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IMMUNOPATHOGENESIS IN PULMONARY TUBERCULOSIS: IMPACT OF IMMUNOMODULATION AND DIABETES CO-MORBIDITY

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Immunopathogenesis in pulmonary tuberculosis: impact of immunomodulation and diabetes co-morbidity
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This thesis is dedicated to the patients who participated in the study

POPULAR SCIENCE SUMMARY OF THE THESIS

Tuberkulos (TB) är en luftburen bakteriell lungsjukdom med ett starkt globalt fäste framför allt i Asien och Afrika. Trots att TB, som orsakas av *Mycobacterium tuberculosis* (Mtb), är en av världens största infektionssjukdomar vet vi förhållandevis lite om infektionsförloppet. Många tror att sjukdomen är på väg att utrotas, men dessvärre tyder statistiken på en ökning av sk. multidrog-resistent TB (MDR-TB). Risken för att utveckla TB ökar betydligt vid en samtidig infektion med HIV men också vid diabetes mellitus (DM). Det är svårt att bota TB på medicinsk väg och befintlig behandling är lång och förknippad med biverkningar, vilket resulterar i en ökning av MDR-TB men också andra former av svårbehandlad sjukdom såsom kavitär TB. Därför är behovet av ny kunskap kring immunsvar och bakteriell patogenes stort för att på sikt skapa basen för nya behandlingsformer som kan stödja befintlig antibiotikabehandling.

Det här avhandlingsarbetet bygger på ett samarbete med forskare i Bangladesh, där förekomsten av TB är vanlig och erfarenheterna av TB är långa, vilket möjliggör både mindre exploratoriska studier och större kliniska prövningar. Delarbete I, syftade till att utvärdera hur daglig behandling med två preparat, fenylbutyrat (PBA) och vitamin D (vit), kan stärka det antimikrobiella immunsvaret i kroppen och bidra till att dämpa patologisk inflammation hos patienter med lung TB. Här hade vi tillgång till provmaterial från TB patienter som ingått i en tidigare klinisk studie. Resultaten tyder på att immunstärkande behandling med PBA och/eller vitD kan minska inflammation och stärka viktiga effektor mekanismer i makrofager, som är den primära immuncellen i lungan som infekteras av Mtb.

Delarbete II, fokuserade på att förstår hur DM typ 2 påverkar sjukdomsutvecklingen och immunsvar vid TB. Här studerades kliniskt och bakteriellt svar hos TB samt TB-DM patienter jämfört med friska kontroller, men också lungröntgenfynd och immunsvar i blod och hostprover vid tiden för diagnos samt vid olika tidpunkter efter påbörjad antibiotikabehandling. Resultaten visar att TB-DM patienter har en fördröjd utläkning av inflammation i den nedre delen av lungan, vilket är associerat med en kvarvarande låggradig inflammation i kroppen. Låga nivåer av ett anti-inflammatoriskt protein, IL-10, är tydlig i lungan och minskat IL-10 korrelerar med förhöjda blodsockernivåer hos TB-DM patienter.

Delarbete III, hade som målsättning att etablera ett experimentellt protokoll för att studera fenotyp och funktion av Mtb-infekterade makrofager med en avancerad teknik för att undersöka celler i vätska med hjälp av laserljus sk. flödescytometri. Resultaten visar att man på experimentell väg kan polarisera makrofager från monocyter i humant blod och noga studera hur olika makrofag populationer påverkas av Mtb infektion.

ABSTRACT

Even in the 21st century, tuberculosis (TB) remains a major global health threat, primarily due to the emergence of antibiotic resistance. Presence of co-morbidities such as diabetes mellitus (DM) has worsened the current situation and made it more difficult to treat this deadly disease, especially in resource-poor settings. It is well-known that Mtb (*Mycobacterium tuberculosis*) bacilli can manipulate both innate and adaptive arms of the human immune system, but how Mtb evade host antimicrobial mechanism is not fully understood. Therefore, a deeper understanding of the immunomodulation caused by Mtb, with and without co-existing illnesses, is essential to develop more effective treatment strategies. The work in this thesis was intended to uncover Mtb-mediated immune alterations, particularly in TB-DM disease, and to examine the feasibility of novel host-directed therapy (HDT).

In Paper I, we set out to study the efficacy of HDT using phenylbutyrate (PBA) and vitamin D (vitD) to strengthen host immune defenses upon administration to pulmonary TB patients. In a randomized controlled trial conducted in Bangladesh, we previously reported positive effects on clinical as well as microbiological TB outcomes upon daily PBA and vitD treatment together with standard chemotherapy for 8 weeks. Stored samples obtained from the clinical trial subjects were now used to assess secondary outcomes including cytokine/chemokine secretion by peripheral blood mononuclear cell (PBMC) cultures (Luminex assay), endoplasmic reticulum (ER) stress markers expressed in monocyte-derived-macrophages (MDMs) (quantitative real-time PCR), and activation of LC3-dependent autophagy in Mtb-infected MDMs (confocal microscopy). We observed a marked reduction in the concentration of inflammatory mediators including tumor necrosis factor (TNF)-α, CC motif chemokine ligand (CCL)-11 and CCL5 after 8 weeks of PBA treatment compared to the placebo group. Similarly, vitD treatment effectively reduced CCL11, C-X-C motif chemokine ligand (CXCL)-10 and PDGF concentrations after 8 weeks of treatment. Both PBA- and vitD-treatment contributed to reduced mRNA levels of the ER stress marker, x-box binding protein1spliced (XBP1sp)-l. Autophagy was enhanced in MDMs obtained from all intervention groups after 8 weeks of treatment as compared to placebo. These findings suggested that the improvement of primary outcomes observed in the clinical trial, were associated with reduced inflammation and ER stress and instead enhanced autophagy in Mtb-infected patient cells.

In paper II, we aimed to explore DM-associated immune alterations of clinical, radiological, and immunological outcomes in TB disease using TB and TB-DM study cohorts collected in Bangladesh. Clinical samples from peripheral blood and sputum from patients and controls were analyzed (blood chemistry, Luminex, quantitative real-time PCR) along with clinical data (composite clinical TB score and demographics) and chest radiography (chest X-ray score) before and after 1, 2 and 6 months of standard anti-TB treatment. TB-DM patients were significantly older, had higher body mass index (BMI), were less anemic and from a better socio-economic background compared to TB patients. Intriguingly, clinical TB symptoms and time to bacterial clearance in sputum were similar comparing TB and TB-DM patients. Even so, TB-DM patients had poorly managed glycemic control throughout the study period and

glycemic status was positively associated with BMI. Importantly, the TB-DM cohort showed reduced resolution of inflammation in the middle and lower lung zones compared with TB patients, which was correlated to plasma leptin concentrations at all time points. These changes were associated with upregulated mRNA expression of inflammatory TNF-α and IL-1β in PBMCs as well as higher CD8 mRNA levels but downregulated CD4 and IL-10 transcripts in sputum cells after standard treatment in TB-DM compared to TB patients. Additionally, glycemic status in TB-DM patients was inversely correlated to sputum IL-10 transcript levels observed after start of anti-TB treatment. These results indicate that TB-DM disease is characterized by low-grade inflammation that persist even after completion of successful anti-TB chemotherapy.

In Paper III, we developed a protocol for assessment of M1/M2 polarization of human myeloid-derived cells using 10-color flow cytometry of adherent macrophages infected with green fluorescent protein (GFP)-expressing Mtb. The experimental protocol involved in vitro polarization of MDMs into classically activated (M1) or alternatively activated (M2) macrophages and assessment of phenotype and function before, and 4 to 24 hours after Mtb infection. M1 or M2 cells were successfully differentiated with granulocyte monocyte colony stimulating factor (GM-CSF) or monocyte colony stimulating factor (M-CSF), followed by polarization with interferon (IFN)-γ and lipopolysaccharide (LPS), or interleukin (IL)-4, respectively. This protocol allowed us to polarize and define M1 cells by elevated levels of CD64 and CD86 co-expression, while M2 cells were characterized by a high CD163 and CD200R co-expression. The level of Mtb infection was generally higher in M2 as compared to M1 cells, although the relative increase in infected cells from 4 to 24 hours was higher in M1- compared with M2-polarized cells. Manual gating as well as unsupervised analysis using dimensionality reduction with Uniform Manifold Approximation and Projection (UMAP) and phenograph clustering, showed that Mtb infection altered the expression of M1 and M2 markers after 24 hours and generated clearly separated cell clusters of different sizes. This M1/M2 flow cytometry protocol could be used as a backbone in Mtb-macrophage research and be adopted for special needs including assessment of cells cultured in vitro or obtained ex vivo from clinical patient samples.

LIST OF SCIENTIFIC PAPERS

I. Rekha RS*, **Mily A***, Sultana T, Haq A, Ahmed S, Mostafa Kamal SM, Annemarie van Schadewijk A, Hiemstra PS, Gudmundsson GH, Agerberth B, Raqib R. Immune responses in the treatment of drug-sensitive pulmonary tuberculosis with phenylbutyrate and vitamin D3 as host directed therapy.
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* Equal contribution

II. **Mily A**, Sarker P, Taznin I, Hossain MD, Haq MA, Kamal SMM, Agerberth B, Brighenti S, Raqib R. Slow radiological improvement and persistent low-grade inflammation after chemotherapy in tuberculosis patients with type 2 diabetes.
BMC Infectious Diseases, 2020 Dec 7;20(1):933.

III. **Mily A**, Kalsum S, Loreti MG, Rekha RS, Muvva JR, Lourda M, Brighenti S. Polarization of M1 and M2 Human Monocyte-Derived Cells and Analysis with Flow Cytometry upon Mycobacterium tuberculosis Infection.
Journal of Visualized Experiments, 2020 Sep 18;(163).

ADDITIONAL PUBLICATIONS

1. Akhtar E, **Mily A**, Haq A, Al-Mahmud A, El-Arifeen S, Hel Baqui A, Roth DE, Raqib R. Prenatal high-dose vitamin D3 supplementation has balanced effects on cord blood Th1 and Th2 responses. ***Nutr J.*** 2016 Aug 9;15(1):75. doi: 10.1186/s12937-016-0194-5.

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6. Al-Mamun A, **Mily A**, Sarker P, Tiash S, Navarro A, Akter M, Talukder KA, Islam MF, Agerberth B, Gudmundsson GH, Cravioto A, Raqib R. Treatment with phenylbutyrate in a pre-clinical trial reduces diarrhea due to enteropathogenic Escherichia coli: link to cathelicidin induction. ***Microbes Infect.*** 2013 Nov 15(13):939-50.

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CONTENTS

LIST OF ABBREVIATIONS

ALS	Antibodies in lymphocyte supernatant
AMP	Antimicrobial peptide
AMPK	Adenosine monophosphate kinase
APC	Antigen presenting cell
Arg1	Arginase 1
BMI	Body mass index
BSL-3	Biosafety level 3
CAMP	Cathelicidin antimicrobial peptide
CCL2	CC motif chemokine ligand 2
CCL5	CC motif chemokine ligand 5
CCR7	Chemokine receptor 7
CD	Cluster of differentiation
CFP-10	Culture filtrate protein 10
CFU	Colony forming unit
CR	Complement receptor
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL10	C-X-C motif chemokine ligand 10
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ESAT-6	Early secretory antigenic target 6
ESR	Erythrocyte sedimentation rate
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FCS	Flow cytometry standard
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus 1
FOHM	Folkhälsomyndigheten

FSC	Forward scatter
γδT cell	Gamma delta T cell
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GM-CSF	Granulocyte monocyte colony stimulating factor
Hb	Hemoglobin
HbA1c	Glycosylated hemoglobin
HBD	Human beta defensin
HDACi	Histone deacetylase inhibitor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA-DR	Human leukocyte antigen DR
IDO1	Indoleamine-Pyrrole 2,3-Dioxygenase
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL-1β	Interlukin-1 beta
IL-1RA	Interlukin-1 receptor antagonist
IL-4	Interlukin-4
IL-10	Interlukin-10
IL-12	Interlukin-12
IL-13	Interlukin-13
iNOS	Inducible nitric oxide synthase
IP-10	Inducible protein 10
IRE1	Inositol-requiring enzyme 1
LAM	Lipoarabinomannan
LC3	Light chain 3
LL-37	Human cathelicidin
LPS	Lipopolysaccharide
M-CSF	Monocyte colony stimulating factor
MAIT cell	Mucosal associated invariant T cell
MDM	Monocyte-derived macrophage
MOI	Multiplicity of infection

MMP9	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
Mtb	*Mycobacterium tuberculosis*
NALC	N-acetyl L-cysteine
NK cell	Natural killer cell
NO	Nitric oxide
OADC	Oleic acid, albumin dextrose and catalase
OD	Optical density
PBA	Phenyl butyric acid
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
qPCR	Quantitative polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PI3K	Phosphatidyl inositol-3 kinase
RANTES	Regulated on activation, normal T cell expressed and secreted
RIF	Rifampicin
ROS	Reactive oxygen species
RT	Room temperature
SiRNA	Small interference RNA
SSC	Side scatter
TB	Tuberculosis
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
TNFα	Tumor necrosis factor alfa
TLR2	Toll like receptor 2
UMAP	Uniform Manifold Approximation and Projection
UPR	Unfolded protein response
UV	Ultraviolet

VDR	Vitamin D receptor
VitD	Vitamin D
WHO	World health organization
XBP1sp1	X-box binding protein 1 spliced 1

1 BACKGROUND

1.1 Tuberculosis

Despite being an ancient disease tuberculosis (TB) continues to be one of the world-leading killers among infectious diseases and a serious global health problem. While active TB is an worrying threat, around 20-25% of the global population have latent TB providing an enormous reservoir for potential spread of the disease [1]. TB is multifaceted, and active disease range from local *Mycobacterium tuberculosis* (Mtb) infection in the lung or other organs, to disseminated and advanced disease including severe, irreversible immunopathology. There is an urgent need of diagnostic methods to diagnose active TB and the only available vaccine, BCG, is old and not very effective in providing protection from the disease [2]. Moreover, standard anti-TB treatment includes multiple antibiotics given daily for many months, which reduce the TB cure rates and enhance development and spread of multidrug-resistant (MDR-) TB. In 2019, around 400,000 people have been diagnosed with MDR-TB globally with an estimated increase in 10% compared to the previous year [3]. Overall, a greater understanding of immunopathogenesis in human TB is required to identify novel correlates of immune protection or disease progression that could be used as diagnostic or prognostic biomarkers and to follow vaccine-induced immunity. There is also a need to find alternative treatment strategies e.g., host-directed therapy, novel immunotherapy or similar, to support or modify currently available chemotherapy with antibiotics to combat this deadly disease without enhancing drug resistance.

1.2 *Mycobacterium tuberculosis*

Mtb is the etiologic agent responsible for TB disease in humans with no known natural reservoir other than the human body. It is a rod-shaped, aerobic, and intracellular bacterium with a length of 2-4 μm and 0.2-0.5 μm of width. The complexity of the cell wall structure of Mtb is the primary virulence factor which is unique among other prokaryotes. Its cell wall consists a large amount of complex lipids (about 60%) which makes the bacteria highly resistant to external biochemical insults [4,5]. The low permeability of the Mtb cell wall has been exploited to develop a staining technique known as Ziehl-Neelsen acid-fast stain to visualize the bacteria using a red dye in clinical samples for rapid diagnosis of TB disease [6]. This feature of Mtb also influence the growth rate of the bacteria (12-24 hr doubling time)[7] resulting in slower growth and longer incubation time in cultures (4-8 weeks) of clinical specimens that causes delayed diagnosis. Pathogenic mycobacterial species that cause disease in humans and other animals are closely related and are termed together as the *Mycobacterium tuberculosis* complex (MTBC) [8]. Important members in this group are Mtb, *M. africanum* and *M. canettii* that cause disease in humans [8]. *M. bovis*, is another member which has a wide range of hosts [8]. The only available vaccine against TB is Bacillus Calmette–Guérin (BCG) and was developed from a live attenuated *M. bovis* strain by the two famous French scientists Albert Calmette and

Camille Guérin in 1921 [9,10]. In the environment, more than 100 mycobacterial species have been identified which are largely non-pathogenic. Mitochondrial DNA analysis revealed the evolution of Mtb occurred most likely about 70,000 years ago [11]. According to the genetic diversity, seven major variants of Mtb exists (L1-L7) [12,13]. Additionally, based on the evolutionary pattern, Mtb can be classified into two lineages: ancestral and modern with the presence or absence of Mtb-specific deletions of DNA loci (TbD1) [8]. The ancient lineages (L1, L5, L6, L7) are mostly limited to specific geographic locations [14], while the modern strains (L2, L3, L4) are distributed all over the world [15].

1.3 The human immune system

1.3.1 An overview of innate and adaptive immunity

Human immune system consists of two key components: innate (common/universal) and adaptive (specific) immunity (Fig. 1). The innate immune system acts rapidly and provides first line protection against invading pathogens or any foreign particle, mostly in the skin and mucus membranes [16,17]. Myeloid-derived cells including monocytes/macrophages, dendritic cells, and granulocytes (neutrophils, eosinophils, and basophils) comprise most of the cellular compartment involved in innate immunity [18]. These cells engulf microbes or their products and respond by secreting effector molecules including lysosomal enzymes, cytokines, chemokines, growth factors, and other inflammatory or anti-inflammatory mediators [19,20]. Natural killer (NK) cells also belong to the innate immune system, but these are of lymphoid origin. The major function of both myeloid and lymphoid innate immune cells is to kill extra- or intracellular pathogens or self-altered cells such as cancer cells, via different mechanisms including oxidative stress and antimicrobial peptides (e.g., myeloid cells) [21,22] or granule-mediated exocytosis (e.g., NK cells) [23].

