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Impact of Mycobacterium tuberculosis and HIV-1 on innate immune mechanisms

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Cover figure: *Mycobacterium tuberculosis*-infected macrophage interacts with uninfected macrophage. Visualized by acid-fast staining, where mycobacteria appear as reddish bacilli and cells are stained blue.

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1), a causative agent of acquired immunodeficiency syndrome (AIDS), and *Mycobacterium tuberculosis* (Mtb), a causative agent of tuberculosis (TB), are among the leading causes of death from infectious disease worldwide. Interplay between HIV-1 and Mtb leads to the detrimental dysregulation of immune system mechanisms, which provides the conditions facilitating progression of the disease in co-infected individuals.

In order to investigate the impact of Mtb and HIV-1 on innate immune responses we set up *in vitro* infection models comprising human monocyte-derived macrophages (Mφs) and dendritic cells (DCs). Firstly, we examined the influence of mycobacterial cell wall-derived glycolipids on the function of DCs. We found that two cell wall components, ManLAM and PIM, modulate DC function in an opposite manner, where ManLAM stimulates the production of pro-inflammatory cytokines, while PIM inhibits cytokine production triggered by activated DCs. Next, we analyzed several clinical Mtb isolates causing a large TB outbreak in Sweden. We found that the clinical isolates are characterized by the ability to trigger increased production of TNF from *in vitro* infected Mφs, above that triggered by the Mtb reference strain. Knowing that different mycobacterial glycolipids may differently impact DC and having several Mtb clinical isolates characterized, we investigated the effects of ongoing Mtb infection on the function of bystander DCs. Here we demonstrated that mycobacteria-infected Mφs create a pro-inflammatory milieu in which DCs undergo partial maturation, produce pro-inflammatory cytokines and additionally increase their ability to mediate HIV-1 *trans*-infection of T cells. Finally, we investigated mechanisms behind the altered cytokine response to Mtb during concurrent HIV-1 infection. We observed that the levels of cytokines released from Mtb-infected Mφs are lower after HIV-1 pre-exposure than those observed from singly Mtb-infected Mφs. Next, we measured levels of miR-146a, a microRNA known to inhibit signaling cascades leading to production of pro-inflammatory cytokines. We found that miR-146a was up-regulated upon Mtb infection and also after HIV-1 exposure, suggesting that HIV-triggered miR-146a expression may be responsible for cross-tolerance of Mφs to following Mtb infection. Furthermore, we showed that exposure to the HIV-1 envelope glycoprotein gp120 is sufficient to up-regulate miR-146a, which in turn is paralleled by down-modulated responsiveness of Mφs to a secondary stimulus, i.e. the Mtb glycolipid ManLAM.

In conclusion, this thesis highlights that several innate immune mechanisms are modulated by either HIV-1 or Mtb, which may hamper adequate immune responses against the two pathogens. These studies also suggest that the effects may be triggered in a bystander manner, where impacted cells are not infected and may be even localized distantly from the site of infection.

LIST OF PUBLICATIONS

I. **Mazurek, J**., Ignatowicz L., Kallenius G., Svenson, S.B., Pawlowski, A., Hamasur, B., *Divergent Effect of Mycobacterial Cell Wall Glycolipids on Maturation and Function of Human Monocyte-Derived Dendritic Cells*, (2012), PLoS One. 2012;7(8):e42515

II. Sandegren, L., Groenheit, R., Koivula, T., Ghebremichael, S., Advani, A., Castro, E., Pennhag, A., Hoffner, S., **Mazurek, J**., Pawlowski, A., Kan, B., Bruchfeld, J., Melefors, O., Kallenius.G., *Genomic Stability over 9 Years of an Isoniazid Resistant Mycobacterium tuberculosis Outbreak Strain in Sweden* PLoS One. 2011 Jan 31;6(1):e16647

III. **Mazurek, J**., Ignatowicz L., Kallenius G., Jansson, M*., Pawlowski, A*., *Mycobacteria-infected bystander macrophages trigger maturation of dendritic cells and enhance their ability to mediate HIV transinfection*, (2012), Eur J Immunol. 2012 May;42(5):1192-202

IV. **Mazurek, J**., Carow, B., Hamasur, B., Rottenberg, M. E., Jansson, M., *HIV-induced miR-146a expression correlates with cross-tolerance to Mtb-triggered responses of macrophages*, Manuscript

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CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ART	Anti-retroviral therapy
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette–Guérin
CE-LIF	Capillary electrophoresis with laser-induced fluorescence detection
CFU	Colony forming unit
CLR	C-type lectin receptor
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing non-integrin
EDTA	Ethylenediaminetetraacetic acid
GALT	Gut-associated lymphoid tissue
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony stimulating factor
HIV	Human immunodeficiency virus
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
imDC	Immature dendritic cell
IRAK	Interleukin-1 receptor-associated kinase
IRIS	Immune reconstitution inflammatory syndrome
IS	Insertion sequence
LAL	Limulus amebocyte lysate
LC	Langerhans cell
LM	Lipomannan
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mAb	Monoclonal antibody
ManLAM	Mannose-capped lipoarabinomannan
M-CSF	Macrophage colony stimulating factor
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
miR	MicroRNA
MR	Mannose receptor
mRNA	Messenger RNA
Mtb	Mycobacterium tuberculosis
MTC	Mycobacterium tuberculosis complex
Mφ	Macrophage
NFκB	Nuclear factor κB (kappa-light-chain-enhancer of activated B cells)
NK	Natural killer
NKT	Natural killer T cell
PAMP	Pathogen-associated molecular pattern

PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PI	Phosphatidyloinositol
PIM	Phosphatidylinositol mannosides
PRR	Pattern recognition receptor
RFLP	Restriction fragment length polymorphism
RISC	RNA-induced silencing complex
rRNA	Ribosomal RNA
SIV	Simian immunodeficiency virus
SNP	Single-nucleotide polymorphism
TB	Tuberculosis
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
WHO	World Health Organization

1 INTRODUCTION

1.1 IMMUNITY TO INFECTIONS

The role of the immune system is to protect the organism from the invasion of broadly defined threats, including pathogens but also dangers derived from within the organism that are the results of malfunctions, including malignant transformation.

The immune system is a network of organs, tissues, cells and soluble factors working together to maintain homeostasis of the body and to ensure a safe environment for other organs to work and perform their functions. Skin and mucosa, which constitute the mechanical barrier, provide a physical protection of the organism from potentially hazardous objects from outside. This barrier is often penetrated by pathogens and then the main task of the immune system is to recognize the threat as quickly as possible and to signal its presence to other components of the immunity network in order to trigger an adequate response. This is possible owing to the different pattern recognition receptors (PRRs) expressed by cells of the immune system and also by other cells [1]. Pathogens and infected cells can be directly recognized or taken up by specialized cells, called antigen presenting cells (APCs), including phagocytes, which process antigens (Ags) and present them for the recognition by specific receptors on the T and B cells. Such recognition of danger signal is accompanied by the release of panels of cytokines and chemokines, signaling molecules that help to recruit more cells to the site of infection and to mobilize additional defense mechanisms [1].

Generally, the immune system is divided into two branches: innate and adaptive immunity. Innate immunity includes evolutionary older mechanisms acting fast but with limited specificity. Cells of innate immunity include dendritic cells (DCs), macrophages (Mφs), granulocytes, NK cells, NKT cells and γδT cells. Adaptive immunity on the other hand is more specific but requires time to develop after recognition of the threat. T cells and B cells belong to this group and they need instructions from APCs in order to function properly [1].

Thus, the first goal of the immune system is to recognize, act and eliminate the threat. Another, equally important aim is to remember the primary insult. After the first encounter of the pathogen the immune system, especially the adaptive immune system, generates a memory, which upon the next invasion of the same danger is mobilized much more quickly and allows the organism to eliminate the threat much faster and without causing too extensive damage. The memory of the immune system is employed in vaccination, where a small amount of inactivated pathogens, subunits or attenuated pathogens are given to the organism. This causes

a mobilization of the immune response, allowing for developing a memory, which upon contact with the real pathogens ensures a quick and effective elimination of the microorganism without causing disease.

Sometimes, however, the immune response is triggered by a minor cause, the response is too extensive relative to the threat or is directed against host antigens, i.e. self-antigens. This may lead to immunopathology and autoimmunity, as seen for instance in rheumatoid arthritis, autoimmune thyroid disorders and many more conditions together called diseases from autoaggression.

1.1.1 Innate immunity

Early concepts in immunology described innate immunity as a non-specific branch of the immune system. However, discovery of Toll-like receptors (TLRs) and other families of PRRs demonstrated that in fact innate immunity receptors specifically recognize pathogen-associated molecular patterns (PAMPs) [2]. The recognition of PAMPs by TLRs occurs in the plasma membrane, endosomes, lysosomes and endolysosomes [3]. In addition, cell type–specific TLR repertoires and cell type-specific signaling pathways activated by specific TLR define their immunological properties [2, 3]. Upon binding of the ligand, adaptor molecules are recruited to cytoplasmic domains of TLRs which results in triggering of a downstream signaling cascade leading to production of pro-inflammatory cytokines and chemokines [3].

Out of ten functional TLRs known in humans several are involved in recognition of PAMPs derived from Mtb or HIV-1. For instance, TLR2, 4 and 9 are known to be engaged in recognition of mycobacteria-derived ligands [4]. Modulation of granuloma progression and cytokine production during Mtb infection has been found to be related to the recognition of the mycobacterial GC-rich genome by TLR9 [4-6]. The mycobacterial cell wall comprises a variety of lipopeptides and liposaccharides that stimulate TLR2 [4, 7], whereas TLR4 is activated by Mtb heat shock proteins [8]. HIV-derived PAMPs are also recognized by TLRs. The HIV genomic single-stranded RNA is able to activate TLR7/8 [9]; double-stranded DNA, which is formed as an intermediate during the HIV replication cycle, may trigger TLR9; whereas double-stranded RNA is recognized by TLR3 [10, 11].

Another important group of PRRs are C-type lectin receptors (CLRs), calcium-dependent carbohydrate-binding proteins. This group includes among others: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), mannose receptor (MR), langerin and dectin 1 and 2 [12]. CLRs are able to trigger a pathogen specific response either by modulating TLR signaling or by direct modulation of gene expression [12]. Both MR and DC-SIGN may bind

highly mannosylated molecules, hence a mycobacterial cell wall component, mannose-capped lipoarabinomannan (ManLAM), and the viral envelope glycoprotein gp120 of HIV-1 strongly interact with these receptors [12-14].

1.1.1.1 Dendritic cells

Dendritic cells are professional APCs with the unique ability to stimulate naïve T cells [1]. They comprise a sparsely distributed, heterogeneous population displaying differences in origins, localization, migratory pathways and function [15]. DCs are the only immune cells that can have either myeloid or lymphoid origin and are found in the blood, skin, mucosa and across many organs in the body [15, 16]. DCs are perceived as immune system sentinels, which sample their environment and upon recognition of a danger signal trigger innate immune responses and initiate adaptive immune responses. Therefore, they are equipped with a large panel of PRRs (including C-type lectins) which allows them to quickly recognize and react to the presence of pathogens and their components [17].

Generally, upon capture of Ag, DCs undergo a maturation process with surface up-regulation of MHC class II and other molecules engaged in Ag presentation. In addition they migrate from their primary location to the lymph nodes and secrete a spectrum of cytokines providing appropriate signals to interact with T cells and elicit an effective immune response. DCs initiate different types of immune responses depending on their origin, location and the type of the signal triggering their activation [17].

There are two main subsets of DCs: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Blood mDCs and pDCs represent 0.5-2% of the total peripheral blood mononuclear cell (PBMC) population [16]. They differ in expression of surface markers, e.g. mDCs express CD11c whereas expression of CD123 and lack of CD11c is specific for pDCs [18, 19]. In addition, mDCs are known to produce large amounts of IL-12, while pDCs secrete important antiviral cytokines, i.e. type I interferons (IFN) [16, 20, 21]. The common feature of DCs is their ability to prime naïve T cells following processing and presentation of Ags.

Human mucosa is well populated by distinct DCs in order to constantly survey surfaces exposed to potential pathogen invasion and to induce an inflammatory response when needed [22]. For instance, mucosal tissue of the reproductive tract includes Langerhans cells (LCs), submucosal mDCs and upon infection it is also rapidly infiltrated by pDCs [22]. Vaginal, submucosal DCs have been demonstrated to migrate to the draining lymph nodes and initiate an anti-viral cellular response upon *Herpes simplex* type 2 infection [23]. Moreover, another study in a similar model has shown that infiltrated pDCs provide large concentrations of anti-viral cytokine IFN-α [24]. Analogous observations have been made for pDCs

attracted to the lymph nodes during acute infection with simian immunodeficiency virus (SIV) in *Rhesus macaques* [25].

LCs are found in the epidermis and mucosal epithelia [26]. They are typical APCs with high capacity to capture, process and present Ags to T cells. LCs are the only known human cells expressing langerin (CD207) that is an important PRR with specificity for mannose, fucose and N-acetyl-glucosamine monosaccharides [27]. This specificity is shared with DC-SIGN that also interacts with mannose and fucose structures, yet it is not expressed on LCs [26]. Ligands bound to langerin may be internalized and directed to processing via the non-classical Ag presentation pathway. It has also been speculated that langerin, similarly to DC-SIGN, upon binding of the ligand might trigger a signaling cascade modulating the cytokine response of LCs [26].

Follicular DCs are a different type of DCs. They are stromal cells of mesenchymal origin, thus not derived from the hematopoietic stem cells, and are not able to process Ags and present them in the context of MHC class II [28]. Follicular DCs reside in the B cell follicles and germinal centers of the peripheral lymphoid tissues where they support B cell survival and proliferation [28]. They retain the opsonized Ag on their surface which has been shown to be a more effective way of B cell stimulation than by soluble Ags [29].

DCs are scarce in the blood and therefore their isolation for *in vitro* studies is difficult. Instead, a widely used, laboratory-adapted model comprises monocyte derived DCs (moDCs). They originate from monocytes isolated from the blood which are differentiated to DCs in the presence of granulocyte/macrophage colony stimulating factor (GM-CSF) and IL-4 [30]. Such moDCs resemble mDCs by expressing CD11c, DC-SIGN and MR, MHC class I and II and co-stimulatory molecules, CD80 and CD86. They are also able to phagocytose, process and present Ags, secrete a panel of cytokines in response to stimulation and acquire a phenotype of mature DCs. Although they are a feasible model they do not fully mimic DCs found *in vivo*.

