTION

Tuberculosis (TB) continues to be a major public health issue in European countries with 340 000 new TB cases per year [1]. In Sweden, modest 835 new cases were reported in 2015, most of these immigrants coming from high TB incidence countries [2]. Three genotyping methods are traditionally used for epidemiological contact tracing of TB, i.e. restriction fragment length polymorphism (RFLP), *Mycobacterium tuberculosis* (*M. tuberculosis*) interspersed variable repetitive unit number tandem repeat (MIRU-VNTR), combined with spoligotyping [3-5]. Some of these techniques have limitations. Molecular typing based on RFLP, that relies on the insertion element IS6110 as a probe, is restricted by the existence of *M. tuberculosis* strains with very low number of copies or no copies of IS6116 [6]. MIRU-VNTR may not distinguish between closely related genotypes and may be suboptimal among immigrants from countries with high incidence of TB, where genetically closely related strains circulate over extended periods of time [7, 8]. Genetic mutations in these strains accumulate resulting often to pairwise SNP distance of <12, but the MIRU-VNTR typing pattern may not change and could wrongly interpret as recent transmission in the country of immigration [9].

Whole genome sequencing (WGS) provide increased resolution over VNTR-based clustering and is considered to be superior in defining the extent and direction of tuberculosis transmission [10-15]. Based on these results, WGS is now rapidly becoming the standard for typing *M. tuberculosis* isolates. However, most of these studies are from high TB incidence settings, while less is known about this technique in low TB incidence countries. Recently, WGS analysis on isoniazid-resistant outbreak in London showed that this technique may not be useful in identifying the direction of transmission as previously reported [16]. Other studies from low TB incidence countries showed that WGS is comparable to MIRU-VNTR in native patients, as opposed to foreign-born patients where standard genotyping overestimates recent TB transmission [14]. In this study, we reanalysed *M. tuberculosis* strains isolated during years 2004-2014 with WGS in order to compare the different genotype techniques in a low TB incidence country.

MATERIAL AND METHODS

Study design and population
We sequenced 100 isolates of *M. tuberculosis* from an archive of more than 800 frozen cultures obtained between 2004 and 2014 that is held at the Regional Mycobacterial Reference Laboratory for Scania County-Sweden. We selected isolates to estimate genomic diversity within and between hosts. TB is a mandatory notifiable to the public health agency of Sweden [2] and relevant diagnostic codes, microbiology results and contact tracing were obtained from Scania university hospital (SUS) in Malmö.

Epidemiologic investigation and Traditional genotyping of bacterial strains
M. tuberculosis isolates were cultured at SUS Malmö. Restriction-fragment–length polymorphism (RFLP) analysis based on insertion sequence 6110 was performed on 77 isolates, and 24-loci MIRU-VNTR combined with spoligotyping analysis was performed on 40 laboratory-confirmed, culture-positive isolates at the Mycobacterial laboratory SUS, Malmö. Clinical, demographic, and microbiological data were available for the isolates. We obtained epidemiological data of interviews with public health teams and supplemented the data by case-record review. Sweden guidelines for contact tracing recommend screening household contacts for every new index case, at-risk individuals and any other pointed contacts in the community when the index case is thought to be infectious.

Bacterial isolates selection and DNA extraction
Retrospectively, all notified TB cases with *M. tuberculosis* culture positive samples were identified. culture positive samples were stored at -80 C. A total of 100 strains were grown on Lowenstein-Jensen medium for up to 4 weeks then approximately single colony was suspended in 0.5x TE buffer, heat inactivated at 80 C for 20 min, sonicated at 35 KHz for 10 min and genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen).

Genomic DNA sequencing and library preparation
Sequencing libraries were prepared from 100 ng DNA using the TruSeq Nano DNA sample preparation kit (cat#FC-121,4001/4002 Illumina inc.) according to the manufacturer instructions guide (#15041110) HiSeq,2500 ,125 bp paired end by SNP&SEQ Technology Platform at Uppsala University. 7 isolates were excluded due to low genomic DNA quantity and quality and the remaining 93 samples were queued for sequencing on 1 lane PE125 and generated 236 M read pair. Raw reads were Archived under accession number (to be determined).

Identification, Annotation, and Confirmation of SNPs
Reads were mapped to the reference genome CDC1551 (NCBI accession GCA_000008585.1_ASM858v1). The median depth of sequencing was (124.7 x), with an average of 99.55% of the reference genome being covered by at least one read after quality control and trimming of reads. The program BWA mem (http://bio-

bwa.sourceforge.net/) [17, 18], version 0.7.13, was used to map the trimmed reads to the reference genome followed by variance calling. Variants were called using freebayes (https://github.com/ekg/freebayes), version 1.0.2. A coverage of at least 8, an alternate allele count of at least two, and an alternate fraction of at least 20% was required evaluate a position. We only retained positions with phred-scaled quality score of ≥ Q20 using vcftools (https://vcftools.github.io/index.html) [19]. The codon sequences for all single nucleotide polymorphisms (SNPs) positions are extracted using information from annotation with SnpEff[20]. The resulting lists of SNPs were merged into a vcf-like file using a custom script, and the variants were then combined with the variants from the 93 samples (unannotated, normalized with vt normalize) using GATK CombineVariants. The resulting vcf file was annotated with SnpEff as previously described, and aligned snp and codon sequences were extracted using custom scripts (Supplementary Figure 1).

