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STUDIES ON THE HOST IMMUNE RESPONSE DURING PULMONARY TB AND DURING *M. TUBERCULOSIS*/HIV CO-INFECTION

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FAC$_t$S are meaningless.

You could use FAC$_t$S to prove anything that's even remotely true!

- Homer Simpson

ABSTRACT

Population studies have shown that HIV-infected individuals co-infected with *Mycobacterium tuberculosis* (MTB) are at up to 10-fold higher risk of developing active tuberculosis (TB) than their seronegative counterparts. Co-infected individuals also progress faster to AIDS than patients infected only with the virus. Therefore, understanding the relation between these two diseases is of a crucial importance. The overall aim of this thesis was to investigate the interaction between HIV and MTB infection from the perspective of host immunity. Using in vitro and in vivo models, we investigated innate and adaptive immune responses to better understand the mechanisms behind the increased susceptibility to MTB and HIV during co-infection.

In the first study we examined regulation of the host immune response during pulmonary TB mediated by CD103+ dendritic cells (αE-DC) and CD4+ Foxp3+ regulatory T (Treg) cells. We found that in resistant mice, the number of lung αE-DCs increased dramatically during MTB infection. In contrast, susceptible DBA/2 mice which failed to recruit αE-DCs even during chronic infection. αE-DCs remained essentially TNF negative but contained a high number of TGFβ-producing cells in the lungs of infected mice. Further, we show that Treg cells in resistant and susceptible mice induced interferon IFNγ during pulmonary TB. We also showed that the Treg cell population was diminished in the lungs, but not in the draining pulmonary lymph nodes, of susceptible mice. The reduced number of lung Treg cells in susceptible mice coincided with an increased bacterial load and exacerbated lung pathology compared to resistant strains. These results demonstrate that immune-regulatory mechanisms may play an important role in protective immune responses during pulmonary TB.

In study II we analyzed the impact of MTB-infected macrophages on DC functionality, including HIV transinfecting ability. An in vitro system was used in which human monocyte-derived DCs were exposed to soluble factors released by MTB- or BCG-infected macrophages. Soluble factors secreted from infected macrophages resulted in the production of proinflammatory cytokines and partial upregulation of maturation markers on DCs. Interestingly, the HIV transinfecting ability of DCs was enhanced In summary, this study shows that DCs respond to soluble factors released by mycobacteria-infected macrophages. These findings highlight the important role of bystander effects mediated by MTB-infected macrophages, and suggest it contributes to DC-mediated HIV spread and amplification in co-infected individuals.

In study III we assessed the impact of MTB infection on the immunogenicity of a HIV vaccine. We found that, depending on the vaccination route, mice infected with MTB before the administration of the HIV vaccine showed impairment in both the magnitude and the quality of antibody and T cell responses directed towards the vaccine. Mice infected with MTB prior to HIV vaccination exhibited reduced IgG and IgA antibody levels and neutralizing activity of serum against the virus In addition mice infected with MTB had significantly lower HIV-specific multifunctional T cells. These results suggest that chronic MTB infection might interfere with the outcome of prospective HIV vaccination in humans.

In study IV we developed a new murine model of MTB/HIV co-infection using conventional, immunocompetent mouse strains. We utilized the chimeric EcoNDK virus together with a low dose MTB aerosol infection. To date, we have observed that the viral burden increased in the spleen and in the lungs of animals infected with MTB prior to virus inoculation. We have also shown that co-infection did not affect control of bacterial growth. During the early stage of co-infection, EcoNDK induced a significantly higher frequency of MTB antigen-specific CD8+ T-cells in the spleen. Also, MTB-specific CD8+ T-cells in both MTB-infected and MTB/EcoNDK co-infected animals were enriched for Tim3- and PD1 expressing cells.

In conclusion, this small animal model may provide a useful tool to increase our understanding of how MTB and HIV influence the host immune response during co-infection in vivo.

List of Publications:

I. Chaniya Leepiyasakulchai, Lech Ignatowicz, Andrzej Pawlowski, Gunilla Källenius, and Markus Sköld
"Failure to Recruit Anti-inflammatory CD103+ Dendritic Cells and a Diminished CD4+ Foxp3+ Regulatory T Cell Pool in Mice That Display Excessive Lung Inflammation and Increased Susceptibility to Mycobacterium Tuberculosis."
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II. Jolanta Mazurek, Lech Ignatowicz, Gunilla Källenius, Marianne Jansson, and Andrzej Pawlowski
"Mycobacteria-infected Bystander Macrophages Trigger Maturation of Dendritic Cells and Enhance Their Ability to Mediate HIV Transinfection."
European Journal of Immunology 42 (5) (May) 2012 : 1192–202.
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III. Lech Ignatowicz, Jolanta Mazurek, Chaniya Leepiyasakulchai, Markus Sköld, Jorma Hinkula, Gunilla Källenius, and Andrzej Pawlowski. 2012.
"Mycobacterium Tuberculosis Infection Interferes with HIV Vaccination in Mice."
PLoS ONE 7 (7) (July 23): e41205.

IV. Lech Ignatowicz, Chaniya Leepiyasakulchai, and Markus Sköld
"A murine model of *Mycobacterium tuberculosis*/HIV co-infection for studies on pathogen-specific T cell responses in vivo."
Preliminary results

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Publications not included in the scope of the thesis:

Berit Carow, Xiang qun Ye, Dolores Gavier-Widén, Sabin Bhuju, Wulf Oehlmann, Mahavir Singh, Markus Sköld, Lech Ignatowicz, Akihiko Yoshimura, Hans Wigzell, and Martin E. Rottenberg
"Silencing Suppressor of Cytokine Signaling-1 (SOCS1) in Macrophages Improves Mycobacterium Tuberculosis Control in an Interferon-gamma (IFN-gamma)-dependent Manner."
The Journal of Biological Chemistry 286 (30) (July 29) 2011: 26873–87.

Jolanta Mazurek, Lech Ignatowicz, Gunilla Kallenius, Stefan B. Svenson, Andrzej Pawlowski, and Beston Hamasur
"Divergent Effects of Mycobacterial Cell Wall Glycolipids on Maturation and Function of Human Monocyte-Derived Dendritic Cells."
PLoS ONE 7 (8) (August 3) 2012: e42515.

LIST OF ABBREVIATIONS

αE	ALPHA-E INTEGRIN, CD103
AFB	ACID-FAST BACILLI
AIDS	ACQUIRED IMMUNODEFICIENCY SYNDROME
ART	ANTIRETROVIRAL THERAPY
BAL	BRONCHOALVEOLAR LAVAGE
BCG	*M. BOVIS BACILLE C*ALMETTE-GUERIN
bDNA	BRANCHED DNA
CCR7	CC-CHEMOKINE RECEPTOR-7
CD	CLUSTER OF DIFFERENTIATION
CFP-10	CULTURE FILTRATE PROTEIN 10KDA
CFU	COLONY FORMING UNIT
CIITA	CLASS II TRANSACTIVATOR
CR	COMPLEMENT RECEPTOR
DC	DENDRITIC CELL
DC-SIGN	DENDRITIC CELL-SPECIFIC INTERCELLULAR ADHESION MOLECULE-3-GRABBING NON-INTEGRIN
DNA	DEOXYRIBONUCLEIC ACID
DOTS	DIRECTLY OBSERVED TREATMENT, SHORT-COURSE
ESAT-6	EARLY SECRETORY ANTIGEN 6KDA
GALT	GUT-ASSOCIATED LYMPHOID TISSUE
HAART	HIGH ACTIVE ANTIRETROVIRAL THERAPY
HIV	HUMAN IMMUNODEFICIENCY VIRUS TYPE 1
IFNγ	INTERFERON GAMMA
IG	IMMUNOGLOBULIN
INH	ISONIAZID
IRIS	IMMUNE RECONSTITUTION INFLAMMATORY SYNDROME
KatG	BACTERIAL CATALASE-PEROXIDASE
LAM	LIPOARABINOMANNAN
MHC	MAJOR HISTOCOMPATIBILITY COMPLEX
MR	MANNOSE RECEPTOR
MTB	*MYCOBACTERIUM TUBERCULOSIS*
NAB	NEUTRALIZING ANTIBODY
NKT	NATURAL KILLER T-CELLS
NLRP3	NOD-, LRR- AND PYRIN DOMAIN-CONTAINING 3
NO	NITRIC OXIDE
NOD2	NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN PROTEIN 2
PBMC	PERIPHERAL BLOOD MONONUCLEAR CELL
PD1	PROGRAMMED CELL DEATH 1
PLN	PULMONARY LYMPH NODE
PPD	TUBERCULIN PURIFED PROTEIN DERIVATIVE
RNA	RIBONUCLEIC ACID
ROI	REACTIVE OXYGEN INTERMEDIATE
RT	REVERSE TRANSCRIPTASE
SCID	SEVERE-COMBINED IMMUNODEFICIENCY
SIV	SIMIAN IMMUNODEFICIENCY VIRUS
TGFβ	TRANSFORMING GROWTH FACTOR BETA
TIM3	T CELL IMMUNOGLOBULIN AND MUCIN DOMAIN 3
TNF	TUMOR NECROSIS FACTOR ALPHA
WHO	WORLD HEATH ORGANIZATION

LIST OF CONTENT

1 INTRODUCTION

1.1 EPIDEMIOLOGY OF *MYCOBACTERIUM TUBERCULOSIS* (MTB), HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND MTB/HIV CO-INFECTION

The earliest evidence that humans developed tuberculosis came from ancient Egyptian mummified remains from 3,400 BC. Recent genetic studies of the whole MTB genome advocate that a common mycobacterial progenitor could have infected our early hominid ancestors as long as 3 million years ago [1], [2]. The first scientific characterization of the agent causing TB was identified by Robert Koch in 1882 and was named *Mycobacterium tuberculosis* (MTB)due to its ability to cause tubercle formations in the lungs. In spite of the discovery of antibiotics, global vaccination programs and populational diagnostic screening, it is estimated that today 2 billion people worldwide are MTB-infected. As a result, TB accounts for 2-3 million deaths annually [3].

The incident rate has dropped greatly in developed countries since Robert Koch's time, when 1 in 7 deaths was attributed to TB that was then incurable. Today, it is estimated that the TB incidence rate is 139/100,000 worldwide, which corresponds to less than 10 million active TB cases globally. Although there is a significant global decline when compared to the predictions from the 1990's, the data represent an 11% increase in the incident rate and a staggering 40% increase in the number of new active TB cases [4]. The majority of newly infected individuals were diagnosed in Sub-Saharan Africa and Asia, as well as certain communities in Europe. The TB incidence and TB-associated deaths in Sub-Saharan Africa has more than doubled in the 90's. The estimated TB-related deaths has tripled compared to the WHO predictions [4]. It was soon realized that new the strength of the TB epidemic was associated with the new emerging HIV pandemic. Although the incident rate has been declining thanks to the introduction of new health programs like Directly Observed Treatment, Short-course (DOTS), or preventive antiviral therapy for acquired immunodeficiency syndrome (AIDS), the overall number of new TB cases is still increasing due to global population growth. This is reflected by the fact that over 50% of all new TB cases are reported in Asia. Over 20% of all screened TB patients has been identified as HIV seropositive [3], [4].

In contrast to TB, HIV was discovered less than 30 years ago and described as the causative agent of AIDS [5], [6]. Today, over 30 million people are estimated to be infected with HIV, with 2.7 million new infections and over 2 million deaths caused by AIDS every year. The fact that over 2.1 million children are infected with the virus is most tragic. As in the case of TB, areas of the highest disease prevalence are located in Sub-Saharan Africa where more than 60% of all infected individuals live [7], [8]. Determining the accurate epidemiological picture of the HIV epidemic is problematic since the majority of individuals infected with HIV are not aware of that fact. In highly

endemic countries, new programs of population screening are being installed. Still, providing robust diagnostic tests is a monumental task due to the logistics of the operation in remote areas. Nevertheless, over 4 million individuals were treated with antiretroviral therapy (ART) or high active antiretroviral therapy (HAART) in 2008 comparing to less than 3 in 2007 [7]. Initially, it was hoped that the antiretroviral treatment would be able to eradicate the virus from the patients and subsequently the pandemic would be controlled within 20 years. Now, it is estimated that eradication of all infected cells from the patients' body would require 60-70 years of treatment. In spite of that, it is promising that with the new generation of drugs, HIV infection is being treated as a chronic disease, and as such kept under control by lifelong therapy [9].

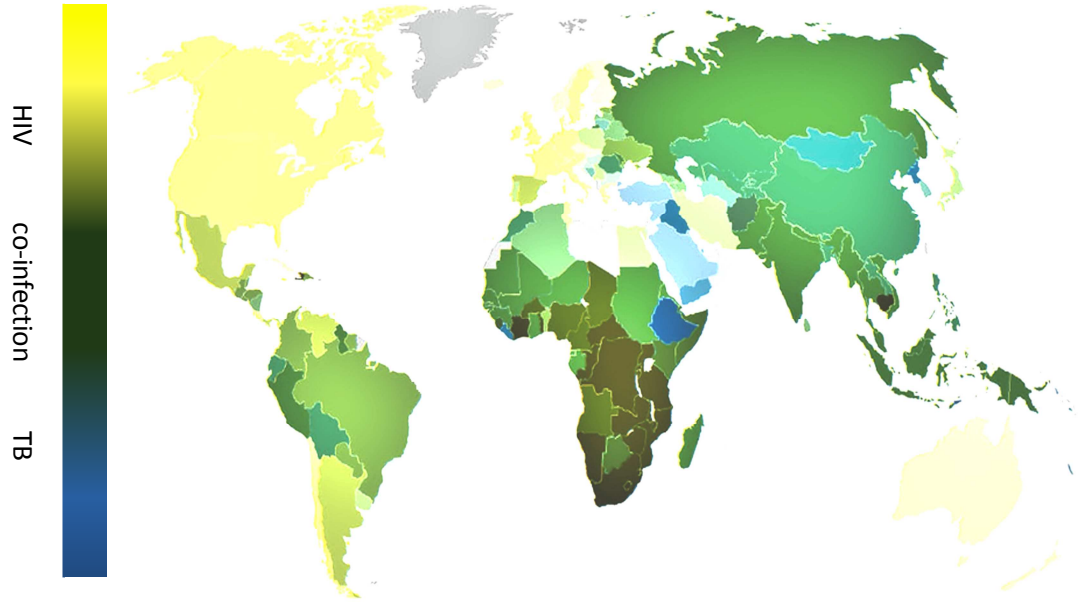

Figure 1. Overlay of the global indidences of HIV and TB in 2009. Based on WHO and UNAIDS maps [3], [7]

1.2 HIV

1.2.1 HIV history and diversity

The probable origins of HIV infection in humans can be found in multiple zoonotic transmissions of simian immunodeficiency virus (SIV) between nonhuman primates and humans in Central-West Africa. More than 30 different species of nonhuman primates are natural hosts of species-specific SIV, Thus, independent, multiple, cross-species infections resulted in different lineages of the virus [10]. There are two main types of HIV: HIV type 1 (referred to as "HIV") being the main cause of the global epidemic and HIV type 2 (HIV-2) that infects only a limited number of individuals, mainly in Central West Africa. HIV-2 is around 40% genetically different from HIV type 1. Within those two types several lineages have been identified: HIV type 1 consists of groups M, N, O and P with a genetic variation close to 30%

between the groups, and HIV-2 groups A–H with half the variation [11]. In the case of HIV type 1, the group M (M for main) is responsible for the global HIV pandemic. Group O has been estimated to be present in not more than 100 000 individuals and groups N and P have only been identified in a few of individuals in Cameroon [12]. Although HIV was identified in 1983, it is speculated that transmission to humans happened already approximately around 1900. Since the two oldest isolates from 1959 and 1960 already differed genetically by ~12% (belonging to two different subtypes: A and D) we now know that fast genetic diversification of group M was already taking place before the explosion of the epidemic [13]. During the course of the HIV pandemic this process accelerated, leading to massive genetic diversity. Within the main group M, nine subtypes were identified: A–D, F–H, J and K with genetic variation levels of 8-17% within the subtypes and reaching up to 42% between them [14]. This astonishing diversity is characteristic for retroviruses and is attributed to their unique replication cycle. The HIV genome consists of two copies of 10kb single-stranded ribonucleic acid (RNA) [9]. As in all retroviruses, the genomic RNA of the virus needs to be transcribed by the viral reverse transcriptase (RT) to deoxyribonucleic acid (DNA), which can then be incorporated into the genome of the host cell. Due to the lack of proofreading activity of the RT, the mutation rate of the virus is extremely high (3,4 x 10^{-5} m/bp for each replication cycle) [15]. This process alone is responsible for generating millions of virus variants in a single infected individual. Additionally, the RT is also responsible for a recombination process occurring between the two strands of RNA leading to additional variation in viral genetic pool and rapid virus evolution [16].

1.2.2 HIV structure and lifecycle

1.2.2.1 Envelope proteins and impact on co-receptor recognition

The genome of HIV is composed of 9 genes, encoding at least 15 viral proteins, which can be divided further into three main groups. Three structural genes: *env*, *gag* and *pol*, two regulatory genes: *tat* and *rev*, and a group of four accessory genes: *nef, vif, vpu* and *vpr*. Non-coding long terminal repeat sequences (LTRs) located at both ends of the genome are required for viral genome integration, and are together with the viral and cellular proteins involved in controlling the production of new viruses [9]. The genetic material of the virus is carried as two positive strands of ssRNA which are encapsulated in the nucleocapsid made of p24 protein and additional enzymes and accessory proteins needed in the first steps of infection, most importantly the RT. The capsid itself is enclosed by the remains of the cellular lipid plasma membrane and the HIV matrix-protein in which the glycoproteins gp120 and gp41 are anchored. Those two products of the *env* gene are derived from the gp160 precursor which is being processed by host cell proteases into gp120, which are the main surface protein interacting with the CD4 receptor of the target cells, and the gp41 protein being the main anchor of the complex in the envelope. The

gp120 protein binding the host cell receptor is responsible for the host-cell specificity of HIV. Therefore, differences in the gp120 protein among the viruses impact their tropism [17]. The CD4 molecule, that is expressed on the surface of T-cells and macrophages, is the main receptor for HIV, although the virus can also bind to the C-type lectin receptor, such as DC-SIGN, on the surface of dendritic cells [18]. Binding only to the CD4 molecule is not sufficient for viral entry. Prior to entry into the cell, the gp120 protein needs to go through conformational changes triggered by binding to the CD4 molecule, and binding to one of various co-receptors, mainly CXCR4 and CCR5 [19]. Subsequently, gp41 also changes its conformation and thus allows for membrane fusion and entry of the virus into the host cell. Huge genetic variation of the virus leads to alterations in the molecular structure of gp120. This leads to recognition of different co-receptors, whereas the CD4 molecule remains the main receptor. R5 viruses, which recognize CCR5 as the co-receptor, are predominantly present in the early stages of infection. In about half of the cases, during the course of the disease, one can observe a shift in the co-receptor variants of the virus. In late stages of the disease, the CXCR4-recognizing viruses (X4 viruses), or viruses being able to recognize both co-receptors (R5X4 viruses), become the dominating variants [20] The CCR5 co-receptor is expressed only on a small fraction of memory CD4+ T-cells, while the expression of CXCR4 is high on naïve T-cells. In the early stages of the infection it is the CCR5 expressing cells which are the main target for the virus. Dominance of the CCR5 strain at this stage is probably associated with the high frequency of the memory CD4+ T-cells in the gut-associated lymphoid tissue (GALT), where robust virus replication takes place soon after infection. In less than a few months, up to 80% of all CD4+ T-cells in the GALT are destroyed, even though only ~20% of the cells are actually HIV-infected [21]. The importance of co-receptor recognition can be best observed in the case of individuals who are homozygous carriers of CCR5 delta 32 receptor mutations. This mutation prevents binding of the virus to the co-receptor making carries immune to R5 virus infection [22].