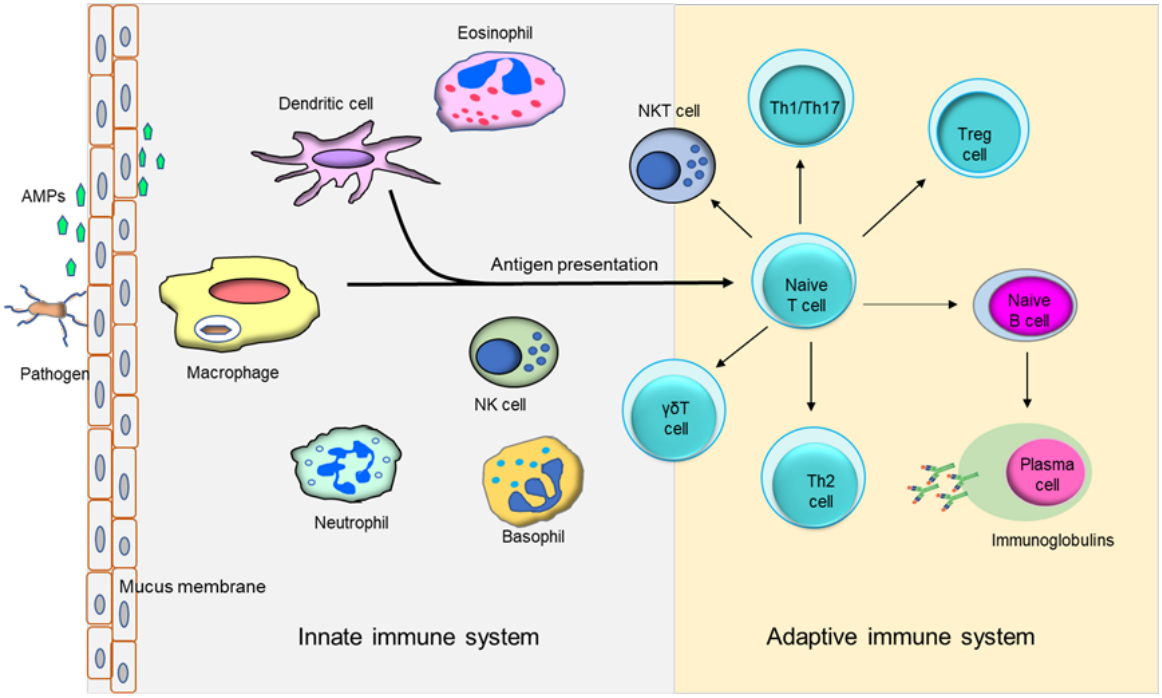

Figure 1: Major cells types involved in the human innate and adaptive immune system.

If the innate immune system fails to eradicate the invading infectious agents, the adaptive immune system is the next line of defence and involves cells with more specific tasks [24,25]. T and B lymphocytes compose the main part of the cellular compartment of our adaptive immunity and are of lymphoid origin. After being produced in the bone marrow, T cells migrate to the thymus for maturation [26]. For antigen recognition, most T cells carry αβ-T cell receptor (TCR) on their surface and form a complex with the CD3 molecule [27]. $CD4^+$ helper T cells (Th) are mostly involved in activation of other immune cells during infection, while cytolytic $CD8^+$ T cells (CTLs) are involved in direct killing of infected host cells [26]. After activation by major histocompatibility complex (MHC) class II molecule on antigen presenting cells (APC), $CD4^+$ T cells divide to generate three different types of effector cells with specific phenotypes and functional characteristics (Fig. 2) [28]. These cell subsets are denoted as Th1, Th2 and Th17 type. Th1 cells provides protection against intracellular pathogens that multiply inside macrophages by secreting interferon gamma (IFN-γ) and tumor necrosis factor (TNF) cytokines [29]. Th2 cells are mostly active against parasitic infections and produce interleukin-4 (IL-4) to stimulate B lymphocytes to secret immunoglobulin E (IgE) to kill the parasite [29]. Th2 cells produce IL-4 and IL-5 cytokines that activate mast cell and eosinophil proliferation and degranulation respectively, and contribute to the development of asthma [29]. Th17 cells secret TNF-α, IL-17 and IL-22 to induce neutrophil-mediated inflammation to fight extracellular bacteria but may also be involved in several autoimmune diseases [30]. Dendritic cells (DCs; professional APC) are essential in triggering naïve T cell responses via production of IL-12, but can also modulate effector T cell responses by secreting anti-inflammatory cytokines such as transforming growth factor β (TGF-β) that give rise to $CD4^+$ regulatory T cells (Treg) [31]. Induced or naturally occurring Treg cells typically express IL-10, TGF-β, cytotoxic T-lymphocyte antigen 4

(CTLA-4), lymphocyte-activation gene 3 (LAG-3) and Glucocorticoid-induced tumor necrosis factor receptor (GITR), and suppress effector T cell responses [32]. Activation of CTL is induced by presentation of MHC class I molecules on the target cells [33]. Activated CTLs produce perforins and granzymes inside cytoplasmic granules and release those in the synapse close to the target cell's surface upon recognition of antigen together with appropriate co-stimulation [33,34]. Activated CTLs and some $CD4^+$ T cells express Fas ligand (FasL) which can bind with Fas (a transmembrane protein that contains a death domain in the cytoplasmic area, essential for apoptosis induction) expressed on the activated lymphocyte surface [35]. Fas-FasL binding activates caspases inside the target cell and initiates programmed cell death (apoptosis) [36,37]. Mutations in Fas or FasL gene could result in excess production of lymphocytes as well as autoimmune disorders and therefore, this mechanism is important to maintain lymphocyte homeostasis [36,38]. Gamma-delta (γδ) T cells use γδTCR for antigen recognition and constitute a major part among intraepithelial lymphocytes in the intestine [39] and exert cytotoxicity by producing perforin and IFNγ[40]. γδT cells are considered as part of both innate and adaptive immune system since they can recognize antigen without MHC recognition mechanism and also can act as APC to prime $CD4^+$ and $CD8^+$ T cell response [41,42].

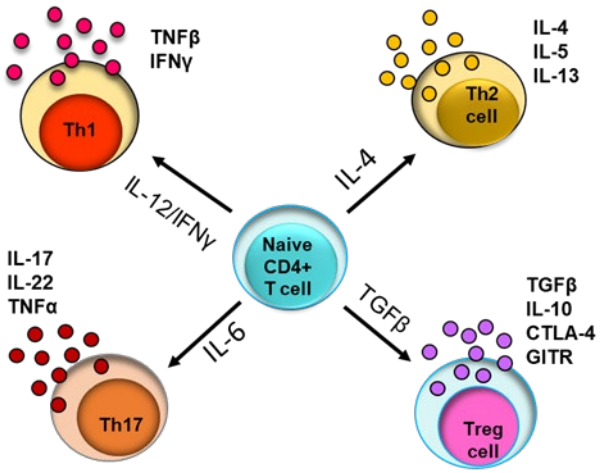

Figure 2: Th1/Th2/Th17/Treg cell induction

B cells are both produced and matured in the bone marrow and ultimately have several functions of adaptive immunity [43], as professional antigen-presenting cells (APCs) [44], cytokine producing cells [45] and foremost cells that can produce specialized molecules immunoglobulins (Ig) which are also known as antibodies [43]. Antibodies are potent soluble mediators to fight extracellular microbes and the most crucial element of humoral immunity being produced by B cells in different stages i.e., short-lived plasmablasts, long-lived plasma cells or circulating memory cells [46]. Immature B cells carry IgD, CD21 and CD22 surface molecules and can produce antibodies in an MHC-restricted T cell independent mode upon LPS activation [47]. During infection, B cells receive activation signals from $CD4^+$ T cells to transform into terminally differentiated plasma cells to produce larger amounts of antibodies to counteract the

intruder. The important function to generate memory after encountering any foreign pathogen for the first time is to produce a fast adaptive response later upon secondary infections.

1.3.2 Macrophages

Macrophages are large phagocytic leukocytes and multifunctional cells derived from the myeloid lineage of hematopoietic cells, which can phagocytise and process many different foreign particles or cellular debris or waste products after receiving signals from internal (cytokines) or external stimuli (microbial cell wall components or other molecules) [48]. Depending on the source of origin, two distinct populations of macrophages have been identified: 1.) a tissue resident population evolved during embryonic development, and 2.) a haematopoietic stem cell-derived population from the bone marrow known as monocytes [49]. In the peripheral blood, approximately 10% of mononuclear cells are monocytes [50].

Macrophages have a key role in lymphocyte activation (APC function and cytokine secretion) [51,52], inflammation (pro-inflammatory cytokines and acute phase response) [51], microbicidal activity (hydrolytic enzymes, reactive nitrogen and oxygen species (RNI and ROS), antimicrobial peptides) [53], tissue reorganization (secretion of proteinases, elastases, collagenases and angiogenesis factors) [54], tumor immunity (secretion of toxic factors, free radicals and hydrolases) and modulation of different responses (secretion of IL-12 for inflammation and IL-10 for anti-inflammatory responses etc.) [53]. Five important cytokines that macrophages produce include TNF-α, IL-1, IL-6, IL-8 and IL-12. These cytokines are involved in initiation of tissue inflammation, increased phagocytosis, and lymphocyte activation, chemoattracts for neutrophils, stimulation of endothelial cells and fibroblasts to form capillaries and connective scar tissue, respectively [54,55]. The pleotropic functions of macrophage are regulated by signals from exogenous stimuli such as bacterial LPS [55,56] as well as signal from endogenous molecules (host defence peptides or cytokines such as IFN-γ or IL-4) [57,58]. These stimulation signals for macrophages modulate the expression of specific genes by epigenetic and or transcriptional reprogramming [50]. A spectrum of phenotypes has been recognized to be associated with the functional ability of macrophages in response to environmental stimuli, a phenomenon also known as macrophage polarization.

1.3.2.1 Macrophage polarization

Macrophage polarization can modulate the expression of specific genes associated with different pathways including lipid mediators, G-protein-coupled receptors, and chemokines at the transcriptional level [59]. Moreover, macrophage polarization involves alteration in metabolic pathways, i.e., glycolysis is the main energy source in M1 cells, while fatty acid β-oxidation and oxidative phosphorylation is more functional in M2 cells, triggering further immunomodulatory consequences [60-64].

Generally, immune polarization of macrophages can be divided into two types: 1.) classically activated M1 macrophages that are inflammatory and induced by LPS and IFN-γ (Th1 cytokines), and 2.) alternatively activated M2 macrophages that are anti-inflammatory and induced by IL-4, IL-13, as well as IL-10 (Th2 cytokines) (Fig. 3). While M1 macrophages are microbicidal and mostly involved in defeating intracellular pathogens [65], M2 macrophages are considered less microbicidal and instead mainly involved in wound healing, tissue repair and/or anti-inflammatory responses [57]. Although this categorization of macrophage polarization is simplified, the M1 and M2 groups could be divided into further subsets depending on the polarizing stimuli and the associated specific phenotypes as well as functions [66]. The M1/M2 polarization states of macrophages are dynamic and could be shifted from one state to the other depending on the stimuli present in the microenvironment after initial polarization [67]. In the steady state, tissue macrophages in the lung and intestine typically exhibit an M2-like polarized state (M0) both phenotypically and functionally, which could shift to M1-type after induction of Th1 cytokines in response to infectious agents [67,68]. The tissue microenvironment could also influence the polarization state of resident macrophages. Tissue macrophages reveal both microbicidal [69] as well as scavenger functions during ex vivo infection [70-72]. Recently, in vitro studies discovered nine distinct categories of macrophage polarization states that were identified through transcriptomic analysis of nearly 300 activation signature genes [73].

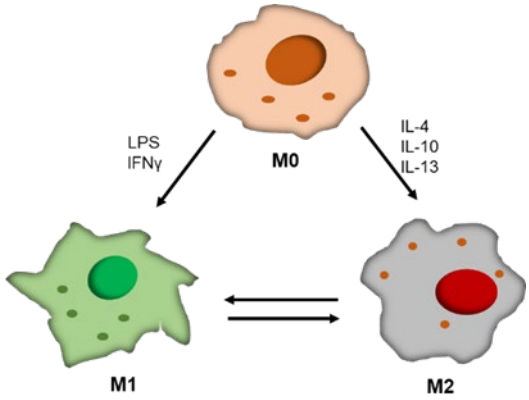

Figure 3: Macrophage polarization and plasticity.

1.3.2.2 Definition of M1/M2 activation

M1 activation of macrophages increases their ability to kill or neutralize invading pathogens and promote antigen presentation to enhance Th1 immune responses responsible for host inflammation [57]. M1 (IFN-γ+LPS induced) cells produce pro-inflammatory cytokines (TNF, IL-1β, IL-6 and IL-12 etc) [57,66] and RNI as well as ROS [67]. Consequently, these oxygen- and nitrogen-derived free-radicals contributes to cell death as well as tissue injury/pathology [51].

Instead, alternatively activated M2 macrophages could be generated by a number of mediators: IL-4, IL-10, TGF-β, immune complexes etc. [57,66]. IL-4 activated M2-derived cells have more diverse forms of activation and functions compared to M1 cells, and give protection from

extracellular parasites as well as viruses [57]. They can act in tissue remodelling, and resolution or modulation of inflammation [51,57,67,71,74]. IL-4-induced M2 cells can produce specific matrix metalloproteinases (MMPs) as well as TGF-β during the tissue-repair process which promotes fibrosis [51,54]. Instead, IL-10-induced M2 cells produce a different group pf MMPs and thus exert modulatory function without causing fibrosis at the site of inflammation [51,54]. IL-10-activated M2 cells are antifibrotic and showed better phagocytic capacity than IL-4 activated M2 cells [75].

1.4 Mtb infection and virulence

Once Mtb enters inside the lung, alveolar macrophages are the main host cell to encounter the bacteria through toll like receptors (TLRs) on their surface (Fig. 4) [76,77]. After recognition, via TLRs, mannose receptors (MR), complement receptors (CR) etc. macrophages engulf Mtb into a cytosolic membranous compartment called the phagosome. Lysosomes fuse to acidify the phagosome (phagolysosome) and lytic enzymes from the lysosome degrade the bacilli [76,78]. Virulent Mtb strains typically inhibit this phagosome-lysosome fusion and thus evade the host killing machinery and persist inside the phagosomal system within macrophages [76,78,79]. A trait of virulent mycobacteria is also their ability to translocate from the phagolysosome into the cytosol of myeloid cells, causing cell death via necrosis [80]. Eventually, Mtb-infected macrophages stick together and fuse to form multinucleated giant cells, which forms the core of TB granulomas that is a well-known histopathological hallmark of TB infection (Fig. 4) [81]. The cell wall structure of Mtb is the primary barrier for the host cell to manage or destroy the bacteria because of its high lipid content. Among several other bacterial virulence factors, lipoarabinomannan (LAM, a cell wall glycolipid), early secreted antigen target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) are the most well-studied and found to be involved in host immune evasion and pathogenesis [82-85].

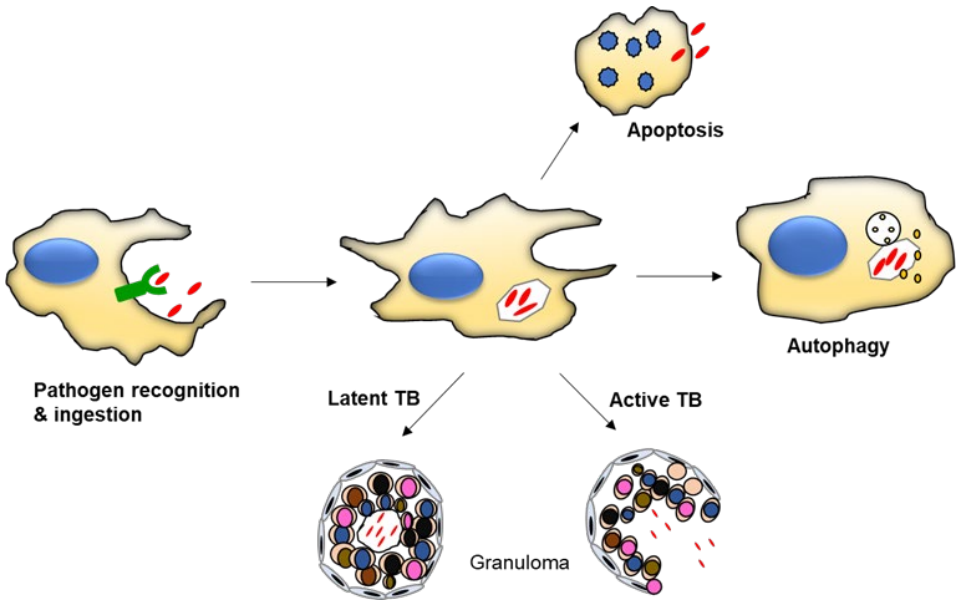

Figure 4: Mtb infection and fate of host macrophages

1.5 Human immune responses in TB infection

Since Mtb is an intracellular pathogen that has developed strategies to survive inside human macrophages, long-term control of TB disease is dependent on cell-mediated responses mainly orchestrated by macrophages, DCs and T cells (Fig. 5). Despite decades of research on human TB, the nature and regulation of protective immune responses remains incomplete [86]. Once Mtb infection is established in tissue macrophages, cells can harbour live bacteria for extended periods of time. The first-line of defence includes production of toxic nitric oxide (NO) via inducible nitric oxide synthase (iNOS) [87] and also antimicrobial peptides, primarily human cathelicidin, LL-37 [88]. LL-37 has an important role in the innate defence mechanism at mucosal surfaces by interacting with the bacteria using ionic strength and killing via osmotic lysis [89,90]. It has been shown that virulent mycobacteria can decrease the production of LL-37 by the infected host cells [91]. Macrophages can also eliminate intracellular bacteria by different cellular mechanisms such as apoptosis [92] or phagocytosis or by activation of autophagy [93]. Th1 cytokines including IFN-γ and TNF-α are mandatory in activation, recruitment and organization of immune cells at the site of Mtb infection, which results in formation of a granuloma [94]. The granuloma is characteristic of human TB and a dynamic structure containing a core of Mtb-infected macrophages surrounded by epithelioid cells (uniquely differentiated macrophages), and multinucleated giant cells (Langerhans cells), B and T lymphocytes, as well as fibroblasts [95,96]. Mtb infection spread through rupture of granulomas into the airways, releasing live mycobacteria that can spread the infection to another host. Persistent Mtb infection can manifest as latent infection or active disease depending on the balance of several host as well as bacterial virulence factors [97]. Recent investigations suggest that IFN-γ producing CD4+ Th1 cells are not sufficient to contain and/or clear Mtb infection [98]. Protective TB immunity may also involve CD8+ CTLs producing antimicrobial effector molecules such as perforin and the antimicrobial peptide, granulysin, that could cooperate in granule-mediated killing of Mtb bacilli and Mtb-infected cells [99]. In addition, non-classical T and NK cell subsets may possess potent protective capacity in TB [98]. Instead, terminally differentiated T cells expressing KLRG-1, PD-1 and IL-27R have been suggested to be pathogenic in TB as these cells are usually associated with reduced proliferative [100] and lung migratory [101] capacities. But also, other inhibitory molecules and immune checkpoint inhibitors may be of potential importance in the modulation of TB immunity, i.e., IDO and LAG-3 [102]. Similarly, an increase of Treg cells [103,104], or regulatory B (Breg) cells but also IgD-CD27- atypical B cells [105] have been observed in TB patients that was normalized after anti-TB treatment.