Phylogenetic analyses and strain diversity
Phylogenetic analysis of the single nucleotide polymorphisms (SNPs) was conducted for both the entire 93 set of isolates as well as for four additional genomes /assemblies (CCDC5180, EAI/OSDD271, Haarlem and CTRI-2) that represent modern *M. tuberculosis* lineages (Lineage 2,3,4,4 respectively) and were aligned to the reference genome Using the Maximum Likelihood method. SNPs located within 12 bp of each other, SNPs in PE/PPE/PGRS genes phage, repeat and transposons were excluded to avoid any concern about errors in the read alignment in those repetitive regions of the genome [21]. Furthermore, SNPs in an additional drug-resistance associated genes [22] were also removed to exclude the possibility that homoplasy of drug resistance mutations would significantly decrease the reliability of phylogeny [23] [24]. As well as, ambiguous positions were excluded and then the concatenated alignment was used to generate a midpoint rooted phylogenetic tree in RAxML version (7.3.4). under GTRCAT substitution model with 100 bootstrap replicates.

Genomic cluster analysis
Genomic clusters were ascertained independently of the epidemiological data and were defined where no more than 12 SNPs separated a patient isolate from that of at least one other patient in the cluster. Twelve SNPs were the previously defined upper threshold of genomic relatedness noted within hosts and between related hosts [11].

Statistical analysis
The linear-by-linear (LBL) association test, with each year treated independently, was used to analyse trends over time. Bonferroni correction for multiple comparisons was performed when necessary. Proportions were compared using the 2-tailed Fisher's exact test (FET) and continuous parameters between groups were compared using the Mann–Whitney *U* test (M-W). Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp., Armonk, NY).

Ethical approval

The research was approved by the Medical Ethics Committee at the Lund University, Lund, Sweden (Dnr. 2014/2).

RESULTS

Study selection

The study population consisted of 801 *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates from eligible TB patients treated between 2004 and 2014 at Scania University Hospital (SUS) located in southern Sweden. The remaining 701 cases during the study period were excluded since they were culture negative, have either unique TB strains or are linked in smaller clusters with cases outside Scania county. Of the remaining 100 patient *M. tuberculosis* isolates, we excluded seven due to low DNA quantity and quality (Figure 1).

Clinical characteristics

The majority of the patients were adults at time of diagnosis (median age, 39 years) presenting with pulmonary tuberculosis (76 %) or extra pulmonary TB (20%) and both pulmonary and extra pulmonary TB (4%) (Table 1). The age range was 1 to 91 years, of which two patients were infants. None of the patients were positive for the human immunodeficiency virus (HIV). Outcomes were recorded through December 2016, with a minimum of 12 months of follow-up for all patients. The majority of patients had favourable outcomes, with 84 patients (91%) cure or treatment completed. Five patient (5%) did not complete treatment, and one patient (1 %) had a relapse of pulmonary tuberculosis. Three TB related deaths (3% of patients) were recorded during the follow-up period.

Transmission clusters

WGS was performed on 93 *M. tuberculosis* isolates routinely analysed by the public health agency of Sweden by RFLP, MIRU-VNTR and spoligotyping. Using the maximum of twelve SNPs as the upper threshold of genomic relatedness noted within hosts [11], we identified 22 cluster comprising 61 patients with pairwise genetic maximum distances of 0 to 9 SNPs (median, 2.5) (Figure 1 and Supplemented Figure 2). 5 clusters comprised of more than 3 patients while the remaining 17 cluster comprised of less than two patients. These 22 transmission clusters included 61/93 patients, corresponding to a clustering proportion of 64 %.

None of the clusters involved only Swedish-born patients, but 13 of the clusters involved only foreign-born patients and 9 clusters contained patients of mixed origin. The largest cluster comprised 11 patients, of which 10 were Swedish-born and one was foreign-born. 9 of these patients belonged to a community of homeless people at the time of the diagnosis. The mean length of time between the first and last isolate in this cluster was 20 months (range 1–112 days).

The phylogenetic tree revealed that four of the seven main global lineages of *M. tuberculosis* circulated in Scania county during the time of sampling [36,49–51]. The vast

majority of isolates belonged to lineage 4 (63%) and 30 % belonged to lineage 2, with lesser representation from lineage 3.

Correlation with epidemiologic investigation

Epidemiological contact tracing complemented with *M. tuberculosis* genotyping is considered to be important for understanding transmissions [16]. In the previously defined clusters linked by RFLP, MIRU-VNTR, spoligotyping and epidemiological analysis, we found pairwise genetic SNPs distances of 0 to 5502. The number of sub-clusters/transmission chains identified by RFLP that overlapped with WGS was 14 of 18 with SNPs difference less than 12 SNPs and (72%) of 93 patients (Table 2). 7 of 8 MIRU-VNTR typing clusters were confirmed as true transmission clusters and consisted of SNP pairs separated by less than 12 (46 %) of 93 patients and the epidemiologically linked cases with max pairwise genetic distance 9 SNPs comprise 74 % of the 93.