HIV is able to infect not only CD4+ T-cells, but also macrophages and DCs. Recent findings suggest that macrophage susceptibility to HIV infection relies on the expression of different chemokine receptors. Both CCR5 and CXCR4 can be expressed by monocyte-derived macrophages but the level of expression is strictly tissue dependent [23]. Intestinal macrophages express very low levels of the CCR5 chemokine receptor in contrast to the macrophages in vaginal mucosa, which have been shown to be highly susceptible to R5 virus infection [24][25]. In the brain tissue, macrophages and microglia mainly express more CXCR4 than CCR5. Thus, in late stages of the infection, neurodegenerative symptoms could be partially associated with the emergence of X4 and R5X4 variants [26]. Similarly, DCs can express both types of co-receptors but their susceptibility to certain variants is skewed towards R5 type viruses [27]. DCs also contribute indirectly to differential propagation of R5 virus strains. By producing CCR5 ligands, vaginal mucosa DCs

attract CD4+ T-cells expressing CCR5 to the site of infection and therefore enrich the pool of T-cell being infected by the R5 viruses [28]. Besides their HIV replication in-*cis*, DCs contribute to viral dissemination into CD4+ T cells via a DC-SIGN trans-infection mechanism [28]. As mentioned before, gp120 can bind not only to the CD4 receptor, but also to a C-type lectin receptor such as DC-SIGN, which is present mainly on the surface of DCs. It normally functions as a receptor in the adhesion process and plays a role in an internalization of the captured antigens. DC-SIGN is able to bind and facilitate internalization of various pathogens like Hepatitis C, Dengue, Ebola, MTB or *Helicobacter pylori* and therefore start the process of phagocytic degradation followed by antigen presentation [22]. Surprisingly, HIV escapes this degradation process and stays intact for the duration of the migration of the DC from the site of infection to secondary lymphoid organs, and thereby facilitates virus transmission to areas rich in main virus targets: CD4+ T-cells [29].

1.2.2.2 Inside the host(ile) cell

Successful virus infection hinges on the ability of the virus to turn the host cell into a submissive state in which the main objective is to turn cellular metabolism into production of new virus particles. To achieve that, viruses depend on a spectrum of accessory and regulatory proteins, which interfere with various cellular pathways. As a result of evolutionary pressure exerted by continuous exposure to viruses, each cell is equipped with a range of restriction mechanisms counteracting viral activity.

After the successful binding of the gp120 and gp41 complex to the CD4 molecule and one of the co-receptors, the envelope of the virus can fuse with the cell membrane and enable the capsid core to enter the cell. The capsid then undergoes uncoating which frees genomic RNA, enzymes and other proteins required for initiation of the reverse transcription [30], [31]. The infected cell attempts to hamper this process with the use of TRIM5α molecules. Initially, it was speculated that this process could be based on an inhibition of decapsidation, but new evidence has shown that it actually promotes premature disassembly of viral capsids and thus restrict productive reverse transcription [32]. In humans, the levels of inhibition of virus replication due to the TRIM5α is relatively low whereas in non-human primates this mechanism seems to be much more effective [33]. In light of the murine HIV model described below, it is worth noticing that Fv1 restriction of MLV in mouse cells, which is known to involve different mechanisms than TRIM5α, also targets decapsidation [34], [35]. Productive uncoating is required for the initiation of reverse transcription of the viral genomic RNA to DNA. This process is conducted with use of an RNA-dependent DNA polymerase, which is simply called RT. RT was carried together with RNA in the capsid of the virus. After the initiation of reverse transcription a RNA/DNA molecule is generated on a RNA template. The RNA part is degraded and after a few more modifications, a second strand of DNA is transcribed. In addition to the RT polymerase lacking proofreading capacity, it can jump from one strand of the template to another, creating new recombinant virus variants [36]. In myeloid cells infected with HIV, the reverse transcription process is being disrupted

by the SAMHD1 protein. SAMHD1 inhibits RT productivity by depleting the pool of nucleotide triphosphates available in the cellular matrix [37]. Another RT counteracting mechanism involves the APOBEC3 family of proteins. Their role as a restriction factor is also based on indirect inhibition of RT activity. APOBEC3 deaminates cytosines in the minus strand of the newly synthesized viral DNA transforming them into uracil. This leads to mutations in the provirus itself and uracil is recognized by the cellular DNA repair system. The unpaired DNA strands containing it are being degraded. Interestingly, APOBEC3 proteins need to be present during the infection. Therefore, they are packed together with the virus proteins into the capsid of the newly formed virus and acts on the next infected cell [38] [39], [40]. Vif is the main viral protein counteracting the activity of APOBEC3 proteins. It triggers degradation of APOBEC3 by the proteasomal pathway. Therefore, the antiviral activity of APOBEC3 can play a substantial role only during infection with *vif*-defective or *vif*-deficient strains of the virus [41].

Freshly transcribed viral double-stranded DNA is transported to the nucleus as a pre-intergation complex. Viral integrase clips open chromosomal DNA and after modification of the 3' ends of the viral DNA, it inserts it to the host cell chromosome [17]. Newly integrated viral DNA is being transcribed by host cell RNA polymerase II and the production of new virus particles can begin. HIV regulatory proteins are the first ones to be synthetized. Their role is to steer the production of new virus particles and counteract other restriction mechanisms. To enhance the transcription of virus RNA, Tat protein interacting with other host cell proteins binds to the LTR's of the integrated virus DNA and severely enhances this process [42]. Transcribed RNA is either spliced and translated into proteins, or is destined to be loaded as genomic RNA into newly produced viral particles. To allow the unspliced mRNA to be taken out of the nucleus, another HIV protein, Rev, binds to the RNA and acts as a transporting chaperon [43], [44].

In the view of murine models of HIV infection, it is worth noting that the inability to sustain Rev and Tat activity is regarded as one of the main obstacles in producing progeny virus by transformed murine cell lines. It was shown that in the 3T3 murine fibroblast cell line, Tat requires presence of human cyclin T1 for proper functioning and Rev depends on human p32 splicing inhibitor [45], [46]. However, in rat fibroblast cell lines, Tat mediates efficient transactivation and Rev activity is also not impaired [47]. The same can be seen in primary murine cells with chimeric virus in vitro and in vivo. Therefore it seems that mechanism Tat mediated transcription is facilitated by other murine protein or proteins and is yet to be described [48], [49].

Nef is another HIV protein taking part in reorganization of cellular signaling and trafficking. Similarly to the other viral proteins it plays more than one role. Nef together with Tat and Rev is responsible for boosting the production of virus proteins, but the main role of Nef is to downregulate expression of MHC class I and II molecules. By this activity Nef protects the host cell from being recognized by the immune system long enough to ensure robust progeny virion production [50].

Once all the required proteins and unspliced RNA are synthesized and reach optimal concentration in the cell, the assembly of the new virions can begin. The gp160 precursor is being cleaved by the furin proteases into gp120 and gp41. After reaching the cellular membrane, trimers of both proteins form a complex. Under the membrane surface, Gag p55 and Gag-Pol proteins, together with two ssRNA molecules, interact with the membrane embedded gp120-gp41 trimers and as newly formed virions bud out of the cell surface. Final modifications are made after budding. A HIV protease, retropepsin protease (PR), is being cleaved from the Pol precursor and cleaves Gag and Pol proteins into p17, p24, p7, p6, RT and IN proteins [43]. The host cell intends to interfere with the last steps of virus production. Membrane protein Tetherin (CD317) binds the newly produced virions to the cell and cross-links them with each other, thereby inhibiting their release. However, the HIV protein Vpu binds and inhibits Thetherin activity. Vpu induce CD317 ubiquitination followed by proteasomal degradation. In the same manner, Vpu is responsible for down regulation of CD4 molecule expression on the cell surface [32].

1.2.2.3 Cellular aspects of HIV latency

Latency is typical for many viruses, bacteria and other infectious agents, but the characteristics are different for each one of them. Latency was initially described as a state of dormancy and was clearly distinguished from the chronic infection with persistent symptoms. With the progress of modern medicine and molecular biology, the line between latency and chronic infection has become very fine and they should be used mainly in describing clinical manifestations of the disease.

In the case of HIV infection, a state of latency can be defined at three different levels: clinical, systemic and cellular. From the clinical viewpoint, it usually takes several years for the infected individual to develop noticeable symptoms. Therefore, on this level we can talk about true latency. On the contrary, on the systemic level, HIV never really enters the latent stage and the virus is constantly present and replicates in the host, mainly in activated T-cells [51]. Despite a virus-specific immune response, the virus can replicate, exceeding 1 billion particles per day in production rate [52]. Even during HAART therapy, viral levels are low but still detectable in the peripheral blood and lymphoid tissues [53–55].

Although all infected cells can become reservoirs of the virus, mainly CD4+ T-cells play that role. A pool of infected and activated CD4+ T-cells turn into memory cells establishing a potential viral reservoir that lasts for life [56].

On the cellular level, the development of latency depends on the virus DNA being integrated or not into the genomic DNA of the host cell. In most cases HIV DNA, freshly transcribed from genomic RNA, does not integrate and resides in the cytoplasm. This state, called pre-integration latency, is abundant in cases of patients with viremia and in individuals which are not treated with ART. Pre-integration latency is found mainly in resting T-cells [39],[57]. Most of the non-integrated DNA is not replication-competent, is easily degradable by the host cell and is almost

undetectable after the course of ART [58]. The main form of virus cellular latency, which allows HIV to be so successful in establishing persistent infection, is stable integration into the genome of the host called post-integration latency or proviral latency. Although the frequency of the latent, replication competent integration is very low (not exceeding 1 in 10^6 cells) in combination with extremely long half-life of the memory cells, it allows for lifelong infection. Additionally, the reservoir of infected memory cells can be replenished by ongoing low-level replication of the virus and infection of new cells [52]. The mechanism explaining why some proviruses become latent is not fully understood, but it is closely related to the site in the host genome in which the virus integrates and the state of activation of the host cell. It is known that the virus preferentially integrates into the introns and that high level of transcription requires LTR regions to be easily accessible for cellular transcription factors. In case of latently integrated provirus, reactivation begins the moment the cell is reactivated. Nuclear factor of activated T-cells (NF-AT) and NF-kappaB upregulate gene expression, which leads to Tat production and together with cellular factors transcription of viral RNA can begin [59]. Rearrangement of chromatin by histone deacetylases can also lead to the reactivation of latent provirus transcription [60]. Since patients still harbor the virus even after several years of HAART, understanding the processes of entering and exiting proviral latency are of great importance for improving the outcome of the therapy. Because immune activation plays a key role in reactivation of provirus transcription, several attempts have been made to boost that process and thus improve the efficacy of HAART. Most of the studies have been based on administration of IL-2 alone [61], or with IFNγ or anti-CD3 activating antibodies [62], [63]. In the first weeks after treatment begins, the levels of viral RNA in plasma drop significantly and T-cell activation is increased. Regrettably, the levels of HIV RNA rebound within weeks after therapy was halted. To purge the reservoir of latently infected memory cells, cyclophosphamide was used together with HAART. It resulted in the elimination of infected and non-infected cells without any impact on the viral levels [64]. Also, therapy with valporic acid, which act as a HDAC inhibitor, inducing relaxation of chromatin and therefore activation of provirus transcription has not been successful [65]. It is unlikely that without tackling the issue of the latently infected memory cell reservoir ART therapies will be successful and achive complete HIV clearance. Additionally those treatments can have a deteriorating impact on the already strained control of latent TB in TB/HIV co-infected individuals.

1.2.3 HIV transmission

HIV can be transmitted in various ways (intravenous, mother-to-child transmission, anal intercourse, etc.) but the major route for infection is through genital mucosal surfaces during heterosexual intercourse. Due to the protective environment of the genital tract being able to neutralize the majority of viral particles, the risk of being infected is less 1:500, although women are twice more likely to be infected than

men. For heterosexual intercourse, more than 80% of infections are caused by only one single virus breaching through the mucosal defenses. For other routes of infection the risk is significantly higher. For example, in the case of *iv* transmission, the risk is almost almost certain [66],[67].

Free and cell bound virus can be found in the semen and vaginal secretions, and both forms were shown to be infectious, which indicates that virus transmission through the mucosa can be achieved by different means [68]. Understanding those mechanisms is crucial for the development of blocking agents, vaccines and therapies. Studies done with SIV infections indicate that although the first cell to be productively infected are CD4+ T-cells, it is DCs that are responsible for transmission of the virus through the mucosa [69]. Since the mucosal secretions and mucosal associated immune cells are the main barrier for viral entry, any disturbance in this environment caused by other infections like HSV2 or *Neisseria* leads to a higher risk of HIV transmission [70].

1.2.4 Development of infection

After successful transmission the initial stage of viral infections begins. Within a week, detectable amounts of viral particles appear in plasma, followed by robust viremia lasting for a few weeks. After that time, the viral loads peaks at 10^7 particles per ml of plasma and the virus widely disseminates and spreads in the body, including to lymphoid organs [71]. Although viral particles circulate in the whole system, the majority of infected T-cells are located in the GALT [43],[21]. After 6-8 weeks, virus-levels begin to decrease. It can be attributed to the developing immune response directed towards the virus. Probably already at this point, a pool of CD4+ T-cells is already substantially diminished [72]. Early control of the infection is associated with the generation of HIV-specific CD8+ T-cells and high antigen-specific CD8+ T-cell numbers correlate with better clinical predictions for the latent phase [73], [74]. In CD8+ T-cell depletion studies, rhesus macaques infected with SIV had several times increased viral plasma levels, which indicates that cytotoxic T-cells play a critical role in controlling virus replication [75], [76]. Although cytolysis plays a major role in maintaining viral control and expression of cytolitic enzymes closely correlates with viral control, cytotoxic T-cells also employ non-cytolytic mechanisms to suppress viral replication. Studies on macaques have demonstrated that secretion of IFNγ, MIP-1β ,IL-1α and TNF strongly suppressed virus replication, but decreased significantly in CD8+ T-cell depleted animals [77–80]. Consequently, viral inhibition mediated by cytotoxic cells is mediated by numerous factors with various effector mechanisms. Polyfunctionality of CD8+ T-cells is characterized by simultaneous production of many effector molecules, usually three or more. The most widely used markers associated with antiviral activity of cytotoxic cells are IFNγ, TNF, IL-2, MIP-1β and perforin. Although correlation of expression does not prove causality, it is clear that individuals harboring more polyfunctional CD8+ T-cells are better in

controlling virus replication [81–83]. In a recent vaccine study, the antiviral activity facilitated by CD8+ T-cell was dependent on IFNγ-production only when IFNγ was expressed together with MIP-1β and CD107a (a marker for degranulation) [84]. IL-2 and TNF were also shown to correlate with virus control in elite controllers and long-term non-progressors [85]. Because of the complexity of the cytotoxic T cell response, and the fact that it is constantly changing due to the emergence of cytotoxic T cell escape variants, it is very difficult to pinpoint which molecules are crucial for virus control. Also, it is not known if the co-expression of several effector functions are required to control viral replication, or if polyfunctionality is only a marker that correlates with successful viral suppression. Importantly it is now clear that a higher percentage of polyfunctional CD8+ T-cells correlates with higher CD4+ T-cell counts. Additionally, expression of perforin by cytokine-producing cells was demonstrated to correlate with better virus control. Unfortunately, unlike perforin, cytokine polyfunctionality of CD8+ T-cells by itself was not associated with lower virus burden [78].

Cytotoxic T-cells isolated from HIV-viremic patients can inhibit replication of the virus in vitro, and thus show that they are functional and specific. Nevertheless, cytotoxic responses are unable to completely stop dissemination of the virus and clear all of the infected cells. This is due to the genetic diversity of the virus, which contribute to constantly create new escape virus variants, as well as to the virus entering post-integration latency. Escape from the cytotoxic activity of CD8+ T-cells is limited by fitness cost to the virus and conserving functionality of vital viral components. This results in some regions of the HIV genome being more conserved than others and emergence of conserved epitopes [86]. Additionally, recently it has been shown that CD8+ T-cells can recognize epitopes derived from alternate reading frames. During early SIV infections, those products are recognized by more than 20% of cytotoxic T cells and their recognition is persistent trough the course of infections [87], [88].

After the initial viremia diminishes the clinical latency period begins. Although the infected individuals do not show any symptoms of the disease, it is not a passive stage in terms of replication of the virus and response of the host immune system. During the next few years after infection, the virus is constantly present in its free form in the plasma. The immune system is constantly activated and the CD4+ T-cell count, after partial recovery in the first few months, continue to go down until it reaches <350 cells per microliter, which is considered to be the beginning of AIDS. For every individual, the period between the control of viremia to manifestations of AIDS depends on personal genetical characteristics, environmental factors, co-infections with other pathogens and the state of the immune system [52], [89–93]. One of the factors, which are thought to be critically contributing to the progress of the decay of immune system, is the chronic immune activation observed during the latent phase. Chronic activation manifests itself as an increased level of pro-inflammatory cytokines, prolonged expression of lymphocyte activation markers and

increased turnover of the lymphocytes themselves [94–96]. The level of T-cell activation measured as CD25 or CD38 expression correlates closely with CD4+ T-cell counts and T-cell [97], [98]. This can be observed even in patients receiving ART treatment and in elite controllers [99].

1.2.5 Decay of the immune system during HIV infection

1.2.5.1 Chronic immune activation and immune exhaustion

Other aspects of chronic activation of the immune system during HIV infection are that the replicative capacity and the quality of T-cell activation by APCs resemble that of an aged person. One of the features associated with this process is reduced expression of the CD28 marker on the surface of T-cells. CD28 is instrumental in activating naïve cells by binding to CD80/CD86 on the surface of APC during antigen presentation. Loss of CD28 occurs naturally during the lifetime and it is associated with inability to respond to vaccinations and greater susceptibility to infections in older age. Not only is the ability of the cells to become activated impaired, but they are also more prone to apoptosis [100], [101].