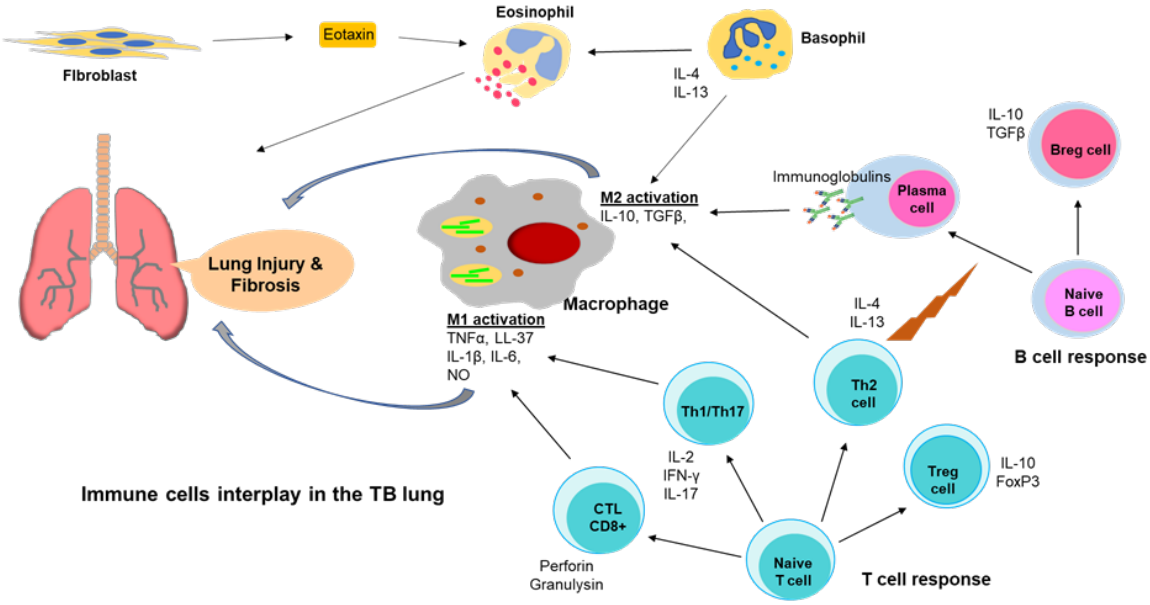

Fig 5: Schematic illustration of cell-mediated immunity in TB infection. Here, two immune cell subsets play a major role in controlling the pathogen: macrophages and T cells. Macrophages can engulf and process the pathogen intracellularly, playing a protective role to limit and confine the infection. In TB disease, a Th1 response characterized by production of IFN-γ and TNF-α to activate CD8+ T cells but also Th17 etc. is crucial to control the pathogen. Instead, a Th2 response characterized by production of anti-inflammatory IL-4 and IL-13 to active antibody-producing B cells, or regulatory IL-10 or TGF-β to activate Treg cells etc. can be less protective and more harmful for the patient. Relatively little is known about B cell mediated immunity against TB, apart from production of antibodies that may neutralize extracellular mycobacteria.

Humoral responses may result from impaired cellular immunity at the local site of infection that contributes to an adverse immune response in chronic TB. Tissue-destruction, necrosis and cavity formation may enhance the release of extracellular Mtb and Mtb products that initiate massive antibody production. Such antibodies may contribute to clearance of extracellular bacteria but will likely have a smaller effect on intracellular Mtb. Accordingly, quantification of antibody-secreting cells in peripheral blood can be used as biomarkers for detection of active and progressive TB disease [106,107]. This novel test, Antibodies in Lymphocyte Supernatants (ALS), was previously developed by our collaborators at the International Center for Diarrheal Disease Research (icddr,b) in Dhaka, Bangladesh and is based on the finding that plasma B cells will only be present in blood from patients with an active TB infection [108], but not in latent TB or healthy controls [106]. Importantly, the ALS is not a serological test, but based on the detection of antibody-producing cells in the circulation. Interestingly, Mtb-specific plasmablast responses decrease after 6 months of successful standard chemotherapy [108], while patients with incurable MDR-TB maintain high IgG titers [107]. We have also found that enhanced B cell responses are associated with low Th1 cell responses and progression of TB [106], which suggest that the ALS represents an adverse immune response resulting from impaired cellular immunity in chronic TB disease.

1.5.1 Autophagy

Autophagy is a major intracellular pathway for lysosomal degradation and turnover of cytoplasmic macromolecules and organelles [109] primarily, in response to starvation [110]. Beclin-1 and microtubule-associated protein 1A/1B-light chain 3 (LC3) are the two mostly studied autophagy related proteins (ATGs) to measure the level of autophagy activation [111]. Autophagy has been implicated in both innate and adaptive immunity [112] since this process can manage and promote killing of several intracellular pathogens including Mtb [113,114]. It has been shown that induction of autophagy by rapamycin in Mtb-infected macrophages can increase the maturation of the autophagosome thereby enhancing intracellular killing of mycobacteria [114]. Similarly, IFN-γ can activate autophagy in Mtb-infected macrophages, which could enhance Mtb killing and protective immunity in TB [115]. Contrary, other reports have shown that Mtb could inhibit TNF-α-induced autophagy in immune cells by blocking of the NF-κB pathway [116,117]. TNFα -mediated activation of autophagy can also be inhibited by the Th2 cytokines, IL-4, IL-10 and IL-13, secreted by Mtb-infected macrophages in an autocrine manner [117,118]. Thus, autophagy could be activated by Th1 cytokines and reduced by Th2 cytokines and promote intracellular killing of Mtb.

1.5.2 ER stress

The endoplasmic reticulum (ER) functions as the major organelle for folding and transportation of proteins after synthesis and is a major storage for intracellular calcium (Ca^{2+}). Mycobacterial infection has been shown to induce ER stress by a Ca^{2+}-mediated pathway, which results in accumulation of unfolded or misfolded proteins in an expanding ER compartment [119,120]. The host cell responds to this ER stress by activation of a pathway that involves removal of unfolded or misfolded proteins from the cytosol [121]. However, constant activation of ER stress triggers downstream pathways that could lead to apoptosis and organ injury [121-123]. Accordingly, among different well-known biomarkers of ER stress, the spliced X-box binding protein-1 (XBP1spl) mRNA has been found to be the most reliable to monitor ER stress response [124]. Mycobacterial secreted antigens such as ESAT-6 has been shown to activate inositol-requiring enzyme 1α (IRE1α) mediated splicing of XBPl mRNA as well as other ER stress genes in epithelial cell lines [123].

1.5.3 Adipokines

Besides the main function of energy storage, adipose tissue produces cytokine-like soluble mediators known as adipokines, which regulate immune cell function especially during infections [125]. Leptin can induce pro-inflammatory cytokine production and thus promotes Th1 response [126]. Conversely, leptin can modulate immune response by inducing IL-4 production [127]. Resistin is another inflammatory adipokine found to be associated with inflammation and has been shown to be eligible as a surrogate biomarker for pulmonary TB disease. Lower levels

of leptin in the plasma have been noted in TB patients than controls [128] and this lower leptin concentration was found to be associated with loss of appetite [129] and increased pulmonary infiltration [130] during TB infection.

1.6 TB and diabetes co-morbidity

While TB is a chronic infectious disease predominantly of the lung, type II Diabetes Mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia due to defects in insulin secretion or function of pancreatic β-cells and insulin resistance in liver, muscle, and adipose tissue [131-134]. Chronic hyperglycemia in DM patients leads to non-enzymatic glycosylation of major serum proteins in the circulation[135] and eventually, measuring glycosylated hemoglobin (HbA1c) levels in the whole blood has been recommended as a diagnostic test to determine long-term glycemic control[136]. DM has been identified as one of the major risk factors for TB disease [137,138] and DM patients pose a 3-fold higher risk of developing active TB infection compared to individuals without DM [137]. It is hypothesised that the immune system of DM-affected individuals become compromised and this situation in turn favours the growth of Mtb inside the host immune cells [139-141]. In contrast, TB disease itself can induce transient DM or impaired glucose tolerance (IGT) in the patients [142,143]. Rifampicin, one of the most important first-line drugs used in anti-TB treatment, has also been known to affect insulin requirement[144] and induce transitional IGT [145]. Conversely, DM may reduce the effect of rifampicin or other anti-TB drugs in TB-DM patients [146]. Therefore, a bi-directional connection exists between TB and DM that is a challenging mystery in TB research [147,148]. In recent times, the convergence of TB and DM has emerged as a serious health threat globally. The countries with a high burden of TB also contain a large population suffering from DM [149]. It has been predicted that by 2030, about 80% of all DM patients will be living in TB endemic countries [149-151]. Without urgent actions to control TB-DM comorbidity, the number of patients with TB-DM dual burden might eventually exceed the number of patients with TB-HIV co-infection around the world[152].

The most important risk factors for development of pulmonary TB among DM patients are decreased body mass index (BMI), poorly managed glycemic status, increased insulin requirements, anemia, higher ESR and higher platelet counts [153]. Epidemiologic studies have revealed delayed treatment response (longer time to sputum conversion) in TB patients with co-existing DM as compared to patients without DM[154]. Lower concentrations of anti-TB drugs in the plasma due to consistently increasing body weight in TB-DM patients at the later phase of treatment, could be responsible for poor treatment outcomes [155]. Impaired absorption of anti-TB drugs as a result of increased glycosylation of plasma proteins [156] or increased free fatty acids in plasma [157] could be the reason for less available drugs in TB-DM patients with poor glycemic control, which could lead to extended therapy or poor treatment outcomes. Increased incidence of drug-resistance in TB-DM patients [158] has also been recognized as important factor for a poor treatment response [159].

1.6.1 Host immunity in TB-DM disease

Clinical progression of TB disease could be synergized by other co-existing illnesses such as HIV infection or DM, but the immunological defects associated with these comorbidities are not fully delineated. Chronic DM typically leads to secondary immunodeficiency disorders by depleting naïve $CD4^+$ T cells and reducing macrophage function, which may lead to an increased incidence and progression of clinical TB disease [139,160,161]. Several studies have shown that DM directly impairs the innate and adaptive immune responses necessary to kill or neutralize intracellular Mtb. Delayed cellular immune responses to Mtb has been reported in DM disease [162], which may result in a persistently higher bacillary burden and more severe disease [159,163]. TB-DM patients may have impaired Th1 immune responses that are crucial to control TB disease [161,164]. Contrary, other studies suggest that progression of TB disease is associated with stronger immune activation and pro-inflammation in chronic DM condition [163,165]. Thus, currently there is no consensus with regards to loss of immune control in TB-DM co-morbidity.

Peripheral blood monocytes from DM patients have been shown to possess reduced capacity to bind or ingest Mtb bacilli due to alterations in the complement opsonisation pathway [141]. It has been reported that DM causes functional changes in macrophages, including reduced phagocytic activity, decreased adhesion and chemotactic activity, skewing towards an anti-inflammatory M2 phenotype, and reduced IL-1β, IL-12p40 and NO production in response to diverse stimuli including LPS and IFN-γ [161,166-168]. IL-12 is known to be associated with autoimmune-mediated β-cell destruction and in the long run, the immunological mechanisms that prevent IL-12-mediated autoimmune reactions in DM patients may simultaneously prevent protective TB immunity by downregulating Th1 responses [169,170]. One recent study revealed that Th1 and Th17 responses are enhanced in TB-DM patients, which may suggest that effector T cell responses [171] and Th1 cytokines [139] contribute to immunopathology in TB-DM. NK cells have also been shown to be increased in both blood and bronchoalveolar lavage from TB-DM patients [172]. In contrast it has been described that CD4+CD25+CD127- Treg cells are expanded in TB-DM patients and selectively accumulate at the site of Mtb infection in the lung [173]. Moreover, diabetic TB patients have been shown to have a lower Th1 to Th2 cytokine ratio in peripheral blood [174] and an impaired Th1 immune response is also evident in Mtb-infected mice with chemically induced chronic DM [163,164]. These conflicting results rationalize more comprehensive analysis of the cellular dynamics and immunopathogenesis in TB-DM disease including aberrant lymphocyte as well as macrophage responses [165,167].

1.7 Conventional anti-TB treatment with antibiotics

Rifampicin and isoniazid are antibiotics that since many years are considered the first-line drugs to be used for treatment of TB disease. Streptomycin, pyrazinamide, and ethambutol are other important anti-TB drugs used in different combinations with rifampicin and isoniazid to treat TB patients. Lately, two new antibiotics, bedaquiline and delamanid, have been found that are used mostly for treatment of MDR-TB. For treatment of drug-susceptible TB, fixed dose

combination (FDC) of four anti-TB drugs: isoniazid, rifampicin, pyrazinamide, and ethambutol are given daily to newly diagnosed patients with pulmonary TB for the first 2 months (intensive phase). Later, a combination of two drugs: rifampicin and isoniazid (2FDC) daily for the next 4 months (continuation phase) are given to treat TB disease [175]. Directly observed therapy short course (DOTS) is a treatment system implemented by the WHO to achieve high cure rates by providing treatment and regular monitoring of treatment results. To date, DOTS is the best curative and cost affordable anti-TB treatment system available for high TB burden countries.

1.7.1 Host-directed therapy as a novel treatment strategy for TB

Despite available antibiotics that are effective against Mtb, drug-resistance is an emerging global problem due to the long, daily treatment with multiple drugs that is associated with poor treatment compliance. To improve treatment of TB, standard chemotherapy could be supported by promising host-directed therapies (HDT) that are designed to boost antimicrobial responses in immune cells to enhance cure, reduce disease severity and side effects, while preventing the emergence of MDR-TB. HDT is a comparatively new concept, aiming to find strategies to enhance immune cell functions that could enhance bacterial control and this way complement drugs with direct antibacterial activities (Fig. 6). Attractive approaches to rejuvenated host immune response in chronic infections such as TB, is based on treatment with immune modulatory compounds. An effective HDT agent, work by modulating several immune pathways involved in bacterial killing or inhibition, and thereby reduce the risk of developing drug resistance, which is a major disadvantage of using antibiotics. Any agent with one single target, will likely promote the development of drug-resistance over time. As such, HDT may not be applicable as a general anti-TB treatment that should be offered to all TB patients, but could be used to for clinical management of more difficult-to-treat cases including MDR-TB and TB-associated comorbidities such as HIV, DM or helminth infections, but also more severe forms of TB such as cavitary forms of pulmonary TB or disseminated or miliary TB [176].

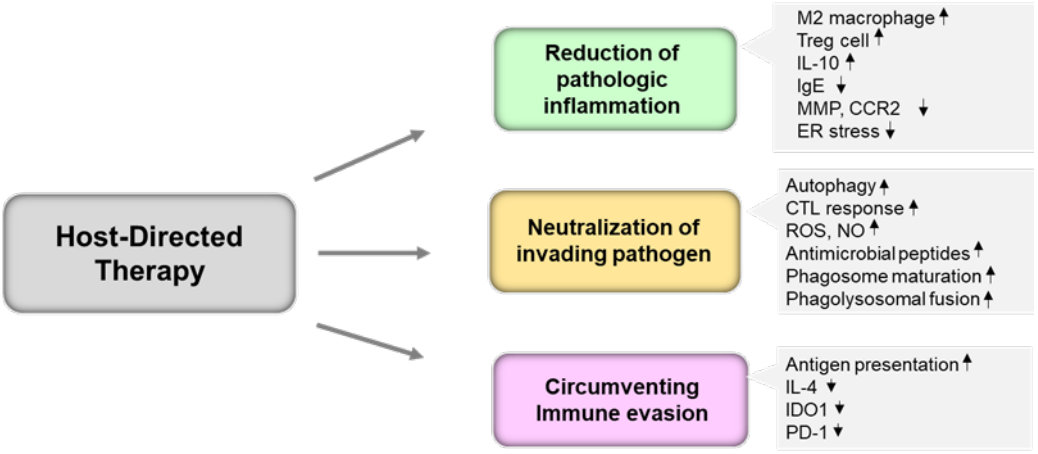

Figure 6: Different approaches of HDT.