Geographic and genetic distances within molecular clusters

Of the 2/47 (4%) epidemiologically linked patients, 2/35 (6%) of the MIRU-VNTR linked patients and 15/58 (26%) of the RFLP clustered patients were separated by more than 12 SNPs. In contrast, 16/46 (35%) of the epidemiologically unlinked patients were separated by less than 12 SNPs.

The ability of genomic clustering to identify unclear outbreaks was most evident in cluster one, in which 9 SNPs or fewer separated the 11 patients with a background of homelessness for whom contact tracing had been difficult. The ability to rule out transmission was particularly evident in one RFLP cluster (represented by SB2, SB11, SC11 and SD3), where more than 12 SNPs separated individuals from a recent immigrant from Somalia. In this cluster, isolates from 4 smear negative patients, with no known epidemiological links, could also not be genetically linked by 12 or fewer SNPs.

DISCUSSION

Since the 70ties, Sweden has been a low TB incidence country with 6,1 cases per 100.000 individuals, with approximately 90% of all new cases coming from countries with high TB burden [2]. The SUS hospital in Scania, which is Sweden's third largest uptake area of new TB cases, enrolled 93 previously genotypically analysed by standard techniques patients into this study. Compared to the standard genotyping, WGS had an overall high match in identifying cluster transmissions in this patient population. When comparing the different techniques individually, WGS and epidemiological data had the highest cluster similarity, while MIRU-VNTR had less cluster transmission. This is in contrast to Stucki et al. that reported higher cluster typing with MIRU-VNTR than with WGS, stating that MIRU-VNTR generally overestimates transmission of *M. tuberculosis* in countries with a low incidence of TB [25]. These results were further supported by the study of Wampade et al. stating that MIRU-VNTR typing may be suboptimal among immigrants from countries with high incidence of TB, where genetically closely related strains circulate over extended periods of time [8]. Our results are so far biased as we lack MIRU-VNTR data from all patients. We are currently analysing all strains to complete the study.

Advances in next generation sequencing technology have provided a whole new chapter in the informative epidemiology and WGS is now a verified technique for investigation of various aspect of TB [26, 27]. The upper threshold of 12-SNP distance to identify transmission was proposed by Walker et al. based on studies in two low incidence TB settings, but these limits has been used in other studies as well [11, 28-30]. Walkers studies furthermore state that isolates separated by five or fewer SNPs are likely a result of recent transmission. WGS was initially shown to provide increased resolution over MIRU-VNTR-based clustering [28], but this technique was recently proven to be insufficient in fully resolving the chains of transmission [16]. An explanation could be that multiple transmissions can occur with no detectable SNP acquisition, and even identical isolate pairs cannot be proven to have resulted from a recent transmission even without supporting epidemiological evidence. WGS was recently recommended in identifying transmission clusters in low TB settings [25]. In good agreement, we observed high correlation in cluster proportion between standard genotyping techniques and WGS. To optimize this method, adequate cluster definition should be adapted according to setting (i.e. low versus high TB incidence), the study population, and the technical specifications of the WGS analysis pipeline (i.e. whether the GC-repetitive sequence, constituting almost 10% of the *M. tuberculosis* genome, was assessed). Stricter definition of transmission clusters and inclusion of additional patient isolates with intermediate genotypes further increased the proportion of true clusters [25, 28].

Another limitation of our study is that we determined the sequence of the patient's dominant genotype, but this approach is recommended as isolating single colonies prior to sequencing is likely lead to overestimation of the SNPs between cases resulting from direct transmission [16]. Compared to other studies, we analysed four of the modern *M. tuberculosis* strains in order to reveal geographic distribution (Comas I). Different *M. tuberculosis* strain lineages have also been associated with variable virulence,

transmissibility, disease phenotypes and drug resistance profiles [31]. Interestingly, we found that vast majority of isolates belonged to lineage 4, suggesting American/European/Middle Easter origin [27].

Level of cluster diversity, 0.8 SNPs per case (270 SNPs/344 isolates) in Casalis study is lower than found in other studies involving large community clusters such as a cluster in Hamburg (1.0 SNPs per case; 86 SNPs/85 isolates) [12, 16], a cluster in Toronto (1.5 SNPs per case; 81 SNPs/55 isolates [32], and a Bernese cluster (2.0 SNPs per case; 133 SNPs/68 isolates) [14]. A lower result could depend on shorter time period two isolates [33]. In an outbreak, lower variation could be due to particular population, i.e. risk factors for transmission and poor adherence to therapy, thus maintaining infectivity and transmission. Alternatively, low variation can also depend to the particular clone.

In contrast to standard genotyping methods, WGS provides better resolution and has been used to study *M. tuberculosis* transmission [34]. We can conclude that is well suited for identifying transmission clusters in settings with low TB incidence. However, without additional epidemiological evidence, centralized routine WGS of TB strains for routine surveillance/outbreak investigation may be misleading and misdirect public health action.

Competing interests
The authors have declared that no conflict of interest exists.

Ethical approval

The study was approved by the Regional Ethical Review Board (Dnr 2014/2), Lund University, Sweden.