1.1.1.2 Macrophages

Macrophages (Mφs) are APCs residing in tissues and characterized by high phagocytic activity. They originate from the myeloid hematopoietic branch and develop from monocytes recruited to the tissue from the blood [31]. Monocytes, constituting 5-10% of blood leukocytes, circulate in the peripheral blood for several days and are able to migrate out to tissue to give rise to a variety of Mφs, but also to some DCs and osteoclasts [15, 32, 33]. Several groups, however, observed that a local proliferation of the Mφ lineage is possible as well [34-36].

Mφs play an important role in the maintenance of tissue homeostasis, through the clearance of apoptotic cells, and remodeling and repair of tissues [31].

They actively participate in sampling of their environment, recognizing pathogens and infected cells and presenting Ags to T cells. Mφs are capable of not only receptor-mediated endocytosis but also internalization of particulate material via membrane ruffling and folding mechanisms [37]. The Mφ population is heterogeneous depending on the tissue they reside in and on the role they play in a specific location [32]. The lung Mφ population, for instance, includes alveolar, pleural, interstitial and intravascular Mφs expressing a wide spectrum of PRRs and other scavenger receptors in order to recognize and react to the presence of danger signals [38]. The respiratory tract is repeatedly exposed to inhaled microorganisms therefore strong and efficient defense mechanisms are so crucial there [36]. Binding of PAMP to a specific receptor triggers a cascade of signals in Mφs leading to their activation. It includes release of a panel of cytokines and chemokines attracting other cells to the site of infection, use of lysosomal enzymes and activation of killing mechanisms involving the release of toxic oxygen and nitrogen species [36, 37].

1.1.2 Interactions between innate and adaptive immunity

Cells of the innate immunity co-operate closely with cells of the adaptive immunity. Antigen presentation by DCs and Mφs is critical to successful development of both cell-mediated and humoral immunity [37]. Fast recognition of pathogen followed by phagocytosis, processing and presentation of Ags to T and B cells is accompanied by release of the appropriate spectrum of cytokines and chemokines. All these requirements must be fulfilled in order to initiate an effective immune response.

A crucial link between innate and adaptive immunity is made when APCs present Ag to T cells in order to initiate their activation leading to development of specific mechanisms directed against invading pathogen or other threats. Interaction between APCs and T cells requires close contact, where the T cell, using its T cell receptor (TCR) recognizes Ag presented by APC [1]. Yet, such recognition alone is not enough to initiate T cell activation. A second signal is needed, which is provided by an interaction between co-stimulatory molecules on the surface of the APC (CD80 and CD86) and the T cell (CD28). Up-regulation of co-stimulatory molecules is observed on DCs during their maturation following recognition of the pathogen, which strengthens their ability to activate T cells. To fully activate the T cell the APC needs to supply a so-called "third signal". This signal comprises a panel of pro-inflammatory cytokines that are delivered to T cells in order to trigger additional pathways leading to activation. A tight controlling mechanism behind T cell activation is essential to avoid accidental triggering and persistent activation, which could lead to unnecessary immune system mobilization and immunopathology.

The interface between the APC and the T cell, created during the priming process, is called the immunological synapse, after neurological synapse that also requires a close contact between two cells. Such proximity of two immune cells is sometimes used by pathogens for efficient spread between cells. An example of such a pathogen is HIV, which makes use of the immunological synapse formation to spread from DC to T cell [39, 40].

B cells are another important component of the adaptive immune response. They are responsible for the humoral immunity, which includes the production and release of antibodies (Abs). B cell activation and differentiation into Ab-secreting plasma cells is driven by Ag recognition and most often requires contact with T helper cells [1]. The immune system makes use of Abs in several ways. Some Abs possess a neutralizing capability; that means that pathogens or their components, including toxins, may be either opsonized or sterically hindered by Abs and thus prevented from reaching or infecting target cells. There are also Abs that instead may facilitate uptake of the pathogens by phagocyting cells, which leads to pathogen degradation and presentation of its Ags. On the other hand, some pathogens have developed mechanisms that use this system to infect the cells. Additionally, pathogens opsonized by Abs can trigger a cascade of complement activation that leads to microorganism degradation by forming pores in their membranes [1].

1.1.3 microRNA

1.1.3.1 General background and biogenesis

MicroRNA (miR) is a class of short, non-coding RNAs first described in *Caenorhabditis elegans* in 1993 [41]. Since then miRs have been extensively studied and their role in controlling many processes, including cell development, proliferation, differentiation, apoptosis, cancerogenesis and immune system regulation, has been widely appreciated.

miRs are about 22 nucleotide-long RNA molecules that can post-transcriptionally down-regulate expression of target messenger RNAs (mRNAs) by imperfect binding to the 3'-untranslated regions of such mRNAs [42]. There are nearly one thousand different human miRs identified to date [43]. Some of them regulate specific individual targets, while others can function as master regulators of a specific process [44]. Some miRs can potentially regulate the expression levels of several hundred distinct mRNAs simultaneously, and many types of miRs regulate their targets cooperatively [44, 45]. Therefore miRs emerged as important regulators of gene expression being responsible for controlling expression of more than 60% of protein coding genes [44].

miRs are encoded within the introns and exons of longer primary transcripts, including protein-coding transcripts [46] and are most often transcribed from the genome by RNA polymerase II giving rise to transcripts which length can extend even over 1 kb fragments forming hairpin structures, primary-miRs (Figure 1) [47, 48]. Primary-miRs are further processed in the nucleus by the enzyme complex Drosha and are cleaved to about 70 nucleotide-long precursor-miRs and thereafter exported to the cytoplasm by the exportin 5 [48]. In the cytoplasm, immature miRs are further cleaved by the enzyme DICER that cleaves the terminal loop structure to form double-stranded miR [47, 48]. After dissociation, single-stranded, mature miR together with Ago protein, forms RISC (RNA-induced silencing complex) that can bind to the target mRNA, leading to inhibition of translation initiation or to the mRNA degradation [44].

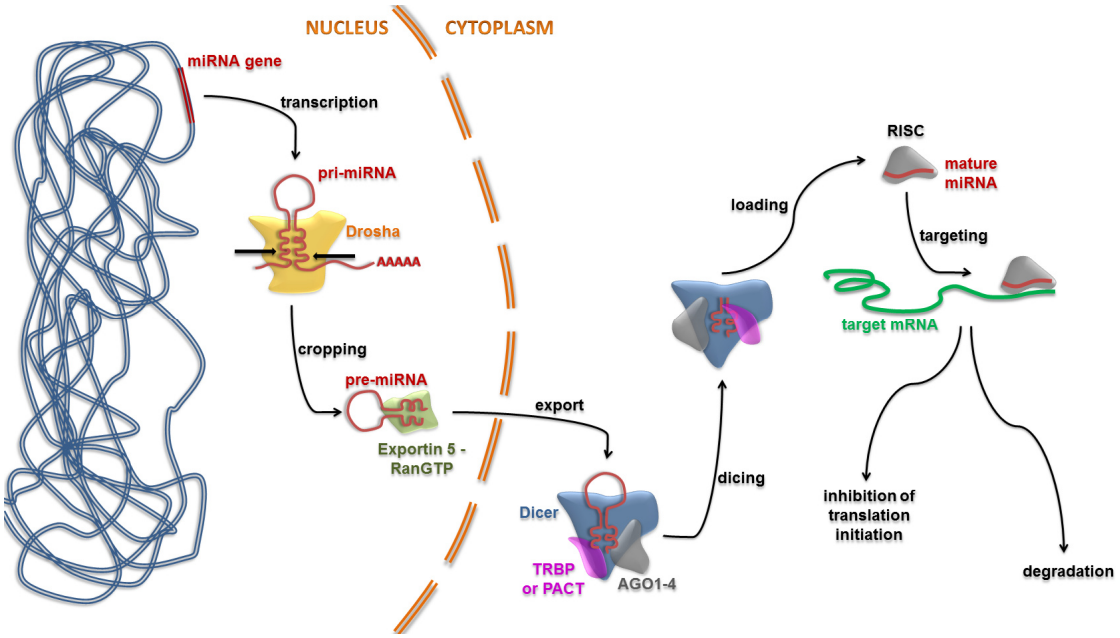

Figure 1. Schematic representation of canonical miR biogenesis. pri-miRNA – primary miR, pre-miRNA – precursor-miR. Adapted with changes from [47].

1.1.3.2 Role of miRs in controlling immune system

miRs have been demonstrated to be crucial for regulation of the immune response. They are involved in fine-tuning of the immune system assuring its well-orchestrated status since too strong or unnecessarily prolonged induction of the immune response can be harmful to the organism. Such quantitative regulation of gene expression has been implicated in controlling both innate and adaptive immune system branches on the several distinct levels, including immune cell

survival and differentiation as well as modulation of signaling downstream from immune receptors [49, 50].

1.1.3.2.1 miR-146a

miR-146a is a widely studied micro-RNA with diverse roles in cancer and immune system regulation [51]. miR-146a is expressed in response to a variety of microbial compounds and pro-inflammatory cytokines, being an NFκB-dependent gene [52]. TRAF6 and IRAK1 were found among the targets of miR-146a thus suggesting an impact on the signaling cascade downstream from TLRs and creating a negative feedback loop to activation of TLRs [52]. Indeed, expression of miR-146a has been correlated with LPS stimulation and found to be responsible for endotoxin self-tolerance [53, 54] and cross-tolerance to other TLR ligands [55]. miR-146a has also been implicated in the development of intestinal tolerance to microbiota in neonates, by regulation of cell survival, differentiation and establishment of mucosal homeostasis [56].

It has been observed that miR-146a is differentially expressed in DC subsets – with higher expression in Langerhans cells as compared with interstitial DCs. In addition, levels of this miR have been associated with DC cytokine production but not maturation state, and inversely correlated with responsiveness of cells to TLR2 signaling [57]. miR-146a has also been described as a negative regulator of IL-12 production in DCs [58] and could be induced by IL-10 in murine bone marrow-derived Mφs [59]. miR-146a promotes development of Mφs from the hematopoietic stem cells and its loss leads to myeloproliferative disorders [60, 61]. Of note, malfunctioning of miR-146a-based negative feedback loops were observed in Mφs isolated from aged mice [62]. Such Mφs displayed abnormally high levels of miR-146a and impaired responsiveness to LPS and pro-inflammatory cytokines [62]. Furthermore, miR-146a has been demonstrated to play a role in adaptive immunity. It was shown to modulate Treg-mediated regulation of Th1 response [63] and to be induced in human [64] and murine [65] T cells upon T cell receptor stimulation.

Recently it has been demonstrated that the expression of miR-146a in mononuclear cells isolated from circulation or pleural fluid from TB patients was decreased and accompanied by augmented levels of IL-6 and IL-1β [66].

1.2 MYCOBACTERIAL INFECTIONS

1.2.1 Tuberculosis

Tuberculosis (TB) is one of the oldest human diseases and its etiology and infectious character was described already in 1882 by Robert Koch [67]. In spite of many advances in understanding its pathogenesis made in past years TB still remains one of the leading cause of death from the infectious disease worldwide [68, 69]. WHO estimated that in 2010 there were 8.8 million TB cases worldwide and 1.5 million deaths from TB, including TB among HIV-positive persons [68]. The risk of developing active TB among Mtb-infected but generally healthy people is estimated to be about 10% during lifetime [70, 71]. In the majority of infected individuals Mtb infection remains latent without giving rise to symptoms of active disease for long periods of time.

TB is an airborne disease and transmission occurs from individuals with developed active pulmonary TB. In such patients TB lesions observed on lung X-rays are the results of lung tissue necrosis and cavity formation [69]. Upon rapture of such cavities and release to adjacent airways, the pathogen is carried by small aerosol droplets that are expelled by coughing, and when inhaled, can reach the lung of another individual [69]. It has been estimated that even single bacterium can give rise to the lung infection [72].

Cough is the most common symptom of pulmonary TB, and other symptoms comprise weight loss, lack of appetite, fever, malaise, and night sweats [71]. Among diagnostic tools available for TB is a tuberculin skin test, that is now being replaced by the blood-based tests assessing release of IFN-γ from Mtb-specific T cells [73]. Radiographic chest examination is also widely used to diagnose active TB and allows the doctors to assess the potential lung abnormalities related to TB lesions [71]. Laboratory diagnostic tools for identification of Mtb comprise direct detection of acid-fast bacilli by sputum smear microscopy, culture of mycobacteria isolated from patients' samples or identification of specific mycobacterial strains by molecular tests based on analysis of bacterial DNA [68, 74].

The mortality rate of non-treated TB is high. Studies from pre-chemotherapy era on smear-positive, HIV-negative population with pulmonary TB showed that almost 70% of those patients died within 10 years [75]. Nowadays, available therapy allows for control the disease fairy well. As estimated by WHO, in 2009 87% of new cases of TB were successfully treated but multi-drug resistant and extensively drug resistant TB emerged as important problems in fighting TB [68].

While the lung is the most common location of TB, extrapulmonary TB is diagnosed in 15-20% of immunocompetent individuals with lymph nodes, pleura and abdomen being the most often affected [71, 76].

1.2.1.1 Primary Mtb infection and latency

The primary Mtb infection starts when inhaled bacilli are taken up by phagocytic cells residing in lung alveoli. Even though Mφs are the main target cells for Mtb, DCs are also infected at the first stage of disease [77]. They get activated and migrate from the site of infection to the draining lymph nodes, where they present Mtb Ags to T cells. Yet, it has been observed that the entire process is delayed during Mtb infection and initiated not earlier than two weeks post-infection [78-80].

When the Mtb-infected cells reach lung tissue additional cells are recruited as a result of the initiated inflammatory response [81]. Structures called granulomas are formed around groups of Mφs infected with Mtb and are the hallmarks of pulmonary TB [82]. Granulomas comprise a core with Mtb-infected Mφs with a central necrotic area surrounded by a dense infiltrating leukocyte layer (CD4+ and CD8+ T cells, NK cells, B cells and neutrophils) [83-85]. Next, the entire structure is encapsulated by fibroblasts, which secrete an extracellular matrix shell providing an additional barrier separating infected cells from the lung tissue [86].

It is believed that containment of the Mtb infection is beneficial for the host as it limits the spread of the pathogens [87]. On the other hand, bacteria make use of granuloma as a site of multiplication and persistence [4, 84]. In addition, experiments on zebrafish embryos revealed that formation of granuloma at the very early stage of infection, before adaptive immunity develops, may contribute to spread of infection by massive influx of Mφs, the main target cells for Mtb [82]. Such Mtb-infected Mφs are able to leave primary granuloma and seed secondary infection foci [82]. Once the granulomas are formed the bacteria are able to persist there for years changing their metabolism to adapt to the hypoxic environment [81]. Maintaining a well-structured granuloma seems to be crucial for controlling the infection and is a result of the balance between host immune response and the potential of bacteria to multiply and disseminate [81].