Immunomodulatory dietary compounds such as vitamin D_3 (vitD) and the histone deacetylase inhibitor, phenylbutyrate (PBA), are attractive therapeutic candidates with the ability to regulate multiple axes of the immune system including chemotactic, antimicrobial, pro-autophagic and anti-inflammatory pathways. *In vitro*, vitD can enhance macrophage-mediated killing of Mtb by inducing the antimicrobial peptide LL-37 [177] and autophagy [178], while PBA regulates inflammatory pathways in macrophages and is bacteriostatic against Mtb bacilli [179]. The combination of vitD and PBA synergistically promotes LL-37 expression in Mtb-infected macrophages [91] and lung epithelial cells [180]. Presumably, PBA opens the chromatin and facilitates binding of the intracellular vitD transcription factor complex that enhances the transcription of LL-37 and genes associated with autophagy. The effects of these compounds have been mainly studied *in vitro* using macrophage infection models. Thus, the role of vitD and PBA in modulation of T cell responses and their role on resolution of inflammation, needs to be further explored after *in vivo* administration of these compounds to patients. Efficacy of PBA+vitD treatment on killing of drug-susceptible Mtb in macrophages has been demonstrated both *in vitro* [91] and *in vivo* after Mtb infection of monocyte-derived macrophages (MDMs) from healthy volunteers [181]. In addition, PBA+vitD improved clinical and bacteriological outcomes that was investigated in randomized controlled trials in Bangladesh [182] and in Ethiopia [183]. In these clinical trials, adjunct treatment with PBA+vitD was shown to support the standard anti-TB drugs, to reduce clinical symptoms and enhance sputum-culture conversion in patients with pulmonary TB [182,183]. In contrast, several clinical trials using vitD only as adjunctive therapy, failed to show overall effects on clinical symptoms or sputum-culture conversion [184-187]. A recent meta-analysis confirms the modest effects of vitD in treatment of drug-susceptible TB, but suggest a greater effect on sputum-culture conversion of MDR-TB [188]. These findings indicated that PBA could enhance the potential protective effects of vitD *in vivo*. The dosing regimen, e.g., bolus versus daily treatment, could also be of importance to the TB treatment outcome upon adjunct vitD therapy as bolus dosing is rarely effective to enhance vitD levels over time [186]. Importantly, adjunct therapy with vitD is likely only effective in individuals who are vitD deficient ($25(OH)D_3$ <50 nmol/L) or insufficient ($25(OH)D_3$ 50-75 nmol/L) [186]. Accordingly, PBA+vitD treatment has been shown to be particularly effective in TB patients with moderate-to-severe TB symptoms and vitD levels <50 nmol/L [183]. In line with these findings, it has been shown that patients with less severe local lymph node TB have significantly better vitD status as compared to patients with pulmonary or pleural TB [189]. Interestingly, plasma levels of vitD correlated to local mRNA expression of the antimicrobial peptide LL-37 at the site of Mtb infection [189]. The effects observed in our clinical trials could involve intracellular growth inhibition of Mtb in the respiratory tract but may also or instead result from a dampened inflammatory response including diminished ER-stress [190]. This would be consistent with the findings that vitD can mediate direct antimicrobial activity in Mtb-infected macrophages via LL-37 [91,177] and simultaneously down-regulates pro-inflammatory responses at high doses [191,192].

1.7.2 Treatment strategies targeting macrophages

Discovery of macrophage heterogeneity regarding both phenotype and associated functions and the role of these different subsets in human health, has driven researchers to investigate the potential of treatment strategies targeting macrophage activation and polarization dynamics. Numerous pre-clinical and clinical studies have been performed with therapeutic manipulation of anti-inflammatory macrophages to obtain better treatment outcomes. Blocking of M-CSF [193] or siRNA mediated silencing of CCR2 mRNA reduced tissue pathology from infiltrating pro-inflammatory monocytes in model animal[194]. Silencing of HIF-1α showed reduced pulmonary fibrosis in a bleomycin-induced inflammatory condition [195]. Blocking M1 polarization of tissue resident macrophages or infiltrating MDMs could be beneficial in treating certain diseases [68,70,196]. Manipulation and transfer of beneficial macrophage populations could be implemented as a novel therapeutic approach. In a pre-clinical trial, adoptive transfer of IL-4-polarized M2 macrophages has been shown to protect mice in a drug-induced colitis model[197]. Another clinical study demonstrated improved acceptance of renal transplants by applying immune-conditioning therapy to the recipient with donor-derived $CD14^{-/low}HLA\text{-}DR^{+}CD80^{-/low}CD86^{+}CD16^{-}TLR2^{-}CD163^{-/low}$ regulatory macrophages [198]. Inhibition of TNF-α, and IL-1β to block M1 macrophage activation could be utilized for resolution of inflammation mediated tissue damage [54]. Whether similar treatment strategies targeting macrophage polarization could also be an option in TB disease, is yet to be discovered.

1.7.3 Implications of HDT in TB-DM co-morbidity

Antidiabetic drugs have shown promising treatment outcomes by enhancing antimicrobial responses by host immune cells which may be utilized to stimulate HDT in TB patients [199]. One of the frequently used oral anti-DM drugs, metformin (biguanide) has been reported to enhance autophagy and ROS-production in macrophages through the activation of AMP-dependent protein kinase pathway [200]. Since TB-DM patients show delayed clearance of Mtb in the sputum [154], restoring autophagy with repurposed drugs (rapamycin, azithromycin and metformin) might be a good choice to successfully treat co-morbid patients [201]. Induction of IL-10 to promote M2 polarized macrophages in the TB-DM lung could be another effective approach to prevent fibrosis without affecting wound healing as well as antimicrobial activity of these cells [202-204].

2 OBJECTIVES, RESEARCH DESIGN AND METHODS

This chapter will present an overview of the aims, experimental design and research methods utilized in this thesis work. A detailed description of individual experiments is described in the Materials and Methods sections in each of the constituent papers included in this thesis (Paper I-III).

2.1 Objectives

The focus of this thesis was to explore the alterations of immune responses involved in the pathogenesis of human pulmonary TB, with and without co-existing type II DM. An improved understanding of the immunopathogenic mechanisms associated with enhanced pulmonary inflammation in active TB disease, could promote the discovery novel treatment strategies for more efficient control of TB disease in more challenging clinical conditions e.g., TB co-morbidies and drug resistance. As such, we aimed to exploit this new knowledge to design more efficient clinical management of TB disease including novel concepts for HDT, for faster remission of clinical symptoms and reduced risk for development of drug resistance by the pathogen.

My thesis project is basically divided into two parts: 1.) To study immune cell responses to HDT using adjunct treatment with PBA and vitD as a new treatment strategy for TB (Paper I), and 2.) To explore immunomodulation of host immune responses in Mtb infection and how this can affect the progression of clinical TB disease in patients with DM (Paper II and III). To address my aims, I have used two different study cohorts recruited in a high prevalence setting in Bangladesh and blood samples obtained from healthy blood donors in Sweden.

2.2 Patients and clinical samples

2.2.1 Study site

All the TB patients included in Paper I and II were recruited from the outpatient department at the National Institute of Diseases of the Chest and Hospital (NIDCH) and Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM). Both the institutes are located inside Dhaka city. Pulmonary TB patients were recruited from NIDCH and TB patients with known concomitant type II DM were recruited from BIRDEM hospital. A group of age-matched healthy individuals was also recruited from Dhaka to be used as endemic controls. Healthy anonymous blood donors (Paper III) were recruited at the Karolinska University Hospital in Huddinge, Sweden. All the clinical, anthropometric, molecular diagnostic (Xpert MTB/RIF Assay) and radiographic examination (Chest X-ray) of the patients were performed at the respective hospital facilities where the patients were enrolled. A significant portion of the laboratory analyses on clinical samples (complete and differential blood count, blood glucose assessment, glycosylated hemoglobin quantification,

sputum microscopy and culture, Luminex multiplex assay, RNA extraction and cDNA preparation) were performed at icddr,b, Dhaka and also at the Center for Infectious Medicine (CIM), Karolinska Institutet, Sweden.

2.2.2 Study cohorts

This thesis was intended to investigate alterations of immune responses in active pulmonary TB patients with or without DM co-morbidity compared to appropriate control groups and to explore how TB immunity can be modulated and used as new strategies for TB treatment:

- **Paper I:** This was a follow-up study of a randomised double-blinded placebo-controlled trial (ClinTrials.gov ID: NCT01580007). In this secondary analyses, a selected number of sputum-smear positive pulmonary TB patients, age 18-55 years (n=127) were allocated into four treatment arms after they had received adjunct daily therapy with PBA and/or vitD.
  - PBA group: n=32
  - VitD group: n=31
  - PBA+vitD group: n=32
  - Placebo group: n=32

- **Paper II:** This was an exploratory study including sputum-smear positive pulmonary TB patients with or without type II DM and also age- and sex-matched healthy controls.
  - Pulmonary TB patients: n=40
  - TB-DM patients: n=40
  - Healthy controls: n=20

- **Paper III:** Swedish healthy controls (n=6)

In Paper I and II, all the TB patients had a history of clinical TB (typical TB symptoms including persistent cough, fever, chest pain, night sweat, anorexia and weight loss), radiological findings (signs of inflammation consistent with TB) and laboratory diagnosis of active TB (sputum AFB positive or Xpert MTB/RIF positive). At the time of enrolment, all patients were treatment naïve for TB (standard anti-TB chemotherapy) and were treated with anti-TB drugs according to the standard treatment regimen provided by the DOTS Center, and longitudinally followed for up for 6 months. TB-DM patients, with a known history of DM for a maximum of 5 years including a glycosylated hemoglobin value $\geq 6.5\%$ during enrolment was considered eligible for the study.

2.2.3 Clinical samples

After enrolment, the patients were advised to fast overnight (~8 hours) and in the next morning first sputum and venous blood was collected. Blood and sputum samples from the patients were collected at baseline (during enrolment), after 1, 2 and 6 months of anti-TB standard therapy provided by DOTS center in the respective hospitals. Fasting blood from healthy controls were collected only during enrolment. Peripheral blood mononuclear cells (PBMCs) isolated from venous blood samples were stored at -150°C in freezing media or at -80°C in RNA later. PMBCs, PBMC culture supernatant, plasma and sputum samples in RNA later, were stored at -80°C until analysis.

2.3 Laboratory methods

Clinical samples collected from TB patients and controls were analyzed by a number of modern and sophisticated laboratory techniques to study microbiological and immunological responses in human TB disease. In summary, the following techniques were used:

- Xpert MTB/RIF Assay: Paper I and II
- Sputum Acid-fast bacilli (AFB) microscopy and culture: Paper I and II
- PBMC separation and culture: Paper I, II and III
- In vitro differentiation of monocyte-derived macrophages: Paper I and III
- Mtb culture: Paper I and III
- Multiplex Luminex Assay: Paper I and II
- Quantitative real-time PCR (qPCR): Paper I and II
- Flow cytometry (FACS): Paper III
- Immunofluorescence (Confocal microscopy): Paper I and III

All the laboratory work involving virulent Mtb or Mtb-contaminated clinical samples (sputum microscopy and culture, Mtb-infected cell and tissue samples) have been performed at a biosafety level 3 (BSL-3) laboratory at icddr,b (Tuberculosis laboratory) in Bangladesh or at the Public Health Agency of Sweden (Folkhälsomyndigheten, FOHM) in Sweden. After chemical inactivation of Mtb, the samples were transferred to a BSL-2 laboratory for continued analysis. A brief description of the key methodologies is given below.

2.3.1 Xpert MTB/RIF Assay

This is a simple, automated, nucleic acid-based technique widely used nowadays for TB diagnosis and determination of rifampicin resistance. In this assay, a specific nucleotide sequence in Mtb genome is amplified by a real-time PCR method. This DNA sequence contains a Mtb-specific RNA polymerase enzyme active site (rpoB gene) at where presence of a mutation indicates resistance to the first line anti-TB drug rifampicin [205,206]. This region is flanked by a conserved DNA sequence specific for Mtb. Thus, the MTB/RIF assay work to detect the presence of Mtb in the sample as well as the resistance of that Mtb strain to the antibiotic rifampicin.

2.3.2 Sputum Acid-fast bacilli (AFB) microscopy and culture

After collection of morning first sputum from the enrolled pulmonary TB patients, the samples were decontaminated by treating with buffer containing N-Acetyl-L-Cysteine (NALC). The sputum was washed and used for smear preparation on a clean glass slide and heated to fix/inactivate the live Mtb present in the sample before Ziehl-Neelsen staining (using carbol fuchsin and counterstaining with methylene blue). The stained slides were then dried and checked under a light microscope with a layer of immersion oil on the smear. The number of Mtb bacilli present per field were then counted and graded. Part of the decontaminated sputum was inoculated with a sterile loop on Lowenstein-Jensen (LJ) solid media (green color) inside a screwcap vial and the vial was incubated at 37ºC for 4-6 weeks until Mtb colonies were visible (yellowish color). The number of Mtb colonies were also graded in a similar way to the microscopy technique. In Paper II, an equal volume of the collected sputum was stored in RNA later for qPCR analysis before decontamination.

2.3.3 PBMC separation and culture

Within 2 hours after collection, whole blood from TB patients and healthy controls were layered over a density gradient medium and thereafter, plasma and PBMCs were separated by a density gradient centrifugation method. The cells were then either used for culture without any stimulation or stored in RNA later for gene expression analysis. One part of the live PBMCs were stored in liquid nitrogen after mixing with freezing media for future analyses at the single cell level.

2.3.4 In vitro differentiation of monocyte-derived macrophages

Isolated PBMCs from TB patients in Paper I, were used to isolate monocytes using plastic adherence. The adhered monocytes were grown for 3 days in autologous RPMI complete media without addition of any cytokine or stimulant since the patients were already receiving PBA and vitD daily as adjunct therapy. These monocyte-derived cells were later used for Mtb killing experiment ex vivo. PBMC culture supernatants were used for cytokine and chemokine detection. In Paper III, isolated PBMCs from Swedish healthy controls were grown in RPMI complete media and monocytes were separated by the similar plastic adhesion technique. The adhered monocytes were then incubated further and differentiated into inflammatory M1 or anti-inflammatory M2 macrophages in the presence of GM-CSF+LPS+IFNγ (M1 cells) or M-CSF+IL-4 (M2 cells) or M-CSF only (M0 cells). All these in vitro cultured cells were monitored regularly under a light microscope.

2.3.5 Mtb culture

The standard Mtb laboratory strain H37Rv was used in all the infection experiments in Paper I and III. In Paper III, the H37Rv strain was labelled with green fluorescent protein (GFP) and used for in vitro infection experiment to facilitate visualization of intracellular bacteria in flow cytometry and confocal microscopy. H37Rv-GFP ATCC strain was stored at -80ºC in the BSL-

3 laboratory at FOHM. Stored bacteria were thawed and grown in Middle Brook 7H9 liquid media supplemented with 10% Middle Brook OADC (oleic acid, albumin, dextrose, and catalase), 0.5% glycerol and 0.05% Tween-80. After growing the bacterial culture for 10-14 days (at log phase), the optical density was taken at 600 nm wavelength to determine the number of colony forming unit (CFU) before MDMs were infected with the bacteria.

2.3.6 Multiplex Luminex assay

Luminex technology is very sensitive (can detect at picogram levels) and utilizes a bead-based immunoassay by which detection of multiple protein analytes is possible in a small volume of sample. In Paper I, we employed this technique to examine the changes in cytokine and chemokine profiles in the PBMC culture supernatants among the TB patients receiving 4 different adjunctive treatments (PBA and/or vitD or placebo). In Paper II, plasma levels of adipokines were determined with this technique in the peripheral circulation in TB, TB-DM patients, and controls. However, Luminex data was not formally included in Paper II.

2.3.7 Quantitative real-time PCR (qPCR)

To quantify mRNA expression of important host immune molecules in the cells, quantitative the real-time PCR (qRT-PCR) method was utilized. Relative expression of transcripts of cytokines, ER stress markers and other immune molecules' genes were calculated applying the ΔΔCt method [207] using healthy control samples as reference and 18s ribosomal RNA (rRNA) gene as endogenous control. qPCR method was used in Paper I and II to assess different inflammatory and effector molecules at the mRNA level in TB patients both before and after start of standard anti-TB treatment. mRNA was extracted from PBMCs preserved in RNA later. We optimized a protocol for decontamination of frozen sputum samples from TB patients using a phenol-based compound (TRI Reagent) at FOHM before RNA extraction, which kills any mycobacteria potentially present in the sample. All the mRNA samples were checked for the concentration and purity in a spectrophotometer (Nanodrop) and converted into cDNA before performing qPCR. A small portion (~5 ng) of the clinical material was utilized to perform the qPCR.