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Table 1. Characteristics of 93 Patients with Laboratory-Confirmed Tuberculosis in Scania region-Sweden between 2003 and 2014 overall and comparing clustered vs unclustered; Data are n (%) or mean ± Standard deviation

Characteristics	All patients (n=93)	Clustered (n=61)	Unclustered (n=32)
Age–(years)			
Mean (± Standard deviation)	40.61(±19.3)	40.24(±18.3)	41.34(±21.1)
Range	1-91	1-91	1-87
Gender			
Male	57 (61)	37 (60)	20 (63)
Female	36 (39)	24 (40)	12 (37)
Place of birth			
Swedish born	28 (30)	18 (30)	10 (31)
Foreign born			
Somalia	17 (18)	8 (13)	9 (28)
Vietnam	8 (9)	4 (6)	4 (13)
Others	40 (43)	31 (51)	9 (28)
Tuberculosis Type			
Pulmonary	71 (76)	49 (80)	22 (69)
Extra pulmonary	19 (20)	10 (17)	9 (28)
Both	3 (4)	2 (3)	1 (3)
Smear positive			
Yes	50 (54)	36 (59)	14 (44)
No	43 (46)	25 (41)	18 (56)
Treatment Outcome			
Completed treatment	84 (91)	55 (90)	29 (91)
Incomplete treatment	5 (5)	3 (5)	2 (6)
TB-related Death	3 (3)	2 (3)	1 (4)
Relapse	1 (1)	1 (2)	0
Risk Factors			
History of TB exposure	4 (4)	2 (3)	2 (6)
Immunosuppressive Rx	4 (4)	2 (3)	2 (6)
Homelessness	10 (11)	10 (16)	0
Newly arrived Immigrants	10 (11)	2 (3)	8 (25)

Table 2. Confirmed traditional clustering compared to WGS

Molecular method	Proportion of the number of clusters vs genomic clusters	Proportion of clustered patient's vs genomic clustered patients
Epidemiologically linked-contact based	77%	74%
RFLP -based	64%	72%
MIRU-VNTR typing	32%	46%
Spoligotyping	36%	54%

Figure legends

Figure 1. Flow chart of sample collection. Graph showing the number of isolates/sequences included in the study.

Figure 2. Maximum likelihood tree of 93 *M. tuberculosis* isolates and 5 reference genomes that represents modern circulating *M. tuberculosis* lineages (2-4) from 5502 single nucleotide polymorphisms. Orange label for clusters with 3 or more isolates, and blue label for clusters with only 2 isolates.

Supplementary Figure 1. Mycobacterium tuberculosis isolates quality control, reference genome coverage and the number of variants.

Figure 1. WGS flow chart

SG2_2012
SF1_2011
SE9_2012
S3433_2013
SD9_2009
SB8_2007
SF2_2011
SD6_2009
SE3_2010
SE4_2010
SA4_2004
S2280_2005
SB5_2008
SC10_2008
SG11_2013
SB1_2005
SB3_2005
SE12_2011
SG9_2013
S2808_2006
S1497_2006
SF3_2011
S114_2006
SC5_2009
SC9_2008
SC8_2008
SF6_2011
SD10_2009
SA9_2005
SE2_2011
SF7_2013
SG5_2013
SF11_2012
S831_2004
SA7_2004
SF5_2011
SC12_2008
SF10_2012
SE5_2010
SG7_2013
CTRI-2_L4
SB9_2007
SG3_2012
SB6_2008
Haarlem_L4
S3394_2005
SA1_2004
SF8_2013
SF12_2012
S613_2013
SE11_2011
SG12_2013
SC4_2009
S845_2006
CDC1551_L4
SD8_2009
SD5_2009
SC3_2010
SD12_2011
SB7_2008
S4439_2005
SB11_2007
SC11_2008
SB2_2005
SD3_2010
SE8_2010
SG8_2013
SA5_2004
SA11_2005
S2563_2005
S3923_2005
SA6_2005
SC2_2006
SD4_2009
SB12_2006
SA12_2004
SA8_2005
SD11_2009
SD2_2010
SD1_2011
SE1_2011
CCDC5180_L2
SG10_2013
SA2_2004
SG1_2012
SE6_2010
SB10_2007
SF9_2013
SA10_2005
SB4_2004
SE10_2011
SF4_2011
SG6_2013
SC7_2008
SC6_2009
S2655_2005
SA3_2004
EAI/OSDD271_L3

Lineage 4

Lineage 2

Lineage 3

5.0 e-5

Whole genome sequencing of clinical iso Mycobacterium tuberculosis

Quality control and trimming of reads
Appendix 1
Number of reads before and after trimming