Exhaustion of the immune system also transpires in increased expression of inhibitory markers on T-cells, like cytotoxic T-lymphocyte antigen 4 (CD158), programmed cell death (PD1) and newly described in contexts of HIV infection T cell, immunoglobulin and mucin domain-containing molecule-3 (TIM3) [102], [103]. Studies where CD158 was blocked by anti-CD158 antibodies have shown no advantageous effects, but when PD1 was inactivated in a similar manner T-cell responses were partially restored [104]. In animals infected with SIV inhibition of the PD1 pathway was followed by a decrease in the virus load and improved survival rate [105]. PD1 upregulation is also associated with increased spontaneous and Fas-mediated apoptosis of cytotoxic and helper T-cells [106–108]. TIM3 is another inhibitory molecule that is found on the surface of exhausted cells. It is known that due to immune exhaustion T-cells lose their capacity to produce proinflamatory cytokines step by step. First, IL-2 production is impaired followed by loss of TNF and finally IFNγ [109].

In the early phase of infection, TIM3 is expressed on the cells producing IFNγ but not TNF. TIM-3 is believed to inhibit Th1-mediated immunity and promotes peripheral tolerance[110], [111]. During the latent phase of HIV infection, TIM3 co-expressed with PD-1 is considered one of the best markers of exhaustion and correlates with reduced number of CD4+ T-cells and increased viremia. Similar to PD-1, when Tim-3 signaling was blocked, both cytotoxic and helper T-cell proliferation and functionality was improved [103].

Interestingly the level of inhibitory receptor expression is not only dependent on the stage of the chronic infection, but also on the specificity toward different antigens. PD-1 expression was compared on the CMV- and HIV-specific CTLs from the same patient. Surprisingly, higher cell surface levels of PD-1 were detected on HIV-specific

T-cells when compared to CMV-specific T-cells. After administration of ART therapy the levels were comparable between CMV- and HIV-specific T-cells [112].

1.2.5.2 Microbial translocation

The level of T-cells activation go hand in hand with the phenomenon called microbial translocation. Early after infection, the majority of infected CD4+ T-cells are located in GALT. CD4+ T-cells by Production of inflammatory cytokines by CD4 cells as well as processing and cross-presentation of antigens derived from apoptotic bodies from dying CD4 cells recruits CD8+ cytotoxic cells to the site [113], [114]. Although epithelial cells are not infected with the virus, the increased numbers of perforin-expressing CD8+ T-cells seem to contribute to the mucosal damage in the gut during the acute phase of infection. Dramatic reduction in the number of resident CD4+ T-cells, and a decrease in the quality of the protective mucosal layer, results in leakage of microbial product [115]. Although the damage to the mucosa is done mainly during the acute phase of infection, elevated levels of bacterial LPS in the system are detected throughout the course of the disease. In addition, it correlates with the progressive decrease in the CD4+ T-cells count [116]. Even after beginning of ART treatment the levels of LPS are elevated comparing to non-infected individuals. This leads to constant activation of monocytes, macrophages and CD4+ T-cells [117][118].

1.2.6 Humoral responses

Although HIV does not directly infect B-cells, the B-cell response is severely affected during HIV infection. Cellular immunity is thought to be a prevailing factor in immune response against HIV infection. Still, antibodies allow for neutralization of new progeny virus particles and facilitate induction of antibody dependent cytotoxicity, phagocytosis and activation of complement [119]. Antibodies against the virus, produced during first few weeks of the immune response, have low or non-neutralizing ability. Even though Ab against neutralizing epitopes emerge later on, new escape variants constantly emerge due to the high mutation rate of the virus [120]. Despite that, some individuals are able to produce broadly neutralizing antibodies (NAbs). Unfortunately, those Ab are generated in the chronic, not the acute phase of the infection. For some time, the ability to elicit NAb by vaccines candidates was considered to be of less importance during preclinical testing as compared to strong cellular responses. Animal studies have demonstrated that partial protection can be achieved with passive administration of anti-HIV NAbs, showing the benefit of a strong hummoral response [121]. Additionally, individuals being able to produce Nabs displayed a reduced virus load in plasma, which lowers chances of virus transmission. For example, in macaques which could elicit moderate plasma levels of Nab more vaginal challenges were needed to achieve infection than in the control group [122], [123]. Although HIV does not infect B-cells, it affects their functions, maturation and the memory compartment. Chronic activation of the immune system also leads to hyper-activated B-cells and hypergammaglobulinemia

is observed in HIV-infected patients [119], [124]. B-cell exhaustion can be observed as the disease the progresses. Although plasma levels of circulating IgG Ab are high, B-cells isolated from infected individuals display impaired capacity to produce Ab in vitro and the percentage of B-cells having a memory phenotype is lower [122], [124], [125]. Normal levels of memory B-cells are restored after HAART has been administrated, but the B-cells are still more prone to Fas-mediated apoptosis [125]. This phenomenon is not HIV-specific and humoral responses against other pathogens, like tetanus or measles are being impaired as well, including the level of antibody production and sustained B-cell memory [126].

1.2.7 Long-term non-progressors and elite controllers

A distinctive group of seropositive individuals was identified who despite being infected with a pathogenic strain of the virus are not progressing towards AIDS. Furthermore, the so-called long-term non-progressors, are maintaining the CD4 count on a relatively high level for prolonged periods of time. Other patient groups can keep virus replication in control even to the point when the virus is undetectable in serum (controllers and elite controllers). These groups develop stronger than average CD8+ T-cell responses, especially toward *gag* epitopes [82], [93], [127]. However, in studies done by Sáez-Cirión *et. al.* five elite controllers, constituting for 25% of the study group, had no CD8+ T-cell-mediated responses [128], [129]. This is an example of how complex and individualistic the successful immune response toward the virus can be. This is important in the context of screening new vaccine candidates, where what is now considered a signature of protective immunity in the future can be shown to be an artifact of an irrelevant correlation.

1.2.8 HIV Animal models

Due to the host-range restriction of HIV, it is challenging to establish suitable models for viral infection and anti-viral immune responses. Although chimpanzees are naturally susceptible to HIV infection, they are usually not used as laboratory animals because of ethical and economic reasons [130]. Instead, smaller non-human primates, like Cynomolgus or Rhesus macaques, took their place. Unfortunately, the further away that we move from *homo sapiens* on the evolutionary tree, the more discrepancies can be seen between human immune responses and those of other primates. Species-specificity of HIV does not allow for infection of macaques. Therefore, various closely related viruses are used instead of HIV. SIV is closely related to HIV and naturally infects sooty mangabeys monkeys. Despite the high levels of viremia in sooty mangabeys, SIV is non-pathogenic. If macaques are being infected with SIV they can develop AIDS-like syndromes (sAIDS – simian AIDS). To overcome the differences between SIV and HIV, especially in vaccine studies, genetically modified chimeric viruses have been constructed with various ratio of SIV and HIV genetic material [130–132]. To lower the cost and shorten the time of the

studies, highly pathogenic strains of the viruses are usually used. For example, SHIV89.6P infection of macaques results in rapid decline of the CD4+ T-cell count and progression to sAIDS in months instead of years [133]. The use of chimeric viruses has proved to be one of the most useful tools in vaccine discovery despite their lack of some HIV components, for example envelope protein.

Several approaches have been made over the years to develop a murine HIV model and the main focus has been on genetically modified mice in which the murine immune system has been replaced with human components. Other strategies have involved adapting the virus to be able to infect murine cells. Soon after the discovery of HIV, the first attempts were made to replicate the virus in murine cell lines and in mice expressing the human CD4 receptor and other co-receptors [134], [135]. Unfortunately, no infectious virus progeny was produced by murine fibroblast cell lines and the infection of transgenic mice was very limited. Consequently, it seem that there is a strong murine restriction not only on the level of viral entry into the cell [136]. Therefore, another approach to establish a productive HIV infection in mice was to substitute components of the murine immune system with their human counterparts. After total body irradiation, mice were transplanted with human bone marrow and human lymphocytes. This allowed for a short-term and limited study spectrum of antibody immunity against the virus *in vivo* [137], [138]. Mice with severe-combined immunodeficiency (SCID) could be used as recipients of a human tissue engraftment. In SCID animals B- and T-cell receptor genes are mutated which result in a defective B and T-cell phenotype [139], [140]. Two models of engraftment in SCID mice have been widely used. In the SCID-hu *thy/liv* model, fragments of fetal liver and thymus are transplanted into a SCID mouse, whereas in the (hu)–SCID-huPBL model, peripheral blood mononuclear cells (PBMCs) isolated from cord blood or from healthy donors are injected intraperitoneally [141], [142]. The two models have been used mainly to study the cytophatic effect of the virus and for antiretroviral drug development, but are not really suitable for long-term studies of pathogenesis or vaccine efficacy [140], [143]. Currently, new SCID humanized models depend on engraftment of presorted CD34+ heamatopetic stem cells, derived directly from fetus liver, cordial blood or isolated from adult donors PBMCs after GM-CSF treatment which induce release of stem cells from bone marrow. The majority of cells that develop after the reconstitution are B-cells and T-cells although NK cells and myeloid cell subsets may also be present. After CD34+ engraftment, the animals develop both primary and secondary lymphoid organs. Although these models present impressive step toward developing a working human immune system in a rodent, the functionality of the immune cells, their localization, numbers, ratios, and interaction with non-hematopoietic cells and host-derived factors are problematic [144–146].

The reversed approach to develop a murine model of HIV infection is to circumvent the CD4-dependent viral entry into host cells. The main advantage of this approach is that it enables the use of conventional, immunocompetent wildtype mice.

Several attempts have been made to genetically modify HIV that would allow it to effectively bind to murine cell surface receptors. One of the early models, developed at Karolinska Institutet, is based on the generation of random chimeric viruses from murine leukemia virus (MuLV) and HIV. In short, human CEM line cells carrying the complete genome of the MuLV virus are infected with HIV and produce four different types of viral particles: MuLV, HIV, MuLV carrying the HIV genome and HIV carrying the MuLV genome. Next, activated murine spleenocytes are infected *in vitro* with a virus mixture and then injected intraperitoneally into recipient mice. Cells are only infected with MuLV enveloped viruses of which a small fraction contain the full HIV genome. After several days the cells are recovered from the peritoneal cavity and co-cultured *in vitro* with a HIV reporter cell line to assess the ability of recovered cells to produce infectious HIV particles [147], [148]. This method allows for a crude assessment of HIV antigen-specific cytotoxic response in the mouse and proves that murine cells can produce viable HIV particles, if limitations to the virus entry are circumvented. However, it does not establish a productive, self-sustaining infection.

Potash and colleagues developed a similar approach of utilizing a MuLV/HIV chimeric virus [48]. Instead of relying on random generation of chimeric viruses, Potash et al constructed two Eco models, EcoHIV and EcoNDK, in which a fragment of the *env* gene in HIV genome was substituted with the *env* gene coding gp80 from ecotropic MuLV virus. The HIV backbone of the constructs were either the common laboratory strain NL4-3 (clade B), or the highly pathogenic isolate NDK (clade D) [6], [149]. Chimeric viruses created this way are able to infect, and successfully replicate in, murine cells but not in human cells. Because the gp120 envelope protein is substituted with gp80 from MuLV, tropism of the chimeric virus is the same or similar to the MuLV virus. The receptor for MuLV virus is a cationic amino acid transporter (mCAT-1) protein which is expressed mainly on hematopoietic cells but can also be found on other cell types, including epithelial cells [150], [151]. The highest concentration of mCAT-1 expression in tissues correlates closely with the level of EcoHIV replication. High virus levels are detected in testicles, thymus, spleen and peritoneal macrophages. Although mCAT-1 is quite prevalent in lymph nodes and lungs EcoHIV is not preferentially replicating in those tissues [unpublished data]. In *in vitro* cultures, EcoHIV has been shown to be able to infect 10% of activated spleenocytes but failed to replicate in cell lines, i.e. 3T3 embryonic fibroblast cell line despite the presence of the mCAT-1 receptor [49]. Both EcoHIV and EcoNDK were shown to be able to establish a systemic infection in approximately 80% of inoculated animals and can be detected fro several weeks after infection [48], [152]. Importantly, from the perspective of vaccine studies EcoHIV infection is broadly disseminated and induces HIV-specific immune responses [153], [154]. Because of the ability of this virus to establish a persistent infection in WT mice we decided to utilize it in developing the first model of MTB/HIV co-infection in immunocompetent mice.

1.2.9 HIV vaccines

1.2.9.1 Early attempts – live attenuated and inactivated vaccines

From clinical, epidemiological and economical points of view, vaccines are the most effective way of preventing the spread of infectious diseases. No other therapeutic or preventive approach will allow for a complete eradication of the disease. The first vaccine ever administered was a crude preparation of non-pathogenic cowpox virus that elicited protection against the closely related, but lethal, smallpox virus. Although the mechanisms of protection achieved with this approach were not understood for more than a century, it has been proven over the following years that even the most dangerous human infections can be successfully prevented. After a rigorous campaign of vaccination, smallpox was eventually officially eradicated. Since then, developing a protective vaccine became the ultimate goal of research on every infectious disease [155].

The sterilizing vaccines that have been developed to date are mainly based on live, attenuated strains, or variants of the pathogenic agent, or on killed or inactivated microorganisms. Live attenuated vaccines were developed essentially against viral infections (polio, measles, mumps or yellow fever). Generation of such vaccines from the current perspective appears to be easy and strait forward. A pathogenic virus is passaged several times through cell cultures to minimize its pathogenicity, even though it may still be able to replicate to some extent in host cells. Similarly, a closely related, naturally occurring strain can be used (chickenpox). Usually, causing asymptomatic or mild infection, live attenuated vaccines are able to initiate both humoral and cellular adaptive immune responses without requiring additional stimulus such as adjuvants. This is a major advantage over the other types of vaccines [156].

This strategy was adapted in early preclinical vaccine trials against HIV. Monkeys infected with a *nef* deficient SIV virus were temporarily protected against an i.v. challenge with fully functional SIV [157]. Unfortunately, the mutation rate of the virus was so high that after several months of controlled infection, non-pathogenic *nef*-deficient strains mutated and created a virulent form of the virus which caused immune degeneration [158]. The same phenomenon was observed in patients infected i.v. during blood transfusions with a *nef*-deficient HIV strains [159], [160]. At the moment, HIV attenuated strains are regarded to be too dangerous to be used as a vaccine, due to their extreme mutation capabilities. Another negative characteristic of live vaccines is that the replication competent viruses or bacteria can cause disease, especially in immune-compromised individuals, although the risk in general public is relatively low. One prime example is the use of oral polio vaccine (OPV) only in areas of high incidence, where a direct risk of vaccine-associated paralytic poliomyelitis (VAPP) caused by OPV strain is smaller than the chance of contracting the disease in a classical way. When the prevalence of polio drops, inactivated poliovirus vaccine (IPV) administered by injection is used [161].

IPV represents another approach to generate vaccines by inactivating infectious strain of the virus or bacteria by physical means. Although several successful inactivated vaccines have been developed, they are inferior to the attenuated vaccines that are inducing broad protective immune responses. The inactivated or killed pathogens are not able to replicate. Therefore, immunization results in induction of strong humoral, but not cytotoxic T-cell mediated responses [162]. An inactivated vaccine candidate against HIV was tested. The Remune vaccine was constructed on the base of inactivated HIV particles stripped of the gp120 protein and formulated in incomplete Freund's adjuvant [163]. When administered to HIV-infected individuals, the CD4 counts increased and the virus levels in plasma decreased [164]. An alternative approach may be to administer Remune with the ALVAC vaccine in hope of prolonging low viral levels in plasma during periods of HAART therapy interruption [165].

1.2.9.2 Recombinant vaccines.

Progress in molecular biology allowed the development of a new generation of vaccines by administration of purified antigens. This made it possible to develop a robust and very flexible composition of vaccines mainly based on proteins. Unfortunately, similarly to the inactivated vaccines, protein vaccines mainly induce antibody and helper T cell responses. Additionally, they are also unable to stimulate innate immunity via pathogen associated molecular patterns (PAMPs) which reflect the low ability of inducing long term memory. To circumvent this problem vaccine subunit are administered with immune stimulators called adjuvants which help to induce broader immune responses [162]. One of the few adjuvants used to boost subunit vaccine responses used in humans is hydrated potassium aluminum sulfate (alum). Despite the fact that alum has been used for decades, the exact mechanism of the immune stimulatory properties is not clear. It is believed that alum forms depots from which the vaccine is slowly released, causing inflammatory responses when recognized or phagocytosed by APCs. Recently it has been shown that stimulation of DCs by alum induce production of ureic acid, which when detected in tissues is known to be an endogenous danger signal that recruits and activates inflammatory monocytes and DCs [166], [167].

Due to the risk associated with attenuated and inactivated strains, the new generation of HIV vaccine candidates are mainly vector or subunit vaccines. Because the latter induces mainly humoral responses the majority of the candidates are based on generating neutralizing antibodies against the gp120 protein, restricting virus entry into the cell. Several studies have also been conducted with the use of recombinant Gag and Tat proteins [168], [169]. The key obstacle in establishing protective immunity is the genetic variability of the virus, even when only the most prevalent and genetically conserved epitopes are being used.

A promising study utilized recombinant gp120 as a vaccine in the AIDSVAX trial [170]. The first phase III trial was conducted mainly in North America with the use of

gp120 subtype B and enrolled 5000 uninfected participants. The second trial was conducted in Thailand with gp120 from subtypes B and E, and included individuals from high risk groups like i.v. drug users and sex workers. Despite very promising results for the earlier trials no protection was observed compared to the group receiving only adjuvant. Despite this drawback, AIDSVAX was used as a boost for the ALVAC trial which showed moderately positive results [171].

1.2.9.3 Genetic vaccines

The latest and most promising approach to tackle the inefficiency of recombinant vaccines for intracellular pathogens is the use of genetic vaccines. By being expressed directly by the host cell, viral antigens can be presented to the immune system in the same manner as during natural infection and thus increasing the chances of eliciting protective immune responses. Genetic vaccines can be administered as purified DNA in an adjuvant formulation or carried as recombinant virus vectors, mainly based on poxviruses and adenoviruses [172].

The only vaccine candidate which has given promising results after a phase III clinical trial (significant protection, p=0,04) is a recombinant canarypox virus, carrying HIV *env* (subtypes B and E), *gag* and protease (subtype B) developed by Sanofil under the commercial name ALVAC. It was used in one of the biggest HIV vaccine placebo controlled trials, RV144, in which 16000 uninfected individuals were enrolled in Thailand. Claims that this trial was successful can be disputed, since the differences between the vaccinated and control group were modest. For example, the viral load in infected individuals did not differ between the patient groups. [165], [171].