2.3.8 Flow cytometry (FACS)

Multicolour flow cytometry was used in Paper III, to identify the phenotypic and functional characteristics of in vitro polarized MDMs with or without Mtb infection. Buffy coat blood derived monocytes from Swedish healthy controls were cultured and differentiated into M1 or M2 macrophages. After infecting the cells with virulent GFP-labelled Mtb strain, these cells were stained with fluorochrome-conjugated antibodies for detection of cell surface or intracellular molecules, fixed with 4% formaldehyde solution in phosphate buffered saline (PBS) and analyzed in a flow cytometer (LSR Fortessa, BD) with FACS Diva software for sample acquisition. Flow cytometry is a laser-based technique where any type of cell can be analyzed based on their size and granularity. This technique also allows simultaneous detection

and analysis of multiple markers expressed on the same cell and can be used to detect rare subsets of cells. Here, FACS analysis of MDM samples included a panel of myeloid markers (CD64, CD86, CD163, CD200R, CD206, CD80, CCR7, TLR2 and HLA-DR) that were evaluated using the FlowJo software for Windows (version 10.6.2). M1 polarized cells were defined as CD64+CD86 double positive cells and M2 cells were defined as cells co-expressing CD163+CD200R. For unsupervised analysis of single-cell data acquired from flow cytometry, uniform manifold approximation and projection (UMAP) algorithm was applied for dimensionality reduction and to visualize the data in a two-dimensional space (X-Y plot). Phenograph clustering tool was involved to identify different subpopulations of polarized MDMs with or without Mtb infection.

2.3.9 Immunofluorescence

This technique was applied in Paper I to assess the induction of autophagy in myeloid cells after in vitro infection with Mtb. MDMs obtained from TB patients were cultured in chamber slides and were infected with the virulent laboratory strain H37Rv. After infection, the cells were fixed with 4% formaldehyde in PBS and stored frozen. The slides were thawed and stained with primary antibody for LC3 protein (autophagy marker) followed by fluorescent dye labelled secondary antibody and the level of expression was assessed using a confocal microscope. In Paper III, we studied the expression of M1 (CD64) and M2 (CD163) phenotype specific cell surface markers on in vitro differentiated monocyte-derived cells before and after infecting them with GFP-labelled H37Rv bacteria.

2.4 Statistical analyses

All the demographic, clinical, and laboratory data were checked first for normality distribution (D'Agostino and Pearson omnibus normality test) and thereafter, subjected to the corresponding parametric (for normally distributed data) or non-parametric (for non-normally distributed data) analyses. Quantitative variables were presented as the mean and standard error of the mean (SEM) or the median and interquartile range (IQR) or 95% confidence interval with an upper and lower range. Categorical variables were presented as frequency or numbers with percentages. An independent sample t-test was utilized to compare the difference between two groups when data were normally distributed, otherwise, Mann-Whitely U test was used for non-normal data sets. Chi-square (χ^2) test was performed to detect difference between groups for categorical variables. One-way repeated measure ANOVA (parametric) or Friedman test with Dunn's multiple comparison test (non-parametric measurements) was performed to observe differences over time in unmatched or matched groups of participants. Kruskal-Wallis test with Dunn's post-test (comparing more than two unmatched groups) was used to determine the difference between more than two independent groups. Spearman's correlation test (non-parametric) was used to detect an association between two numeric variables. In correlation analysis, a value of r=1 indicates a perfect positive correlation and r=1 indicates a perfect negative correlation. To evaluate changes in outcomes (TBscore, BMI, radiological features,

blood glucose, HbA1c, and CBC) both between the groups and within-group over time, two-way repeated measure Analysis of variance (ANOVA) was performed. A p-value of <0.05 was considered as significant (*) and a p-value of <0.001 was considered highly significant (***). Stata/IC (v.13, Stata Corp., LP, College Station, Texas, USA) and GraphPad Prism 8.3.0 were used for statistical analysis.

To quantify the difference in outcome variables (both categorical and numeric) between two or more groups, multivariate regression model and generalized estimating equation (GEE) analysis were performed in case of repeated measures at multiple time points. The odds ratio was calculated to express the effect of adjunct therapy or the presence of DM as co-morbidity in the treatment outcomes in TB disease at three different aspects (clinical, microbiological, and immunological). Age, sex, BCG vaccination status, baseline body mass index (BMI) and socio-economic status (SES) were considered as covariates and were adjusted for in these sophisticated analyses. The GEE model (with 95% confidence interval) was created to analyze the interaction between groups and time (baseline and follow-up time points) in blood hemogram markers and percent lung involvement. Covariates that influenced the model R^2 by 5% or more were adjusted to avoid collinearity.

2.5 Ethical considerations

Blood and sputum samples from Bangladeshi TB patients, Bangladeshi healthy controls were obtained after receiving ethical approval from icddr,b (PR-16078 and PR-13074) and BIRDEM in Bangladesh (Paper I and II). Analyses of clinical samples were done at Karolinska Institutet after obtaining ethical approval from Sweden (EPN, dnr 2019-01622). Patients were recruited in the studies after receiving signed informed consent (both in English and the native language Bangla). The Center for infectious medicine (CIM) has obtained ethical approval (EPN, dnr 2010/603-31/4) to work with blood from Swedish healthy donors (Paper III).

3 RESULTS AND DISCUSSION

My thesis project is based on both clinical and experimental work in Bangladesh and Sweden including two different study cohorts recruited in a high prevalence setting in Bangladesh, and also studies using an in vitro macrophage infection model. The major aims of my thesis work have been to explore how 1.) Mtb infection modulate host immunity in the presence or absence of DM2 (Paper II and III), and how 2.) host immunity can be modulated using adjunct therapy with immunomodulatory compounds to enhance immune cell function and TB cure (Paper I).

3.1 Paper I

Immune responses in the treatment of drug-sensitive pulmonary tuberculosis with phenylbutyrate and vitamin D3 as host directed therapy

3.1.1 Background

VitD deficiency is common in TB patients, which has been reported in the last century by several epidemiological studies [208-213]. Accordingly, vitD levels ($25(OH)D_3$) in plasma has previously been associated to severity of TB disease as well as the local expression of the antimicrobial peptide LL-37 [189,214]. It is not known whether vitD deficiency is a cause or an effect of TB infection, or if vitD deficiency is a consequence of poor nutrition or other factors. Considering the immunomodulatory effects of vitD, numerous attempts have been made to study if an improved vitD status could be used to prevent infections, primarily in the respiratory tract. Several randomized controlled trials have been conducted in different geographical locations using various dosages of vitD, administrated as bolus doses or as daily supplementation, to test if vitD can improve outcomes of i.e., TB [188] or acute respiratory infections [215]. Although, different studies have shown different results, it seems as if daily supplementation of high doses vitD could be effective to reduce symptoms in MDR-TB as well as ARI [188,215]. VitD supplementation showed positive effects in reducing disease severity in HIV-negative TB patients after 2 months of adjunct therapy in one randomized controlled trial [185]. In another study, significant reduction in sputum culture conversion time was registered in a subgroup having a specific genotype variant of the vitD receptor (VDR) after high-dose bolus vitD supplementation during the intensive phase of anti-TB therapy [184]. It has also been reported that clinical symptoms were significantly improved after daily vitD adjunct therapy in combination with PBA, especially in patients with severe TB disease and more severe vitD deficiency [183]. Our group also showed promising results of vitD adjunct therapy alone or together with PBA in reducing clinical symptoms and rapid sputum culture conversion [183].

In Paper I, the aim was to study if host-directed therapy (HDT) using adjunct treatment with PBA and vitD could be effective as a new treatment strategy for TB. This was a follow up study to assess secondary outcomes in a sub-sample of TB patients enrolled into a clinical trial that we had previously conducted in Bangladesh [182]. Year 2010-2014, we performed a

randomized controlled trial on newly diagnosed pulmonary TB patients to test if daily adjunctive therapy using (1) PBA, (2) vitD, (3) PBA+vitD or (4) placebo for 2 months, could support standard short-course anti-TB therapy and enhance clinical recovery and bacteriological outcomes (Clinical trial registration number: NCT01580007) [182]. In this trial, we observed that a significantly higher proportion of TB patients became sputum culture negative after 4 weeks of adjunctive treatment with vitD alone or in combination with PBA compared with placebo. In parallel, we also noticed only PBA treatment significantly reduced clinical score, increased LL-37 transcript expression in the monocyte-derived macrophages (MDM) as well as enhanced killing capacity of MDMs. Improved clinical and bacteriological responses in the TB patients who received treatment with PBA+vitD were associated with an enhanced expression of the antimicrobial peptide LL-37 in MDMs [182]. Importantly, only a few patients who received adjunct treatment with PBA and/or vitD showed adverse effects and most of these were mild adverse symptoms which could possibly be due to the anti-TB drugs used in DOTS regimen. Subsequently, in Paper I, we sought to explore in more detail the immunomodulatory effects of this adjunct therapy on immune cell function using clinical samples that had been collected from the study subjects in the clinical trial. One hypothesis was that PBA and vitD could decrease disease pathology in the lung by reducing harmful inflammatory responses exerted by various immune cells. Therefore, we planned to determine changes of different immune pathways that could be involved in the inflammatory process to further understand the mechanisms by which these compounds could ameliorate TB disease. We aimed to investigate if HDT in TB patients using PBA and/or vitD might be associated with more effective cytokines/chemokines responses, bolstering autophagy in macrophages and a simultaneous causing a decline in persistent ER stress. In these experiments, we analyzed microscopic slides with MDMs from the trial subjects using confocal microscopy, supernatants from in vitro cultures of patient´s PBMCs using multiplex assays, and mRNA obtained from MDMs with qPCR. The experimental results from Paper 1 were related to the clinical and experimental data obtained from the clinical trial i.e., clinical response/severity assessed by TB scores, LL-37 expression by various cell types, and macrophage mediated Mtb-killing.

3.1.2 Results and Discussion

3.1.2.1 PBA and vitD decreased the expression of inflammatory cytokine/chemokine responses with concomitant reduction of ER stress in MDMs obtained from TB patients

In TB disease, uncontrolled inflammatory responses contribute to tissue pathology and cavitation in the lung [96]. Immune cells secret pro-inflammatory cytokines and enzymes, which trigger different downstream pathways involving activation of potent effector molecules such as matrix degrading enzymes (MMPs) that eventually leads to lung injury [216,217]. Our results from Paper I, showed that daily treatment with PBA for 8 weeks significantly reduced the ex vivo production of TNFα, IL-17, eotaxin (CCL11) and RANTES (CCL5) by patient´s PBMCs as compared to PBMCs obtained from the placebo controls. In parallel to maintaining TB

granuloma structure and integrity during TB infection [218], TNFα induces mitochondrial ROS-production in endothelial cells [219] and neutrophils [220] and subsequent tissue damage. Furthermore, IL-17 enhances excessive influx of leukocytes that can contribute to organ pathology [221]. Exploratory analysis revealed, a marked reduction in pro-inflammatory TNFα and IL-17 production by PBMCs after 8 weeks of adjunctive therapy with PBA alone compared to placebo. VitD treatment also showed better efficacy than placebo in reducing the levels of eotaxin, IP-10 (CXCL10) and PDGF production by patient´s PBMCs. In a study with asthma patients, eotaxin was found to be associated with lung damage by stimulating degranulation of infiltrating eosinophils[222]. RANTES and IP-10 have been shown to have the potential to be used as both diagnostic and prognostic biomarkers to distinguish TB patients from healthy controls as well as to in monitor efficacy of standard anti-TB treatment [223-225]. Moreover, IP-10 has also been found to be negatively associated with pulmonary cavitation in TB-HIV co-infection, which suggests that IP-10 contributes to chronic inflammation and tissue destruction[226]. RANTES has been shown to act as a potent chemoattractant for eosinophils [227] and inflammatory T cells [228] in other diseases, which ultimately led to lung pathology [228]. Altogether, reduced production of these pro-inflammatory cytokines and chemokines in the PBMCs cultures after adjunct therapy indicated favourable effects of PBA and vitD adjunct therapy in dampening of systemic inflammation and downstream immunopathogenesis in the TB patients.

ER is a major organelle in eukaryotic cells and involved in processing and folding of proteins and a reservoir of intracellular Ca^{2+}. Increased cellular demands for protein folding or the presence of mutations in the amino acid sequences of proteins could impede the folding capacity of the ER and these unfolded proteins accumulate inside ER and increases ER size [229]. Conditions that promote an increase in protein misfolding including infections that have been found to induce ER stress, can result in cellular dysfunction, cell death and disease pathogenesis [229]. Accordingly, induction of ER stress has been identified in macrophages surrounding lung granulomas during Mtb infection in experimental animals [230]. The mycobacterial virulence protein ESAT-6 has been found to be responsible for ER stress-mediated apoptotic cell death in human epithelial cells, which contributes to enhanced tissue pathology [123] (Fig. 7). As such, enhanced enzymatic degradation of unfolded or misfolded proteins, or inhibition of the effector molecules or enzymes that get activated in response to an accumulation of misfolded proteins (such as IRE-1α), could be a clever approach to restore normal cellular functions to fight infection-mediated ER stress conditions. The active metabolite of vitD ($1,25(OH)_2D_3$) has been found to reduce ER stress in human umbilical endothelial cells[231]. Likewise, PBA has been reported to reduce ER stress and associated neuronal cell death[232]. Reduction of ER stress after cerebral ischemic injury, have been observed after PBA treatment that have anti-inflammatory effects by downregulating iNOS and TNFα expression in primary cultured glial cells [233].

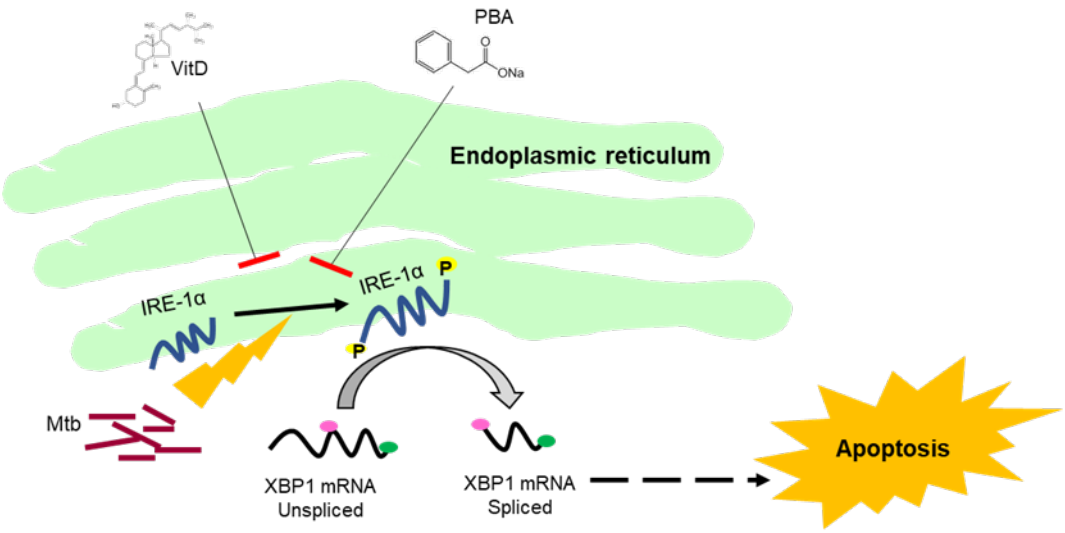

Figure 7: PBA and vitD reduces ER stress.

We investigated the expression of XBP1spl (spliced X-box binding protein-1) and (Growth Arrest and DNA Damage-Inducible Protein) GADD34 transcripts as surrogate markers for ER stress, in TB patients' MDMs obtained before and after treatment with PBA and vitD. After 8 weeks of treatment, a significant reduction of XBP1spl transcript was detected in PBA and vitD$_3$ treated groups compared to the placebo group. Surprisingly, we did not find any significant effects of the combined treatment with PBA+vitD on ER stress in the MDMs of TB patients. All four treatment groups showed a downregulation of GADD34 transcripts in MDMs assessed at baseline to week 8, and no significant difference between the groups was observed.

3.1.2.2 PBA and vitD alone or in combination activated autophagy in Mtb-infected macrophages obtained from TB patients

The importance of autophagy as a mechanism that destroy intracellular pathogens has been described by many research groups [91,234-237]. In this physiological process, part of the cytoplasm is captured into a double membrane wrapped compartment to quarantine the intracellular infectious material and thereafter is presented to the lysosome for enzymatic degradation [238]. Microtubule-associated protein 1 light chain 3 (LC3) is an important device in the autophagy machinery, which is translocated from cytosolic form of LC3 (I) to the membrane bound form (LC3II) in conjugation with phosphatidylinositol-3 kinase (PI3K) upon TLR activation [239]. Eventually, LC3II promotes autophagosome maturation and finally fusion with lysosomes for degradation. The conversion of LC3 isoforms (LC3-I to LC3-II) is a reliable and widely used method to quantify autophagy [91,240,241]. Previous studies have demonstrated the ability of vitD to induce LL-37-dependent autophagy (the conversion of LC3I to LC3II), which results in reduced intracellular Mtb growth [236]. Consequently, our group has discovered that the combination of PBA+vitD$_3$ can counteract Mtb-mediated reduction of LL-37 expression in

human MDMs and induce autophagy using an LL-37-dependent mechanism that is associated to enhanced killing of intracellular Mtb (H37Rv) in infected cells [91] (Fig. 8).