Input Read	Both Surviving	
	Pairs	
Sample_114	2181410	2127951 (97.55%)
Sample_1497	2505245	2412907 (96.31%)
Sample_2280	1913995	1854667 (96.90%)
Sample_2563	2179946	2104004 (96.52%)
Sample_2655	1954051	1829233 (93.61%)
Sample_2808	2905012	2766449 (95.23%)
Sample_3394	1651813	1597845 (96.73%)
Sample_3433	2158510	2056277 (95.26%)
Sample_3923	2242485	2164970 (96.54%)
Sample_4439	2418004	2328965 (96.32%)
Sample_613	1899333	1852542 (97.54%)
Sample_831	702111	658378 (93.77%)
Sample_845	2706868	2604424 (96.22%)
Sample_A1	2366915	2279202 (96.29%)
Sample_A10	2364349	2306945 (97.57%)
Sample_A11	2772645	2620010 (94.49%)
Sample_A12	2696447	2595227 (96.25%)
Sample_A2	1101180	1054015 (95.72%)
Sample_A3	2155524	2109104 (97.85%)
Sample_A4	1531083	1443653 (94.29%)
Sample_A5	2165690	2089139 (96.47%)
Sample_A6	2224898	2148632 (96.57%)
Sample_A7	2606828	2534806 (97.24%)
Sample_A8	2746513	2668974 (97.18%)
Sample_A9	3362312	3244554 (96.50%)
Sample_B1	2370228	2264789 (95.55%)
Sample_B10	2904605	2791659 (96.11%)
Sample_B11	873452	836459 (95.76%)
Sample_B12	3072121	2961427 (96.40%)
2016-11-22		
Sample_B2	2243809	2175963 (96.98%)

Sample_B3	2481759	2412549 (97.21%)
Sample_B4	1829940	1791710 (97.91%)
Sample_B5	2512137	2451193 (97.57%)
Sample_B6	1630966	1542942 (94.60%)
Sample_B7	2732943	2640014 (96.60%)
Sample_B8	2327972	2246961 (96.52%)
Sample_B9	7011333	6778915 (96.69%)
Sample_C10	2148582	2088744 (97.22%)
Sample_C11	2016987	1950216 (96.69%)
Sample_C12	2275195	2228643 (97.95%)
Sample_C2	872165	791438 (90.74%)
Sample_C3	1198936	1151997 (96.08%)
Sample_C4	1817857	1751123 (96.33%)
Sample_C5	2728372	2611044 (95.70%)
Sample_C6	3160285	3074073 (97.27%)
Sample_C7	2866688	2767981 (96.56%)
Sample_C8	2854942	2760484 (96.69%)
Sample_C9	2463299	2387527 (96.92%)
Sample_D1	3364205	3231236 (96.05%)
Sample_D10	692759	563413 (81.33%)
Sample_D11	1105724	1057433 (95.63%)
Sample_D12	1395265	1322549 (94.79%)
Sample_D2	1698256	1593068 (93.81%)
Sample_D3	2497586	2378038 (95.21%)
Sample_D4	1980505	1914112 (96.65%)
Sample_D5	2105810	2055568 (97.61%)
Sample_D6	3427420	3280504 (95.71%)
Sample_D8	1605917	1530850 (95.33%)
Sample_D9	979078	880723 (89.95%)
Sample_E1	2243764	2177348 (97.04%)
Sample_E10	2263212	2189214 (96.73%)
Sample_E11	2551312	2491442 (97.65%)
Sample_E12	2628176	2568150 (97.72%)
Sample_E2	2693146	2586848 (96.05%)
Sample_E3	2813002	2697096 (95.88%)
Sample_E4	2525548	2461435 (97.46%)
Sample_E5	1136501	1067988 (93.97%)
Sample_E6	1417208	1375137 (97.03%)
Sample_E8	694573	639577 (92.08%)
Sample_E9	2562196	2478347 (96.73%)
Sample_F1	1992170	1915477 (96.15%)
Sample_F10	3774372	3635931 (96.33%)

Sample_F11	3764438	3624095 (96.27%)
Sample_F12	2941065	2864424 (97.39%)
Sample_F2	2679214	2582548 (96.39%)
Sample_F3	2055122	2004912 (97.56%)
Sample_F4	2014817	1964483 (97.50%)
Sample_F5	2348919	2290121 (97.50%)
Sample_F6	1941779	1890684 (97.37%)
Sample_F7	1983946	1942291 (97.90%)
Sample_F8	2782413	2667917 (95.89%)
Sample_F9	3638979	3535191 (97.15%)
Sample_G1	2078849	2029089 (97.61%)
Sample_G10	3059523	2978497 (97.35%)
Sample_G11	2536805	2449083 (96.54%)
Sample_G12	4115903	3949050 (95.95%)
Sample_G2	2307566	2220166 (96.21%)
Sample_G3	2285414	2220520 (97.16%)
Sample_G5	2440903	2369001 (97.05%)
Sample_G6	2147561	2090270 (97.33%)
Sample_G7	2948362	2850573 (96.68%)
Sample_G8	2116409	2066124 (97.62%)
Sample_G9	2535442	2440159 (96.24%)

Appendix 2
Fractions of reads that could be mapped to reference, predicted fraction of reads that are duplicates, and mean coverage after mapping