The significance of the ALVAC trial is even more clear when compared to the Merck STEP study, which had to be discontinued due to futility. Over 3000 uninfected individuals were enrolled in this study, 49 of the 914 men in the vaccine group got infected with HIV when compared to 33 men of 922 in the placebo group. Since the vaccine candidate was based on the adenovirus vector (Ad5) carrying *gag*, *pol* and *nef* genes of HIV it was speculated that pre-existing immunity to Ad5 could be to blame. Although no association could be found, it has lowered the level of confidence in the safety of adenovirus-based vectors [173–175].

One of the ways to overcome the pre-existing immunity toward the vector is administration of the vaccine in its pure DNA form. Additionally advantages are easy manufacturing, stability and flexibility of this system. DNA immunizations are highly antigen specific, but are unable to elicit strong response by themselves. Therefore, the vaccine candidate needs to be administrated together with adjuvants and in prime-boost regimens. Nevertheless, DNA vaccines are able to stimulate innate immune responses much better than protein subunit vaccines. Because plasmid vectors are propagated in bacteria they carry unmethylated cystidine-phosphate-guanosine (CpG) motifs which stimulate toll-like receptor 9 (TLR9). DNA-dependent activator of IFN regulatory factors (DAI) and the TANK-binding kinase-1 (TBK-1) also activate innate immune system after recognizing plasmid double stranded DNA

[176–178]. Adjuvants not only facilitate strong stimulus for the immune system, but also play a role as carriers of the vaccine, they enhance phagocytosis as well as protect the vaccine from premature degradation. In DNA vaccine formulations one of the most efficient adjuvants are lipid emulsions in the form of liposomes, or more complex nano- and microparticles which bind DNA and by neutralizing the charge of the plasmid enhance transport of DNA across the cell membrane [179]. Emulsions are used to augment humoral responses also in protein based subunit vaccines. The oldest and most commonly used emulsion in laboratory setting is mineral oil based Freund's adjuvant. However, new adjuvants based on squalene have been recently developed and are now used commercially: MF59 by Novartis and AS03 by GlaxoSmithKline [180], [181]. In study III we have successfully used two new adjuvant formulations 2% mono-olein and oleylamine (N3) for DNA prime and 2% mono-olein and oleic acid for protein booster formulation (L3).

To further improve DNA-based vaccinations, a regimen of prime and boost has been adapted where usually the prime is delivered in form of DNA followed by one or more recombinant protein, or viral vector, boosts (as described earlier). It has been demonstrated that highly specific cytotoxic and humoral immune responses elicited by a DNA vaccine can be significantly enhanced by protein boosts [182], [183]. The efficacy of a DNA prime-boost strategy was shown in the study of a recombinant modified Vaccinia Ankara virus (MVA) vaccine candidate carrying the HIV genes *env*, *gag* and *pol*, which was developed at Karolinska Institutet. When the vector was administrated alone it induced only moderate immune responses, mainly against envelope protein, but after the introduction of a DNA prime – vector boost regime, it became highly immunogenic for *gag* and *env* epitopes [184]. In the RV144 trial, another prime-boost regimen was adapted by boosting vector prime (ALVAC) with protein formulation (AIDSVAX). Encouraging results from this study provide convincing arguments in favor of combining genetic and recombinant vaccine candidates [185].

To further enhance the potency of DNA-based vaccines various ways of delivery are being explored. Vaccines can be injected intradermally (i.d.) or intramuscularly (i.m.), or administered at mucosal surfaces (intranasally, intravaginally, etc,) or delivered with gene gun or electroporated directly into the tissue [186]. Different ways of delivery of the same vaccine can modulate quality and scale of the immune response (as shown in Paper III).

Taken together, various ways of constructing genetic vaccines, combined with different delivery methods and adjuvants provide a vast spectrum of accessible solutions for the problems we face with vaccine development against HIV. Although a vaccine providing sterilizing immunity will probably remain elusive for a long time, improving quality of the acute immune response to the virus by vaccination can have a significant impact on the resolve of the epidemic by reducing the transmission by highly viremic patients and therefore slowing down infection.

1.3 MYCOBACTERIUM TUBERCULOSIS (MTB)

1.3.1 Structure and genetic diversity

MTB is a microaerophilic, rod shaped bacteria. Unlike other bacteria, the MTB cell wall contains significant amounts of lipids (60%), which determine resistance to conventional staining (such as the Gram staining). It can also be visualized after staining with the Ziehl-Neelsen method. After a classical staining of all bacteria with fucsin, other bacteria in the culture are discolored with 3% HCl solution in ethanol, whereas mycobacteria retain the pigment. Hence, mycobacteria are commonly referred to as acid-fast bacilli (AFB). The mycobacterial cell is composed of complex macromolecular polymer containing peptidoglycan, arabinogalactan and mycolic acids, which makes it unique among other bacterial species. It plays a critical role both as an impermeable barrier and in bacterial virulence. The mycobacterial cell undergoes dynamic architectural modifications during infection that correlate with both the active and the dormant state [187], [188]. When in a dormant form, due to its cell wall, mycobacteria can resist many different environmental factors such as desiccation, elevated and low temperatures, high and low pH. Interestingly, it can persist in the environment for a long time while still being infectious. One of the main physiological characteristics of MTB is an average division time of 15-20 hours, which results in particularily long culturing time (a colony which could be visible to a naked eye requires over 3 weeks of culture on solid media).

Mycobacterial strains that are human pathogens are usually described as belonging to the MTB complex consisting of several closely related species with minimal genetic variation and includes *Mycobacterium tuberculosis* (MTB), *Mycobacterium canettii*, *Mycobacterium africanum*, *Mycobacterium microti* and *Mycobacterium bovis,* also as attenuated vaccine strain *M. bovis bacille C*almette-Guerin (BCG). MTB is a major pathogen in human populations. In contrast, other species are usually causing opportunistic diseases in immunodeficient individuals. Other pathogenic mycobacteria which do not belond to MTB complex are *Mycobacterium leprae* and *Mycobacterium marinum* [189].

In 1998, the genome of the MTB H37Rv strain was sequenced. It consists of 4 million bps coding for 3,959 genes. Until now, only about 50% of the genes have been characterized. Since the genome was sequenced, comparative genomic studies have identified several MTB-specific genomic regions of difference (RDs) absent from the genome of avirulent BCG [190], [191]. The importance of this finding lays not only in the prospective analysis of virulence factors but also in the possibility of developing better diagnostic tools based on immunological readouts that are able to distinguish people latently infected with MTB from BCG-vaccinated individuals. The first discovered region - RD1, codes for the most studied proteins. Early secretory antigen 6kDa (ESAT-6) and culture filtrate protein 10kDa (CFP-10) were identified as promising diagnostic antigens as well as possible vaccine candidates. Currently,

more than 11 RDs have been identified and the number of proteins that are being tested for their usefulness as diagnostic markers has incresased [192–194].

Taking into account the importance and complexity of the mycobacterial cell wall, it is not surprising that more than 250 genes are found to be related to the metabolism of fatty acids and 40 are directly associated with the construction of the cell wall. These genes are highly conserved, demonstrating the evolutionary importance of the lipid wall for the survival of MTB [188].

As a result of pressure exerted on HIV by the immune system, immense genetic variability occurs in a single host, which leads to constant development of new escape variants of the virus. In contrast, a recent study of MTB-derived human T-cell epitope diversity have shown that most prevalent epitopes recognized by the human immune system are also the most conserved elements of the MTB genome. The study included over 500 experimentally verified human T cell epitopes derived from a collection of geographically and genetically diverse isolates. The data suggest that at least some MTB-derived proteins do not change to avoid MHC-restricted T cell immunity. It is tempting to speculate that MTB may actually benefit from T cell recognition under some circumstances and provide an evolutionary advantage [195]. It has been suggested that MTB uses excessive inflammation caused by the immune response to generate tissue damage and lung cavitation, which promotes transmission of the bacteria. This demonstrates how complex and unique the host-pathogen interactions are between infected humans and MTB.

1.3.2 Transmission and cell entry

Bacilli of MTB are transmitted via airborne person-to-person contact. Actively infected hosts cough and expell bacteria, which are then inhaled by another individual. Acute pulmonary TB is relatively rare since only about 10% of infected individuals develop disease symptoms. Primary TB pneumonia is most often diagnosed in young children, elders or in immunosuppressed individuals such as HIV-infected and AIDS patients. In addition, it can also affect patients on long-term corticosteroid therapy. The most common forms of active disease are miliary and cavitary TB. In miliary TB many small nodules can be seen on a chest X-ray distributed throughout the lungs, whereas in a later form mainly highly oxygenated upper lobes are affected. Excessive immune reactions, necrosis of infected cells and remodeling of the connective tissue by the bacteria, cause destruction of the lung tissue and formation of large air pockets (cavities). This type of TB is highly infectious and occurs mainly during reactivation from latent TB [189]. After being inhaled and deposited in pulmonary alveoli, the bacteria are phagocytosed by alveolar macrophages, neutrophils, monocytes or DCs [196]. Due to the specific environment in which alveolar macrophages reside and the constant exposure to foreign airborne particles, these cells do not induce as strong inflammatory responses as macrophages in other tissues [197].

MTB does not have any active mechanisms of cell entry and this process is facilitated by receptor-mediated phagocytosis. There are two receptor families which mediate recognition and phagocytosis of MTB [189]. Complement receptors (CRs) expressed on the surface of macrophages recognize molecular complexes formed by complement and secreted bacterial proteins and shed cell wall components. Opsonized with C3b and iC3b components, MTB activates the alternative complement pathway and can be recognized by CR1 and CR3/CR4, respectively. The mechanism of generating opsonizing complement C3 peptides is specific for mycobacterial strains. A cell wall component of MTB can associate with complement fragment C2a to form a C3 convertase and produce opsonic C3b recognized by the CR3 receptor [198], [199]. One additional advantage of preferential CR-mediated MTB phagocytosis is the low level of superoxide inducted in alveolar macrophages during this process compared to MR dependent phagocytosis [200]. In a second mechanism of recognition, mannose receptors (MR) recognize mannose components of the most prevalent bacterial cell wall lipoarabinomannan (ManLAM). Although less prevalent than CR-mediated phagocytosis, MR recognition plays a crucial role in survival of MTB inside the host cell, since phagosomes created in this process have limited ability to fuse with lysosomes [201]

DCs use the C-type lectin receptor DC-SIGN to recognize and take up mycobacteria. Similar to MRs, DC-SIGN binds the mannose cap of ManLAM and its progenitors like PIM. In activated DCs, ManLAM augments pro-inflammatory TNF, IL-12, IL-6 cytokine production and expression of the co-stimulatory molecules CD80 and CD86. PIM acts as an inhibitor of these effects and also inhibits DC maturation [202]. Although, when C-type lectin receptor genes were deleted, no significant change was noted in the progress of the infection. Nevertheless, when a component of downstream signaling cascades of DC-SIGN, CARD9, was deleted in mice, an excessive neutrophil influx into the lungs resulted in accelerated mortality [203].

1.3.3 Prevention of phagosome maturation.

An important element of MTB pathogenesis is the ability to evade phagosome degradation inside infected macrophages or DCs. MTB prevents fusion of phagosomes with lysosomes by inhibiting the maturation. Understanding this process is essential for discovery of new drug targets as well as development of potential attenuated vaccine strains. Since the phagosome maturation process is linked to changes in the pH, it was initially proposed that the bacterial urease enzyme producing ammonia counteracts lowering of the pH inside the vesicles. Studies with urease-deficient strains have shown that these strains are still able to arrest phagosome maturation [204]. Recent studies suggest that the process of evading phagosome maturation is facilitated by inhibition of Rab7. Rab7 is a signaling molecule for phagocyte fusion with lysosomes. Additionally, markers of early phagosomal stage, like Rab5, and cell membrane proteins are retained in the membrane. This allows for nutrient and microelements acquisition which is essential for MTB metabolism and replication [205]. ManLam plays an important role in

inhibition of phagosome maturation. It disrupts the association between the Golgi apparatus and phagosomes harboring bacteria. In studies with latex beads coated with ManLam, phagosomes were unable to recruit SNARE protein syntaxin 6 (responsible for spontaneous calcium independent fusion of vesicles) and to accumulate cathepsin D and lysosomal hydrolase [206]. ManLAM by itself does not fully inhibit phagosomal maturation. Other bacterial products may be required to comple the process, or that not all phagosomes in the infected cell are being affected [207]. Despite the ability of MTB to interfere with phagosome maturation to avoid degradation, bacteria-derived antigens are eventually presented to the adaptive immune system.

Toll-like receptor 2 (TLR) is the main pattern-recognition receptor involved in the process of pro-inflammatory signaling during MTB infection. TLR2 recognizes over a hundred agonists produced by mycobacteria, including lipoproteins, mannams and lipomannans. Bacterial DNA can be recognized by TLR9, which leads to cytokine production by macrophages and DCs. Similar to DC-SIGN, deletion of TLR2 or TLR9 does not lead to severe exacerbation of the disease. However, deletion of both receptors in mice leads to increased susceptibility to MTB infection [208]. Additional, deletion of downstream signaling molecule MyD88, shared by TLR2, 4 and 9 receptors also leads to rapid mortality in MTB infected animals [209].

Other pattern-recognition receptors are also involved in detecting MTB components. Nucleotide-binding oligomerization domain protein 2 (NOD2) recognizes peptidoglycan components of the mycobacterial wall and NOD-, LRR- and pyrin domain-containing 3 (NLRP3) can recognize secretory protein ESAT6 and the secretion system ESX-1 [210], [211].

As with HIV, induction of the immune response at the site of infection can be a two-edged sword. Influx of monocyte-derived macrophages and DCs into the lungs provides additional targets for infection for MTB [196], [212], [213]. For example, induction of a pro-inflammatory response by virulent MTB via NLRP3 leads to necrotic, rather than apoptotic, cell death. This results in release of bacteria and immediate infection of freshly recruited macrophages and other myeloid cells. Mycobacteria does not have its own mechanism of cell to cell transmission, therefore induction of cell death followed by acquisition of cell bound or unconfined bacteria allows for efficient MTB expansion and dissemination [214].

1.3.4 Modulation of antigen presentation

MTB modulates the development of the adaptive immune response mostly by interfering with antigen presentation, thus postponing priming and proliferation of antigen specific T-cells. Animal models have shown that during the initial stage of MTB infection, the onset of the adaptive response is delayed by weeks compared to other bacterial diseases [215].

MTB antigens are mainly processed via three antigen presentation pathways: MHC I, MHC II and CD1. One mechanism which MTB utilizes to inhibit antigen presentation

to T-cells is reduction of MHC II cell surface levels on APCs. In a standard pathway, antigens are degraded to peptides in phagolysosomes and loaded on the MHC II complex, which, if stable, is transported to the cell surface. Otherwise it is degraded via the endosomal pathway [216]. Inhibition of phagolysosome formation impair MHC II presentation. This mechanism plays a major role in the early phase of cell infection [217]. Other MHC II inhibitory mechanisms are also responsible for interfering with MHC II complex synthesis. MTB can downregulate formation of functional peptide MHC complex by inhibiting expression of class II transactivator (CIITA) [218], [219].

Bacteria appear to inhibit the IFN-γ-induced up-regulation of MHC II expression in infected APCs. The degree of inhibition is higher when viable bacteria are being used compared to heat-killed bacilli, indicating that the mechanism, at least partially, relies on metabolic activity by the bacteria [220].

The ESAT6 protein is able to inhibit the expression of MHC Class II molecules (particularly upon IFN-γ activation) by downregulating the expression of CIITA independently of TLR2 signaling, whereas the 19-kDa lipoprotein inhibits induced class II presentation through a TLR2-dependent pathway [221], [222]. ManLAM signaling trough MR can also counteract IFNγ induced expression *in vitro*. Taking into account that mycobacteria shed significant quantities of ManLAM, which could be found in substantial amounts in circulation, this can have an effect on bystander, non-infected cells [223–225].

MHC I presentation has not been shown to be affected by MTB evasion mechanisms. Bacterial antigens are usually presented on MHC I in a process of cross-presentation but due to the fact that MTB can escape from the phagosome to the cytosol, some of the antigens are presented via classical MHC I pathway. ESAT6 and CFP10 bacterial secretory proteins can also cause disruption in the phagosomal membrane and leak into the cytosol, where degraded, can be loaded onto the MHC I molecules [226].

Mycobacteria have a complex cell wall compared to many other pathogenic bacteria. The main component is a glycolipid layer consisting of mycolic acid, peptidoglycan, arabinoglycan and LAM, as well as variety of glycol and lipoproteins. Hence, in addition to presentation of peptide antigens on MHC molecules, presentation of lipid antigens by CD1 antigen presenting molecules to CD1-restricted T cells may play a role in developing protective MTB-specific adaptive immune responses during pulmonary TB [227–229].

Previous studies have shown that lipid antigen presentation can occur by the detour pathway characteristic for MHC class I cross-presentation. This new 'detour' pathway for presentation of antigens from bacteria contained in phagosomes, demonstrates the significance of infection-induced apoptosis in the activation of CD8 T-cells specific for glycolipids and lipoproteins. Therefore, the ability of MTB to block apoptosis can be considered an evasion mechanism in this context [230].

Similarly to MHC II molecules, MTB can also inhibit expression of CD1 molecules in DCs *in vitro* [231]. After *in vitro* infection of monocytes, which were stimulated to differentiate into DCs, the cells failed to induce CD1 expression. This shows that the infection of monocytes can impair their differentiation in vitro [232], [233].

Natural killer T-cells (NKT) are also involved in recognition of lipid antigens presented on CD1d molecules. Destined to be one of the first lines of defense, activated NKT cells produce large amounts of IFNγ after T cell receptor recognition of antigens presented on CD1d molecules. It has been shown that the NKT cells stimulation with α-galactosylceramide, can lower their susceptibility to MTB infection [234], [235]. On the other hand, no downregulation of CD1d expression in infected cells was observed and adoptively transferred macrophages upregulate the cell surface expression of CD1d in MTB-infected lungs tissue [236]. Finally, mice which lack CD1d do not display augmented disease progression showing that CD1d-restricted NKT cells are not absolutely required for control of MTB growth during the natural course of infection [237], [238].