The latent TB may last for years and never be reactivated [88]. However, upon immunosuppression the delicate equilibrium between immune response and bacteria may be disturbed and granulomas, being dynamic structures where the instant influx of new cells is necessary, become disrupted [87]. Such dysfunctional granulomas allow the bacteria to escape, spread, infect new cells and cause the reactivation of TB [84].

1.2.2 Molecular typing of mycobacteria

Mycobacterium tuberculosis complex (MTC) is a group of seven mycobacterial species causing TB, with Mtb as a major causative agent of human TB. The MTC consists of bacteria with identical 16S rRNA sequence and nucleotide identity higher than 99.9% [89]. Numerous different molecular tests were developed throughout the years in order to characterize strains belonging to this group.

Spoligotyping, or spacer oligonucleotide typing, is a widely used method to identify members of MTC. It is based on the hybridization of the sequences amplified by PCR to membrane-immobilized probes, and detects the presence of specific DNA spacer sequences in the direct repeat region in mycobacterial genome [90]. As result a pattern with possible 43 dots is obtained and is used to allocate an isolate to the specific cluster [90]. Spoligotyping is relatively cheap, allows for digitalization of the data (presence or absence of the specific spacer), may be employed in high-throughput screenings and possesses a fair discriminatory capacity. Yet, this method is not able to differentiate isolates within large families, requires specially tailored membranes and advanced laboratory equipment and a potential convergent evolution leading to obtaining same patterns for different strains may be a problem [90].

Restriction fragment length polymorphism (RFLP) with the insertion sequence 6110 (IS6110) as a probe, is another typing method to characterize MTC. Genomic DNA of mycobacteria is digested by restriction enzyme PvuII [91, 92]. Afterwards, obtained fragments are separated by electrophoresis and transferred to a membrane by Southern blotting. Hybridization with IS6110 probe allows for visualization of the results [91, 92]. IS6110 RFLP has a very good discriminatory power, however such an analysis requires a large amount of mycobacterial DNA therefore a long culture is necessary [90]. Additionally, some Mtb strains lack IS6110 and others have only a few copies that is too little to provide sufficient resolution [91].

Whole genome sequencing has an excellent discriminatory capacity and could be used as a high-resolution tool, where standard typing approaches are incapable of differentiating between strains. By whole genome sequencing minor differences between Mtb strains may be revealed, which is particularly important in case of organisms characterized by a general low genetic diversity such as MTC members [93, 94]. The correct interpretation of the sequences covering repetitive regions may however become a challenge [94]. Although this method still remains a research tool, the rapidly decreasing costs make it an interesting future genotyping method.

1.2.3 Mycobacterium tuberculosis

1.2.3.1 Bacterium

Mtb, a rod-shaped bacillus, is a major causative agent of TB. It is an obligate aerobe that requires oxygen to grow but has the ability to survive in hypoxic conditions [95]. It belongs to the genus *Mycobacteria*, which is divided into nontuberculous species and *Mycobacterium tuberculosis* complex (MTC) including *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microtii* and *M. canettii,* that cause disease in humans [96]. The Mtb genome is ~4.4 Mbp-long and was first fully sequenced in 1998 [97]. It is GC-rich and encodes approximately 4000 genes [97]. The characteristic feature of the mycobacterial genome when compared with other bacteria is a high content of sequences coding for enzymes engaged in lipid metabolism [97].

Mtb bacilli are slow growing bacteria with a long doubling time, about 24 hours, that contributes to the chronic character of disease caused by Mtb [95, 98]. Mtb is an intracellular bacterium, able to infect mononuclear phagocytes, namely Mφs and DCs [99]. Yet, it is still not clear if Mtb can actively multiply in DCs as it does in Mφs [99, 100]. Interestingly, Mtb DNA has been found in adipose tissue surrounding kidneys, stomach, lymph nodes or heart of the TB patients [101]. Additionally, mycobacterial DNA was detected in alveolar and interstitial Mφs as well as in type II pneumocytes, endothelial cells and fibroblasts of the samples from patients without tuberculous lung lesions [102].

1.2.3.2 Mycobacterial cell wall

The mycobacterial cell wall contains complex waxes and glycolipids. Different mycobacterial species show distinct sugar substitutions in the cell wall glycolipids or peptidoglycolipids [96]. Such a thick and impermeable cell wall is a passive barrier protecting a bacterium from potentially harmful water-soluble substances and it is physically and functionally different from bacterial cell membrane [71]. Its synthesis requires a panel of specialized enzymes that are engaged in lipogenesis and lipolysis [97].

The mycobacterial cell wall is acid-fast, which means that it retains carbolfuchsin dye during decolorizing with acid-ethanol that allows for identification of mycobacteria in host tissues and visualizing them in laboratory specimens (Figure 2). This method was first developed in 1882 as Ziehl-Neelsen staining [103] and is still used in diagnosis of TB.

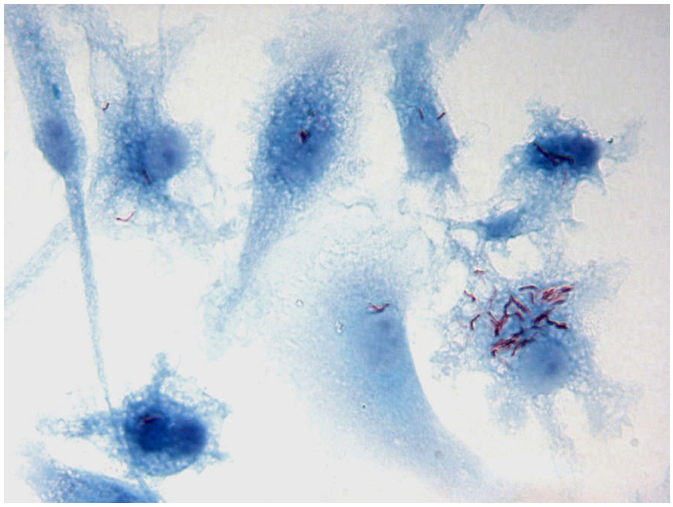

Figure 2. Acid fast staining of Mφs infected with Mtb. Mycobacteria are visualized as red rods that retained carbolfuchsin dye during destaining with ethanol-acid. Next, cells were counterstained with methylene blue.

A distinctive feature of the mycobacterial cell wall is a presence of highly structured macromolecules, peptidoglycan-arabinogalactan-mycolic acids and also different glycolipids, including phosphatidylinositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) [104] (Figure 3). A rigid scaffold is made of peptidoglycan linked to arabinogalactan, which is esterified at its distal, non-reducing end by the mycolic acids [105]. Glycolipids and proteins are localized between peptidoglycan and mycolic acid layers [105]. Phosphatidyloinositol (PI) anchors form foundations for mycobacterial glycolipids [105]. PIMs are PIs with several mannoses attached, and di- (PIM_2) and hexa-mannosylated (PIM_6) forms are the most abundant in the mycobacterial cell wall [106, 107]. LM is a PI with longer and branched mannose chain, while LAM possesses an arabinnan part [105, 108]. The cell wall of slow growing mycobacteria, e.g. *M. tuberculosis* and *M. bovis,* contains LAMs with mannose caps at the end of arabinnan branches – mannose-capped LAMs (ManLAMs) [109]. It has been observed that ManLAM from a single source is heterogeneous with regard to size, branching and acylation [104].

Structurally LAMs can be divided into ManLAM, PILAM and AraLAM. PILAM is LAM capped with phosphoinositides and is a part of the cell wall of fast growing mycobacteria (*M. smegmatis, M. fortuitum*), whereas AraLAM, specific for *M. chelonae,* is LAM lacking any capping motifs [110, 111]. PILAM binds to CD14 and TLR2 and induces production of chemokines and pro-inflammatory cytokines [110, 112-114]. It is also known to be cytotoxic, by inhibiting protein kinase C and impairing several intracellular pathways [115]. AraLAM is not as immuno-stimulatory and cytotoxic as PILAM, and the lack of the capping motifs has been associated with its lesser activity [116, 117]. Interestingly, both PILAM and AraLAM weakly interact with DC-SIGN, which confirms an affinity of this C-type lectin for highly mannosylated structures [118].

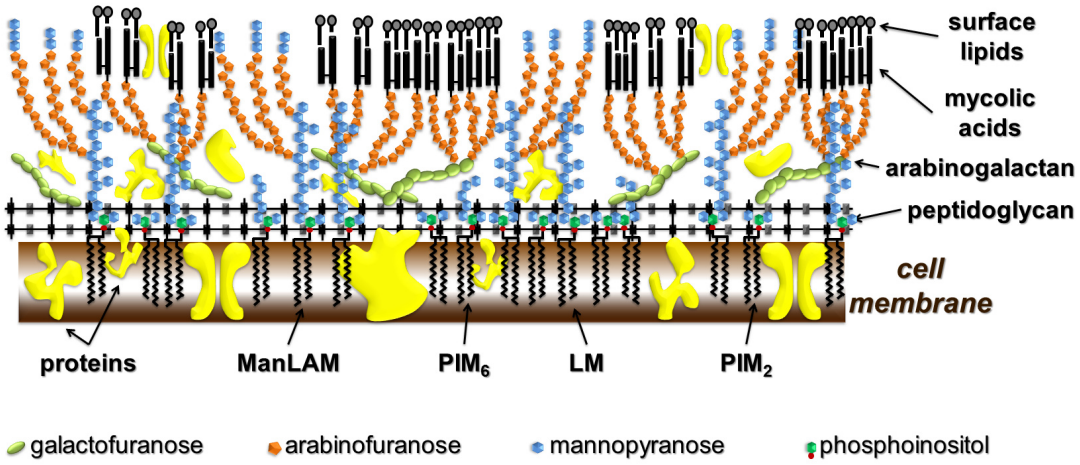

Figure 3. Schematic representation of mycobacterial cell wall structure. Adapted with changes from [104].

Many cell wall compounds were found to play a role in regulating the immune response to Mtb infection or to be engaged in modulating host cell mechanisms to facilitate bacterial growth and persistence [119-123]. The mycobacterial cell wall is also a challenge for development of new drugs as they have to penetrate through this thick, non-permeable, hydrophobic barrier [124].

Receptors on the host cell surface specifically interact with distinct mycobacterial cell wall components. MR, for instance, associates favorably with higher-order PIMs and this interaction is dependent on PIM acylation degree [125]. On the contrary, DC-SIGN equally well recognizes LM, ManLAM and PIMs of all mannosylation and acylation degrees [125]. Thus, structural differences between mycobacterial cell wall compounds influence the interaction of bacterium with host cell.

1.2.4 Immune system and mycobacteria

1.2.4.1 Activation and modulation of immunity in Mtb infection

The host defense against Mtb requires both a vigorous innate and cellular immune responses. Both Mφs and DCs are target cells for Mtb [71, 81, 126-128], and mycobacteria interact with receptors on the surface of these cells, which leads to internalization and infection. The main receptors engaged in such interactions on Mφs are MR and complement receptor-3 (CR-3), whereas DC-SIGN, which is expressed by freshly isolated lung DCs, plays a major role in infection of DCs [129].

It has been shown in the mouse model that DCs undergo functional activation following infection with bacillus Calmette–Guérin (BCG) *in vivo* and that they are able to initiate immune response to mycobacteria during early phase of infection [126]. At this phase Mtb are transported from the lungs to the draining lymph nodes by infected lung myeloid DCs [77]. However, such DCs are not fully efficient in initiating Mtb-specific CD4+ T cell responses, as presentation of mycobacterial Ags by MHC class II is limited without affecting expression level of MHC class II [77]. Moreover, Mtb has been demonstrated to interact with TLRs, namely, TLR2, TLR4 and TLR1/6 and thus to trigger a cascade of intracellular signaling leading to production of pro-inflammatory cytokines [130-132].

Importantly, the effect of mycobacterial infection is not limited merely to infected cells but may also in a bystander manner extend to remotely localized cells. For example, mycobacterial lipids were found in extracellular vesicles released by infected Mφs and also in uninfected bystander cells [133]. Mycobacterial Ags were found to be cross-presented to T cells by uninfected DCs [134]. Furthermore, it has been demonstrated *in vitro* that uninfected bystander DCs were preferentially matured during Mtb infection, whereas Mtb-infected DCs from the same culture displayed only minimal phenotypic maturation [135].

Another important feature of mycobacteria is their ability to manipulate the processes leading to the infected cell death [136]. The virulent strains predominantly cause necrosis rather than apoptosis [137, 138]. This is beneficial for mycobacteria because death by apoptosis maintains continuity of cell membrane and therefore restricts bacterial spread, while necrosis is associated with cell disruption and release of bacteria to the extracellular compartments [136, 139]. It is widely accepted that preventing apoptosis is related to prolonged bacterial survival in infected cells [140] possibly due to delayed activation of T cells [141-143]. Induction of apoptosis in infected cells is more pronounced for non-virulent mycobacteria, e.g. *M. bovis* BCG, *M. smegmatis* or the attenuated *M. tuberculosis* H37Ra strain [137, 140, 144, 145]. Interestingly, the avirulent Mtb strain H37Ra is able to induce apoptosis in bystander Mφs, however such a reaction is dependent on the direct contact of bystander Mφs with infected cell [146]. The apoptosis triggered by mycobacteria is controlled by miRs, such as let-7e or miR-155 [147, 148]. For example, induction of miR-155 in response to *M. bovis* BCG activates caspase 3 and triggers apoptosis in Mφs [147].

Mycobacteria are able to prevent acidification of phagosomes and fusion of phagosome with lysosomes [149]. Therefore mycobacteria residing in such phagosomes are protected from harsh conditions of the lysosome and able to avoid degradation, subsequent processing and presentation of mycobacterial Ags [150]. Mycobacterial glycolipid, ManLAM, is responsible for phagosomal maturation block [119, 120]. Such phagosomal maturation arrest creates a niche for mycobacteria to live in and multiply inside the phagocyte [150]. Interestingly, it is not quite clear whether mycobacteria multiply inside the phagosome. Some groups reported that mycobacteria are strictly confined to the phagosomal compartment, while others observed their translocation to the cytosol [128, 151-155]. These two scenarios are not

mutually exclusive and it is possible that both co-exist widening a niche in which Mtb may reside inside target cells.

In most cases a sufficient immune response develops to control the infection and to prevent from development of active TB disease. Dominant response of Th1-type seems to be critical and it is associated with activation of specific cells and production of cytokines that act as both effectors and regulators of immunity to mycobacterial infection [156]. Hence, an impaired immune system may allow for reactivation of latent infection or result in rapid progression of primary Mtb infection to disease [157]. This is often observed in latently infected patients undergoing cancer chemotherapy, HIV-positive individuals and those with compromised immune systems due to other pathological conditions or malnutrition. Conversely, an excessive and unbalanced immune response is also detrimental. It causes immunopathology with extensive necrosis in granulomas resulting in failure to contain and control Mtb infection and exacerbation of the disease [158-161].