	Percent mapped	Percent duplicated	Mean coverage
Sample_114	99,65	0,8138	118,97
Sample_1497	99,66	0,9127	134,56
Sample_2280	99,71	0,6703	103,53
Sample_2563	99,42	0,9616	116,83
Sample_2655	99,20	0,9259	100,64
Sample_2808	99,53	1,4429	153,95
Sample_3394	99,71	0,6453	88,92
Sample_3433	99,69	0,747	114,64
Sample_3923	99,43	0,8662	120,20
Sample_4439	99,42	1,0804	129,29
Sample_613	99,62	0,7478	103,40
Sample_831	99,38	1,0059	36,54
Sample_845	99,88	0,8766	145,72
Sample_A1	99,71	1,0181	127,11
Sample_A10	99,44	0,7203	128,36
Sample_A11	99,48	0,9425	145,51
Sample_A12	99,48	0,8292	144,65
Sample_A2	99,36	0,9994	58,44
Sample_A3	99,34	0,8496	117,43
Sample_A4	99,66	0,9956	80,30
Sample_A5	99,36	1,1058	115,73
Sample_A6	99,49	0,7102	119,59
Sample_A7	99,61	0,7377	141,44
Sample_A8	99,45	0,9242	148,33
Sample_A9	99,72	0,9884	180,73
Sample_B1	99,68	0,7382	126,01
Sample_B10	99,34	0,9431	154,80
Sample_B11	99,06	0,8478	46,23
Sample_B12	99,50	0,87	164,93
Sample_B2	99,46	0,7952	120,98
Sample_B3	99,72	0,705	134,77
Sample_B4	99,47	0,6435	99,88
Sample_B5	99,55	0,7678	136,85
Sample_B6	99,55	1,1348	85,65
Sample_B7	99,64	0,7971	147,04

Sample_B8	99,67	0,9745	125,22
Sample_B9	99,72	1,1837	377,83
Sample_C10	99,57	0,6909	116,30
Sample_C11	99,49	0,656	108,34
Sample_C12	99,77	0,6255	124,68
Sample_C2	99,23	1,0292	43,66
Sample_C3	99,60	0,9954	64,23
Sample_C4	99,79	0,9646	97,74
Sample_C5	99,63	0,8996	145,56
Sample_C6	99,36	1,0433	170,98
Sample_C7	99,26	0,9745	153,33
Sample_C8	99,61	0,8529	153,61
Sample_C9	99,62	0,658	133,15
Sample_D1	99,47	1,2923	179,50
Sample_D10	99,11	0,9857	31,11
Sample_D11	99,29	1,0128	58,38
Sample_D12	99,51	0,995	73,18
Sample_D2	99,36	1,1078	88,15
Sample_D3	99,40	1,0821	132,24
Sample_D4	99,42	1,0218	106,04
Sample_D5	99,59	0,8914	114,70
Sample_D6	99,50	0,9603	182,49
Sample_D8	99,83	1,1129	85,18
Sample_D9	99,57	1,0237	48,70
Sample_E1	99,51	0,8902	121,37
Sample_E10	99,44	0,6842	121,72
Sample_E11	99,65	0,6554	139,11
Sample_E12	99,56	0,7709	143,24
Sample_E2	99,68	0,9687	144,16
Sample_E3	99,68	0,9861	150,44
Sample_E4	99,71	0,9016	137,45
Sample_E5	99,61	0,9354	59,38
Sample_E6	99,31	1,0568	76,10
Sample_E8	99,33	0,9209	35,47
Sample_E9	99,71	0,7305	138,07
Sample_F1	99,71	0,6687	106,93
Sample_F10	99,73	1,007	202,56
Sample_F11	99,60	0,9879	201,61
Sample_F12	99,64	0,74	159,84
Sample_F2	99,72	1,22	144,42
Sample_F3	99,69	0,7433	112,03
Sample_F4	99,43	0,6842	109,30

Sample_F5	99,76	0,7077	128,08
Sample_F6	99,62	0,6812	105,38
Sample_F7	99,71	0,6415	108,55
Sample_F8	99,64	0,779	148,72
Sample_F9	99,44	1,1974	196,76
Sample_G1	99,45	0,7523	113,00
Sample_G10	99,47	0,8357	165,94
Sample_G11	99,64	0,7061	136,26
Sample_G12	99,57	1,2132	219,69
Sample_G2	99,69	0,7972	123,97
Sample_G3	99,72	0,935	123,93
Sample_G5	99,59	0,6809	132,23
Sample_G6	99,37	0,7145	116,18
Sample_G7	99,71	0,962	158,82
Sample_G8	99,47	0,6977	115,01
Sample_G9	99,58	0,7227	135,84