1.3.5 Modulation of effector adaptive immune response

During the initial stage of infection, or reactivation from the latent phase, multiple molecular and cellular innate immune events take place, but induction of adaptive immune response is severely delayed. From skin test studies using purified protein derivative (PPD), it is known that the T-cell response in humans require over 40 days to develop comparing to only a few in the case of other pulmonary bacterial diseases [239]. In mice, this period is shorter and does not exceed three weeks [234]. Interestingly, adoptive transfer of antigen specific CD4+ T-cells into infected animals does not have an effect on bacterial load in the lungs or overall survival of the mice [240–242]. Late development of T-cell responses can be attributed to delayed priming having its origin in inhibition of APC maturation. It has been shown that in resistant mice bacteria are disseminated earlier to the PLN than in susceptible strains allowing for earlier T-cell priming [240]. Another important factor is an exceptionally slow replication of MTB with the doubling time being 20-24h, compared to less than one hour in the case of other common bacterial pathogens [243]. The exact mechanism of the delayed DC migration to the draining lymph nodes is yet unknown. One of the proposed explanations is the inhibition of CC-chemokine receptor-7 (CCR7) signaling by MTB. Also interleukin-10 was shown to contribute to this effect [244],[245].

It is not defined which population of dendritic cells are responsible for the delivery of bacteria to the lymph nodes but it is clear that arrival of DCs infected with viable bacteria correlated with emergence of CD4+ MTB specific T-cells [240]. Since T-cell priming and proliferation coincides with dissemination of the disease to the LN it was suggested that this can affect migration of T-cell to the site of primary infection. It was shown that naive T cells upregulate CD44 and downregulate CD62L, and begin to accumulate in the lungs with similar kinetics as when T-cells were primed with

soluble protein antigens [246]. However, during the course of infection, development of regulatory T-cells is observed which contributes to delayed priming and migration of CD4+ T cells to the lungs. Importantly those T regulatory cells were shown to contain MTB antigen-specific cells [247]. On the other hand BCG vaccination also was shown to induce MTB specific Tregs therefore accumulation of these cells may not be the direct reason why the immune system is unable to clear MTB infection [248–250]

The capacity of CD4+ T-cells to mediate protection is not fully understood. It has been demonstrated that production of IFNγ by CD4 cells is critical for the induction of bactericidal activity after phagocytosis by macrophages and IFNγ knock-out animals succumb to the infection extremely rapidly [251]. Production of other pro-inflammatory cytokines like TNF has also been shown to play an important role in controlling TB infection [252]. As mentioned before in chapter 1.2.6, effector CD4+ T-cell can produce more than one cytokine at a time and multi-functionality are associated with better protection against MTB infection [253], [254]. CD4+ and CD8 T-cells producing several cytokines have been identified in newly BCG vaccinated children and in TB patients. Therefore, eliciting T cells able to produce several pro-inflammatory cytokines simultaneously by new TB vaccine candidates is considered to be a major goal [254], [255].

It is very important to note that in the case of TB, most of the infected individuals either become latently infected for life, or are able to clear the infection without noticeable symptoms. Therefore, information gathered studying MTB-infected individuals comes mainly from the minority of infected patient that are unable to control the infection. This may skew our understanding of what is protective and what is disruptive in the anti-MTB immune response.

1.3.6 Granuloma formation

Granuloma formation is one component of the immune response against MTB that is constantly under debate. It is a characteristic feature of immune responses against pathogens that cannot be controlled and eradicated by the immune system. When phagocytic and cytotoxic cells are unable to kill the pathogen, other innate and adaptive immune cells accumulate around the infected cells. During MTB granuloma formation, undifferentiated monocytes congregate around infected macrophages and DCs. and after activation with IFNγ produced by CD4+ T-cells activation increase in size and cell organelles creating so called “foamy cells” [256]. IFNγ also acts on neighboring macrophages which fuse their cell membranes creating polynuclear cells (giant cells) [257], [258]. Membranes of the giant cells overlap with each other creating a multilayer, zipper barrier that restrains physical dissemination of the bacteria outside the granuloma center. The core of a granuloma is surrounded by T-cells and B-cells, together with neutrophil and NK cells. Epithelial cells create a sack, which is further surrounding and reinforcing formation of the lesion. TNF-α is

believed to be a crucial cytokine maintaining the integrity of the granuloma structure [214], [252], [259], [260].

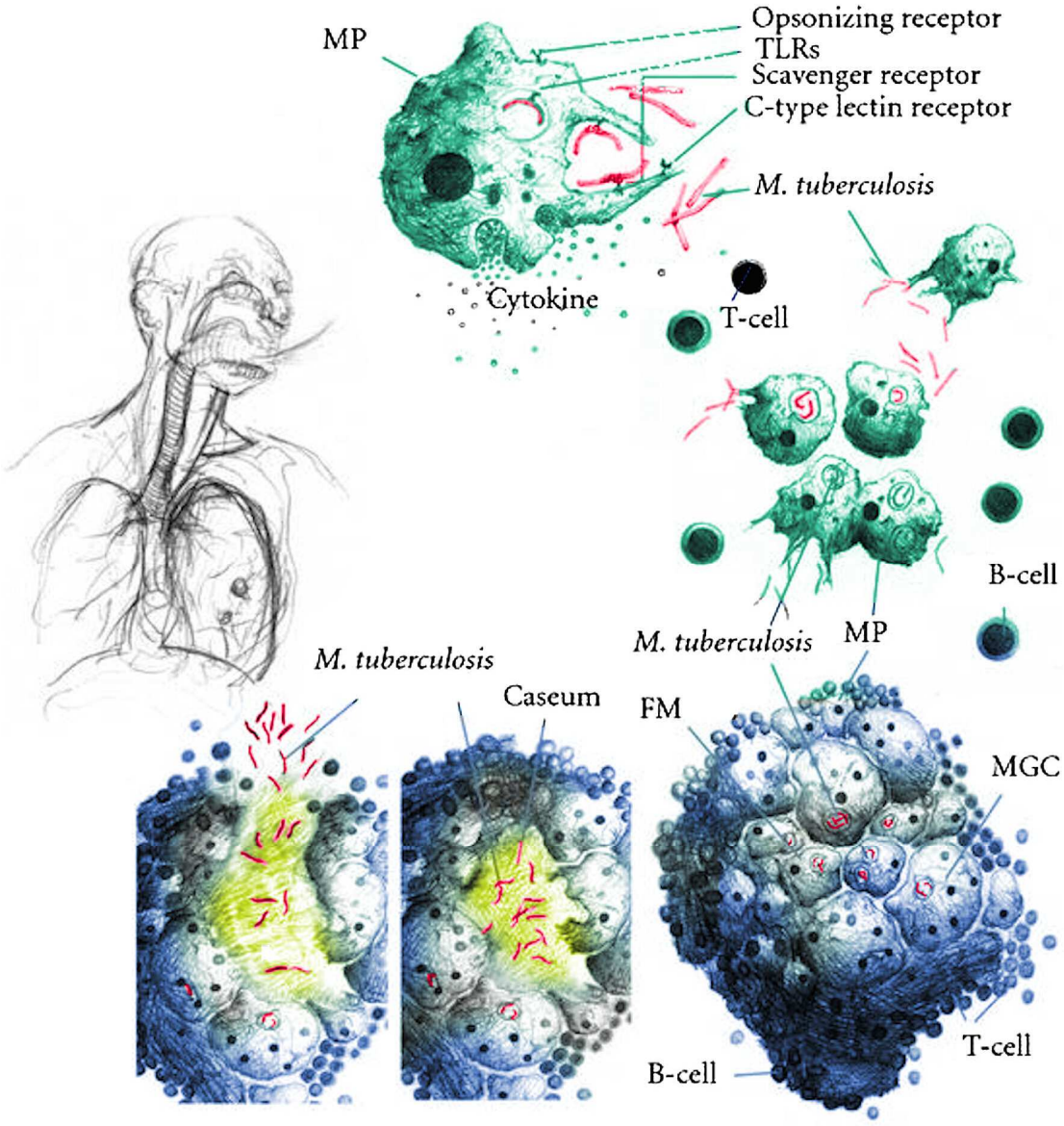

Figure 3. Formation and maturation of lung tuberculous granulomas. Adapted from *Silva Miranda* et. al. [403]

Human granulomas are more organized than murine lesions and contain calcified aggregates of necrotic macrophages. Due to the dense structure, the center of granuloma is anaerobic and partially deprived of nutrients required for MTB replication. It is believed that by combining a growth-restricting environment surrounded by a tight cellular barrier, the host organism can control dissemination of the infection. Latently infected, asymptomatic individuals can control the infection indefinitely. However, due to the limitedations in probing methods the amount of bacteria being harbored in granulomas of infected patients is unknown [214], [261–263]. Although granulomas may play an important role in controlling the infection, it is possible that granuloma formation is part of a long-term survival strategy for the bacteria. Due to its structure, granulomas can become a niche for

dormant bacilli, with giant cells and foamy macrophages creating a barrier. Whilst the macrophage barrier may prevent dissemination of bacteria, it also prevents an influx of antigen specific T-cell into the granuloma, which could activate infected cells to kill phagocytosed bacteria [264], [265]. It is debated whether the TB granuloma is a host defensive mechanism, or a bacteria-induced survival feature. We should keep in mind that humans and other natural hosts co-evolved together with MTB for millions of years. Thus, granuloma formation may be beneficial for both organisms on a population level. In the majority of the 2 billion people infected with MTB, reactivation rarely (10%) happens and it is usually associated with immune deficiencies. Despite that, TB is one of the most prevalent bacterial diseases. The available knowledge on how granulomas develop in healthy and asymptomatic individuals, and what characterizes successful control of the infection, is still limited.

In recent years evidence showing that TB granuloma is not a static construction and that not all bacilli inside it are in a dormant metabolic state [214]. Studies in resistant mice strains have demonstrated that, although CFU (colony forming unit) load in the lungs remains stable after 6 weeks a subpopulation of bacteria continues to replicate while other bacilli are being killed. Those two processes balance themselves out creating a state of bacterial load equilibrium [266]. Another study of whole bacterial genome sequencing in monkeys has shown that MTB steadily accumulates mutations during latency [267].

On the other hand it seems that not all bacteria are fully metabolically active during active phase of infection. Monotherapy with isoniazid (INH) has been shown to be ineffective not only due to the development of resistance but also due to the fact that INH is not an active form of a drug. Hence, it needs to be activated by bacterial catalase-peroxidase (KatG) to its active form of isonicotinic acyl-NADH complex. Because only active bacteria can be eliminated by INH, dormant forms survive unaffected suggesting that bacteria develop isoniazid resistance. Nevertheless, long lasting monotherapy with INS in latently infected individuals leads to sterility which provides further evidence that bacteria previously described as dormant, actually switch between active and latent state [268], [269].

A new interesting model of dynamic latency and its environment-dependent reactivation has been proposed. In this model, the majority of the bacilli lay dormant in the granuloma and only a few are metabolically active. Those “scouts” are able to “sense” the attractiveness of environment. If oxygen and nutrient conditions in the granuloma improve as a result of loss of structural integrity, the scouts can induce reactivation of dormant bacilli by secreting resuscitation-promoting factors (Rpfs). This kind of bacterial communication is common in other species like *Pseudomonas* [270–272]. One of the indirect evidence supporting this concept is increased bacterial recovery form sputum of actively infected patients before administration of MTB antibiotic therapy [273].

1.3.7 Humoral response during TB infection

Humoral responses against MTB have been considered to play a minor role in controlling the infection. Nevertheless, the largest population of lymphocytes in the granulomas are B220+ cells [274]. The reason why the B220+ cells accumulate is not fully understood, but it has been shown that they can play a role in regulating immunity within the lesions [275]. Also B-cell deficient mice show alteration in granuloma formation. Although those mice are not more susceptible to infection with MTB via the aerosol route, they succumb faster to infection than WT mice after intravenous inoculation [276–278]. *Hamasur* et. al have shown that passive immunization with LAM specific F'ab fragments of Abs results in lowered bacterial burden after intravenous infection [279]. Consequently, the role and significance of humoral immunity during MTB infection may need to be reassessed.

1.3.8 Animal models of tuberculosis

1.3.8.1 Non murine rodents

In contrast to HIV most mammals are naturally susceptible to TB. Thus, finding a suitable model of human MTB infection should theoretically not be as demanding as in the case of HIV. In very early studies of TB, guinea pigs quickly became an animal of choice and were widely used. The reasons for choosing guinea pigs by Koch and others were the close similarity of tubercles (granulomas) and progression of the disease to that observed in people [280]. Other similarities which make guinea pigs a favorable TB model of human disease are mature lymphomyeloid complex in new-born animals and similarities in the physiology of the pulmonary tract, especially the response of the lungs to inflammatory stimuli. Importantly, unlike mice and rabbits, guinea pigs can transmit TB between themselves via aerosol [280], [281]. Unfortunately out of the 20 inbred strains of guinea pigs developed in the first half of the XX century, there are only two are commercially available (strain 2 and 13) [282].

Another rodent model is the rabbit. Rabbits are quite susceptible to TB which causes excessive lung damage. Inbred strains with different level of susceptibility to TB were developed. TB resistant rabbits are excellent model for study of cavitary TB whereas in most susceptible strains disseminated, systemic disease is a characteristic trait. [283], [284], rabbits are usually not inbred anymore therefore their usefulness is limited.

1.3.8.2 Non-human primates

The use of primates in studies of TB has the same limitations as described for animal models of HIV infection. Similarly, in the case of TB, most studies are performed with the use of rhesus monkeys which are highly susceptible to TB and other virulent mycobacteria [285].

Bronchial infection results in a rapid progression of primary pulmonary TB followed by quick dissemination. When a low dose of inoculum is administered, only half of

the animals develop symptoms immediately, whereas the other half is considered latently infected (positive quantiferon assay and TST). Interestingly, latency described in those monkeys is not uniform. Animals that do not progress to active TB have significantly lower CFU and pathology score in the lungs. Additionally, some of the animals with low CFU revert spontaneously to an active form of TB, while some harbor intermediate levels of bacteria. Although non-symptomatic, these animals described as percolators, have significant levels of replicating MTB. In monkeys with active, TB extensive caseous necrosis along with liquefaction of the caseous have been shown to result in cavity formation, whereas in latent monkeys solid fibrotic and solid cellular granulomas are observed [286–289]. It is worth noticing that while it is not fully understood why certain monkeys develop active pulmonary TB and others do not, the animals are not inbred. However, the genetic variability is partially limited compared to wild animals.

Similar results were obtained from Philippine cynomolgus monkeys which are considered more resistant to TB than rhesus macaques, although no cavity was observed [290]. In both species direct delivery of a small MTB inoculum into the lungs is critical in achieving human-like progress of the infection. Nevertheless, these animals are still more susceptible to TB than most humans.

1.3.8.3 Mice

Although other rodent models of TB infection seem to mimic human TB infection better, due to the many tools that are available, mice have been the model of choice in the majority of the studies. Two strains of inbred mice which are mainly used are C57BL/6 (B6) and BALB/c. The former is considered to be one of the most genetically resistant to TB while latter is highly susceptible to *i.v.* infection but has a relatively good resistance against aerosol infections [291]. These strains develop disease that could be described as an intermediate or chronic disease. After initial exponential growth of the bacteria for 5-7 weeks, replication is controlled and reaches plateau at ~10^6 CFU per lobe. This coincides with accumulation of antigen specific effector CD4+ and CD8+ T-cells and formation of granulomas. The number of bacteria is maintained at this level until the mice die one year or one year and a half later. The granulomas that form in MTB-infected B6 and BALB/c mice lack necrotic centers and the lesions do not show signs of hypoxia. Data indicate that innate and adaptive immune responses in these mice are sufficient to arrest the growth of MTB, but their ability to eliminate bacteria is limited [292–294].

Despite the large number of mouse strains available, they are not all being commonly used in TB research. The mouse model is criticized for lacking many of the features of human TB, but while this is true for B6 and BALB/c mice, in other strains some certain human-like characteristics are better represented. For example C3Heb/FeJ, one of the sub-strains of the well-characterized C3H strain, has been shown to develop highly organized necrotic lung granulomas after a low-dose

aerosol challenge with pathogenic MTB Erdman strain, and was shown to be more susceptible to infection that the parental strain [295], [296].

It was determined that the sst1 locus plays a key role in the development of pathogenesis of necrotizing lung granulomas. It has been shown that congenic mice on the B6 strain background which carry the sst1 locus derived from C3HeB/FeJ animals are more susceptible to TB than the parental B6 strain [297].

It was shown that TB-caused cachexia is controlled by combinations of different alleles, therefore introducing more genetic diversity into the inbred strains can result in improved phenotypic models [298]. Latency is one of the most important features of human TB, which is still not properly represented by any of the murine models. A partially successful model, which is still being used, was developed in 1950s at Cornell University. Resistant mice were infected with MTB and after the bacterial load reached a plateau, the animals were treated with isoniazid and pyrazinamide to kill replicating but not dormant bacteria. Approximately three months after cessation of the antibiotic treatment revival of active infection occurs in two thirds of animals [299]. This approach is laborious and although metabolically active bacteria are not detectable this model for some time, it only roughly resembles the physiology of latency, including the immune response and mechanisms of bacterial containment in latently infected humans [300], [301].

Similarly to HIV infection, it will probably be impossible to model all features of the immune response toward TB in a single mouse model. Additionally, the choice of inbred strains that carry unique combinations of genetic variants, which are not typically representative of the whole species, can result in missing or omitting results.

1.4 CO-INFECTION OF MTB AND HIV

The most prominent symptom of HIV/TB co-infection is the reactivation of latent TB due to the development of AIDS in patients. In the classical model of reactivation, the slow decline in CD4+ T-cell mediated immunity against MTB triggers disruption of granulomas and bacterial replication is no longer controlled. In co-infected individuals, the CD4+ T-cell count decline is a leading factor in TB progression. Nevertheless other factors play an important role in driving progression of MTB- and HIV-mediated disease.

1.4.1 Induction of active TB during co-infection

Population studies have demonstrated that the chance of developing an active form of TB is estimated to be between 5-10% in a lifetime. However, this risk increases more than 10 fold in HIV positive individuals [302]. Additionally, in studies of mine workers in Malawi and South Africa it has been shown that HIV positive individuals were two to three times more likely to develop symptoms of active TB only two years after estimated HIV transmission. Due to the high prevalence of TB in this

community and working conditions favorable for aerosol infection transmission, these incidences were a mixture of reactivated and newly acquired TB. Clinical discrimination between re-infection and re-activation of MTB is very difficult. Nevertheless, because mechanisms of MTB re-infection and re-activation are not identical, grouping subjects with re-activated latent and acute infection together during studies can distort the relevancy of the findings. By restriction fragment length polymorphism typing of the MTB insert sequence 6110 (IS6110) it has been shown that in seropositive workers the chance of developing an active form of pulmonary TB resulting from a newly acquired infection was 2.2%-5.5% higher than in HIV negative individuals [303–306].