Thus, the controlled inflammatory reaction to the mycobacterial infection is crucial for the formation and maintenance of granulomas, typical TB-related structures [162]. Well-sealed non-progressive granulomas ensure Mtb isolation and prevent from its spread but also provide a relatively favorable environment in which bacilli can survive for years [87].

1.2.4.1.1 Role of the cytokines

Mtb infection triggers the production of a large number of soluble mediators of inflammation. Many cytokines released in response to Mtb infection are essential for controlling the infection, but their over-expression may also lead to immunopathology [157]. Cytokine patterns expressed by distinct cell types differ and play complementary roles often balancing each other functions. Mφs, for instance, release mainly TNF, IL-1, IL-6 and IL-18, whereas DCs produce large amounts of TNF and IL-12 [127]. Both DCs and Mφs secrete IL-10, a potent inhibitor of IL-12 production [127, 163].

TNF is critical for controlling Mtb infection and its role is non-redundant. However, excessive production of TNF is detrimental and causes immunopathology [164], while its insufficient levels may lead to reactivation of latent Mtb infection [165]. The latter phenomenon is observed in rheumatoid arthritis patients undergoing TNF-neutralizing therapy [166]. Deficiency in production of TNF leads to inadequate activation of phagocytes and lack of proper production of chemokines which in turns impairs migration of cells to the site of infection, malformation of granulomas and spread of bacteria [156, 167].

Mtb induces IL-12 but its expression in the infected lung is not high. Still, local production of IL-12 within the draining lymph nodes seems to be crucial for initiation of optimal IFN-γ production that is critical for the control of the Mtb

infection [156]. Interestingly, people with congenital deficiencies in the IL-12/23-IFN-γ circuit displayed increased susceptibility to infections caused even by less virulent mycobacteria [168].

The role of IFN-γ in Mtb infection is widely acknowledged, as its protective effects have been observed both *in vitro* [169] and *in vivo* [170, 171]. This cytokine is produced mainly by CD4+ and CD8+ T cells but also by cells of innate immunity, including γδT cells, NKT cells, and NK cells. IFN-γ produced by innate cells may be especially important in Mtb-HIV co-infected individuals with a severe depletion in T cell compartments [156].

Another cytokine playing a major role in Mtb infection is IL-1. Experiments with knock-out mice lacking either IL-1 receptors or IL-1β itself revealed a high susceptibility of such animals to Mtb infection resulting in increased pulmonary bacterial loads and decreased survival [172-174]. In humans, the role of IL-1 signaling in host resistance to Mtb has also been appreciated. Studies investigating polymorphisms in IL-1 and its receptor genes revealed their correlation with TB progression and course of the disease [175, 176]. Blocking of IL-1 receptor is a part of rheumatoid arthritis therapy and, similarly to anti-TNF treatment, leads to increased risk of reactivation of TB [177, 178].

Mtb infection also induces abundant amount of IL-6. Its role has been associated with initiation of the appropriate T cell activation and production of IFN-γ [156]. Surprisingly, while Mtb infection of IL-6 knock-out mice first led to increase in bacterial burden, these mice were still able to control infection and develop protective immunity to secondary Mtb infection [179]. Also rheumatoid arthritis therapy with antibodies against IL-6R was not associated with an increased risk for TB incidence among treated patients [180].

IL-10 is an immuno-regulatory cytokine produced by many T cell subsets, B cells, neutrophils, Mφs, and some DC subsets and it plays a not yet fully defined role during Mtb infection [163]. Elevated IL-10 levels may lead to decreased production of TNF and IL-12p40, which in turn results in failure to control Mtb infection [156, 163, 181, 182]. Studies in IL-10-deficient mice generated contradictory results where either TB progression [183] or reduction [184] in bacterial load in both the lungs and spleens were observed.

1.2.4.1.2 Immunomodulatory roles of mycobacterial cell wall components

Surface-exposed mycobacterial glycolipids and glycopeptidolipids are known to interact with receptors on Mφs and DCs and facilitate phagocytosis and subsequent infection of such cells [107, 185].

ManLAM interacts with C-type lectins, such as mannose receptor (MR) and DC-SIGN [125]. The high content of ManLAM in the Mtb cell wall and its systemic presence during TB suggest that it may exert important immuno-modulatory

functions [186, 187]. Indeed, ManLAM stimulates phagocytosis by interacting with MR on Mφs and thus mediates binding and entry of Mtb to the host cell [188-190]. Another important feature of ManLAM is its ability to interfere with phagosome-lysosome fusion that in turn allows bacteria to survive inside phagocytes [119, 191]. ManLAM derived from the cell wall of virulent Mtb promotes NO production by activated Mφs [192]. Furthermore, such ManLAM specifically stimulated production of IL-1β and TNF by monocytic cells (THP-1) [193], it was able to trigger TNF release from human blood monocytes and murine Mφs as well as TNF and IL-1β expression in mice *in vivo* [122, 194, 195]. The ability of ManLAM to regulate IL-12 release from DCs is controversial. Some authors found this glycolipid to be unable to trigger IL-12, or even to inhibit IL-12 production induced by distinct signals [196, 197]. Other groups, including ours, found that ManLAM is actually a potent inducer of IL-12 from DCs and enhances effects of additional pro-inflammatory stimulus, i.e. LPS [198] (see also the Results and Discussion Section and *Paper I*). Of note, experiments with *M. bovis* BCG mutants lacking ManLAM capping motifs revealed that both mutated and wild type strains induce comparable levels of IL-12p40 and IL-10 from stimulated DCs [199].

The role of ManLAM has also been investigated in the context of apoptosis modulation. ManLAM was found to inhibit apoptosis caused by Mtb infection without interfering with NO and TNF production [123]. Moreover, ManLAM affects levels of calcium ions in Mφs in CD14- and MR-dependent manner [200], modulates DC function [198] and acts as chemoattractant to T cells, monocytes and Mφs [121, 201].

LMs derived from distinct mycobacterial species are able to induce apoptosis and production of TNF, IL-12 and IL-8 [116, 202]. Such a strong pro-inflammatory response triggered by LM results from its interaction and signaling through TLR2 [110, 203]. Interestingly, a recent finding identified Mtb LM as an inhibitor of TNF release rather than its inducer [204].

PIM-induced production of immuno-regulatory cytokine, IL-10, has been linked to suppression of the inflammatory response during allergic airway disease [205]. Additionally, PIM_2 is a chemoattractant for NKT cells [206]. Of note, PIMs, similarly to PILAM or LM, signal through TLR2 [207]. Furthermore, PIM_6, but not PIMs of lower mannosylation degree, interact with MR while PIMs of all orders could bind to DC-SIGN [125, 199]. Mycobacterial PIMs, independent of TLR2, are able to inhibit signaling from TLR4 leading to the production of NO, chemokines and pro-inflammatory cytokines [7, 208].

Finally, it has been postulated that a ratio between different glycolipids in the mycobacterial cell wall may be responsible for the virulence of specific bacterial species or strains [110]. Moreover, alterations in the cell wall composition related to the latent state of bacteria were observed [209]. Thus, it suggests that the mycobacterial cell wall is a dynamic structure and its composition may impact the immunogenicity and immunomodulatory properties of mycobacteria.

1.3 HUMAN IMMUNODEFICIENCY VIRUS (HIV)

1.3.1 HIV and AIDS – epidemiology

According to WHO, it was estimated that worldwide a total of 34 million people were living with HIV in 2010, and that yearly 2.7 million individuals were newly infected [210]. Globally there is a declining trend in the HIV incidence; however, there are clear differences between geographical regions. The incidence of HIV in highly affected Sub-Saharan regions of Africa noted a decreasing trend during the past years, whereas in other locations the incidence of HIV infection is still on the rise [210]. Annually, about 1.8 million people die from acquired immunodeficiency syndrome (AIDS)-related diseases, even though introduction of anti-retroviral therapy (ART) has been estimated to avert about 2.5 million deaths in low- and middle-income countries [210]. Thus, HIV is still a leading infectious cause of death worldwide [68]. There are two related types of HIV (HIV-1 and HIV-2) and both cause AIDS. HIV-1 is more pathogenic and infection caused by HIV-1 has resulted in the pandemic spread of HIV, while HIV-2, mainly endemic in West Africa, is the cause of AIDS in a reduced number of individuals with a slower disease progression rate [211-213].

1.3.2 HIV-1

1.3.2.1 Genome and structure

The HIV-1 is a member of the *Lentivirus* genus and the *Retroviridae* family, and is globally the major causative agent of AIDS [214]. The genome of HIV-1 consists of ~10 kb single-stranded RNA with nine open reading frames [215]. The HIV-1 genome codes for structural proteins (encoded by the *gag* gene), envelope proteins (encoded by the *env* gene), three enzymes (encoded by the *pol* gene) and six regulatory and accessory proteins (Tat, Rev, Nef, Vif, Vpr and Vpu) [216, 217] (Figure 4A).

The HIV-1 virion is enveloped by a lipid bilayer that is derived from the membrane of the infected host cell and is acquired by the virus during a budding process [218] (Figure 4B). Among proteins found in the viral particles are host-derived proteins, e.g. actin, ubiquitin, MHC molecules, and the viral envelope

glycoprotein complex, consisting of surface gp120 and trans-membrane gp41 proteins in trimeric form [217, 219]. Under the envelope there is a matrix layer made up by the matrix protein p17 that in turn surrounds a conical capsid built up by the capsid protein p24 [217]. The capsid contains two copies of viral genomic RNA [216]. HIV-1 RNAs are stabilized by nucleocapsid proteins p7 [217]. The capsid also contains three viral enzymes: protease, reverse transcriptase and integrase. During the life cycle HIV-1 produces also accessory and regulatory proteins engaged in regulation of virus replication and in host immune response evasion [214, 220].

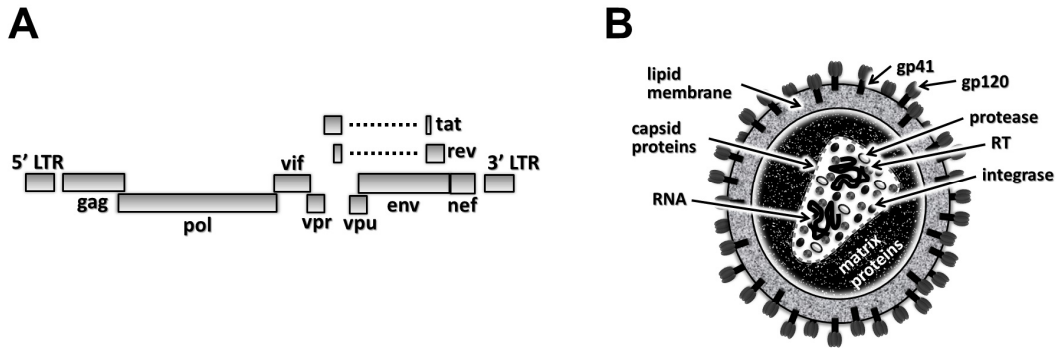

Figure 4. Schematic representations of HIV genome arrangement (A) and virion structure (B). Adapted with changes from [212, 216].

1.3.2.2 Life cycle

The very first stage of the HIV-1 infection involves virus interaction with a potential target cell. The primary receptor for HIV is CD4, but host cell entry also requires virus interaction with a co-receptor, usually CCR5 or CXCR4 [221]. The involvement of other receptors, such as C-type lectins, syndecans or galactosyl ceramide, has also been demonstrated to play role in virus attachment to the target cell that in turn may lead to enhancement of viral entry and infection [16, 214, 222-224].

Upon entering the host cell viral RNA is retro-transcribed into double-stranded DNA (dsDNA) by the viral reverse transcriptase enzyme. Then the dsDNA is translocated to the nucleus and subsequently inserted by the viral integrase into the host genome, where it can remain latent, as a provirus, as long as the cell is alive [216, 218]. With the help of the host cell transcription machinery the viral replication starts at the viral promoter region, long-terminal repeats (LTRs) (Figure 4A) [218]. The rate of the replication depends greatly on the activation state of infected cell, and therefore HIV-1 in proviral form may persist for years in resting cells and be activated upon their stimulation [225, 226]. From HIV-1 mRNAs new copies of viral genomic RNA and viral proteins are generated in a highly regulated manner in order to produce new viral particles [218]. Viral envelope proteins are processed by

the cellular machinery: endoplasmic reticulum and Golgi apparatus, and are eventually targeted to the cell membrane [218]. Two copies of genomic RNA are associated with nucleocapsid proteins and precursor Gag and Pol proteins, and then transported to the cell surface [215, 216]. There, the viral particle acquires its envelope consisting of cellular lipid bilayer and proteins which are anchored in it, including the viral envelope glycoproteins [215]. Lastly the viral protease enzyme cleaves the Gag and Pol precursor proteins allowing for the capsid formation and maturation of the budding virus and its release into the extracellular compartment [216].

HIV regulatory proteins play a major role in the HIV replication process. Tat is a powerful transactivator of the viral gene expression and Rev participates in stabilization and transport of singly- or unspliced viral transcripts from the nucleus to the cytoplasm allowing for the full synthesis of the virus [215, 216, 227]. In addition, Tat is secreted from infected cells and influences the neighboring cells in a bystander manner in order to modulate their functions, e.g. by their activation and increasing the expression of HIV co-receptors [228]. The accessory proteins, Nef, Vpu, Vif and Vpr, also participate in regulation of the virus replication but also counteract immune response mechanisms. Nef has been implicated in CD4 and MHC class I down-regulation on the infected cells affecting their function [215, 229]. Vpu participates in the release of newly produced viral particles from the cell surface, but also promotes CD4 degradation and interferes with CD1d expression and antigen presentation [216, 220, 230]. Vif neutralizes anti-viral cellular mechanisms, including APOBEC3 [220], while Vpr plays a role in nuclear translocation of the provirus, modulates the cell cycle and causes the cell division arrest or cell death [220].

1.3.2.3 Course of the HIV-1 infection

HIV-1 is most often transmitted upon sexual exposure, but the transmission may also occur upon contact with blood of infected persons e.g. during blood transfusion or by needle sharing between intravenous drug users [210]. Generally, virus particles and HIV-1-infected cells in blood and genital secretions are the main source of virus transmission [214]. Other body fluids, such as saliva, tears, urine and sweat may contain virus particles but due to antiviral immune components these viruses appear to be non-infectious, or markedly less infectious [214]. HIV-1 may also be transmitted from infected mothers to their children, either before birth in the uterus, during delivery or through breast milk at nursing of the newborn.