Appendix 3
Number of variants predicted from individual samples

	Total number of variants	snp	ins	del	mnp	complex
Sample_114	1055	852	95	54	21	33
Sample_1497	1056	838	104	48	27	39
Sample_2280	1094	868	114	47	25	40
Sample_2563	1555	1274	133	69	32	47
Sample_2655	2292	1972	146	81	34	59
Sample_2808	978	788	95	43	21	31
Sample_3394	913	685	113	45	29	41
Sample_3433	1072	844	114	53	24	37
Sample_3923	1550	1283	129	71	24	43
Sample_4439	1062	833	111	52	29	37
Sample_613	833	631	98	39	26	39
Sample_831	965	786	94	41	19	25
Sample_845	434	265	94	27	22	26
Sample_A1	878	667	105	40	30	36
Sample_A10	1589	1264	142	82	41	60
Sample_A11	1640	1333	142	76	30	59
Sample_A12	1593	1299	136	79	29	50
Sample_A2	1481	1200	123	74	31	53
Sample_A3	2204	1872	154	82	34	62
Sample_A4	1133	890	124	56	25	38
Sample_A5	1518	1257	124	69	26	42
Sample_A6	1637	1327	144	81	29	56
Sample_A7	1176	924	122	60	29	41
Sample_A8	1576	1290	133	76	31	46
Sample_A9	1100	870	111	53	28	38
Sample_B1	1146	900	118	62	26	40
Sample_B10	1503	1214	135	74	32	48
Sample_B11	1536	1265	133	69	28	41
Sample_B12	1596	1303	132	79	31	51
Sample_B2	1602	1305	135	77	30	55
Sample_B3	1131	892	117	60	27	35
Sample_B4	1498	1225	130	70	32	41
Sample_B5	1099	865	118	51	28	37
Sample_B6	825	632	96	38	28	31
Sample_B7	1114	860	118	55	34	47
Sample_B8	1119	878	122	57	24	38
Sample_B9	1264	981	129	62	38	54
Sample_C10	1124	883	120	52	27	42
Sample_C11	1599	1306	138	76	29	50
Sample_C12	1138	883	119	58	29	49

Sample_C2	1470	1213	120	76	24	37
Sample_C3	1017	811	96	47	28	35
Sample_C4	438	267	93	28	23	27
Sample_C5	1156	921	119	48	29	39
Sample_C6	2258	1931	148	84	33	62
Sample_C7	2246	1897	155	95	36	63
Sample_C8	1141	912	114	49	28	38
Sample_C9	1150	914	118	50	29	39
Sample_D1	1564	1262	137	77	34	54
Sample_D10	1008	806	102	49	19	32
Sample_D11	1496	1234	125	69	24	44
Sample_D12	1056	830	102	53	30	41
Sample_D2	1555	1283	131	67	28	46
Sample_D3	1598	1310	137	78	29	44
Sample_D4	1585	1303	134	77	29	42
Sample_D5	1093	851	110	54	33	45
Sample_D6	1141	887	126	59	26	43
Sample_D8	405	252	85	24	16	28
Sample_D9	1069	843	117	54	23	32
Sample_E1	1521	1235	132	70	32	52
Sample_E10	2315	1953	163	95	38	66
Sample_E11	899	683	102	42	25	47
Sample_E12	1116	888	116	49	30	33
Sample_E2	1107	878	112	53	29	35
Sample_E3	1129	880	122	58	27	42
Sample_E4	1140	894	123	58	27	38
Sample_E5	1001	794	100	49	21	37
Sample_E6	1518	1226	137	71	33	51
Sample_E8	1466	1216	122	66	25	37
Sample_E9	1098	862	118	55	26	37
Sample_F1	1114	870	124	57	26	37
Sample_F10	1127	882	117	52	29	47
Sample_F11	1177	921	124	58	30	44
Sample_F12	918	693	113	44	25	43
Sample_F2	1088	856	117	54	25	36
Sample_F3	1059	849	105	44	26	35
Sample_F4	2265	1916	159	91	38	61
Sample_F5	1117	875	119	55	27	41
Sample_F6	1092	879	111	46	26	30
Sample_F7	1113	886	116	49	26	36
Sample_F8	890	671	110	42	30	37
Sample_F9	1534	1233	138	75	36	52
Sample_G1	1588	1279	137	80	34	58
Sample_G10	1553	1250	138	74	36	55
Sample_G11	1211	942	127	61	34	47
Sample_G12	907	687	111	42	24	43

Sample_G2	1112	872	120	57	27	36
Sample_G3	836	638	102	37	28	31
Sample_G5	1147	901	121	56	28	41
Sample_G6	2257	1902	154	93	43	65
Sample_G7	1125	888	112	50	33	42
Sample_G8	1577	1292	138	72	30	45
Sample_G9	1174	924	122	53	31	44

Sample	#SNPs Q20	#INDELs Q20	#Private variants Q20	Count Q20	#SNPs Q30	#INDELs Q30	#Private variants Q30	Count Q30
all	12993	1049	n/a	14062	12931	1040	n/a	13986
Sample_114	1024	187	180	13588	1022	186	179	13551
Sample_1497	1008	184	0	13634	1007	184	0	13602
Sample_2280	1023	192	114	13636	1022	192	113	13610
Sample_2563	1505	235	0	13731	1505	235	0	13698
Sample_2655	2199	256	504	13820	2194	256	500	13773
Sample_2808	971	179	1	13560	969	179	1	13523
Sample_3394	822	177	1	13878	822	177	1	13828
Sample_3433	1027	196	1	13629	1027	196	1	13607
Sample_3923	1463	235	0	13672	1463	235	0	13644
Sample_4439	1002	190	185	13887	1000	189	184	13832
Sample_613	764	166	27	13605	764	165	27	13585
Sample_831	1057	196	3	13753	1057	195	3	13707
Sample_845	373	149	42	13764	373	149	42	13730
Sample_A1	800	171	0	13798	800	171	0	13757
Sample_A10	1428	249	49	13767	1427	249	48	13726
Sample_A11	1489	243	0	13719	1489	243	0	13692
Sample_A12	1464	239	1	13758	1464	239	1	13720
Sample_A2	1495	245	106	13843	1491	245	105	13786
Sample_A3	2097	270	430	13680	2095	269	429	13652
Sample_A4	1056	215	14	13874	1056	215	14	13814