1.4.2 Bacterial transmission during co-infection

One of the main anatomical manifestations of active TB is severe lung tissue destruction and creation of cavities (cavitary TB). As described before, this form of TB is the most infectious and generates the highest concentration of bacilli in sputum samples [307]. It has been shown in numerous studies that aggravated immune responses contributes to tissue damage during development of active MTB, and it is speculated that MTB induces this inflammatory reaction to allow for transmission between hosts. In the lungs of co-infected individuals, the number of tubercle lesions as well as the frequency of MTB-induced cavities is lower than in patients infected with MTB alone. Additionally, the CD4+ T-cell count in blood of co-infected individuals correlated with the probability of cavitary development. In patients with a CD4+ T-cell count below 200 per µl, the likelihood of cavitary TB was four times lower than in patients with a CD4+ T-cell count above 500 per µl [308].

As a result, the number of bacteria in sputum samples of co-infected patients is lower. Whether this reflects on the infectivity of sputum from those patients has not been tested. Due to that fact that most TB diagnostics is still based on acid-fast staining, the lower CFU results in poor detection of MTB cases among seropositive individuals [89], [309].

These findings are unexpected since in the majority of other pulmonary pathogens like influenza virus or *pneumococci*, immune suppression is usually associated with an increased pathogen load in sputum or mucus as well as prolonged infection [310].

1.4.3 Exhaustion in co-infection

T-cell exhaustion is another mechanism that may help explain the failure of the host immune response and could be associated with increased speed in which latent TB develops in seropositive individuals. It is not clear weather the exhausted phenotype of HIV antigen-specific CD4+ T-cells can be observed for the MTB-specific T-cells in the lungs of co-infected individuals. As previously described, during HIV infection PD1 is considered a marker of immune exhaustion. PD1 and Tim3 can be expressed on cells that are unable to produce cytokines and proliferate upon antigen

stimulation. Strikingly, in mice deficient in PD1, MTB infection causes an excessive pro-inflammatory response in the lung tissue that leads to premature death of the animals. Following depletion of CD4+ T-cell in these mice partially attenuates the excessive inflammation [311]. Additionally, it has been shown that PD1 is expressed by functional MTB antigen-specific T-cells in the lungs of mice. CD4+ PD1+ T-cells isolated from MTB infected animals were able to proliferate and produce pro-inflammatory cytokines *in vitro* [312]. On the other hand, it was shown that a high blood count of pro-inflammatory MTB-specific CD4+ T-cells that produce multiple cytokines correlates with a lower CFU in the lungs of MTB-infected individuals. In patients with pulmonary TB, the majority of the cells were able to produce only TNF and IFNγ. 6 months after antibiotic treatment and significant reduction of the CFU in the lungs, both the CD4+ and the CD8+ antigen-specific T-cells regained the ability to proliferate and produce multiple cytokines. [253]. This finding suggest that the exhaustion mechanism being responsible for the failure to control HIV infection does not need to be directly detrimental for the immune response against MTB, and that expression of inhibitory molecules in different locations play a different role in host immunity.

1.4.4 Lungs as a harbor of HIV replication

Two interesting observations have been made in studies of bronchoalveolar lavage (BAL) from MTB/HIV co-infected individuals. Samples collected from the area of the lung affected by MTB contained higher levels of HIV branched DNA (bDNA) and p24 protein than samples collected from relatively unaffected tissue (localization was based on chest X-ray analysis). Additionally, the viral load in the lungs was higher than in plasma from the same patient that was not yet treated for TB. After antibiotic treatment, the virus load in the lungs decreased. Interestingly another correlation was made between elevated levels of TNF in the areas containing high number of granulomas and p24 levelsa [313]. Another study has confirmed the increased virus load in lungs compared to plasma. The median HIV concentration in lung pleural fluid was four times higher than in plasma. Again, increased expression of pro-inflammatory cytokines was detected which correlated with virus loads in the lungs. Interestingly, it was shown that in the lungs a substantial amount of the virus is replicating in CD14+ macrophages, whereas in blood the virus is almost exclusively lymphocyte-derived [314], [315]. These results show that the virus replicates preferentially at the site of MTB infection which can be a substantial virus reservoir.

Interestingly in co-infected individuals had lower CD4+ T-cell levels than ones infected with only MTB. CD4+ T-cell levels were also lower in comparison to seropositive patients living in the same community but with no confirmed MTB infection [316]. This could be an inclination that virus replication in the co-infected lungs is not mainly lymphocyte dependent. On the other hand, it could be associated with the different structure of the lesions in co-infected patients. CD4+ cell T-cell depletion, resembling the one occurring in the gut could be the reason for observed

differences. Additionally, yield of broncholavache sampling may not be a good representation of cell arrangement in the lung tissue.

In the perspective of animal models of co-infection it is worth noting that in cynomolgus macaques latently infected with TB and SIV there was no increase in peripheral virus replication described. Nevertheless, acute and transient decrease in the number of peripheral CD4+ T-cells correlated with time of TB reactivation [317].

Lawn et al. showed that HIV propagates in macrophages from co-infected patients, which underlines the importance of these cells, together with monocytes and DCs, as common hosts for HIV and MTB replication [314]. Co-infecting the same cell is yet another dimension of how these two pathogens could interact with each other. It has been shown that *in vitro* infection of alveolar macrophages with MTB caused reduced induction of apoptosis in cells isolated from seropositive individuals comparing to healthy donors [300]. This was associated with elevated levels of IL-10 rerelease. Direct co-infection of monocyte-derived macrophages in vitro has been shown to have a detrimental effect on the control of the replication of both HIV and MTB. Co-infection also resulted in a decreased viability of the cells. Increased levels of interleukin 1β, -6 and -8 (IL-1β, IL-6, IL-8) in co-infected cultures comparing to MTB infected ones indicates that co-infection can have a by-stander effect on other cells in a form of additional cytokine stimulation[318].

It is worth noting that MTB/HIV co-infection of the same cell is probably a rare event. However, the micro-environment of the TB granuloma may increase the chances of it occurring. Additionally, the high number of potential target cells in the granuloma may enhance the effect of released cytokines, mycobacterial and HIV antigens. Additionally, as described before, HIV proteins can cause an exogenous effect on none infected cells. Macrophages infected with MTB released less TNF when purified Nef was added to the in vitro culture. This lead to decrease in bacterial killing and TNF-dependent apoptosis [319]. The effect of mycobacterial products on enhanced maturation and increased ability to mediate trans-infection by DCs have been described by us in study I. It will be described in more detail in the following section.

1.4.5 Impact on the development of active TB before AIDS

One intriguing question regarding HIV infection in MTB co-infected individuals, is the scale of modulation of immune response against MTB before the development of AIDS. Addressing this issue in clinical settings is particularly difficult due to the inability to determine the exact time of HIV transmission. Since the symptoms of the acute phase after virus infection are not specific enough, the only way to determine the time of infection is to monitor seroconversion of individuals at high risk. *Geldmacher* et al. tackled this problem by monitoring the HIV status of 114 healthy individuals that were considered to be latently infected with MTB. Latency was confirmed by a PPD skin test, an IFNγ release assay and restimulation of PBMC with

the MTB-derived ESAT-6 protein. In comparison to the HIV positive latently infected individuals, seronegative controls had higher levels of MTB-specific CD4+ T-cells in circulation. In four out of five individuals that seroconverted, acute infection with HIV resulted in immediate depletion of CD4+ T-cells. Importantly, those individuals did not show symptoms of TB reactivation and remained asymptomatic until the end of the study. Soon after HIV transmission, an active form of TB was diagnosed in patients where the CD4+ T-cell population did not decreased after seroconversion. Interestingly, this phenomenon was associated with TB but not with chronic CMV infection [320], [321]. Although these studies show a drastic impact of acute HIV infection on the systemic CD4+ T-cell response against MTB, it is yet to be determined to what extent this CD4+ T-cell depletion in peripheral blood translates to depletion of antigen specific cells at the site of the infection.

1.4.6 Granuloma as a site of co-infection

It has been shown that MTB/HIV co-infected patients have a similar number of tubercle lesions as in the patients latently infected with MTB alone. Interestingly, in the granulomas of co-infected patients the expression of three major pro-inflammatory cytokines IFNγ, IL-12 and TNF was increased. Also TNF expression coincided with necrotic granulomas [322]. One needs to bear in mind that the increased expression of cytokine mRNA in the tubercles does not imply whether these cells are HIV or MTB-specific. A partially contradictory study was conducted with the use of cells isolated from BAL of seropositive BCG-vaccinated individuals. Cells re-stimulated with BCG antigens produced less IL-2, TNF and IFNγ in comparison to HIV negative individuals. These individuals were not infected with MTB, although BCG vaccination is in fact infection with an attenuated *Mycobacterium bovis* strain. The inability of the CD4+ T-cells to produce pro-inflammatory cytokines does not prove that the same defect would occur in case of an infection with a pathogenic mycobacterial strain [316]. Another contradictory study has shown decreased levels of TNF production in lesions of co-infected individuals compared to MTB-infected controls. The granulomas displayed extensive necrosis, reduced TNF production and to be unorganized [323]. An important fact when comparing these two studies is the fact that the latter was performed as a retrospective necropsy. Lungs were obtained from AIDS patients who died between 1995 and 2001, and their CD4+ T-cell count was not stated. Whether or not they had been treated with ART therapy is also unknown. Thus, they represent an end stage of the disease rather than reflect an immunological situation in the granulomas of latently co-infected patients.

Not surprisingly, also IFNγ production has been shown to be decreased in AIDS patients with active pulmonary MTB compared to patients with latent TB [324]. It is worth noting that although low levels of IFNγ was detected after restimulation of PBMC with PPD antigen, the percentage of MTB-specific IFNγ-producing T-cells in BAL fluid was significantly higher in patients with AIDS [325]. These studies clearly

illustrate that the process of TB reactivation, due to HIV infection and progression of the disease is a dynamic, localized process. Hence, it is important to better understand how MTB and HIV influence host immunity in vivo at early and late stages of co-infection.

1.4.7 Immune reconstitution inflammatory syndrome (IRIS)

One of the unexpected results of the development of antiretroviral therapy was the development of IRIS in co-infected patients. TB is the underlying factor most of IRIS manifestations, but other bacteria like *cocci*, non-tuberculosis *mycobacteria*, or viruses like CMV, Herpes simplex or Hepatitis virus C also provoke the development of this condition. Restoration of the immune response to combat the infections results in a robust inflammatory response, which in other circumstances would be beneficial to the host but in severely immunosuppressed individuals results in life threatening symptoms. The main manifestations of IRIS are severe fewer, enlargement of lymph nodes and reactivation and dissemination of TB lesions which cause respiratory dysfunction, hepatomegaly and splenomegaly [326]. Diagnosis of IRIS is problematical and mainly based on clinical observations. The vast number of possible symptoms in co-infected patients makes diagnosis difficult even for experienced clinical staff and it can be an additional obstacle to ART treatment programs in high TB endemic countries [327]. Although IRIS depends on the restoration of a functional immune system, it is the individuals with the lowest CD4+ T-cell counts that are prone to developing the syndrome. For the majority of co-infected patients, the risk of developing IRIS after initiation of ART varies between less than 10% to over 40% and the risk decreases over time. Due to the different pace in which individual patients react to ART and how fast the immune system recovers, IRIS can manifest itself even 3 months after the beginning of the treatment [326]. Even though the risks of IRIS, ART or HAART are recommended to be initiated in all AIDS patients with identified opportunistic infections not later than 3 weeks after the beginning of the antibiotic treatment [327].

1.4.8 Co-infection after HAART and antibiotic treatment

When MTB is the co-infecting pathogen of HIV+ patients, the majority of the detrimental immune responses are mediated by newly proliferated MTB antigen-specific CD4+ T-cells producing IFNγ, IL-2 and IL-12 [328]. This leads to activation of macrophages and DCs in the granulomas, which induces reactivation and granuloma disruption [329]. There is limited knowledge on how the immune system responds to and controls MTB during long timeperiods after ART. One of the few studies addressing this question was conducted by Wilkinson et. al. PBMC from seropositive patients who were infected with MTB. The patients were treated with HAART, and antibiotics were regularly administered up to a year after the initiation of HAART therapy. Out of the MTB antigen specific CD4+ T-cells identified, central memory cells and terminal effector cells were the first cells to expand by week 12, followed

by appearance of greater numbers of naïve cells by week 36. Although the overall CD4+ T-cell counts increased steadily, the percentage of MTB antigen-specific T-cells declined within the total CD4+ T-cell population [330]. Although most of AIDS patients who are being diagnosed with and MTB are currently being treated for both infections, there is that they will be re-infected after successful antibiotic treatment. There is also a chance of patients who were not infected with MTB prior to ART therapy to become infected. Due to the fact that more and more patients in MTB endemic areas are being successfully treated with ART this issue needs to be addressed in additional studies.

2 AIMS

The overall aim of this thesis was to investigate interaction between HIV and *M.tuberculosis* infection from the perspective of hosts immune system and modulation of which mechanisms of immune response directed against these two pathogens could be responsible for increased susceptibility to MTB and HIV during co-infection.

Specific aims of studies presented in this thesis were:

- To develop murine MTB/HIV co-infection model in immunocompetent animals (Study IV)
- To gain insight into role of immmunomodulatory cells in the natural differential susceptibility to *M.tuberculosis* (Study I)
- To assess impact of chronic *M.tuberculosis* infection on immunogenicity of HIV vaccine (Study III)
- To investigate effects of M.tuberculosis infection of macrophages on Ability of dendritic cells to facilitate HIV transmission (study II)

3 RESULTS AND DISCUSSION

3.1 STUDY I

Failure to recruit anti-inflammatory CD103+ dendritic cells and a diminished CD4+ Foxp3+ regulatory T cell pool in mice that display excessive lung inflammation and increased susceptibility to Mycobacterium tuberculosis.

3.1.1 The importance of controlling host immune responses during MTB infection

While innate and adaptive immune effector functions are required for control of MTB growth, excessive immune activation can lead to tissue damage and loss of function. In this study, we established a low dose nose-only MTB aerosol infection model and took advantage of WT inbred mouse strains that are either resistant (C57BL/6 and BALB/c) or susceptible (DBA/2) to MTB infection, in order to increase our understanding of the inflammatory response during pulmonary TB [291], [331], [332]. Comparisons between different inbred WT mouse strains have identified numerous features of the host immune response that may help explain naturally occurring susceptibility [240], [291], [332–337].

3.1.2 Recruitment of myeloid cells in resistant and susceptible mice

We took advantage of wild-type (WT) mice that are either resistant (C57BL/6 and BALB/c) or susceptible (DBA/2) to MTB infection [291], [331], [332]. To mimic the natural route of infection, we established an aerosol infection protocol in which the mice could be infected with a low dose(20 to 200 CFU) of aerosolized bacteria directly into the lungs. As expected, nine weeks after the infection, the lungs of susceptible DBA/2 mice harbored the highest number of bacteria (>10^7 CFU), and hematoxylin/eosin staining showed that these mice had larger lesions and more extensive loss of alveolar architecture than the resistant strains. Because excessive inflammation in the lungs of susceptible mice seems to play a critical role in increased mortality, we investigated the cellular infiltrate in the lungs and in the pulmonary lymph nodes (PLN) draining the lung tissue. As expected, the total number of cells in the lungs and PLN increased dramatically after MTB infection in all mouse strains tested. Already three weeks post infection there was a significant difference in the total number of cells in the lungs of BALB/c mice compared to C57BL/6 and DBA/2 mice. We characterized influx of myeloid cells at various time points after MTB infection by examining expression of the cell surface markers CD11b, CD11c, Ly6C and Ly6G. Despite the differences in total lung cell numbers, the number of recruited Ly6C+ inflammatory monocytes was comparable between resistant and susceptible mice. Neutrophils were recruited in comparable numbers to the lungs of susceptible DBA/2 and resistant BALB/c mice, but because of the

lower number of total lung cells in infected DBA/2 mice, neutrophils accounted for a larger percentage of all cells. In resistant C57BL/6 mice numbers of neutrophils remained relatively low during the course of the infection.

Inflammatory monocytes recruited into MTB-infected lungs rapidly upregulate CD11c [213]. Therefore, we enumerated the CD11b+CD11c+ cell subset, which contain dendritic cells and macrophages, in infected resistant and susceptible mice and determined the number of iNOS-producing cells within this subset. The number of both CD11b+CD11c+ cells and iNOS+ macrophages increased during the first six to nine weeks post infection before it reached a plateau. We did not detect a significant difference between the three mouse strains tested.

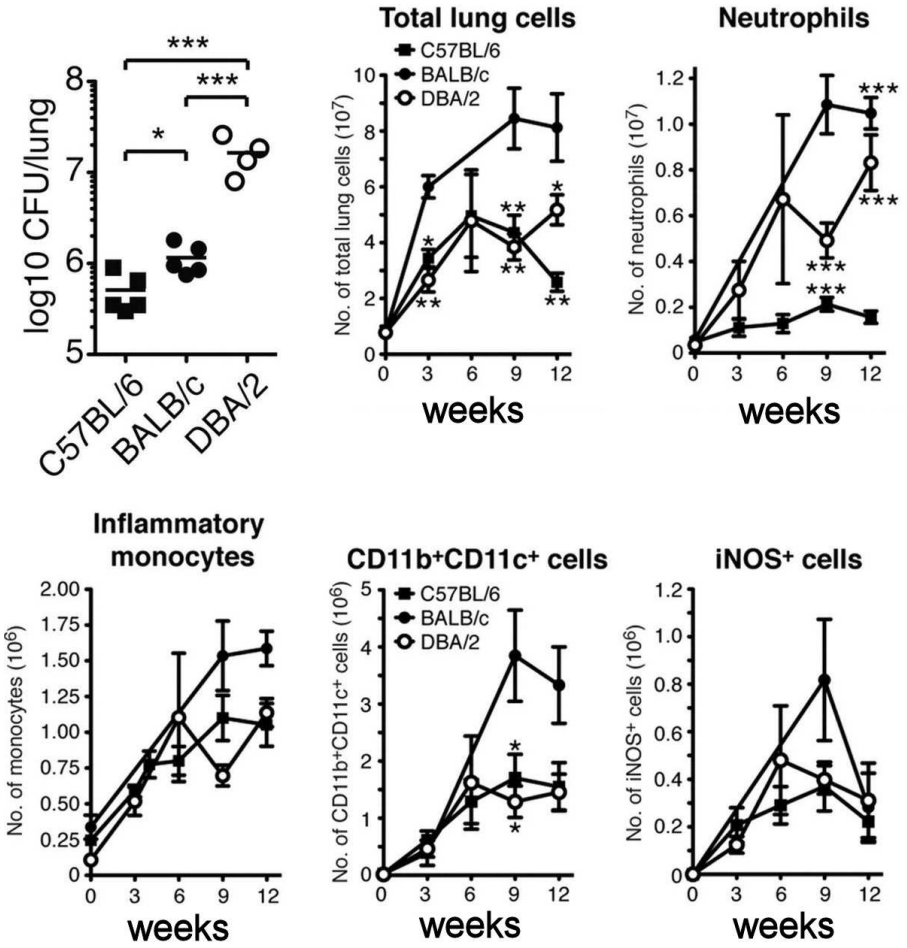

Figure 5. Bacterial burden and myeloid cell recruitment into the lungs of MTB infected mice.