In principle, the course of the HIV infection can be divided into three stages:

1) The primary, or acute, infection phase with high levels of circulating virus in the peripheral blood before fully developed immunity;
2) The chronic infection phase, when the virus level is maintained at a stable level partially controlled by the immune system;
3) The AIDS phase, defined as immunodeficiency following CD4+ T cell number reduction below 200 cells/mm^3 and the onset of opportunistic infections and malignancies [214].

Primary HIV infection may be asymptomatic or manifested by a panel of flu-like symptoms, including fever, rash, muscle ache, and nausea [231]. Viral RNA can be found in the blood as early as two days after infection, and peak viremia appears about two to three weeks later [214]. Primary infection lasts for 4-6 months and is characterized by high HIV levels in the blood accompanied by immune system activation. During this time the number of CD4+ T cells in peripheral blood is transiently decreased while amount of CD8+ T cells is elevated [214, 232]. Next, CD4+ T cell level in the blood may return to almost normal value and then again steadily decrease over the course of HIV infection and progression to AIDS [214]. The fraction of infected, circulating CD4+ T cells rarely reaches 1% [233]. However, it has been demonstrated that CD4+ T cells in gut-associated lymphoid tissue (GALT) are not fully recovered in patients successfully treated with ART [234]. Furthermore, the same study revealed that the proportion of CD4+ T cells infected with HIV-1 was higher in GALT than among PBMCs and that a cross-infection between GALT and blood compartment was noted [234]. Yet, an overall CD4+ T depletion is not solely related to direct HIV infection of these cells. In addition, hyperactivation of the immune system caused by chronic HIV-1 Ag exposure and persistent inflammation, together with impaired mechanisms responsible for virus eradication, lead to increased CD4+ cell turnover and CD4+ cell depletion [235-237]. Abs against HIV proteins usually appear 1-2 weeks post-infection [214]. Among Abs produced as a result of HIV infection are neutralizing Abs, however, virus escape mutants constantly emerge making these Abs less effective [238]. Also non-neutralizing and even enhancing Abs, that may opsonize the virus and facilitate its entry to the phagocytic cells, develop [239, 240].

The chronic stage of the infection, with clinical latency, defined as an asymptomatic phase with no AIDS symptoms usually lasts around ten years in untreated individuals, but upon initiation of antiretroviral therapy (ART) the asymptomatic phase may be significantly prolonged [241, 242]. According to WHO guidelines, AIDS is diagnosed when blood CD4+ T cell count is less than 200/mm^3 and opportunistic infections or malignancies develop, including pulmonary tuberculosis, recurrent pneumonia, invasive cervical cancer and several other clinical conditions [243].

The virus phenotype may influence the course of HIV-1 infection (reviewed in [244]). The switch in HIV-1 co-receptor use, from CCR5 to CXCR4, as observed in 30-80% of AIDS patients depending on the HIV-1 subtype, has been associated with accelerated disease progression [245]. However, the remaining proportion of infected individuals may eventually also progress to AIDS despite harboring virus only restricted to CCR5 [244].

Nowadays, upon implementation of ART the progress to AIDS is slowed down. Such a therapy includes several anti-retroviral drugs targeting distinct stages of the HIV-1 replication cycle. Recommended drug combination should include at least three drugs targeting more than one step of the HIV-1 replication cycle [246]. Commonly used drugs belong to reverse transcriptase inhibitors, integrase inhibitors, protease inhibitors and HIV-1 co-receptor inhibitors [246, 247]. Drug resistance, either primary, when an individual is infected with resistant HIV, or acquired during ART, is an emerging problem [247, 248]. In addition, possible side effects, drug-related toxicity and development of malignancies must be taken into consideration when choosing the therapy [242]. Successful ART results in reduction of viral load to residual low levels, but at present there is no therapy available that leads to complete elimination of the virus [249, 250].

1.3.2.4 Cells of innate immunity and their interactions with the virus

The main target cells for HIV-1 are the CD4+ T cells which efficiently support viral replication and virus spread to the new cells. Still, the spectrum of cells infected by HIV-1 is broad, including Mφs and DCs. Even though the route of HIV-1 transmission may vary, APCs can often be the first cells to encounter the virus. These cells may either be infected or the virus can also bind to them for transfer from the periphery to lymph nodes and subsequent transmission to CD4+ T cells, in a process called *trans*-infection. Other innate immune cells, including NK, NKT and γδT cells may also play important roles as HIV targets, HIV reservoirs and by modulating immune response to the viral infection.

1.3.2.4.1 Dendritic cells and HIV-1

Langerhans cells and other DCs present in the skin, in the mucosa and in the blood express CD4, CCR5 and CXCR4 and therefore they often become infected with HIV-1. These cells may then spread the virus to other cell types. Studies on simian immunodeficiency virus (SIV) infection in *Rhesus macaques* revealed that mucosa-associated DCs bound or were infected by the virus and subsequently could transmit the virus to CD4+ T cells [251, 252]. Additionally, vaginal and ectocervical DCs, but not Mφs or lymphocytes from the same localization, have been shown to be the first

cells infected with HIV-1, whereas four days later viral replication was mostly associated with lymphocytes [253]. It has also been reported that both types of DC subsets, mDCs and pDCs, are susceptible to infection with CCR5- and CXCR4-restricted viruses *in vitro* [254]. The replication in DCs appears, however, to be much less productive than in the CD4+ T cells and number of infected DCs *in vivo* is much smaller as compared with HIV-infected CD4+ T cells [255, 256]. The lower virus production of DC has been ascribed to relatively low levels of HIV-1 receptors on the DC surface, fast degradation of internalized virions and existence of intrinsic HIV-limiting cellular factors, e.g. APOBEC3G [16, 257-259] and SAMHD1 [260, 261].

Interestingly, DCs can efficiently mediate HIV-1 infection of T cells. This mechanism, known as DC-mediated *trans*-infection or *trans*-enhancement, is a multi-stage process. DCs may transmit HIV-1 to target cells without being infected, which is possible when virus is taken up by DCs, while not degraded, and later released. It has been suggested that C-type lectins, including DC-SIGN and MR, play role in the DC-mediated HIV *trans*-infection [259, 262]. Transmission of the virus usually occurs when DCs get into close contact with CD4+ T cells during formation of the immunological synapse, in this case known as the virological synapse. As reported, productive HIV infection of DCs is required for long-term virus transmission, while shortly after virus exposure DCs mediate *trans*-infection to T cells without being infected [258, 263, 264].

DCs are also known as virus reservoirs. This is particularly well established for follicular DCs, which reside in the lymph nodes and may capture virus without getting productively infected with HIV-1. In spite of that, they may play a major role in HIV-1 pathogenesis because of their exceptionally high pathogen harboring capacity [265]. Soon after infection large quantities of virions can be trapped on the surface of follicular DCs and retained there, while maintaining their high infectivity even in the presence of neutralizing Abs [266]. Furthermore, location of follicular DCs in the lymph nodes in proximity to a pool of CD4+ T cells that are particularly susceptible to HIV infection helps to spread the virus over long periods of time [16]. Such an accumulation of the virus occurs rapidly, before the symptoms related to infection are recognized and treatment is implemented [267].

Notably, DC function is modulated by HIV-1 contributing to HIV-associated immune system dysregulation. DCs isolated from HIV-positive individuals display down-regulated levels of CD80 and CD86 co-stimulatory molecules which results in impaired efficiency in stimulation of T cells [16]. In addition, HIV-1 accessory proteins are engaged in HIV immune system evasion and DC-mediated spread of the virus, e.g. Tat induces release of chemoattractants facilitating interaction of T cells with HIV-1-infected DCs and Nef is able to down-modulate Ag presenting molecules on the DC surface [16].

1.3.2.4.2 Macrophages and HIV-1

Upon sexual contact Mφs residing in the subepithelial mucosa can also be infected by HIV-1 [268]. Level of virus replication in Mφs is rather low owing to intracellular limiting mechanisms. Despite this, it is thought that Mφs may constitute an important virus reservoir. Although it is a resting memory CD4+ T cell population that is a main HIV reservoir, also Mφs are considered to harbor infectious viral particles for a long time [249]. Studies have suggested that even during successful ART HIV-1 may remain hidden in Mφs [269]. HIV-infected Mφs can survive in the body for several weeks and even after prolonged periods of time efficiently transmit the virus to the CD4+ T cells [270, 271]. Chronically infected Mφs are able to start producing new viral particles upon stimulation with pro-inflammatory cytokines, namely TNF, IL-6, IL-1β or IL-18 [272-274].

Interestingly, a model where Mφs could be differently activated during progression of HIV infection has been proposed. According to this concept first stage of infection is accompanied by the presence of classically activated Mφs with rapid formation of viral reservoirs [275]. Subsequently, alternatively activated Mφs appear followed by augmented production of IL-10 resulting in deactivation stage of Mφs as seen at the late stages of AIDS [275].

The ability of primary Mφs to support HIV replication *in vitro* is variable and donor-dependent [276]. Studies on HIV isolates derived from the patients in whom there was no co-receptor switch and the virus remained strictly CCR5-tropic have shown that viruses isolated at the later stage were more prone to active replication in Mφs than viruses appearing early on [277].

It has been observed that infection with HIV-1 results in manipulation of the functions of Mφs to facilitate viral spread and hampers Mφ antimicrobial mechanisms [276]. For instance, Nef protein induces release of MIP-1α and MIP-1β, which trigger recruitment of T cell to the site of infection and enable spread of the infection [278]. Interestingly, HIV-1 has been suggested to make use of cellular autophagosomal machinery in Mφs to process Gag protein while blocking later stages of autophagy in order to protect viral Ags from presentation in MHC class II context [279]. Moreover, the ability of Mφs to phagocytose opsonized pathogens has been shown to be impaired during HIV infection [280].

1.3.3 Pathogenesis of HIV-1 infection

HIV-1 infection eventually leads to CD4+ T cell depletion, which may be explained by dysregulation of the immune system that results in malfunctions of the tight control of the activation and survival of cells, followed by chronic immune

activation and exhaustion. In addition HIV-1 infection has been linked to suppression of hematopoiesis, changes in phenotypes of circulating lymphocytes and B cell abnormalities such as polyclonal activation or lack of Ab response to new antigens [214]. Cells of innate immunity are also severely affected, as observed by reduced phagocytosis, decreased Ag presentation capacity and modified cytokine and chemokine profiles [281].

General dysregulation of the immune system leads not only to impaired response to the pathogens, but also affects the normal function of many organs, including heart, kidneys, lungs and joints [214]. In addition, HIV-1 infection has been associated with several malignancies caused either by loss of anti-cancer immunity or by increased proliferation of cancerous cells triggered by the pro-inflammatory conditions [282, 283]. Kaposi's sarcoma is a very common malignancy seen during HIV infection and is associated with human herpesvirus 8 [284]. Other dysplasia often found in HIV patients is human papilloma virus-associated cervical cancer and Epstein-Barr virus-associated lymphoma that both are AIDS-defining conditions [243].

Another common complication related to the HIV-1 infection is an injury of the nervous system. Neurological dysfunctions might derive from a direct viral infection, toxicity of viral proteins, emergence of immunosuppression-related opportunistic infections, HIV-associated cancers, side effects of ART, but most often they display multiple etiologies [285].

Overall the time from the infection to the development of AIDS may vary and depends on many factors, including genetic host factors, viral factors, other co-infecting pathogens, e.g. Mtb, affecting the immune system of the HIV-infected individual, and also so-called life-style factors such as alcohol abuse, smoking and drug use [214].

1.4 CO-INFECTIONS WITH MTB AND HIV-1

1.4.1 Epidemiology and clinical considerations

According to WHO in 2010 there was 0.35 million deaths from TB associated with HIV infection and 13% of TB cases occurred among HIV-infected people. The likelihood of developing active TB among HIV-positive individuals is 21-34 times greater than among those that are not HIV-infected [286]. In other words, the risk of developing active TB in immunocompetent adults is estimated to be 5-10% during lifetime, while in HIV-infected people it is increased to 5-15% annually [287]. Generally, HIV infection is the strongest risk factor for developing TB [288]. Also the likelihood of re-infection with Mtb is augmented in HIV-positive individuals due to an impaired ability to mount long-lasting protective immunity [289].

The diagnosis of TB is challenging in the HIV co-infection setting. The sensitivity of conventional tests diagnosing Mtb infection is decreased and additionally the clinical presentation of TB might be atypical [290]. Implementation of ART has resulted in a rise of CD4+ T cell count and partial immune system restoration, that in turn has significantly decreased TB incidence [288]. ART also correlates with drop in number of extrapulmonary TB cases [291]. Nonetheless, the overall TB incidence rate for HIV-positive individuals undergoing successful ART still remains at least two-fold higher than for those without HIV [288].

Paradoxically, introduction of ART for some co-infected patients may result in clinical deteriorations. Immune reconstitution inflammatory syndrome (IRIS) appears when ART-related recovery of the immune system leads to excessive inflammatory responses against pathogens and/or tumors [288]. It is manifested by high fever, respiratory and renal failures, lymphadenopathies, and generally worsening of the infection symptoms [288, 292]. TB-associated IRIS either accelerates inflammatory reaction to ongoing active TB or activates latent, so far undiagnosed Mtb infection [293]. The incidence of TB-associated IRIS reaches 40% depending on many associated conditions, and appears usually within first 4-8 weeks after ART initiation [294-296]. In addition to common manifestations of IRIS, a TB-associated one is related to augmentation of pulmonary infiltrates and expansion of lung lesions [297, 298].

The general dysregulation of the immune system during Mtb-HIV-1 co-infection leads to rapid progression of both diseases. Disrupted homeostasis between pro- and anti-inflammatory cytokines, manipulation of apoptosis processes and impaired effector functions of immune cells account for detrimental pathogen synergy in people concurrently infected with HIV-1 and Mtb [299].

1.4.2 Mechanisms of Mtb-HIV-1 synergy

1.4.2.1 Impact of HIV-1 on TB progression

HIV infection has a great impact on general immune system homeostasis that leads to immunosuppression and failure in controlling Mtb infection. This includes altering of the cytokine and chemokine production and Ag presentation, apoptosis dysregulation, disruption of granuloma structures and control of dormant Mtb infection [300].

The risk of developing active TB in HIV-1-positive patients is higher than in HIV-uninfected individuals and is inversely correlated with CD4+ T cell count [301, 302]. In addition, not only the number, but also functionality of CD4 T cells in bronchoalveolar lavage (BAL) of co-infected patients is impaired, i.e. their ability to release cytokines upon mycobacteria-specific stimulation [303].