Sample								
Sample_A5	1512	235	0	13858	1511	235	0	13800
Sample_A6	1487	240	0	13722	1487	240	0	13692
Sample_A7	1088	201	5	13793	1088	201	5	13757
Sample_A8	1477	234	1	13757	1477	234	1	13722
Sample_A9	1007	190	312	13791	1007	190	312	13753
Sample_B1	1056	203	4	13799	1055	203	3	13763
Sample_B10	1417	235	1	13765	1416	235	0	13718
Sample_B11	1467	232	23	13685	1467	232	23	13648
Sample_B12	1500	245	2	13739	1500	245	2	13709
Sample_B2	1492	237	41	13685	1491	237	40	13654
Sample_B3	1049	196	1	13706	1049	196	1	13679
Sample_B4	1405	225	423	13595	1405	225	423	13563
Sample_B5	1036	197	0	13699	1036	197	0	13670
Sample_B6	790	174	0	13763	788	174	0	13723
Sample_B7	1001	191	60	13900	1000	190	58	13842
Sample_B8	1036	201	1	13838	1034	201	0	13789
Sample_B9	1119	202	242	14025	1119	202	242	13951
Sample_C10	1058	197	3	13747	1058	197	3	13720
Sample_C11	1477	243	19	13768	1477	243	19	13735
Sample_C12	1020	191	0	13741	1019	191	0	13705
Sample_C2	1469	231	2	13749	1466	231	0	13696
Sample_C3	958	178	8	13610	957	178	8	13588
Sample_C4	381	146	39	13799	381	146	39	13753
Sample_C5	1051	185	1	13801	1051	185	1	13762
Sample_C6	2173	268	519	13749	2172	267	518	13715
Sample_C7	2145	282	53	13866	2144	281	53	13811
Sample_C8	1069	194	3	13820	1068	194	2	13787

Sample							
Sample_C9	1059	186	0	13804	1059	185	0	13759
Sample_D1	1466	235	3	13764	1464	235	3	13720
Sample_D10	988	176	205	13618	986	176	202	13585
Sample_D11	1458	225	35	13830	1457	225	35	13776
Sample_D12	988	191	30	13860	985	189	29	13800
Sample_D2	1468	236	45	13855	1468	236	45	13798
Sample_D3	1512	240	28	13883	1511	239	27	13831
Sample_D4	1489	238	3	13843	1488	238	2	13791
Sample_D5	983	187	3	13801	979	185	0	13760
Sample_D6	1038	203	0	13746	1038	203	0	13706
Sample_D8	368	143	107	13881	364	143	104	13820
Sample_D9	1008	198	5	13713	1008	198	5	13675
Sample_E1	1456	231	0	13605	1456	231	0	13580
Sample_E10	2133	279	1	13855	2131	278	1	13808
Sample_E11	800	165	44	13829	798	163	42	13783
Sample_E12	1049	190	4	13770	1046	190	2	13723
Sample_E2	1025	192	37	13828	1023	192	37	13781
Sample_E3	1034	204	0	13728	1034	204	0	13694
Sample_E4	1040	200	3	13852	1039	200	3	13808
Sample_E5	972	190	0	13616	972	190	0	13590
Sample_E6	1425	238	1	13821	1424	238	0	13779
Sample_E8	1458	230	32	13594	1456	230	31	13567
Sample_E9	1028	201	0	13750	1028	201	0	13716
Sample_F1	1031	203	0	13700	1030	203	0	13672
Sample_F10	1031	197	3	13861	1029	197	1	13816
Sample_F11	1054	198	3	13848	1053	198	2	13805
Sample_F12	810	175	205	13753	810	174	205	13721

Sample_F2	1037	197	3	13723	1034	196	2	13683
Sample_F3	999	175	114	13704	998	175	114	13672
Sample_F4	2126	277	2	13834	2122	277	1	13782
Sample_F5	1021	192	4	13835	1020	191	3	13785
Sample_F6	1017	183	3	13703	1013	183	1	13670
Sample_F7	1030	186	43	13701	1030	186	43	13668
Sample_F8	800	172	103	13799	800	172	102	13754
Sample_F9	1406	237	19	13752	1406	237	19	13718
Sample_G1	1457	249	120	13728	1457	249	120	13696
Sample_G10	1449	239	88	13780	1448	239	87	13744
Sample_G11	1068	202	109	13840	1068	202	109	13797
Sample_G12	825	171	280	13830	823	171	278	13794
Sample_G2	1026	195	1	13700	1026	195	1	13672
Sample_G3	801	166	1	13693	801	166	1	13660
Sample_G5	1035	196	0	13749	1033	195	0	13716
Sample_G6	2129	274	63	13716	2129	274	63	13686
Sample_G7	1038	190	214	13673	1036	190	213	13635
Sample_G8	1470	242	37	13740	1470	242	37	13710
Sample_G9	1053	189	0	13741	1053	189	0	13707