Because myeloid cells are recruited early after MTB aerosol infection, a defect in the ability to recruit these cells may help exaplin susceptibility to MTB infection. In this study, we have shown that the increased susceptibility of DBA/2 mice is not caused by defective inflammatory monocyte-, neutrophil- or CD11b+CD11c+ DC- and macrophage recruitment to the infected lungs. We also showed that macrophage activation in the lungs is not different in resistant and susceptible mice.

3.1.3 A diminished population of CD103+ dendritic cells (αE-DC) in susceptible mice

We also analyzed the myeloid CD11b-CD11c+ cell compartment, focusing on αE-DC in infected lungs and PLN. These cells have been shown to preferentially acquire and cross-present antigens acquired from apoptotic bodies to CD4 cell in draining lymph nodes and to have regulatory properties in the gut mucosa [338–343]. In this study, we showed that the number of αE-DCs increased dramatically in response to MTB infection in resistant, but not in susceptible mice. We found that the number of lung αE-DCs was approximately six-fold higher in C57BL/6 and BALB/c mice compared to DBA/2 mice 12 weeks post infection. The difference between mice and BALB/c mice, and the difference between BALB/c mice and DBA/2 mice, was not statistically significant. Nevertheless a higher number of αE-DCs were present in the more resistant BALB/c mice than in the DBA/2 mice. The reduced number of αE-DCs in the draining PLN may reflect reduced αE-DC migration from the MTB-infected lungs in susceptible mice.

It has previously been shown that αE-DCs are derived from Ly6C+ monocytes [344]. We have shown that the influx of inflammatory monocytes is not defective in infected DBA/2 lung tissue, which suggests that monocyte differentiation into αE-DC is altered in the susceptible animals. Additionally, while the number of αE-DCs was reduced in infected susceptible mice, the number of CD11b+CD11c+ cells, which consist of mixture of monocyte-derived DCs and iNOS-producing macrophages, was not [213], [331], [345].

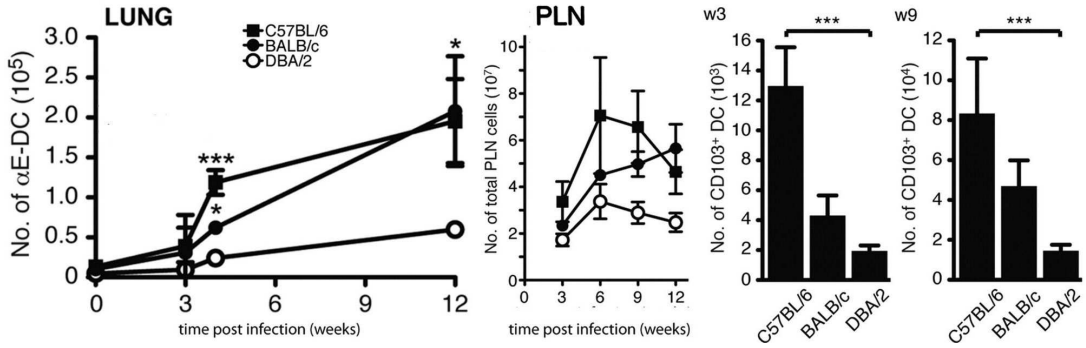

Figure 6. Number of αE-DC recruited to the lungs and to the PLN after MTB infection

To start delineating the role of αE-DC in host immunity during pulmonary TB, we investigated cytokine αE-DC production after MTB infection and compared it to the cytokine profile of other myeloid cells [338], [339], [341], [343]. Total lung cells were divided into four populations: The CD11b− CD11c+ population, which was further divided into αE-DCs and CD103− cells, which may contain alveolar macrophages; CD11b+CD11c+ cells (activated macrophages and DCs); and CD11b+CD11c− cells (inflammatory monocytes and granulocytes) [212], [213]. After stimulation with an MTB cell-wall extract, or with LPS, a high percentage of alveolar macrophages,

inflammatory monocytes/granulocytes and DC/macrophages produced TNF. In contrast, αE-DCs remained essentially TNF negative, but contined the highest percentage of TGFβ producers. this cytokine profile was observed in the early phase of infection (week 3 post infection) and in the chronic stage (week 12 post infection). Absolute number of TGFβ αE-DCs producing cells in susceptible mice were lower comparing to other strains. This clearly showed that comparing to other types of myeloid cells, αE-DCs have anti-inflammatory profile.

3.1.4 A diminished CD4+Foxp3+ regulatory (Treg) cell population in MTB-infected lungs of susceptible mice

The increased susceptibility and inflammatory response observed in DBA/2 mice caused by MTB infection correlated with a reduced lung αE-DC population. Because αE-DC in the gtu mucosa play a role in Treg cell induction, we wanted to determine if the susceptible mice also had a defective Treg cell response in the MTB infected lungs. Since the Foxp3 marker is unique to Treg cells, we enumerated the number of Foxp3-expressing CD4+ in the lungs and in the PLN of infected mice.

It is worth noting that Foxp3 mRNA levels were the same in uninfected lungs of naïve C57BL/6 and DBA/2 [346] as well as Treg cells number in lungs of both strains (data not shown).

We have found that CD4+ T cell population in the lungs of C57BL/6 and BALB/c mice contained a significant proportion of Treg cells that increased as the infection progressed. We made similar observations in the PLN, where the absolute number

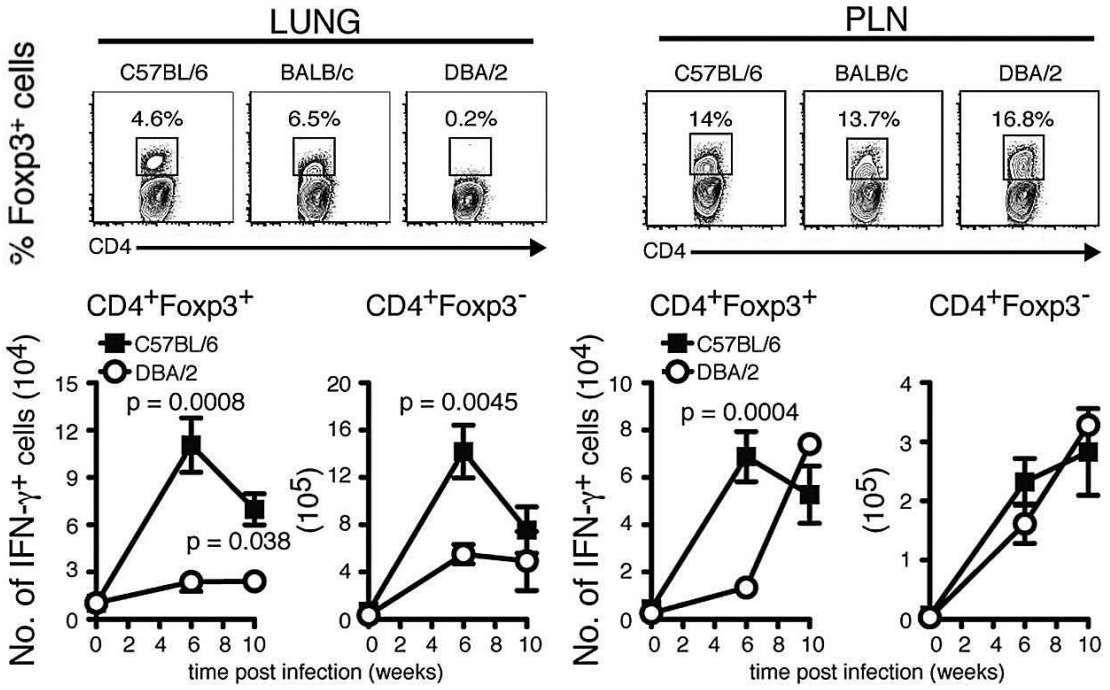

Figure 7. Recruitment of Treg cells (Foxp3+ T-cells) into the lungs and PLNs and ability of these cells to produce IFNγ

of Treg cells increased significantly by week 12 post infection compared to week 3 post infection. Importantly, the frequency and absolute number of Treg cells in the lungs of DBA/2 mice were dramatically reduced early after MTB infection as well as during chronic TB. In contrast, in the PLN the frequency and absolute numbers of Treg cells were comparable to resistant C57BL/6 and BALB/c mice, showing that MTB can have an organ-specific effect on Treg cells.

In previous studies it has been shown that a reduction in Treg cell numbers in *T. gondii*-infected mice was preceeded by the induction of IFNγ in the Treg cells [347]. Therefore we investigated the functional potential of Treg cells in response to MTB infection.

Total lung and PLN cells isolated from MTB infected C57BL/6 and DBA/2 mice were polyclonally re-stimulated in vitro. In the lungs and in the PLN of naïve and chronically infected animals, we found a similar percentage of IFNγ-producing CD4+ cells in both strains. After infection, a large fraction of Foxp3+ and Foxp3- CD4+ T-cells produced IFNγ in lungs and PLN. At weeks 6 and 10 p.i we observed a significantly higher number of Treg cells producing IFNγ in the lungs of C57BL/6 in comparison to DBA/2 mice. In PLN the absolute number of IFNγ+ Treg cells was significantly higher only at week 6 p.i.. Among CD4+ Foxp3− T cells, we did not observe any significant differences in the PLNs but we did find a significantly higher number of IFNγ producers in the lungs of C57BL/6 mice at week 6 p.i.

In summary, the reduced number of TGFβ-producing αE-DCs in lungs of MTB-susceptible mice correlated with a diminished pool of Treg cells during the peak of the immune response (week 3 post infection) as well as during the chronic phase of the disease. Additionally, Treg cells in resistant and susceptible animals acquire the ability to produce IFNγ in response to infection with a clinical MTB isolate (Harlingen strain).

3.1.5 Discussion

In majority of pulmonary diseases, like the ones caused by pneumococcus or influenza infections, the immune response is able to clear the infection while not causing excessive damage to the lung tissue. In the case of pulmonary TB, excessive immune activation in untreated patients can lead to tissue damage in the lungs and loss of the physiological function. Hence, a balance between inflammatory and tolerogenic immune reactions must be maintained to control the infection and maintain organ function. The significance of controlling the inflammation caused by MTB is illustrated by the extensive lymphocyte- and myeloid cell infiltration into the lungs of Foxp3-deficient mice that lack Treg cells [348]. Here, we showed that the numbers of lung Treg cells with anti-inflammatory effector functions are significantly reduced during MTB infection of susceptible mice. By comparing animals displaying different level of susceptibility against MTB, we may model differences in MTB-resistance in humans.

Recent studies using the mouse model have shown that Treg cells are able to inhibit the antigen-specific T cell response during pulmonary TB and reduce the ability of the immune system to control bacterial growth [247], [349], [350]. Also, in TB patients, the number of Treg cells increases in infected tissues which when isolated are able to suppress antigen-specific T cell responses *in vitro* [351].

Studies performed in mice do not present a clear picture of the role of Treg cells duirng the course of the infection. It has been shown that Treg cell depletion reduces bacterial growth early after MTB infection [350], but not when Treg cells were depleted in later stages of the disease [352]. Depletion of regulatory cells was also associated with increased frequency of IFNγ- and IL-2-producing cells, but it had no effect on bacterial growth or lung lesions early after infection [248]. Adoptive transfer of Treg cells to infected animals resulted in increased T cell responses and slowed bacterial growth although this effect was temporary [247].

While Treg cells are associated with production of anti-inflammatory cytokines, our data show that during MTB infection, Treg cells are able to induce IFNγ production. This findings suggest a change in functional potential of the Treg cells during disease progression.

In light of previous findings, we expected to observe an increased percentage of Treg cells in infected susceptible lung tissue. Instead, we found that the frequency of Treg cells was significantly reduced three weeks post infection, and that they were almost completely absent in chronically infected susceptible mice. Despite the differences in the infected lungs, the frequency of Treg cells in the PLN was similar in susceptible and resistant animals. Therefore, the lack of Treg cells in the chronically infected DBA/2 lungs was not due to systemic failure to generate this T cell subset.

One of the reasons why Treg cells are not being recruited into the lungs could be low number or αE-DCs which were shown to be able to produce CCL22 chemokine (although under steady-state conditions and during allergic airway inflammation) [340]. The CCL22 receptor, CCR4, is expressed on Treg cells and induces Treg cell migration [353]. Moreover, a large fraction of lung αE-DCs are TGFβ+ during TB (Fig. 5), and TGFβ is important for peripheral Treg cell function and homeostasis (43). Therefore, the inability of susceptible mice to induce αE-DC differentiation may help exaplin the low number of Treg cells. Overall, our results suggest that addressing regulatory role of αE-DCs and Treg cells at different stages of MTB infection may increase our understanding of susceptibility to TB.

SUMMARY

Mice susceptible to tuberculosis versus resistant mice have:

- higher bacterial burden in lungs and worse lung lesions
- significantly reduced numbers of αE-DCs lung in lungs after MTB but not other myeloid cell subsets
- lung αE-DCs have an anti-inflammatory cytokine profile
- diminished pool of Treg cells in the lungs, but not in the draining pulmonary lymph nodes
- MTB infection changes the functional potential of CD4+ Foxp3+ regulatory T (Treg) cells, which induce gamma interferon (IFN-γ)

3.2 STUDY II

Mycobacteria-infected bystander macrophages trigger maturation of dendritic cells and enhance their ability to mediate HIV transinfection.

3.2.1 Induction of cytokine production in DC by MTB-infected macrophages

With the aim to mimic bystander effects mediated by an ongoing mycobacteria infection, and to investigate the cross-talk between DCs and mycobacteria-infected macrophages, we devised an in vitro model system comprised of human monocyte-derived DCs and monocyte-derived macrophages. To asses the impact of different mycobacterial strains on macrophages, we infected them with BCG, MTB strain Harlingen or the highly pathogenic S96–129 isolate. Infected cells were either in direct contact with DCs, or isolated by a permeable membrane (0.2 µm) in order to assess the contribution of soluble mediators on macrophage function. DCs in direct contact with non-infected macrophages did produce cytokines. We found that exposure of DCs to soluble factors released by infected macrophages resulted in increased production of TNF, IL-12p40 and IL-6 in the cultures. When the cells were in direct contact with macrophages, cytokine production was two- to fivefold higher and seemed augmented depending on the mycobacterial strain used. To determine which cells were the main sources of the produced cytokines, we used fixed mycobacteria-infected macrophages. We observed that DCs were the main source of IL-12p40, whereas macrophages were main producers of TNF in the co-cultures. Regardless of whether the macrophages were live or fixed, different levels of

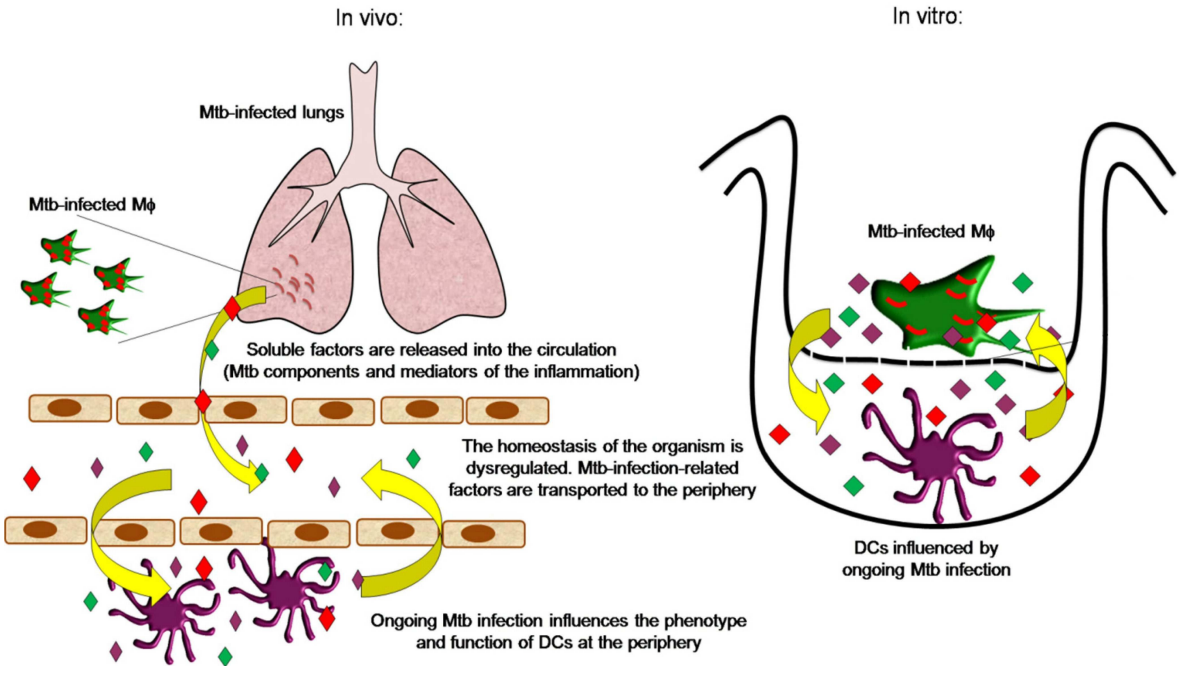

Figure 8. Experimental setup of experiment measuring bystander impact of MTB infection of macrophages on DCs ability to mature and facilitate HIV transinfection.

cytokines were produced depending on mycobacterial strain. Taken together, these observations suggest that mycobacteria-infected macrophages stimulate DCs to produce pro-inflammatory cytokines after direct cell-to-cell contact as well as in bystander manner.

3.2.2 Soluble factors released by mycobacteria-infected macrophages cause partial maturation of DCs

To determine the effects of soluble factors on maturation of DCs, we assessed upregulation of CD86 and MHC class II. In comparison to LPS stimulation, which was considered 100% stimuli, upregulation of CD86 and MHC class II was higher in DCs that were co-cultured with infected macrophages without direct cell-to-cell contact. The effects of soluble factors were mycobacterial strain-dependent. With an exception for macrophages infected with the MTB S96–129 isolate, no changes in expression of the MR and DC-SIGN were observed. In contrast, full maturation of DCs and downregulation of the MR and DC-SIGN were observed when the cells were co-cultured with infected macrophages in direct contact. Interestingly BCG-infected macrophages induced less profound DC maturation as compared to infection with virulent MTB strains. In summary, we showed that DCs undergo partial maturation when exposed to soluble factors from infected macrophages, whereas direct contact between these cells leads to full DC maturation.