Production of cytokines in response to Mtb infection is highly modulated by HIV-1. It has been demonstrated that TNF production by Mtb-infected alveolar Mφs isolated from HIV-1-positive patients was reduced as compared with cells from HIV-negative individuals [304]. Impaired TNF production was also noted *in situ* in lung granulomas of Mtb-HIV-1 co-infected individuals and this was accompanied by the presence of abnormally formed granulomas [305]. This effect was assigned to HIV protein Nef that dampens the Mtb-triggered production of TNF in Mφs [306]. Also IFN-γ production is defective in HIV-positive persons, which may explain the decreased sensitivity of TB diagnostic tests based on Mtb-specific IFN-γ release [307, 308]. IL-10 is known to be released as the result of high levels of IL-6 and TNF, and in this way inhibit excessive inflammatory response in a negative feedback manner [309]. Interestingly, the IL-10 level in BAL fluid from HIV-positive individuals is elevated when compared with HIV-negative BAL, which suggests that Th1-dependent control of Mtb infection is down-regulated [310]. IL-10 prevents phagosome maturation during Mtb infection [311], suggesting that HIV-triggered conditions may help Mtb to survive and multiply in the lungs.

Strikingly, Mtb-specific CD4+ T cells are highly susceptible to infection with HIV and are preferentially depleted during course of the co-infection [312, 313]. Also HIV-infected Mφs facilitate intracellular growth of mycobacteria [314, 315]. In case of alveolar Mφs it could be the result of Mtb-induced apoptosis observed in cells obtained from HIV-1-positive individuals when compared with cells obtained from patients infected with Mtb only [304, 310]. Of note, the ability of Mtb to arrest phagosome-lysosome fusion is maintained in Mtb-HIV-1 co-infected patients [128].

It is well established that the architecture of granuloma in co-infected individuals is impaired [300, 305]. In addition, comparative *in situ* cytokine staining of granulomas derived from Mtb-infected versus Mtb-HIV-1 co-infected patients showed reduced levels of TNF in granulomas from co-infected individuals [305].

There are several factors affecting granuloma containment of Mtb that may lead to reactivation of latent TB in HIV-1 co-infected individuals. Firstly, systemic CD4+ T cell depletion during HIV-1 infection impairs T cell trafficking into the granuloma and destabilizes its organization [316]. Secondly, function of granuloma Mφs is affected in the context of cytokine production, e.g. TNF, which results in dysproportions between pro- and anti-inflammatory milieu inside granuloma [317]. Thirdly, HIV-1 preference to replicate inside activated cells makes granuloma an ideal environment for the virus to be produced in T cells and Mφs. This results in an increased HIV-1-induced cell death and exhaustion and hence, failure in controlling Mtb infection [300].

1.4.2.2 Impact of Mtb on HIV-1 pathogenesis

Mtb infection accelerates the course of HIV-1 infection mainly by dysregulation of the immune system. Perturbation of the balance between pro- and anti-inflammatory cytokine production, chemokines and impaired apoptosis processes account for the dissemination of both pathogens in the co-infected host [299, 318].

Clinical observations suggested that inflammatory conditions accompanying Mtb infection promote replication of HIV-1 [319]. Higher HIV-1 protein levels were noted in the BAL fluid from TB-involved lungs compared with uninvolved segments, and they were correlated with high TNF concentration [320]. In addition, the viral load in BAL fluid was higher than that measured in the plasma, which suggested Mtb-infected lungs to be a site of active and extensive viral replication [320]. Similar observations were made when viral burden in pleural fluid was compared with that in plasma of patients with pleural TB; increased viral loads were accompanied by elevated concentrations of pro-inflammatory cytokines in the pleural fluid [321].

Detailed *in vitro* studies provided information about the impact of pro-inflammatory cytokines on activation of HIV-1 LTR. TNF, IL-6 and IL-1β and a direct contact between activated lymphocytes and Mφs were found to be responsible for enhanced HIV-1 replication [322]. For example, TNF-activated NFκB can bind to HIV-1 LTRs and thus enhance transcription of viral proteins [323].

Interestingly, not only viral load but also virus heterogeneity is augmented both at the site of Mtb infection and systemically in co-infected individuals [324]. In HIV-1 samples obtained from dually infected patients frequencies of mutations are higher than in virus isolates from HIV-1-infected individuals without active TB [325]. Moreover, the spectrum of HIV-1 may be diversified even within one organ. This has been shown by Nakata *et al*. who discovered that viruses isolated from the lung segments that harbored Mtb contained a more diverse quasi-species than viruses from Mtb-free parts of the lung [320].

DCs infected with Mtb have also been shown to be more efficient in mediating HIV *trans*-infection to T cells than uninfected DCs [326]. Furthermore, we recently showed that in the presence of Mtb-infected Mφs DCs increase their HIV *trans*-infecting ability in a bystander fashion, i.e. without coming into direct contact with either bacteria or bacteria-infected Mφs (*Paper III*) [327]. Thus, such an increase is merely dependent on the impact of soluble factors released by Mtb-infected Mφs [327].

The Mtb infection also affects the expression of HIV co-receptors. CCR5 on PBMCs from dually infected individuals is overexpressed when compared with cells from individuals with HIV infection alone [328]. Furthermore, the mycobacterial cell wall compound, ManLAM, induces CCR5 and CXCR4 up-regulation on CD4+ T cells *in vitro* [329]. Interestingly, CXCR4 is also elevated on alveolar Mφs isolated from TB patients [330]. Such an alteration has been associated with augmented susceptibility of cells to CXCR4-, but not CCR5-using HIV viruses, which suggests that concurrent Mtb infection may accelerate progress to AIDS by promoting switch from CCR5 to CXCR4 viruses. Finally, a recent study in a mouse model of Mtb infection showed that both Ab and cell-mediated responses to HIV Ags, used as a potential vaccine, are impaired by a concurrent Mtb infection [331].

2 THESIS AIMS

The main goal of this thesis was to examine the interactions between *Mycobacterium tuberculosis* (Mtb) and human immunodeficiency virus type 1 (HIV-1), with the special focus on the interplay between these pathogens and the innate immune system.

More specifically the aims of the projects included:

- characterization of glycolipids isolated from the mycobacterial cell wall and their effects on dendritic cell maturation and secreted cytokine profiles using an *in vitro* model based on monocyte-derived dendritic cells (*paper I*);

- studies of Mtb clinical isolates and their ability to infect and induce cytokine production in monocyte-derived macrophages (*papers II and III*);

- investigation of the influence of mycobacteria-infected bystander macrophages on the function of dendritic cells, especially in the context of maturation and their ability to mediate HIV-1 *trans*-infection of T cells in an *in vitro* co-infection model (*paper III*);

- examination of the response of HIV-1-pre-exposed macrophages to subsequent Mtb infection; including mechanisms behind impaired cytokine response and its correlation with HIV-induced miR-146a expression (*paper IV).*

3 MATERIALS AND METHODS

Detailed descriptions of the methods used in the projects are given in the respective papers and in the manuscript. Here, descriptions of the methods specific for this thesis and non-commercially available reagents are given.

3.1 MYCOBACTERIAL GLYCOLIPIDS

ManLAM and PIMs from the Mtb strain H37Rv or from *M. bovis* were purified using a dry cell wall as a starting material. Such dry cell walls were obtained from a large scale mycobacterial culture in the Middlebrook 7H9 medium. Dry material was rehydrated in PBS and sonicated. Next, an extraction in 40% phenol for 1 h at 70 ^{o}C resulted in obtaining two phases. The water phase was dialyzed against water and subjected to affinity chromatography on the Concanavalin A-Sepharose column. The retained fraction, the mannose-rich material, was eluted and next subjected to hydrophobic interaction chromatography on the Phenyl-Sepharose column. After elution the hydrophobic material containing aggregated ManLAM and PIMs was subjected to a dissociation step using 0.5% sodium deoxycholate for 24 h. Such material was thereafter fractionated by gel filtration on a Sephacryl S-100 column in the presence of 0.25% sodium deoxycholate. Fractions containing either ManLAM or PIMs were then identified by immunodot with the specific mAbs, pooled and dialyzed against water in order to remove sodium deoxycholate from the preparations.

The hydrophobic phase generated during phenol extraction of the cell wall was a rich source of PIMs. The hydrophobic phase was washed three times with PBS and mixed with an equal volume of 2% SDS in PBS and incubated overnight with a constant stirring. Next, the solution was centrifuged and water phase was collected and mixed with 9 volumes of ice-cold ethanol. After centrifugation a precipitate was obtained and solubilized with 10 mM Tris-HCl, 0.5 M NaCl, 2 mM EDTA and 0.25% sodium deoxycholate buffer. The material was then subjected to gel filtration on a Sephacryl S-100 column. Fractions containing PIMs were identified by immunodot with anti-PIMs mAbs, pooled and dialyzed against water.

3.2 MYCOBACTERIAL STRAINS

Mycobacterial strains used in the studies are listed in Table 1.

Table 1. Mycobacterial strains.

Name	Species	Description	Reference	Included in Paper:
BCG	*M. bovis* BCG	Attenuated vaccine strain, bacillus Calmette-Guerin	[332]	III
BTB05-552	*M. tuberculosis*	Clinical isolate of the Swedish outbreak caused by the cluster SMI-049, isolated in 2005	[333]	II and III
BTB05-559	*M. tuberculosis*	Clinical isolate of the Swedish outbreak caused by the cluster SMI-049, isolated in 2005	[333]	II
H37Rv	*M. tuberculosis*	Frequently used as reference laboratory-adapted Mtb strain, first isolated in 1905	[334, 335]	I
H37Rv-GFP	*M. tuberculosis*	H37Rv strain expressing green fluorescent protein (GFP)	[336, 337]	IV
Harlingen	*M. tuberculosis*	Clinical isolate of the tuberculosis outbreak in the Netherlands	[338]	III
M. bovis	*M. bovis*	Member of Mtb complex, major causative agent of bovine tuberculosis	[339]	I
S96-129	*M. tuberculosis*	First clinical isolate of the Swedish outbreak caused by the cluster SMI-049, isolated in 1996	[333]	II and III

3.3 HIV-1 ISOLATE

The primary HIV-1 isolate used in the studies described in *papers III* and *IV* originated from an asymptomatic patient [340]. This virus had been characterized to be CCR5-restricted and since its isolation has been passaged limited number of times in the laboratory.

3.4 BYSTANDER IN VITRO MODEL

In order to study bystander effects of an ongoing Mtb infection on the function of DCs an *in vitro* model was established. It consisted of Mφs pre-infected with different mycobacterial strains and of DCs exposed to such Mφs. By the use of the semi-permeable membrane, pore size 0.2 µm, separating DCs from Mφs investigation of the interplay between DCs and infected Mφs compartments was enabled. Such a DCs-Mφs cross-talk was only dependent on the exchange of soluble factors released by cells or derived from bacteria, but without possibility for cells or entire bacteria to cross the membrane. The model and its *in vivo* context are depicted in Figure 5.

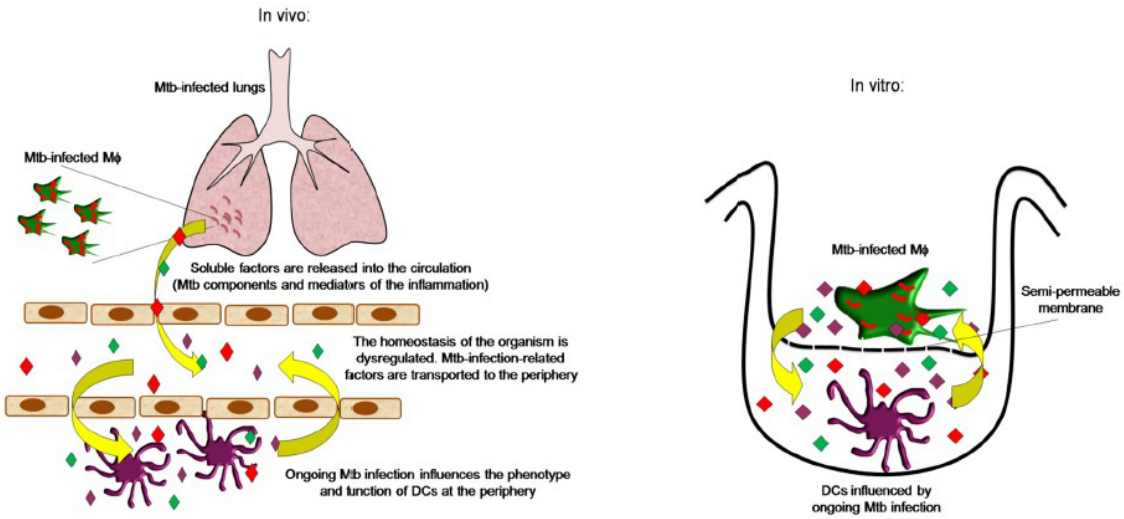

Figure 5. Schematic representation of the *in vivo* context and the bystander *in vitro* model.

4 RESULTS AND DISCUSSION

4.1 MYCOBACTERIAL GLYCOLIPIDS AND THEIR INTERACTIONS WITH DENDRITIC CELLS (*PAPER I*)

Mannose-capped lipoarabinomannan (ManLAM) and PIMs are glycolipids present in the mycobacterial cell wall. Both have been demonstrated to modulate immune response to Mtb [7, 110, 122, 199, 208]. However, some discrepancies have emerged throughout the years of studying ManLAM as for its ability to influence immune cell function. Some groups have reported pro-inflammatory properties of ManLAM [194, 341, 342], while others demonstrated an anti-inflammatory activity [197, 343-345].

In this study we used highly purified ManLAM and PIMs in order to dissect their properties and study their abilities to modulate DC function. We found that ManLAM was able to trigger production of pro-inflammatory cytokines from human monocyte-derived DCs and caused their maturation (Figure 6A-D). Interestingly, release of TNF and IL-12p40 by DCs exposed to ManLAM in combination with LPS was further potentiated as compared to production triggered by LPS alone (Figure 6A-B). PIMs on the other hand were not able to activate DCs. Instead, PIMs added together with LPS could inhibit production of pro-inflammatory cytokines as compared with amounts released upon stimulation with LPS alone (Figure 6A-B). The ability of ManLAM to induce IL-12 is especially striking. In our study ManLAM alone and in combination with LPS was a potent IL-12p40 inducer (Figure 6B), while others reported ManLAM-dependent inhibition of IL-12 released from human DCs upon LPS stimulation [197] or, alternatively, that ManLAM was unable to induce IL-12p40 production from murine Mφs [346]. Yet, recently another report demonstrated that levels of IL-12 produced upon stimulation of DCs with ManLAM in combination with LPS were higher than those observed for LPS treatment alone [198]. Gringhuis *et al*. also mentioned, without showing the data however, that exposure of cells to ManLAM alone did not result in IL-12 induction [198].