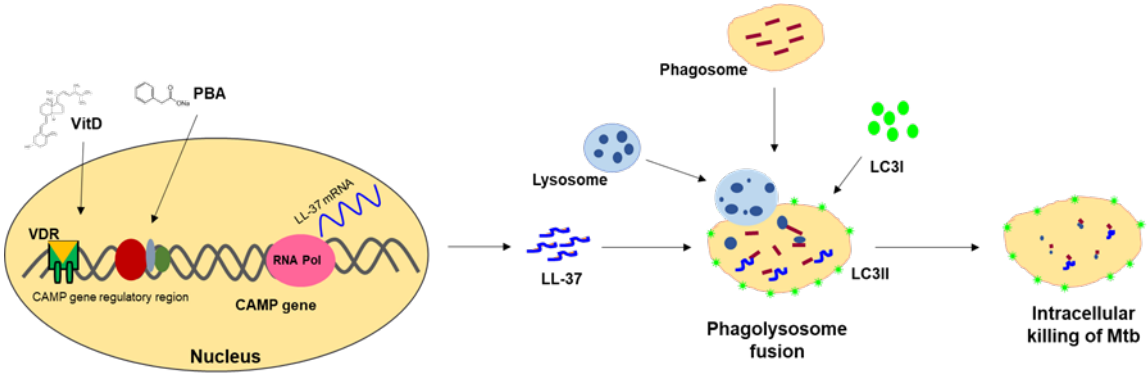

Figure 8: Autophagy process in Mtb-infected MDM.

In Paper I, we demonstrated that adjunct therapy with PBA and vitD, alone or in combination, could enhance the induction of autophagy in Mtb-infected MDMs obtained from the TB patients [190]. Presence of LC3II positive puncta inside Mtb-infected MDMs was determined by immunofluorescence and confocal microscopy (Fig. 9). While no difference was observed in LC3 activation/conversion in uninfected MDMs from the TB patients, Mtb-infected MDMs obtained from all the treatment groups (i.e., PBA, vitD or PBA+vitD) showed a gradual increase in the activation of autophagy as compared to the placebo control (GEE model analysis) (Fig. 9). Enhanced autophagy with the adjunctive treatment was maintained for an additional 4 weeks after the completion of PBA and/or vitD treatment. The induction of autophagy was associated with an upregulation of LL-37 production by the immune cells as well as clinical recovery of the patients [182]. The active vitD metabolite, $1,25(OH)_2D_3$, has been demonstrated to induce autophagy in human macrophages using an LL-37 dependent mechanism [178]. Reports are also available showing stimulation of autophagy and increased killing of intracellular bacteria after PBA treatment in an in vitro MDM-Mtb infection model [91]. In a pre-clinical study, vitD intervention was observed to accelerate alternative activation of macrophage (M2) via an autophagy-dependent mechanism in parallel with a reduction in UV light-mediated skin inflammation and apoptosis [242]. These data indicate that PBA and vitD might cooperate to enhance LL-37-mediated autophagy that kills intracellular Mtb in MDMs and contributes to an earlier decline of Mtb bacilli in the sputum of TB patients. This notion is also in line with the previously published findings from our group, where we demonstrated that induction of LL-37 in the immune cells after PBA+vitD treatment correlated with enhanced antimycobacterial activity in MDMs in healthy participants who received PBA and/or vitD [181].

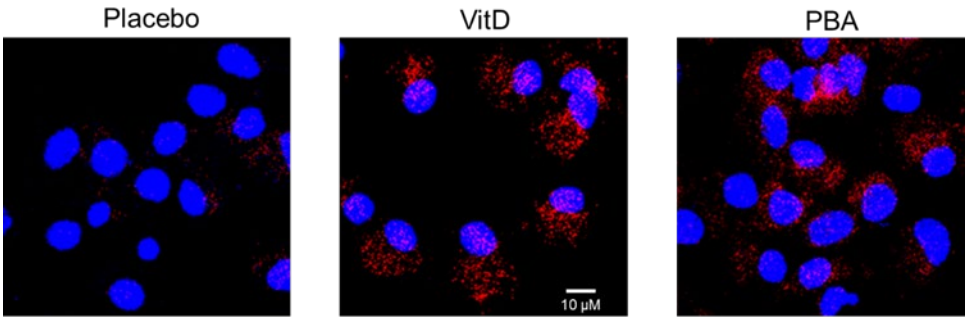

Figure 9: Detection of LC3II-positive MDMs from TB patients after 8 weeks of adjunctive treatment with vitD or PBA. LC3 is visualized in red and cell nuclei in blue.

3.2 PAPER II

Slow radiological improvement and persistent low-grade inflammation after chemotherapy in TB patients with type 2 diabetes

3.2.1 Background

In Paper II, the aim was to explore the immunopathogenic evens in pulmonary TB disease and the immune alterations observed in TB patients with DM. DM-associated impairment of cell-mediated immune responses involved in the progression of TB disease have not been fully delineated. Previous studies described that monocytes from DM patients showed reduced capacity to neutralize and control intracellular Mtb growth [243].This also included defective expression of the antimicrobial peptide LL-37 [166]. Thus, DM-mediated impairment of macrophage functions may impede generation of effective immune responses which are crucial to control development of as well as progression of clinical TB disease. In this study, we aimed to evaluate DM-mediated alterations associated with clinical, radiological, and immunological outcomes of TB disease. For this purpose, we collected clinical and anthropometric data as well as clinical samples from TB and TB-DM patients and controls, to obtain a better view of the clinical and immunological status of the patients before and after 1, 2 and 4 months of standard anti-TB treatment, provided by DOTS Center of the respective hospitals. Blood chemistry parameters and glycemic status was determined from whole blood samples, while plasma samples were examined for insulin, C-peptide and adipokines. mRNA samples from PBMC and sputum samples were assessed for gene expression of relevant immune molecules. We also analysed blood components from healthy controls to use as reference for analysis of the patient samples.

3.2.2 Results and Discussion

3.2.2.1 Baseline characteristics revealed that TB-DM patients had a better socio-economic background compared to TB patients

Socio-economic status (SES) is a combined measure of different variables such as income, education and employment, health knowledge, housing, nutrition/diet, and health care that may affect disease outcomes in different ways [244]. Interestingly, the TB-DM cohort had an overall better SES as well as higher BMI, higher rate of BCG vaccination and lower frequency of anemia as compared to the TB patients at the time of TB diagnosis and study enrolment. Interestingly, majority of the enrolled subjects were males, which may be explained by males being the more social gender group due to occupational reasons, who naturally may be at more risk of getting exposed to contagious TB patients in public areas etc. Some of the demographic observations in our TB-DM cohort were unexpected and inconsistent with previously published reports where this patient group were also shown to be more susceptible to TB disease [137,245]. Although higher BMI has been documented in TB-DM patients by other research groups [246]. A significant association was noted between HbA1c levels and BMI (Fig. 10A) in the TB and TB-DM cohorts. Additionally, an inverse correlation was found between hemoglobin concentrations and erythrocyte sedimentation rate (ESR) values combining both patient groups (Fig. 10B).

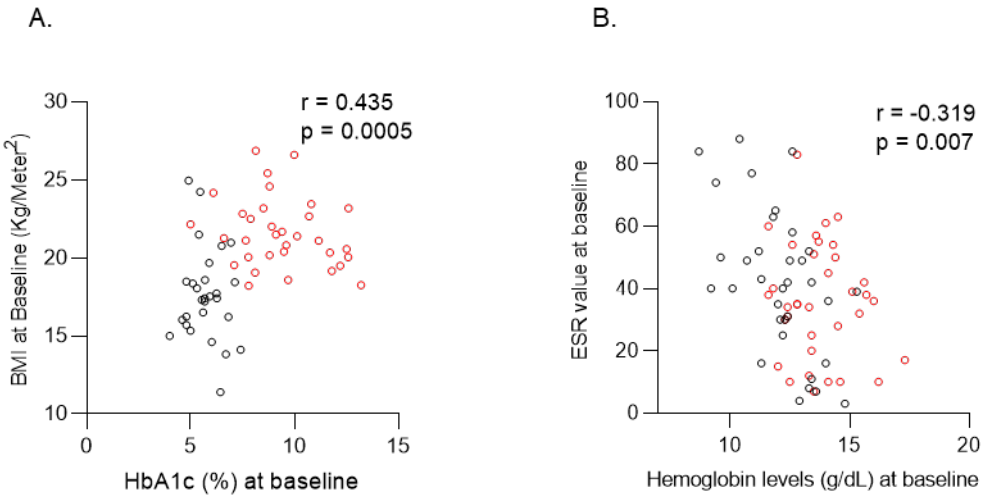

Figure 10: Association between (A) HbA1c and BMI, (B) Hemoglobin levels and ESR in TB and TB-DM patients. Spearman correlation coefficient (r) is calculated for non-parametric distribution of data. Black and red coloured symbols indicate TB and TB-DM patients, respectively.

3.2.2.2 Dysregulated glycemic control in TB-DM patients was evident before and after start of standard anti-TB treatment.

Overall DM patients have a greater risk of developing TB disease [247] and poor glycemic control has been shown to aggravate this situation in DM patients [247,248]. Moreover, it has been predicted that improving glycemic status in DM patients could potentially reduce the risk of TB among DM patients [247]. Dysregulated glycemic status was evident in the TB-DM patients

throughout the study period (mean HbA1c >8.7±2.2) (Fig. 11A). Fasting blood glucose data also supported poorly managed glycemic control in TB-DM patients (Fig. 11B). Another measurement of DM control is insulin resistance. Consequently, peripheral insulin resistance was evident in TB-DM disease as reflected by significantly higher insulin levels in TB-DM patients at 2 months after start of anti-TB treatment that decreased rapidly after 6 months of treatment. Contrary, TB patients consistently maintained glycemic control. Overall, TB-DM patients showed dysregulated glycemic status at TB diagnosis that was not improved despite administration of DM medication along with standard anti-TB treatment (Fig. 11A-B).

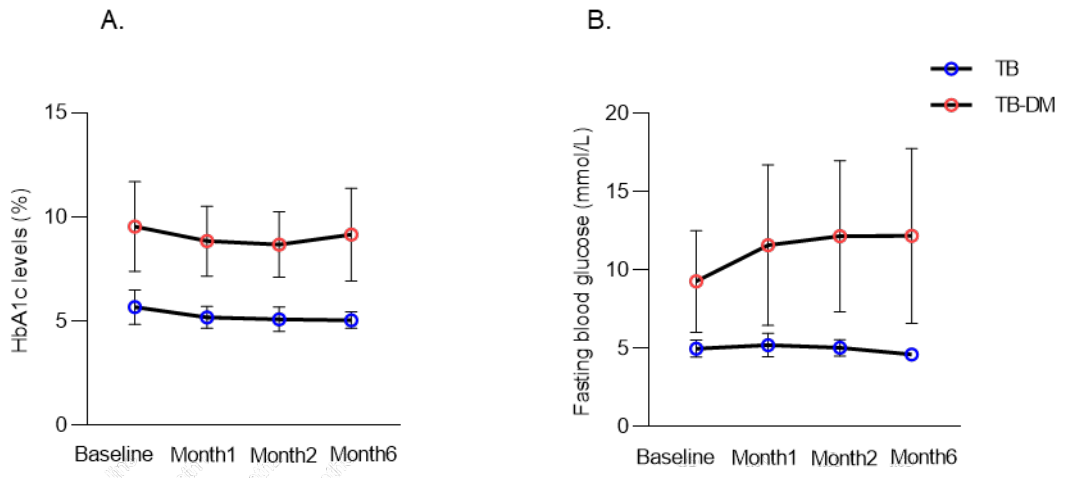

Figure 11: (A) HbA1c levels (%) and (B) Fasting blood glucose concentration (mmol/L) in TB and TB-DM patients both before and after anti-TB treatment (Mean ± standard deviation).

3.2.2.3 Enhanced pulmonary pathology in TB-DM patients was associated with higher age

Several studies have reported a positive association between higher age and progression of lower lung disease in TB patients [249,250]. Aging has also been documented as a risk factor for TB disease [251,252], although TB is most prevalent in the reproductive age-groups. In our study, more than half of the TB-DM patients (52%) were aged ≥40 years, which has been reported previously as a risk factor for TB disease [252]. On the other hand, only two TB patients (5.7%) were ≥40 years. TB patients were mostly (74%) less than 30 years of age, while only 8% of TB-DM patients were less than 30 years of age. It has been suggested that aging facilitates increased availability of O_2 through alveolar ventilation in the lower lung region, which might promote Mtb growth and survival in that area [250,253,254]. It has also been described that DM induce microangiopathy and downstream histological and functional alterations comparable to aging-related complications in the lower lung lobes that could enhance Mtb-mediated lower lung infiltrations in TB-DM patients [250,255]. Consistently, TB and TB-DM patients showed a positive association between age and inflammatory involvement in the total lung region determined at baseline (Fig. 12). Most our TB-DM patients were ≥ 40 years and therefore, we speculate that both uncontrolled DM and aging could contribute to enhanced Mtb-induced

inflammatory infiltrates in the lower lung area, which were observed in the chest radiographs even after 2 months of standard treatment.

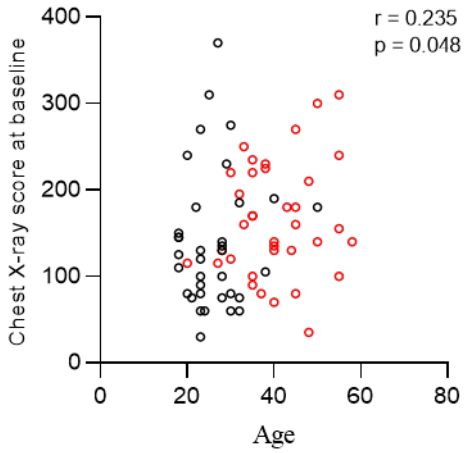

Figure 12: Association between age and chest radiography score in TB and TB-DM patients at the time of enrolment. Spearman correlation coefficient (r) is calculated for non-parametric distribution of data. Black and red coloured symbols indicate TB and TB-DM patients, respectively.

3.2.2.4 Similar clinical and microbiological features were recorded in TB and TB-DM patients

Higher bacterial burden in sputum have been demonstrated in TB-DM patients before anti-TB treatment [256]. TB-DM co-morbidity has been described to increase susceptibility to TB as well as the severity of TB disease [252], with longer time to sputum-culture conversion and delayed responses to anti-TB drugs [257]. However, other reports observed no differences in sputum conversion rates or treatment outcome between TB and TB-DM patients [256]. In our study, we also failed to detect any major differences in sputum-microscopy or culture conversion as well as differences in clinical symptoms (assessed as a composite TBscore) comparing TB and TB-DM patients before and after standard anti-TB treatment. TB-DM patients may metabolize anti-TB drugs differently and therefore it could have been interesting to study TB drug concentration e.g., rifampicin and/or isoniazid, in blood samples from this cohort. However, this would have required coordinated sampling at specific time-points after drug intake [258,259] as the half-life of these drugs are rather short with a serum peak concentration around 2 h after administration [260].

Interestingly, in the TB-DM group, Mtb-culture conversion rates at 1 month differed depending on the type of diabetic treatment and was most efficient in patients receiving metformin alone or in combination with other drugs (80% culture conversion, n=8) compared to patients receiving non-metformin hypoglycemic agents (63.6% culture conversion, n=14). We did not calculate the number of patients who received insulin because all except two of the patients who received metformin were also prescribed insulin. Though the difference in median time to culture conversion between the different antidiabetic treatments were not significant, metformin users showed relatively shorter sputum AFB and culture conversion times (33.0±3.0

days and 37.0±4.0 days, respectively) compared to the non-metformin drug users (54.0±9.2 days and 46.4±7.0 days respectively). In addition, TB-DM patients who received metformin had a lower average acid-fast bacilli (AFB) grade (<2+) compared to the non-metformin treatments. It is difficult to make conclusions because of the small sample size in each group, but these results may suggest that the response to anti-TB treatment in TB-DM patients is influenced by the antidiabetic medication. It has been shown that metformin has immunomodulatory functions on innate immune responses in TB including beneficial effects on cellular metabolism [261]. Likewise, a small observational study demonstrated that enhanced sputum conversion rates in TB-DM patients treated with metformin could be associated to enhanced autophagy [262]. Recent data also suggest that metformin enhance CD8+ T cells with antimycobacterial properties that could have protective effects against Mtb in patients with TB-DM disease [199].

3.2.2.5 Enhanced inflammatory involvement and middle-to-lower lung pathology in TB-DM compared to TB patients that persisted after start of anti-TB treatment

Over the years, many studies have provided evidence that abnormal radiologic features are significantly more common in TB-DM patients compared to TB patients without DM such as increased infiltration in the lower lung region and/or higher frequency of cavitary TB also including multiple cavities [263-271]. Increased Mtb growth in the lower lung lobes might be favoured through increased oxygen supply from alveolar ventilation in TB-DM patients [250,253] which could explain the observation of enhanced inflammation in the lower lung of this patient cohort. The TB-DM cohort in Paper II, exhibited significantly higher inflammatory involvement in the total lung area compared to the TB cohort at baseline, but not after start of anti-TB treatment. However, pulmonary involvement in TB-DM patients increased significantly in the middle lung zone at baseline but also at 1 and 2 months after start of anti-TB treatment as compared to the TB group. Additionally, pulmonary pathology was significantly higher in the lower lung zone of TB-DM patients after 6 months of chemotherapy. Consistently, longitudinal analysis confirmed that TB-DM patients showed elevated pulmonary involvement in the middle and lower zones as well as in the total lung region but not in the upper zone. Altogether these results are consistent with previously published reports on atypical radiologic features in TB-DM co-morbidity [269,271,272]. Apparently, it could be misleading to assess total lung involvement only, but pathological involvement should be quantified in the individual lung lobes. Lung pathology manifested in chest radiographs was also associated with HbA1c levels, total WBC and basophil counts even after start of anti-TB treatment, which indicates that high blood glucose levels fuel persistent infiltration of pro-inflammatory cells into the lung of TB patients even after clearance of the pathogen.