Furthermore, in our study, ManLAM triggered up-regulation of DC maturation markers, namely CD80, CD86 and MHC class II and co-stimulation of DCs with ManLAM and LPS resulted in even higher levels of these markers on DC surface. PIMs were not able to activate DCs in this context and DCs exposed to both PIMs and LPS displayed less MHC class II molecules than cells exposed to LPS alone.

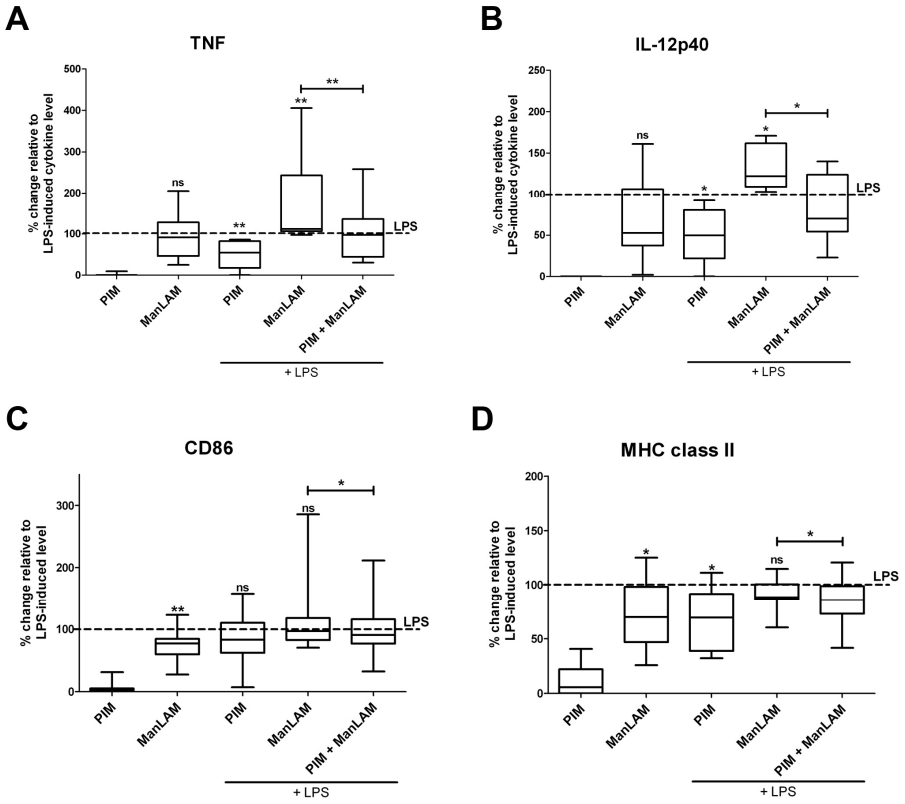

Figure 6. Cytokine release by DCs exposed to glycolipids. Normalized levels of (A) TNF, (B) IL-12p40, (C) CD86 and (D) MHC class II released during 24 hours upon exposure to ManLAM, PIMs and/or LPS at the concentration of 10 µg/ml, 5 µg/ml and 100 ng/ml, respectively. The level of cytokines released upon exposure to LPS (100 ng/ml) is shown as 100% of activation (dashed line). Background levels (not-treated cells) are 0%. The median percentage change in cytokine production is shown as a line. The box defines the 75th and 25th percentiles and the whiskers define the maximum and minimum values of 3–9 donors/group. Groups significantly different from LPS-treated control are labeled with asterisks. Vertical bars designate significant differences between treatment groups. Wilcoxon matched pair test was used to assess statistical significance (* $p < 0.05$, ** $p < 0.01$).

Several additional experiments were performed in order to rule out contamination of our ManLAM preparation, thus excluding that LPS could be responsible for stimulatory effects observed in our study. ManLAM prepared in our laboratory was found to be of high quality as demonstrated by silver staining (Figure 7A) and Western blotting with ManLAM-specific mAbs (Figure 7B). Additionally, analysis by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) revealed characteristic for ManLAM sugar content (Figure 7C) and capping pattern (Figure 7D). Also, pre-incubation of LPS or ManLAM with ManLAM-specific mAbs revealed that observed immuno-stimulatory feature of ManLAM was not LPS-dependent (Figure 7E). Furthermore, TLR-4 activation triggered by ManLAM was much weaker than that mediated by LPS and comparable to that displayed by PIMs (Figure 7F). Similarly, endotoxin-like activity observed for ManLAM and LPS in the limulus amebocyte lysate (LAL) test could be inhibited by anti-ManLAM mAbs for

ManLAM. These mAbs were not inhibitory for LPS, thereby showing that LAL positive result for ManLAM was not LPS-derived (data not shown).

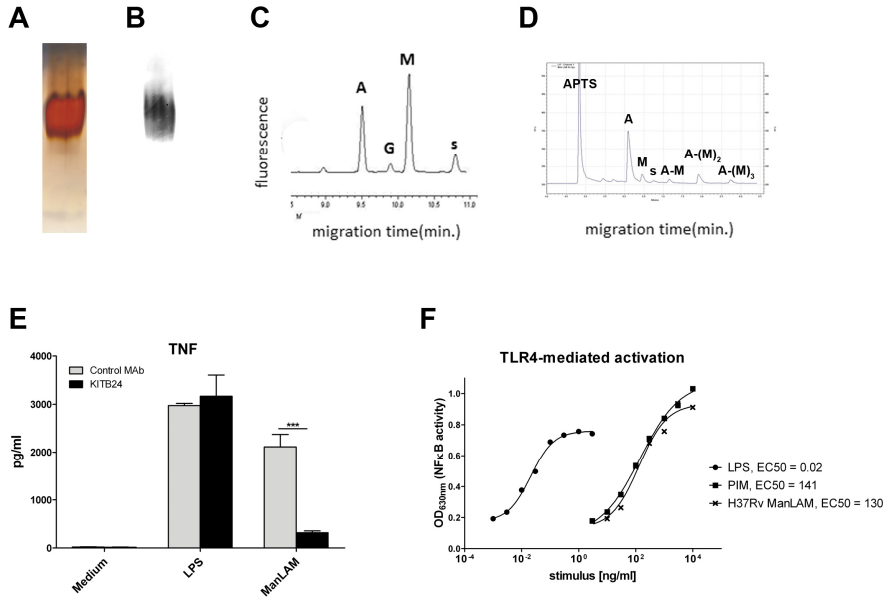

Figure 7. Characterization of ManLAM prepared in our laboratory. (A) ManLAM subjected to SDS-PAGE followed by staining with periodic acid-silver nitrate method. (B) Western blot analysis of ManLAM with a mix of ManLAM-specific and PIM-specific mAbs. CE-LIF analysis of ManLAM-derived sugars after total (C) and mild (D) acid hydrolysis: A – arabinose, G – glucose, M – mannose, s – internal standard (heptose), ManLAM-derived capping mono-, di-, and tri-mannoside oligosaccharides are designated as A-M, A-$(M)_2$, and A-$(M)_3$, respectively. (E) TNF release from DCs exposed to ManLAM and LPS pre-incubated with ManLAM-specific mAbs. Experiment was carried out in triplicates, values are mean +/- SD. Two-tailed, unpaired t-test was used to assess the statistical significance (*** $p < 0.001$). (F) TLR-4-mediated activation of reporter cells exposed to LPS, PIMs and ManLAM.

In summary, this study demonstrated that two glycolipids derived from the mycobacterial cell wall possess divergent immuno-modulatory properties, where ManLAM displays pro-inflammatory properties and PIMs are immuno-inhibitory. Furthermore, these studies showed that the glycolipids prepared in our laboratory were of high purity. Thus, contradictory results obtained by other groups could arise from different impurities present in ManLAM preparations. Additionally, conflicting reports may also derive from distinct strains used as starting material or even different culture conditions. Interestingly, it has been demonstrated that distinct Mtb isolates are characterized by specific cell wall composition that in turn may affect the immune response against Mtb [347]. Additionally, ManLAM is a heterogeneous family of related molecules sharing the same basic structure. Recently, a study demonstrated the existence of several isoforms of ManLAM differing by arabinnan chain length and number of fatty acids [348]. This was associated with differences in ability of those specific ManLAM isoforms to trigger immune response. Possibly,

those subtle differences in composition of specific ManLAM preparations may also contribute to discrepancies that are noted when investigating ManLAM derived from different laboratories.

4.2 CHARACTERIZATION OF CLINICAL ISOLATES OF MYCOBACTERIUM TUBERCULOSIS (*PAPERS II AND III*)

The outbreak of isoniazid-resistant Mtb in Sweden was first reported in 1996. The strain that caused this outbreak was characterized and more isolates, collected during next years, were annotated to the same group. This group was described as cluster SMI-049, based on the RFLP pattern, and by 2010 consisted of 115 isolates [333].

In the study presented in *paper II* three isolates of cluster SMI-049 were chosen and characterized in detail. Isolate S96-129 was the first to be identified in this cluster therefore it is the index isolate. Additionally two isolates obtained nine years later and found to go through long chains of transmission between patients were included in the study. All three isolates were analyzed by spoligotyping, RFLP and massive parallel DNA sequencing. Sequencing revealed that the analyzed strain remained genetically very stable despite being present in the population for a long period of time and several passages between infected patients. None of the late isolates differ extensively from the index case, no large sequence polymorphisms were observed. Notably, only a single four amino acid in-frame deletion was found in the BTB05-552 isolate and four SNPs in BTB05-559 as compared with the genome of the isolate S96-129. Yet, major differences were found when SMI-049 isolates were compared with a reference, laboratory H37Rv strain.

Next, an index case isolate and one of the later isolates (BTB05-552) together with a laboratory Mtb strain (H37Rv) were used to infect human monocyte-derived Mφs. All tested mycobacterial strains were able to infect and grow inside the Mφs as determined by the CFU count in the cell lysate harvested over time (Figure 8A). Additionally, Mtb-infected Mφs were counted and the number of bacteria per cell assessed after acid fast staining. No significant differences were noted for clinical strains and a reference laboratory strain or between isolates. Interestingly, TNF release from infected Mφs was found to be similar for all bacteria in the beginning of the infection, however, TNF concentrations on day 4 and 6 tended to be higher in cultures infected with clinical isolates than with the H37Rv laboratory strain (Figure 8B).

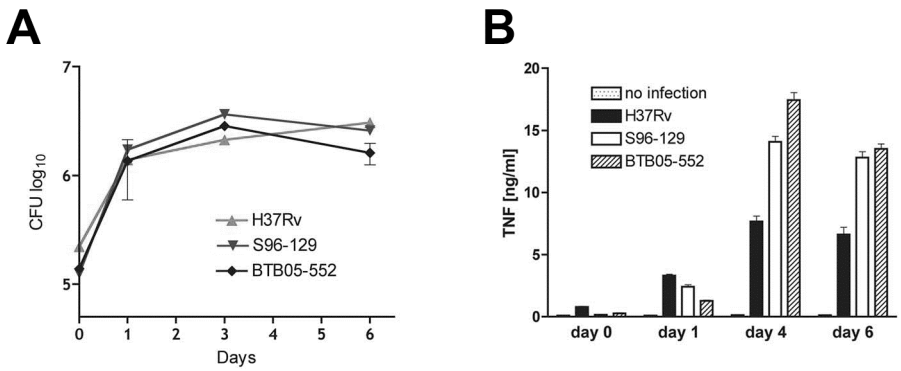

Figure 8. Infection of Mφs with clinical Mtb isolates and a laboratory strain. (A) CFU assessed at the different time points for lysates of Mφ infected with isolates of cluster SMI-049 (S96-129 and BTB05-552) and a reference Mtb strain (H37Rv). (B) TNF production by Mtb-infected Mφs assayed for different time points post-infection.

In the next study, described in *paper III*, we used two clinical isolates of cluster SMI-049: S96-129 and BTB05-552. Here, the effects of Mφs infected with Mtb on the function of DCs were investigated. In this study we also used another clinical isolate, a Harlingen strain, and the avirulent, vaccine strain of *Mycobacterium bovis*, bacillus Calmette-Guerin (BCG). We found that production of pro-inflammatory cytokines, namely TNF, IL-12p40 and IL-6, was augmented upon infection with all tested strains and the virulent Mtb strains tended to trigger more extensive responses than the avirulent BCG strain (Figure 9A-C).

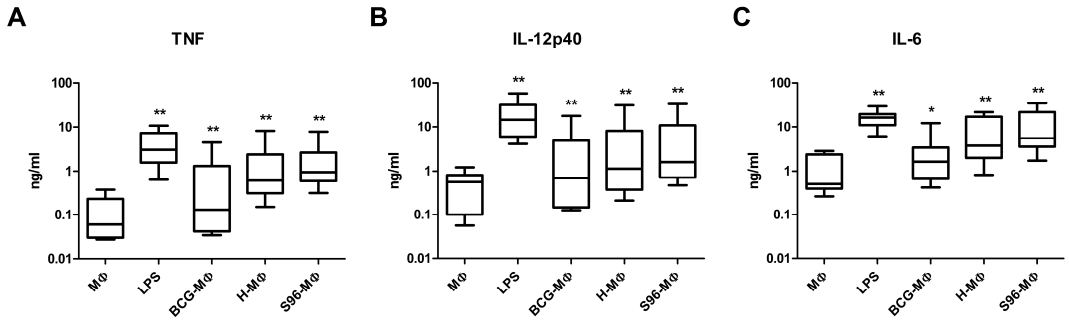

Figure 9. Cytokine production from co-cultures of DCs and mycobacteria-infected Mφs. Mφs were infected with the indicated mycobacteria for 3 h and after that co-cultured with DCs separated from the Mφs by a semi-permeable membrane. Concentrations of (A) TNF, (B) IL-12p40 and (C) IL-6 in cell culture supernatants were assayed after 48 h of co-culture. Box plots show the median, 25–75% interquartile range, and whiskers denoting full range data from n = 9 donors. * $p < 0.05$, ** $p < 0.01$ as determined by Wilcoxon matched pairs test in relation to non-infected Mφs.

Overall, these studies reveal that clinical Mtb isolates of the SMI-049 cluster are characterized by genetic stability. Lack of major differences in the isolate genomes was paralleled by similar properties tested in the *in vitro* Mφ model.

Importantly, no antibiotic resistance was developed throughout the years when the isolates were collected. Similar studies performed on the isolates from Uzbekistan and Estonia revealed that mycobacteria belonging to the same lineage may however display substantial genomic diversity [349, 350]. The genetic stability of the cluster SMI-049, also in the context of stability in the antibiotic susceptibility, was observed despite the fact that patients were not diagnosed and treated at the onset of the infection. In spite of the low rate of genetic alteration, cluster SMI-049 still contributes to one of largest TB outbrakes ever reported for low endemic countries.

Additionally, the amount of pro-inflammatory cytokines released upon infection with cluster SMI049 isolates tended to be augmented when compared with the laboratory Mtb strain and avirulent BCG. Of note, it has been demonstrated that the amount of TNF produced during TB must be tightly regulated and the outcome of the disease depends on this regulation. TNF released in excess causes immunopathology in the infected tissue and leads to a general dysregulation of immune homeostasis, while deficiency in TNF production has been associated with TB reactivation [164, 165].

4.3 BYSTANDER EFFECTS OF MYCOBACTERIAL INFECTION ON DENDRITIC CELL FUNCTION (*PAPER III*)

In this study we investigated bystander effects that mycobacteria-infected Mφs have on the function of DCs. Epidemiological studies have reported that progression of the disease caused by either HIV-1 or Mtb is faster in the co-infected host [288, 299]. It is known that mycobacterial components can be found systemically in Mtb-infected individuals [186, 187, 351]. Furthermore, immunological homeostasis of Mtb-infected patients is largely dysregulated [352]. We hypothesized that the milieu created during Mtb infection may contribute to altered ability of DCs to mediate HIV-1 *trans*-infection of T cells.

An *in vitro* model comprising DCs separated from the mycobacteria-infected Mφs by a semi-permeable membrane was set up. It was designed to mimic a bystander exposure of DCs to ongoing Mtb infection (see Bystander in vitro model in the Material and Methods section, page 35).

In the co-cultures of DCs and Mφs infected with different mycobacteria including avirulent *Mycobacterium bovis* BCG strain and two virulent clinical isolates of Mtb, concentrations of pro-inflammatory cytokines, TNF, IL-12p40 and IL-6, were found to be elevated (Figure 9). Similar observations were made when cell-to-cell contact between Mφs and DCs was allowed, however, concentrations of the cytokines were significantly higher than in the bystander model. In order to check which cell type produces which cytokine in the co-cultures we performed several

additional experiments: i) mycobacteria-infected Mφs were fixed with paraformaldehyde and then added to DCs cultures; ii) DCs were cultured in the conditioned medium derived from mycobacteria-infected Mφs and the concentrations of the cytokines were compared; iii) cytokines were detected intracellularly in co-cultured DCs and Mφs. These experiments led us to the conclusion that whereas TNF and IL-6 were produced by both DCs and Mφs, IL-12p40 was mainly DC-derived.

Next, the bystander influence of mycobacteria-infected Mφs on DC phenotype was examined. Up-regulation of the CD86 and MHC class II molecules was noted on the DCs exposed to Mφs infected with mycobacteria and this effect tended to be mycobacterial strain dependent. Levels of MR on DC surface were significantly reduced only for Mtb strain S96-129. Interestingly, expression of DC-SIGN on DCs was unchanged upon exposure to any mycobacteria-infected Mφs.

Finally, the ability of DCs to infect T cells with HIV-1 *in trans* was studied. It was previously demonstrated that Mtb infection has an enhancing effect on HIV infection by increasing virus replication and its genetic heterogeneity [324, 353]. We here demonstrated that also ability of DCs to mediate HIV-1 *trans*-infection of T cells is affected by ongoing Mtb infection. We observed a significant increase in DC *trans*-infecting ability upon exposure to S96-129, the virulent Mtb strain that exhibited the strongest cytokine response from exposed DCs (Figure 10A). Enhanced ability to mediate HIV-1 *trans*-infection was also noted in DC cultures exposed to soluble factors released by Mφs infected with another clinical Mtb isolate, BTB05-552 (Figure 10A). Moreover, in order to analyze which stage of the *trans*-infection process was affected we tested the ability of DC to capture the virus, and found that the virus associated better with DCs exposed to Mtb-infection (Figure 10B). In addition, we demonstrated that the observed increase in HIV-1 capture and the *trans*-infection ability could be inhibited by mannan, known to block the interaction between DCs and C-type lectins [354] (Figure 10A-B).

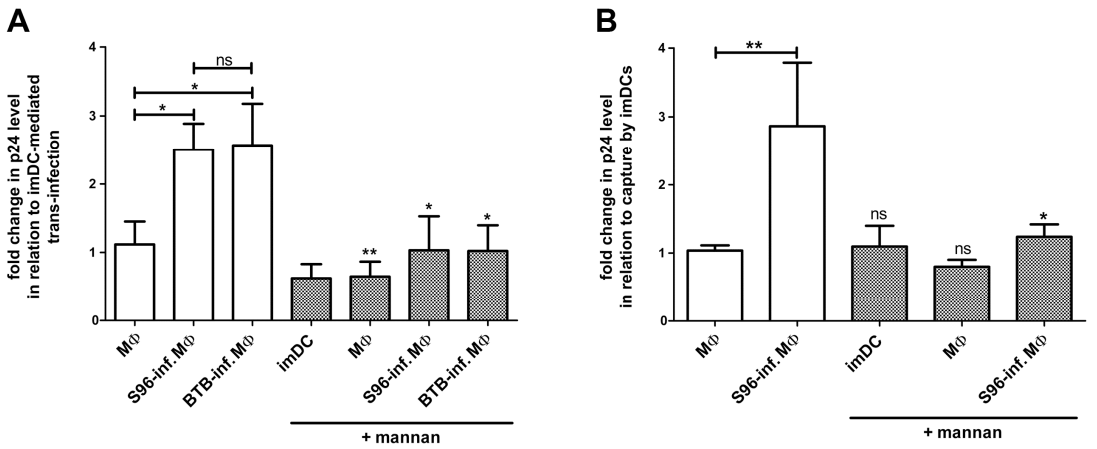

Figure 10. HIV-1 *trans*-infection and capture ability of Mtb-exposed DCs. (A) *Trans*-infecting ability of DCs cultured in the supernatant derived from Mtb-infected Mφs is presented as p24 levels detected in *trans*-infected PBMC cultures. (B) p24 levels detected in lysates of DCs exposed to soluble factors released by Mtb-infected Mφs. Shaded bars represent DCs pre-exposed to mannan in (A) and (B). Data are shown as mean + SD from four donors. Statistical significance was assessed by paired t-test. Statistical differences between mannan-treated and untreated counterparts are shown above bars illustrating mannan-treated cells, and other significances are calculated for group pairs as indicated in the graphs and * $p < 0.05$, ** $p < 0.01$.

In summary, these studies revealed that soluble factors released by mycobacteria-infected Mφs affect several DC functions. Accordingly, we found that the pro-inflammatory cytokine production in the DC-infected-Mφs co-cultures was increased and accompanied by the partial maturation of DCs. Furthermore, we demonstrated that conditions created during Mtb infection potentiated DC-mediated *trans*-infection of T cell with HIV-1. Thus, these studies suggest that Mtb infection may create an environment that, in a bystander manner, may promote HIV-1 spread and replication in the co-infected individual.

4.4 MECHANISMS BEHIND IMPAIRED IMMUNE RESPONSE TO MTB IN HIV-1 CO-INFECTION SETTING (*PAPER IV*)

It has been demonstrated that control of Mtb infection is deficient in HIV-infected individuals [288]. HIV-triggered dysregulation of the immune balance has been found to contribute to this situation [288]. Indeed, the release of TNF in response to Mtb and Mtb-induced apoptosis have been shown to be impaired during HIV-1 infection [304, 305, 310]. However, the mechanism responsible for this defect is still unknown.

Here we tested whether the response of human monocyte-derived Mφs to Mtb infection is impaired also in regard to production of other cytokines. In fact such deficiency was noted not only for TNF but also for IL-10 and IL-6 (Figure 11A-C).

This is even more intriguing given the fact that the susceptibility of HIV-exposed Mφs to subsequent Mtb infection is augmented together with an increased Mtb growth in HIV-exposed cells (our own observations and [314]). Next, the Mtb-derived glycolipid, mannose-capped lipoarabinomannan (ManLAM), was used to stimulate cells and high levels of all cytokines tested were secreted. When Mφs were pre-exposed to HIV-1 envelope glycoprotein, gp120, and then treated with ManLAM their response to the second stimulus was inhibited in a similar manner as in the infection experiment.

In order to dissect the mechanism of inhibited cytokine response to Mtb infection in HIV-infected cells we investigated the expression pattern of miR-146a, a miR known to regulate pro-inflammatory responses, in Mφs. Results showed that miR-146a was up-regulated, both upon exposure of Mφs to HIV-1 and also to Mtb (Figure 11D). Notably, experiments where cytokine release was blocked by brefeldin A upon Mtb infection still resulted in miR-146a up-regulation, thus demonstrating that induction of this miR was pathogen-specific. Furthermore, cells pre-exposed to HIV-1 and then infected with Mtb produced significantly higher levels of miR-146a than cells infected with Mtb alone (Figure 11D). In addition, experiments with the pathogen compounds, gp120 and ManLAM, suggest that a mere exposure of Mφs to HIV-1 gp120 is sufficient to induce miR-146a and to impact the response of the cells to a secondary stimulus – in this case mycobacterial ManLAM.

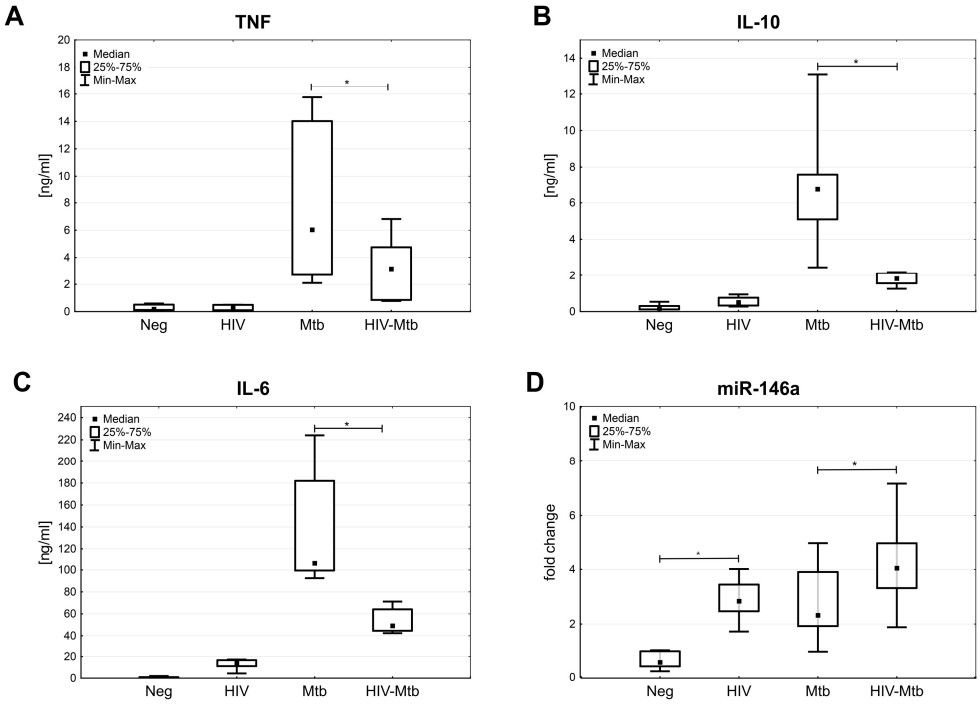

Figure 11. Cytokine profile and miR-146a expression of Mφs pre-exposed to HIV-1 and infected with Mtb. (A-C) Cytokine production and (D) miR-146a expression levels in Mφs infected by either HIV-1, Mtb or both pathogens. (D) Fold change of miR-146a expression in relation to U6 gene used as a reference was calculated. (A-D) Box plots show the median, 25–75% interquartile range, and whiskers denoting full range data from n = 6 independent donors and * $p < 0.05$ as determined by Wilcoxon matched pairs test.

In conclusion, we found that HIV-1 is able to trigger up-regulation of miR-146a in human Mφs. This was paralleled by an impaired cytokine response of such cells to the second pathogen, Mtb. A similar phenomenon was previously described for monocytic cells which were anergic to triggering by a secondary TLR ligand after LPS stimulation [53-55]. Thus, these findings suggest that HIV-induced cross-tolerance in Mφs may contribute to dysregulated responses to Mtb infection in co-infected individuals.

5 CONCLUDING REMARKS

Dysregulation of the immune system is observed in Mtb-HIV-1 co-infected individuals. As demonstrated previously, either excessive or limited reaction of the immune system to the invading pathogen may lead to disease progression and may cause immunopathology [164, 165]. In this thesis the causes of such unbalanced immune reactions were investigated.

First, we demonstrated that mycobacterial cell wall contains glycolipids that may display opposite effects on the function of human DCs (*paper I*). Accordingly, ManLAM was found to be immuno-stimulatory, while PIMs were able to inhibit pro-inflammatory reactions in DCs, triggered by other stimuli including LPS. These observations are of particular importance given the fact that mycobacterial glycolipids are found systemically during TB. Thus, their presence may trigger reaction of cells located distantly from the site of Mtb infection. Such an influence may have a significant impact on the outcome of the infection, in line with the observation that the cell wall composition of Mtb relates to the virulence of the strains [347]. Indeed, the bystander effects of ongoing Mtb infection were further supported by findings described in *paper III*. Here we found that Mtb-infected Mφs influenced DCs without coming into direct contact with them. DCs exposed to Mtb infection secreted pro-inflammatory cytokines and underwent partial maturation. Moreover, their ability to mediate HIV-1 T cell infection *in trans* was augmented. Additionally, in this study several different mycobacteria were used in order to test their distinct impact on *in vitro* infection outcome. Indeed, we observed strain-related differences, with avirulent BCG being the least potent inducer of pro-inflammatory reactions in DCs and Mφs, and clinical isolates triggering stronger effects. We did not, however, see a difference between the two Mtb isolates belonging to the same cluster, SMI-049, which was identified in the Swedish outbreak (*paper II)*. In agreement with similar impact on DC and Mφ immune reactions (*papers II and III)*, whole genome sequencing did not reveal any major genetic differences between the clinical isolates of cluster SMI-049. Finally, we pursued the reason for impaired responsiveness of Mφs to Mtb infection observed during co-infection with HIV-1. Here the data suggest that the compromised response of HIV-1 pre-exposed Mφs to Mtb infection is associated with up-regulation of miR-146a, and could be a consequence of HIV-triggered cross-tolerance (*paper IV*).

Further studies are necessary to gain a full insight into the mechanisms behind dysregulated immune responses during Mtb-HIV-1 co-infection. Studies on clinical samples from patients would be particularly valuable and could confirm our observations from the *in vitro* models. The approach where the features of infecting pathogen are studied and simultaneously related to disease progression (monitored in easily accessible patient material, e.g. in blood) would be of a particular

importance. In the future, such an approach could lead to better understanding of challenges associated with the co-infection and eventually lead to more efficient strategies in therapy and hopefully also vaccination.

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