Proinflammatory cytokines such as IL-β and TNF-α can enhance MMP9 expression in immune cells at disease sites [273,274]. Consistently, we observed a trend of increased MMP9 expression in sputum cells and PBMCs from TB-DM as compared to TB patients before and after anti-TB treatment (Fig. 13A-B). Likewise, low IL-10 levels is correlated with increased expression and

activity of MMP9 [274,275] that has been found in pulmonary TB lesions in vivo, particularly in necrotic tissues with cavitation [276]. Increased levels of MMP9 in serum and cerebrospinal fluid was also associated with progression of TB meningitis to a more advanced stage [277]. MMP9 seems to be involved in macrophage recruitment and early granuloma formation [277], which could also contribute to dissemination of Mtb bacilli in the lung [278]. Consistently, it has been shown that Mtb increased in vivo expression of MMP1, which in turn promoted collagen breakdown resulting in alveolar destruction and lung pathology in TB [279]. Therefore, low IL-10 levels but enhanced proinflammatory cytokines and MMP levels could fuel lung pathology in TB-DM patients and may promote the progression of more severe cavitary disease compared to TB patients, especially if anti-TB treatment is delayed.

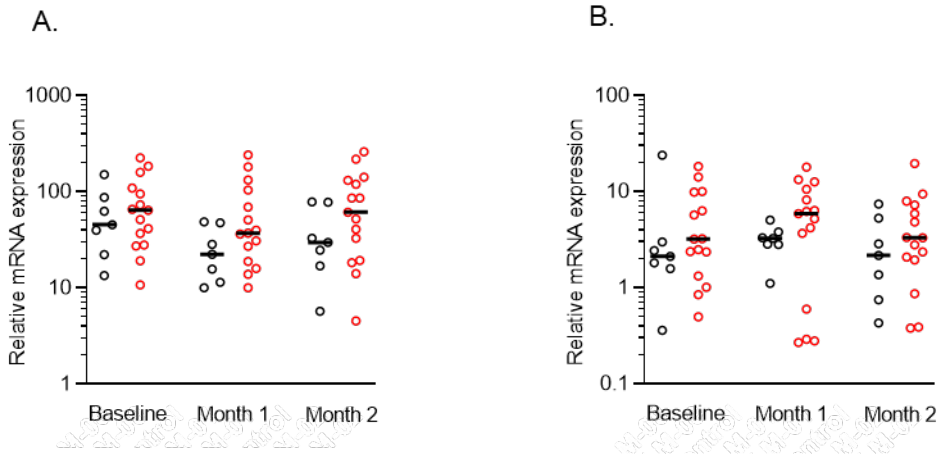

Figure 13: MMP9 mRNA expression in (A) sputum cells and (B) PBMCs in TB (black) and TB-DM (red) patients before and after anti-TB treatment.

Our TB-DM cohort had approximately 75 days duration of TB symptoms, most patients had around 5 years history of DM, and most importantly, no cavitation was recorded [280]. It would have been interesting to study corresponding data on immune markers and inflammation in TB-DM patients with more severe forms of lung disease including cavitary forms of TB. Perhaps TB-DM patients with more severe TB would also have shown significantly enhanced TB symptoms and reduced sputum conversion rates compared to TB patients. These results suggest that the TB-DM patients were in the early phase of TB disease progression during study enrolment.

The inhibitory action of anti-inflammatory IL-10 has been thoroughly described to involve dampening of both innate and adaptive immune responses in TB [281,282]. IL-10 has been shown to inhibit proinflammatory cytokine production in LPS-stimulated macrophage cell lines [283]. In vitro experiments also report that IL-10 can dampen intracellular killing activity of IFN-γ-activated macrophages by reducing the production of nitric oxide intermediates [284]. IL-10 inhibits phagosome maturation [285] and antigen presentation by blocking expression of MHC class II molecules [286]. Conversely, IL-10 has also been shown to reduce host-mediated pathology by inhibiting inflammatory and tissue-destructive immune responses [287-289]. On top

of the reduced mRNA levels of IL-10 found in sputum samples from TB-DM patients, an inverse association was recorded between sputum IL-10 mRNA and both fasting blood glucose and HbA1c levels in TB and TB-DM patients after 1 and 2 months of anti-TB treatment. Altogether, the mRNA expression profile of immune molecules suggested that Mtb-induced inflammatory responses were prolonged in the TB-DM patients even after the start of standard anti-TB treatment compared to TB patients, supporting low-grade persistent inflammation in TB-DM disease.

3.2.2.6 Persistent inflammation in TB-DM disease could contribute to adipokine dysregulation

Adipokines are secreted by adipocytes and inflammatory cells that have functions both as energy regulatory hormones and cytokines including direct effects on T cell polarization and T cell apoptosis [290-292]. In TB-DM patients, increased inflammatory adipokines suppress production of ROS, which affects intracellular Mtb control by macrophages [293]. Adipokine dysregulation in DM patients may influence development of insulin resistance, which has been found to be associated with TB [291,294]. There is growing evidence that TB patients have significantly lower circulatory leptin levels than controls [295,296]. Leptin and plasminogen activator inhibitor 1 (PAI-1) has been described to be associated with HbA1c and random blood glucose levels in earlier studies [295]. Similarly, we also noted lower circulatory leptin concentration in both TB and TB-DM patients compared to the controls (Fig. 14A). Plasma resistin (Fig. 14C) and PAI-1 (Fig. 14D) were increased in both patient cohorts, while ghrelin levels were lower in the TB-DM cohort compared to the controls at baseline (Fig 14B).

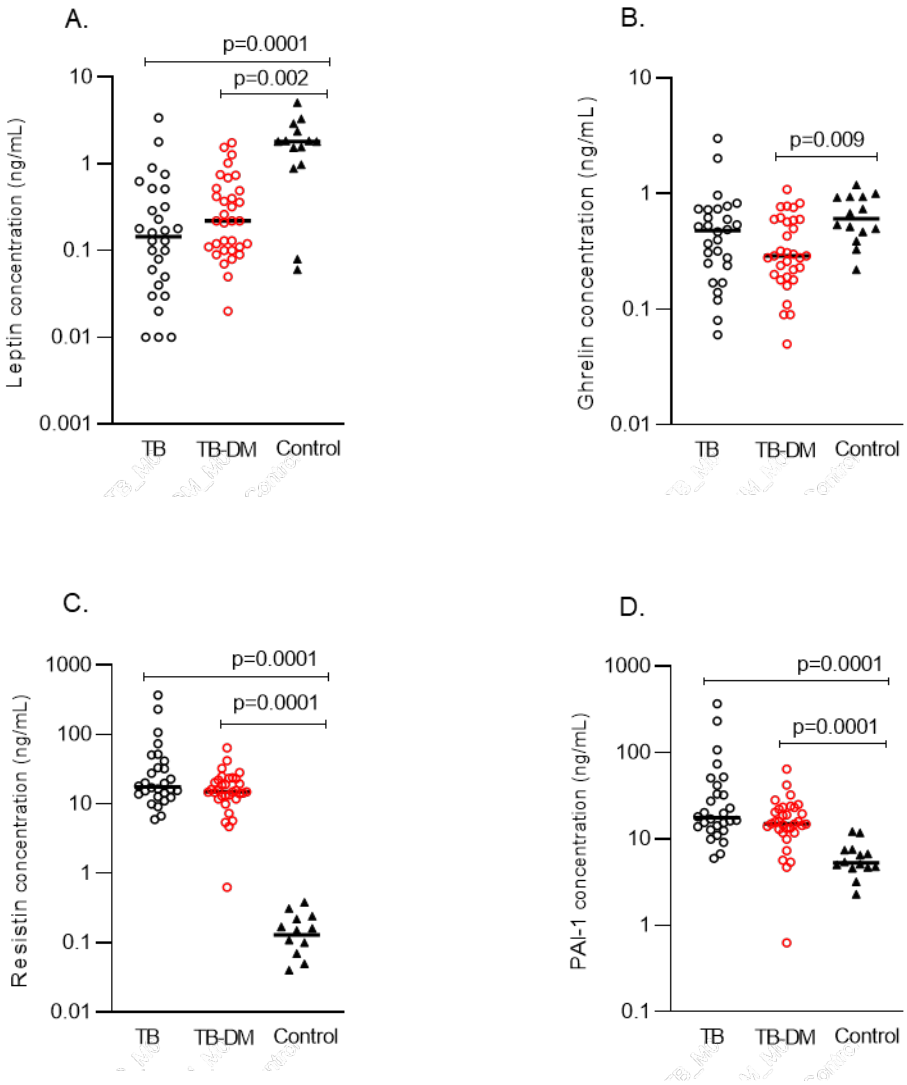

Figure 14: Plasma concentrations of leptin, Ghrelin, resistin and PAI-1 at baseline in TB and TB-DM patients as well as healthy controls. Kruskal-Wallis test was used to determine the indicated p-values.

In our study, TB-DM patients showed a significant association between circulating leptin concentrations and lower lung inflammation up to 6 months after anti-TB treatment, although we did not see any significant association at baseline (Fig. 15A-B). Moreover, consistent with other reports [297], plasma leptin levels in TB-DM patients were positively associated with BMI at baseline and until 6 months after anti-TB treatment (Fig. 15C-D). Contrary, TB patients showed no association between leptin levels and BMI at baseline but after 1 and 2 months of receiving standard therapy. Anti-TB treatment promoted an increase in the concentrations of leptin and a reduction in resistin levels back to normal in both the patient groups compared to the controls, while PAI-1 remained relatively higher in the patients. These results suggest that TB-induced inflammation was resolved after successful anti-TB treatment, while DM-associated adipokine imbalances were prolonged in TB-DM patients.

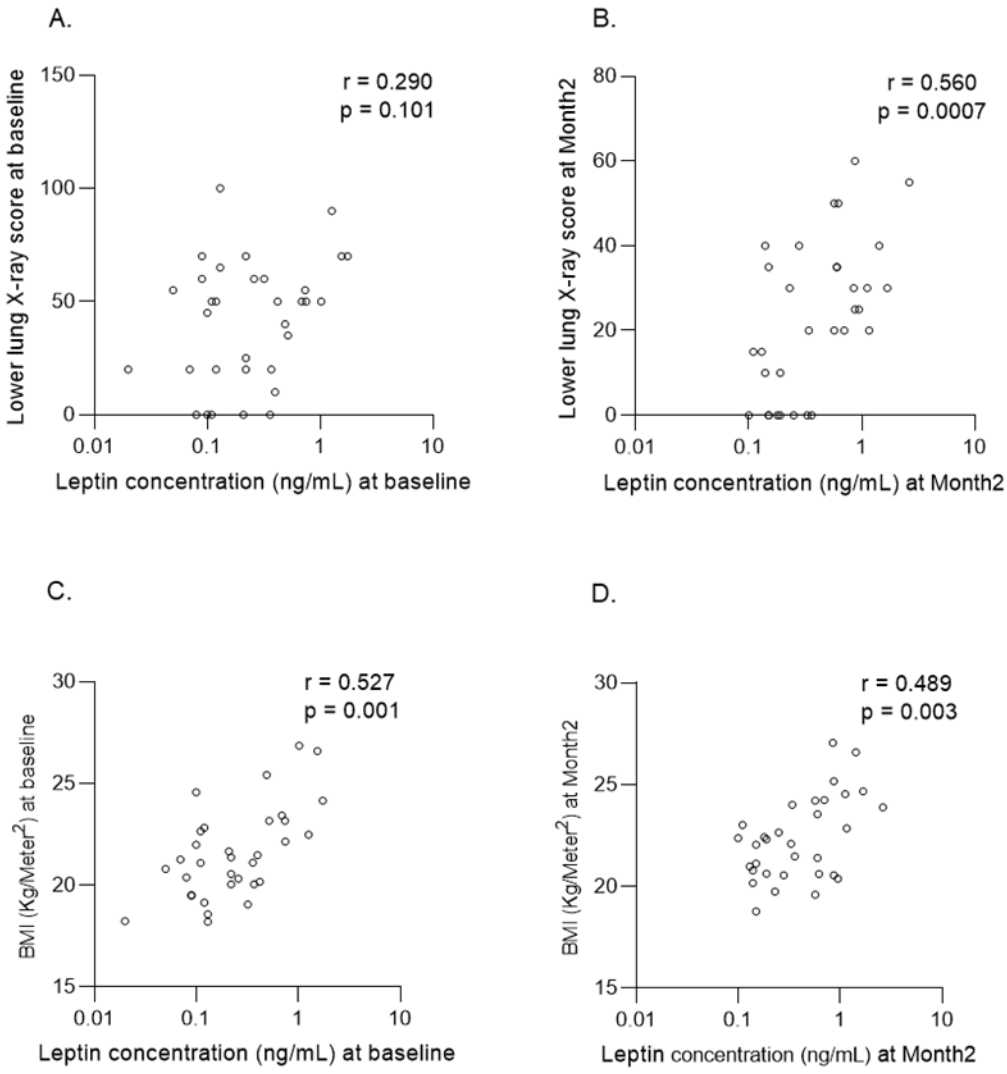

Figure 15: Association between (A-B) chest radiography score and plasma leptin concentrations and (C-D) BMI and plasma leptin concentration in TB-DM patients before and at 2 months after anti-TB treatment. Spearman correlation coefficient (r) is calculated for non-parametric distribution of data.

3.3 Paper III

Polarization of M1 and M2 human monocyte-derived cells and analysis with flow cytometry upon Mtb infection

3.3.1 Background

In Paper III, the aim was to use an in vitro macrophage infection model to investigate how different polarization protocols would affect Mtb infection and vice versa. As described in the introduction of this thesis, human macrophages are the primary host cells of intracellular Mtb infection and thus have a central role in immune control of TB disease. Using an in vitro macrophage infection model, our group has previously shown that vitD-polarized macrophages

had superior capacity to restrict growth of intracellular Mtb as compared with conventional M1 (classically activated) and M2 (alternatively activated) macrophages [202]. As expected, this property of vitD-polarized cells was attributed to enhanced levels of LL-37 and inflammatory IL-1β expression, but also reduced induction of the IL-1 receptor antagonist (IL-RA) as well as the immunosuppressive enzyme IDO1 in Mtb-infected macrophages [202]. Initially, we planned to use this Mtb infection model to study how hyperglycemic conditions would affect macrophage polarization and Mtb infection in vitro. A similar model has previously been described in which it was shown that high glucose concentrations could enhance pro-inflammatory cytokine responses but did not influence macrophage phagocytosis nor intracellular Mtb growth [298]. In line with these findings, we could not detect any differences in macrophage polarization or Mtb killing capacity in MDMs differentiated using low (5.5 mM), intermediate (11 mM) or high (25 mM) glucose concentrations (unpublished observations). Instead, we decided to develop an experimental protocol for flow cytometry of M1 and M2 macrophages that we could use to characterize myeloid cell subsets in the TB-DM cohort described in Paper II (ongoing work). For M1 and M2 polarization, we used GM-CSF-stimulated monocytes and IFNγ plus LPS to push the differentiation of fully polarized M1 cells, while M-CSF-stimulated monocytes and IL-4 were used to induce fully polarized M2 cells (Fig. 16) [299]. Multi-colour flow cytometry allowed visualization and deep-characterization of diverse phenotype and functional markers of adherent M1 and M2 macrophages subsets utilizing GFP-labelled Mtb (H37Rv) infection model.

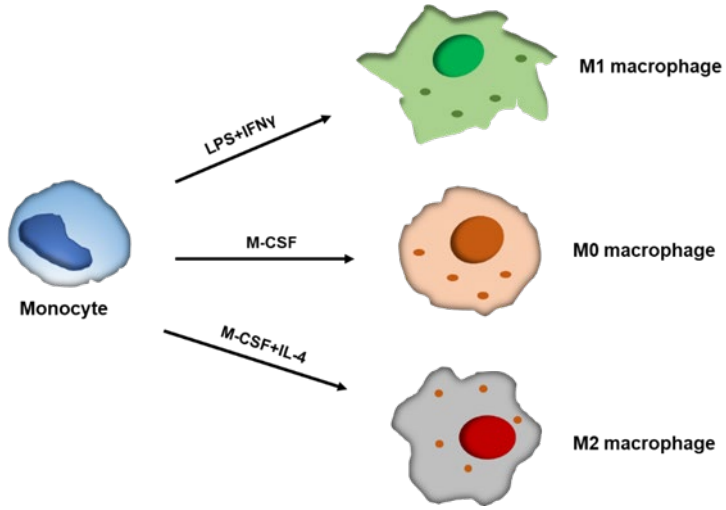

Figure 16: Polarization of M0, M1 and M2 macrophages from blood monocytes.

3.3.2 Results and Discussion

3.3.2.1 Phenotypic characterization of uninfected and Mtb-infected M1- and M2-polarized macrophages

This protocol represented an effective and reproducible method to study M1 and or M2 polarized cells and the assessment of their phenotypes using 10-colour flow cytometry. The panel of phenotypic markers was selected from the previously published paper from our group

[202]. In Paper III, we developed this staining protocol and analyses to identify M1 cells based on the co-expression of Fcγ receptor1 of immunoglobulin (CD64) [300,301] and the co-stimulatory molecule CD86 [302] (Fig. 17A), while M2 cells were recognized by co-expression of the scavenger receptor CD163 [303] and the inhibitory receptor CD200R [304] (Fig. 17B).

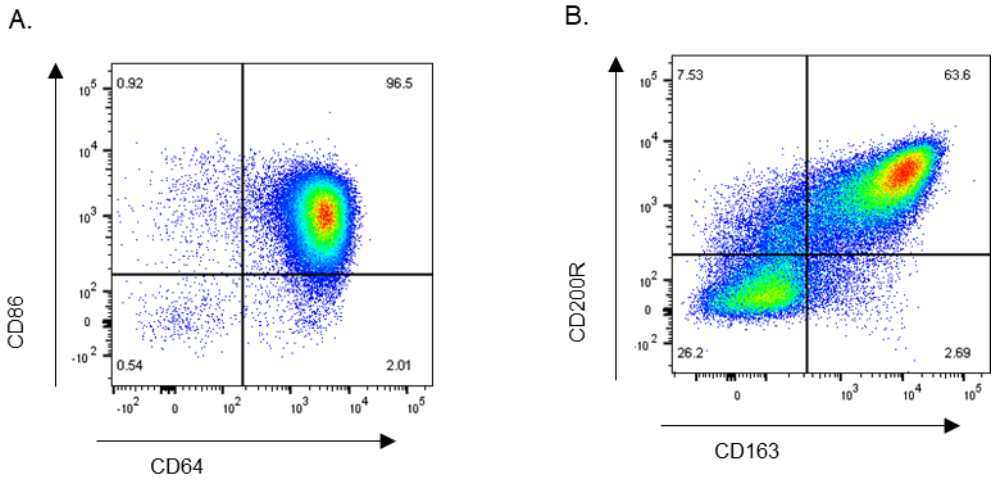

Figure 17: Identification of (A) M1 and (B) M2 macrophages based on co-expression of the phenotypic markers CD64/CD86 and CD163/CD200R, respectively.

We further showed that uninfected M1-polarized macrophages had improved antigen recognition as well as antigen presentation capacity, which was determined by enhanced TLR2 and HLA-DR expression as compared to uninfected M2-polarized cells. M1 cells also had an enhanced expression of the chemokine receptor CCR7 compared to M2 cells, which may indicate enhanced chemotactic capacity of M1 cells [305]. Instead, uninfected M2 cells displayed upregulation of the mannose receptor CD206 [306] and the co-stimulatory molecule CD80 [202] expression. However, Mtb infection altered the expression of these phenotype markers and downregulated M1 as well as M2 markers, resulting in a mixed M1/M2 phenotype of Mtb-infected cells, similar to what has been observed by other groups [202,299,307].

Previous studies have shown that virulent Mtb infection may promote M2-polarization of infected macrophages that may be more permissive of bacterial growth [308]. We observed that Mtb infection increased expression of the M2-marker CD200R in both M1- and M2-polarized cells (Fig. 18A-D). This might indicate that intracellular Mtb triggers expression of inhibitory CD200R on infected macrophages to suppress the production of pro-inflammatory cytokines and effector molecules essential to neutralize or eliminate the pathogen [309]. Mtb-infected M2 cells displayed a reduction in CD163 expression, which may imply that increased inflammation in TB disease is also aided by impaired scavenger function of M2 macrophages.

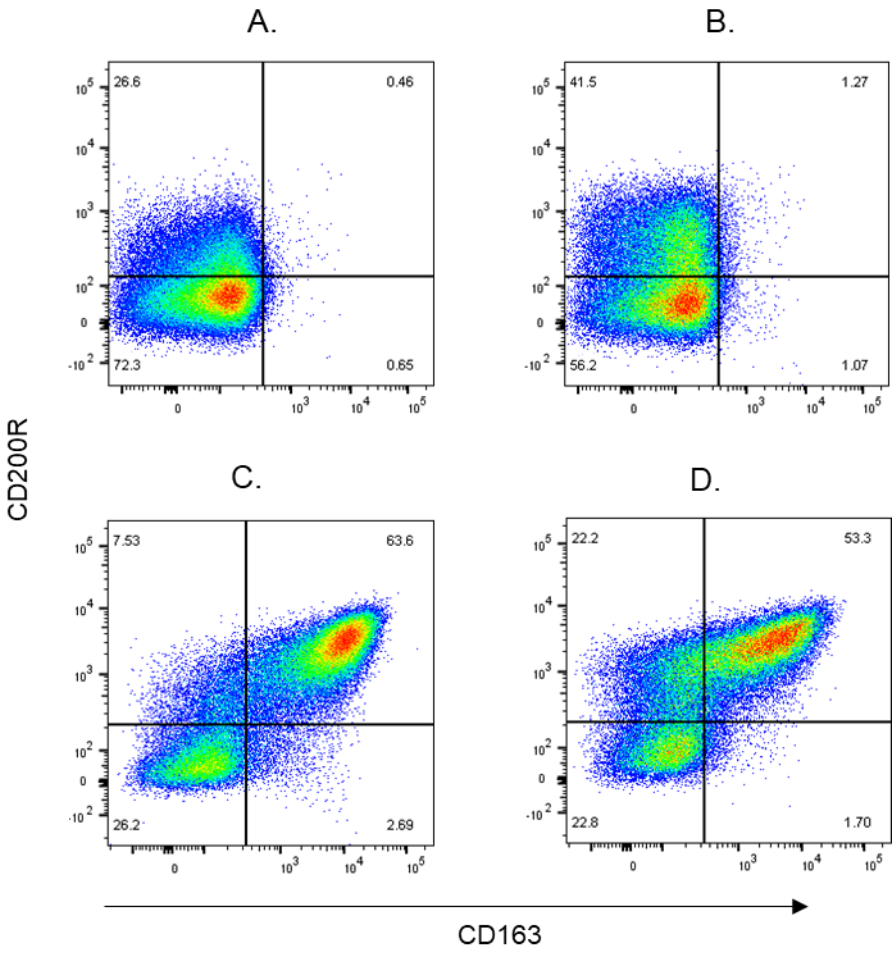

Figure 18: CD163/200R expression in (A) uninfected and (B) Mtb-infected M1-polarized MDMs as well as (C) uninfected and (D) Mtb-infected M2-polarized MDMs.

Although the infectivity was significantly higher in M2 compared to M1 cells, M2-polarized macrophages showed superior capacity to contain intracellular Mtb over time as compared to M1-polarized cells (77% vs 19% GFP-positive cells) (Fig. 19A-D). Consequently, the increase in GFP-expression from 4 to 24 hours was relatively higher in M1 compared to M2 cells (Fig. 19A-D), which could imply that bacterial replication is enhanced in M1 cells.

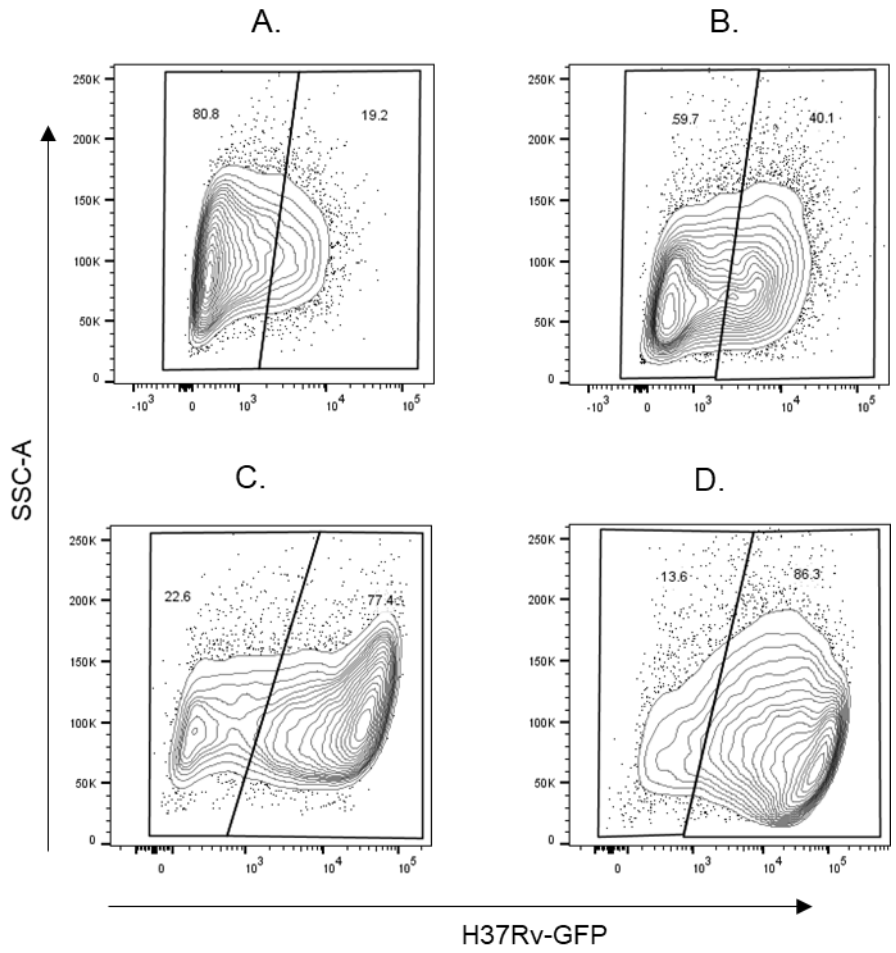

Figure 19: GFP-expression in M1-polarized macrophages after (A) 4 hours and (B) 24 hours post-Mtb infection, and GFP expression in M2-polarized macrophages after (C) 4 hours and (D) 24 hours post-Mtb infection.

We further performed unsupervised analysis of the flow cytometry data of the M1- and M2-polarized macrophages in the presence or absence of Mtb infection using Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction method and used a Phenograph clustering tool to identify phenotypically distinct cell clusters to describe their properties. With UMAP analysis, M1 and M2 cells formed well-separated clusters after 24 hours of Mtb infection. Consistent with the manual analysis in FlowJo, UMAP analysis also revealed higher expression of M1- and M2-phenotypic markers on the respective cell types. Presence of 13 distinct cell clusters in M1 cells and 11 clusters in M2 cells were detected following phenograph analysis.

3.3.2.2 M1- and M2-polarization protocols and assessment of myeloid cells in patient samples using flow cytometry

This protocol to generate phenotypically and functionally diverse M1- and M2-polarized cells in vitro could potentially be implemented in other areas of research, e.g., studies of anti-tumour responses. Pursuing macrophage polarization could also be an effective strategy to reinforce HDT in treatment of a variety of diseases [310]. Reprograming of tumor-associated macrophages towards M1 phenotype has been shown to be highly efficient in recognizing and destroying cancer cells and reduced metastasis in certain tumor models [311,312]. Instead, the anti-inflammatory and protective capacity of M2 macrophages could be exploited in treatment of inflammatory diseases [313]. Therefore, in vitro polarization models provide the opportunity for in depth understanding of specific macrophage phenotypes and associated functions [202]. Thorough assessment of both intrinsic and extrinsic mediators that contribute to macrophage polarization towards M1 or M2 as well as other macrophage subtypes could serve to develop successful anti-tumor therapies [314], but perhaps also HDT in chronic infections such as TB. In vitro polarization studies including similar flow cytometry methods provides opportunities to study a variety of diseases [315], which could be utilized for effective drug screening.

We are now in the phase of extending our 10-color flow cytometry panel to include additional myeloid cells markers used to identify different monocyte subsets and immune polarization in patient samples. These studies of circulatory myeloid-derived cells and their association with clinical, microbiological, and radiologic features determined in Paper II, may add new information about potential therapeutic targets for TB and/or TB-DM disease [299]. Preliminary data shows an increase in intermediate and non-classical monocytes in the TB-DM group compared to the TB group or healthy controls (Fig. 20, work in progress), which supports our previous observation of increased systemic inflammation in TB-DM co-morbidity after initiation of standard anti-TB treatment [280,316]. Additionally, as part of this extended staining panel, we plan to assess the frequency and contribution of so called myeloid-derived suppressor cells (MDSC) that have been shown to be increased in active TB disease and to suppress imperative T cells responses[317].

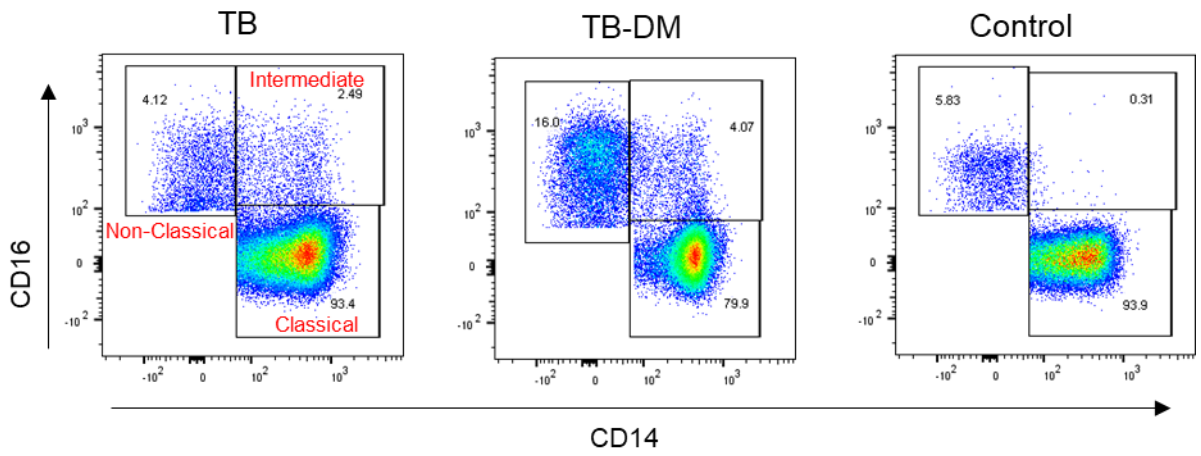

Figure 20: Myeloid cell phenotyping in PBMCs from TB and TB-DM patients as well as controls after 2 months of standard anti-TB treatment.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

The pathological features and mechanisms related to development and progression of TB disease is most accurately studied in human patient cohorts. This includes TB-associated comorbidities such as TB-HIV or TB-helminth co-infections as well as TB-DM disease that adds complexity to the TB disease spectrum and its features. This thesis work is a result of a long-term collaboration between researchers at Karolinska Institutet in Sweden and the icddr,b in Bangladesh. Over the years, we have built a strong collaborative network focusing on clinically relevant aspects of TB disease including host-pathogen interactions, TB diagnosis and host-directed therapies. The B cell-based ALS assay was initially invented at icddr,b [108,318] and later, the ALS assay was also tested in a high-prevalence setting in Ethiopia with positive results to diagnose active TB in TB-HIV co-infected individuals [106]. The ALS assay has also turned out to be beneficial for TB diagnosis is young children [319] and to follow disease prognosis in patients with MDR-TB [107].

In parallel, ground-breaking research from this research constellation provided proof-of-concept that oral treatment with butyrate could restore mucosal expression of LL-37 that improved the outcome of Shigellosis [320]. These findings paved the way for a new concept of host-directed therapy in pulmonary TB using the derivative of butyrate, PBA, in combination with vitD, another potent inducer of LL-37 expression in human lung epithelial cells and macrophages [180]. In vitro studies confirmed that PBA and vitD has a synergistic or additive effect on LL-37 expression and LL-37-dependent induction of autophagy in human macrophages, which was associated to intracellular killing of Mtb [91]. In 2013, Mily et.al. performed a pilot study in healthy volunteers who received oral PBA and/or vitD in different doses to determine the therapeutic dose required to induce LL-37 expression in immune cells and to enhance the killing capacity of Mtb-infected MDMs [181]. This study was followed by major efforts to design the randomized controlled trials in Bangladesh [182] and Ethiopia [183], where combination treatment with PBA and vitD was tested in patients with active pulmonary TB. The Bangladeshi study performed by Mily et.al., was the seed to Paper I of this thesis work, which supported the notion that PBA and/or vitD could enhance antimicrobial effects in Mtb-infected MDMs but also reduce inflammation and ER stress.

The preparations and clinical work of Paper II of this thesis, started already in 2014-2015 and were initially designed to entail a major part related to assessment of TB disease progression in the TB-DM cohort using the ALS assay. This was also done, however, we failed to detect any significant differences in the IgG titers comparing TB and TB-DM patients. This would be consistent with the findings in Paper II, revealing no differences in clinical or bacteriological outcomes comparing TB to TB-DM patients. Furthermore, patients in both the TB and TB-DM group were vitD deficient (median vitD level of approximately 30 nmol/L) at baseline and follow-up, although there were no differences in vitD status comparing the groups. Interestingly, we found a strongly significant correlation between ALS IgG titers and vitD levels in plasma of TB-DM patients at baseline and at 6 months after start of chemotherapy.

These results may suggest that TB-DM patients may be a suitable target group for HDT using PBA and vitD.

While the main part of this thesis work was devoted to Paper I and II, the work with Paper III involved more basic laboratory work using an in vitro infection model and training of advanced multicolor flow cytometry, which is a powerful technology for assessment of phenotype and function including surface as well as intracellular proteins, production of soluble mediators, and signaling pathways. Nowadays, advanced flow cytometry can contain staining panels of up to 30-40 colors also including dimension reduction methods such as UMAP that becomes an essential tool to understand and interpret complex data by visualizing clusters or groups of data. Whereas the attempts to set up a hyperglycemia model in vitro was not successful, the knowledge and experience obtained for the work with this M1/M2 staining protocol has enabled new adventures exploring an extended panel for assessment of myeloid cells in the TB-DM cohort.

Overall, this thesis contributed to the understanding of how human immune responses can be modulated by virulent Mtb and how human immunity can be modulated or restored to enhance eradication of TB infection. A delicate balance between inflammatory and tissue repair functions exerted by macrophages is one of the key determinants for protection from excessive inflammation triggered by pathogenic microbes. Therefore, using natural compounds or repurposed drugs to prime myeloid-derived cells that possess antimicrobial immunity but simultaneously reduce inflammation is attractive. Future research should continue to explore whether HDT with such immunomodulatory compounds could be designed to bridge effective myeloid responses to triggering of adaptive T cell effector functions that could be effective in adjunct treatment of difficult-to-treat cases such as TB-DM disease.

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