3.2.3 DCs exposed to MTB-infected bystander macrophages display enhanced ability to mediate HIV trans-infection

Maturation of DCs has been shown to affect their ability to mediate trans-infection of HIV to T-cells. Therefore, we asked whether DCs can still facilitate trans-infection under the influence of MTB infected macrophages. We pulsed DCs recovered from the co-cultures (both direct and membrane-separated) with HIV, washed and incubated the cells with activated PBMCs or CD4+ T-cells as target cells for the virus. Viral replication in CD4+ cells was assessed by quantification of p24 antigen in the culture supernatants.

The DCs exposed to soluble factors in indirect cultures did not have enhanced ability to mediate HIV trans-infection of PBMCs, except when macrophages were exposed to the MTB isolate S96–129. Nevertheless, when immature DCs were exposed to supernatants from infected macrophage cultures their trans-infection ability was significantly enhanced as compared to supernatants from non-infected macrophages. Taken together, these findings suggest that DCs exposed to Mtb-infected macrophages in bystander manner display augmented ability to capture HIV and consequently mediate trans-infection to CD4+ T-cells.

3.2.4 Discussion

It has been previously shown that under non-inflammatory conditions, DCs precursors are recruited to the lungs and to the draining lymph nodes, and show rapid turnover [354]. As described in the introduction and discussion to paper I, when exposed to antigens during MTB infection, DCs migrate to the PLN and are responsible for T-cell priming. With the onset of an infection, this process changes dramatically. During the early phase of infection, DCs accumulate in the PLN [355]. Phagocytosis of antigens and direct interaction with other cells is the main stimulating factor for DC. We have demonstrated that DCs can be affected by components released from infected macrophages indirectly. In addition DCs exposed to these factors undergo partial maturation, including upregulation of co-stimulatory molecules and MHC class II, and downregulation of the MR, while DC-SIGN expression is sustained. Maturation of DCs is accompanied by increased production of the pro-inflammatory cytokine IL-12. Similar observations were made by *Giacomini* et al. who found that DCs and macrophages responded to MTB infection with distinct cytokine profiles, suggesting different and complementary functions of these two cell populations during the course of MTB infection [356]. TNF is one of the identified soluble factors released by MTB infected macrophages, which affects DCs. TNF has previously been shown to induce DCs maturation [357], [358].

We showed that the strength of the maturation signals mediated by soluble factors are less efficient than the signals received via direct interaction with infected cells. In direct contact, maturation of DCs was on the same level as when stimulated with LPS which is in agreement with previous findings by other investigators [359]. Interestingly, the observed effects are related to the virulence of the mycobacterial strains that were used to infect the macrophages.

LAM is one of the soluble factors released by infected macrophages, which could enhance DC maturation. In a recent study, we have shown that LAM facilitates DCs maturation as measured by CD80, CD86 and MCHII up regulation. ManLAM also induced production of IL-12 by DC, which could be blocked by LAM specific monoclonal antibodies. In the current study we show only partial maturation of DCs in comparison to the exposure of DCs directly to the LAM [202]. It is worth noting that concentration of LAM released by infected macrophages in culture was lower than one used with direct application of purified LAM. Additionally, LAM is usually accompanied by phosphatidylinositol mannoside PIM, which we show has an inhibitory effect on the DCs maturation. In the light of our new findings, partial maturation of DCs is not only the result of cytokines release by infected macrophages, but also products shedded from the bacterial cell wall which can play a role in DC differentiation [202]. On the other hand, binding of HIV by DC-SIGN can be blocked by a high concentration of mannan. Although the highest concentration of mycobacteria-derived compounds is expected to be found at sites with the highest bacterial burden, their presence is not limited to the lungs and the PLN. For

example, ManLAM can be detected in blood and urine [360], [361]. Also mycobacterial proteins can be found in sera of TB patients [362]. Quantities of LAM in sera and urine are high enough to be used in the development of diagnostic tools for TB [363]. This suggests that mycobacterial components can induce bystander effects in organs and tissues distant from the lungs.

DC maturation has been demonstrated to enhance the ability of DCs to transfer HIV to T cells [364]. As described in the introduction section, ability of DCs to mediate trans–infection of HIV virus is dependent on DC-SIGN receptor. Because some of the components of mycobacterial wall can bind DC-SIGN and inhibits its function we investigated possible effects of soluble factors on ability of DC-sign to mediate trans-infection. It has been shown that indirect activation of DCs by inflammatory mediators resulted in partial DC activation as compared to DCs matured by direct exposure to pathogens [365]. We have shown that partial maturation increased the ability to mediate HIV trans-infection to T cells, as compared with immature DCs.

Studies have shown that upregulation of MHC II and co-stimulatory molecules on the surface of DCs enables close contact between DCs and T cells during formation of the immunological synapse, which enables DC to pass on the engulfed DC-SIGN-bound HIV virus to CD4+ T cells [18], [366], [367]. Therefore it could contribute to virus spread from DCs to T cells. The importance of trans-infection was demonstrated in studies showing that HIV variants evolving during chronic infection conserve the ability to utilize DC-SIGN for efficient spread to CD4+ T cells [368].

Conceivably, our findings suggest that mycobacterial infection alters immunological homeostasis and could affect cells in distant tissues. However, further studies are necessary to explore the complex interactions between DCs and other cell types that take place during MTB/HIV co-infection in order to uderstand the mechanisms that lead to deleterious disease progression in co-infected hosts.

SUMMARY

DCs exposed to soluble factors from MTB infected macrophages:

- produce substantial amounts of IL-12 but not TNF
- mature partially by upregulating CD86 and MHC II
- can facilitate higher levels of HIV trans-infection via DC-SIGN receptor
- direct contact with infected macrophages is a stronger maturation and cytokine release stimuli

3.3 STUDY III

Mycobacterium tuberculosis Infection Interferes with HIV Vaccination in Mice.

One of the neglected issues in the development of HIV vaccines is the fact that areas of the highest prevalence of HIV overlap geographically with MTB endemic regions. This will result in administration of prospective HIV vaccines to a population harboring latent as well as undiagnosed active TB. Other pathogens as well as physical fitness factors, such as age or malnutrition, have been shown to interfere with unrelated vaccinations. Hence, we decided to investigate the impact of chronic MTB infection on the efficacy of one of new HIV vaccine candidates [369–375].
We chose the most immunogenic variant of the new MultiHIV DNA vaccine candidate delivered in an efficient DNA prime and double protein boost immunization regimen [184]. The vaccine was administered both intranasaly (i.n.) and intramuscularly (i.m.) to animals which had previously been infected with a low dose of aerosolized MTB (Harlingen strain) or to uninfected controls. It is important to note that C57BL/6 mice are considered resistant to aerosol MTB infection in comparison to other strains (as shown in study I). Animals were vaccinated 7 weeks after aerosol infection, which is 3-4 weeks after the bacterial burden in the lungs reaches a plateau and the adaptive immune response toward MTB is well established.

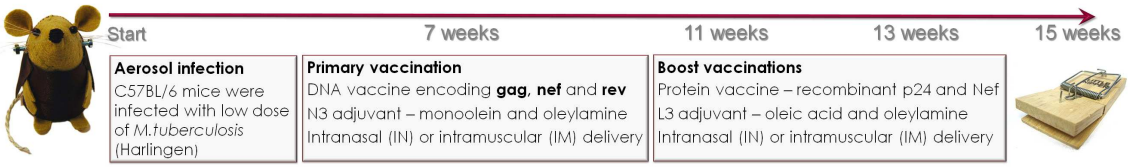

Figure 9. Infection, vaccination and sample collection schedule

We tested both the cellular and the humoral immunogenicity of the vaccine candidate, including the ability to induce antigen-specific humoral responses in the vaginal mucosal surface, which is the main entry site for the virus during sexual transmission [376].

3.3.1 MTB-infection impairs humoral responses induced by HIV vaccination

Vaccination via the i.n. route resulted in moderate HIV-specific vaginal IgA levels four weeks later. In comparison, i.m. vaccination induced minimal levels of HIV-specific IgA. After i.n. vaccination of MTB-infected mice, IgA titers in vaginal secretions were not reduced compared to uninfected controls. However, we observed a significant reduction in serum IgA levels. I.m. vaccination elicited high levels of IgA in serum of both MTB-infected and in uninfected animals. Even though

i.m. vaccination elicited high levels of IgA in serum, HIV-specific antibodies were not detectable in vaginal mucosa.

To assess HIV-specific IgG production we collected blood samples from mice two and four weeks after the second protein boost was administered. In mice vaccinated i.m., we detected high levels of HIV-specific IgGs at both time points, irrespective of previous MTB infection. After vaccination via the nasal route and two weeks after the last protein boost, uninfected mice had high levels of IgGs, whereas MTB-infected animals induced significantly lower antibody levels. Unexpectedly, IgG titers in i.n. vaccinated mice were diminished over two-fold two weeks later.

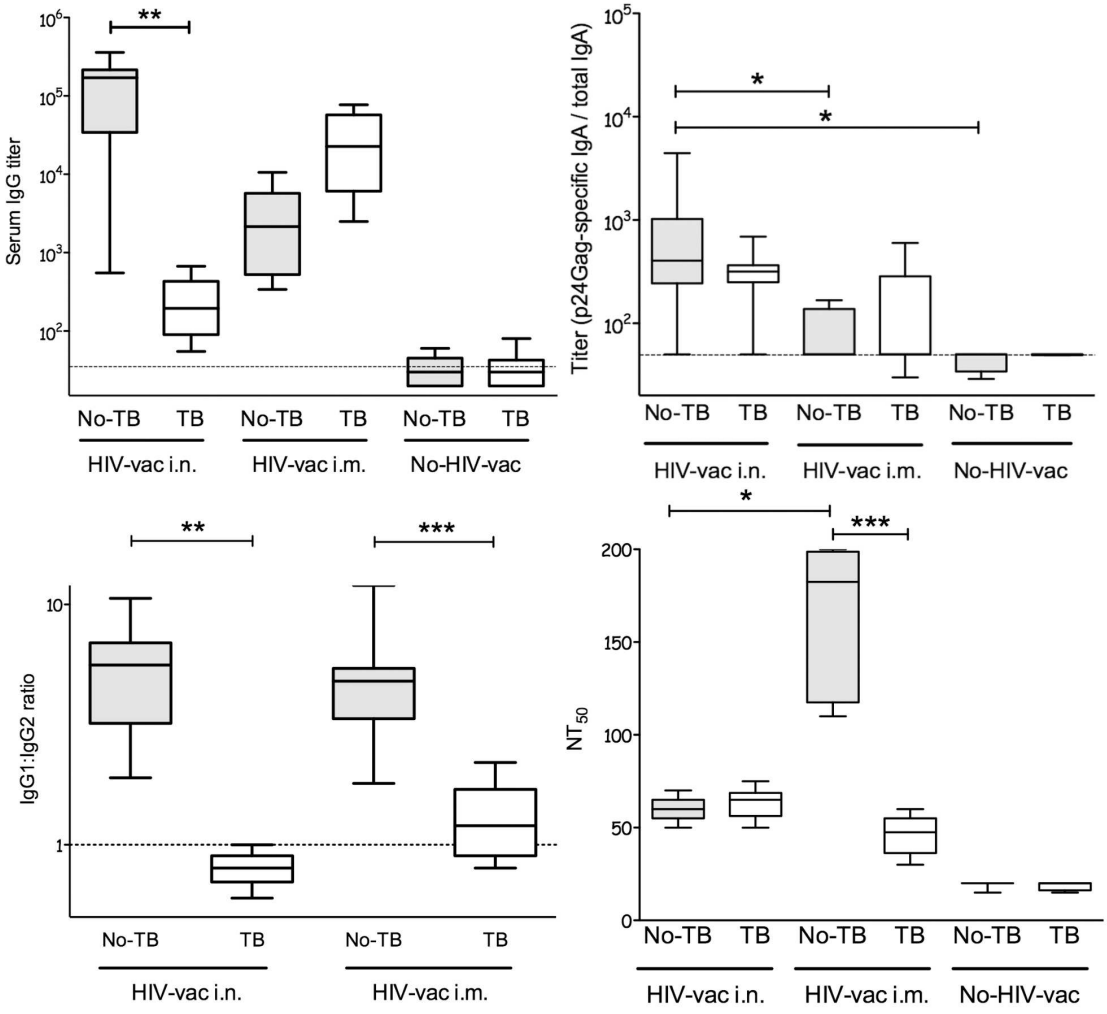

Figure 10. Quantity and quality assessment of humoral responses toward the vaccine

Neutralization of viral particles is essential for quality assessment of induced humoral responses. Serum collected from both uninfected and MTB-infected animals, vaccinated i.n. at week 4 post infection and had the same neutralization capability. Serum from healthy mice vaccinated i.m. was characterized by high neutralization activity, whereas the serum collected from i.m. vaccinated MTB-infected animals was significantly inferior in neutralizing the virus.

In order to investigate in more detail the degenerative impact of MTB-infection on the neutralization capability of serum, we characterized the IgG1 versus IgG2a profile of HIV-specific IgG antibodies. We observed, that in healthy animals a significantly higher portion of HIV-specific antibodies were of the IgG1 type, whereas the IgG1 to IgG2 ratio in MTB-infected mice was significantly lower regardless of vaccination route.

Overall, our results showed that MTB infection strongly affects the development of humoral immunity towards vaccine components, including mucosal IgA secretions, IgG levels, neutralizing capability and the IgG1/IgG2a-profile. These effects vary depending on the route of vaccination.

3.3.2 MTB infection impairs cellular responses induced by the HIV vaccination

HIV-specific multifunctional T cells that simultaneously produce more than one pro-inflammatory cytokines (IFNγ, IL-2, or TNF) were recently suggested to play an important role in the control of HIV infection. Therefore, new HIV-vaccine candidates aim at eliciting these type of T-cell responses [171], [377]. We assessed the ability of the MultiHIV vaccine to induce Th1 responses in CD4+ and CD8+ T-cells following antigen stimulation of spleen cells *in vitro* and measuring the cytokine profile by flow cytometry.

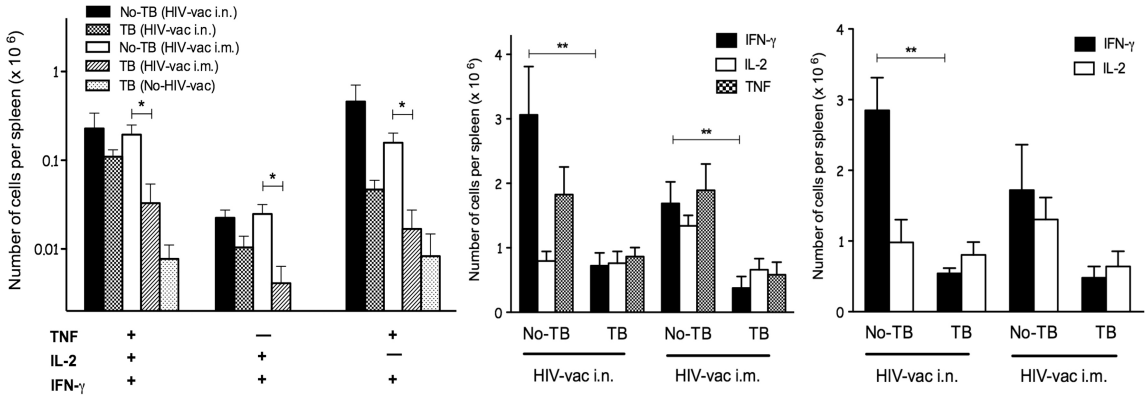

Figure 11. Quantity and quality assessment of polifunctional cellular responses toward vaccine

When the cells were grouped as IFNγ-, IL-2- or TNF monoproducers we observed that the number of IFNγ-producing MultiHIV-specific CD4+ T-cells cells was significantly reduced in animals that were MTB-infected, A similar trend was seen for TNF production, but no statistical significance was observed. In CD8+ T-cells compartment significant differences were noted between MTB infected and vaccinated-only animals in amount of IFNγ producing cells after i.n. vaccination .

We also found that mice that were infected with MTB and vaccinated i.m. had a significantly lower amount of CD4+ T-cells producing all three cytokines simultaneously, as well as fewer CD4+ T-cells that produced IFNγ together with TNF,

or with IL-2. No significant differences were observed between the i.n. vaccinated groups.

In conclusion, our data show that MTB negatively influences induction of IFNγ-producing CD4+ and CD8+ T-cells, as well as diminishes multiple effector functions elicited by i.m. vaccination believed to be vital for efficient control of HIV infection [377], [378].

3.3.3 Discussion

Studies of long-term non-progressors and elite controllers have identified certain characteristics of host immune responses that can successfully control virus replication and prevent AIDS development:

- high number of multifunctional T cells that produce multiple cytokines in response to HIV antigens
- early development of broadly virus-neutralizing antibodies
- strong immune responses associated with genital mucosal surfaces to prevent, or lower, the transmission rate [376], [377], [379–383].

Currently, most of the new vaccine candidates are screened against these requirements [384]. Although all recent vaccine candidates have shown protection in animal studies, and some of them induced protection in small groups of healthy volunteers during early clinical trials, they have failed in large-scale studies. Although we do not know exactly why substantial levels of protection has not yet been achieved, one of the important details worth noting is that large-scale trials are usually located in high MTB endemic countries [164], [165], [385]. Additionally, if an effective vaccine will be developed, due to co-localization of HIV and MTB in high epidemic regions, the HIV vaccine will be administered to a population in which MTB infection is frequent or sometimes omnipresent [8]. Although other pathogens have been shown to disturb the induction of immunity against vaccine components, MTB poses one particular clinical problem. In the majority of other pathogens, the infection time-span is short and infections are symptomatic. During TB, most latently infected individuals will never be clinically diagnosed due to the lack of symptoms and the fact that MTB can persist in a host throughout life [369–375]. Therefore, we chose to assess the efficacy of the vaccine in mice which develop chronic MTB infection and are able to control the bacterial burden in the lungs for over a year[294].

In this study we found that chronic MTB infection impairs both humoral and cellular immune responses directed towards an HIV vaccine. Hence, we suspect that more than one mechanism is involved and we suspect that the inhibitory effect of MTB infection would be transient for other DNA vaccines. Previous studies indicate that MTB-induced specific Treg cells could contribute to the inhibition of the immune response toward the vaccine. Speculatively, these cells may suppress unrelated immune responses in a non-antigen specific manner [386–388]. As shown in Study I,

Treg cells are present in substantial quantities in the lungs and in PLN of MTB-infected C57BL/6 mice at the time when the vaccine was administered [389]. Additionally, the impairment of humoral and T cell responses to intranasal vaccination could result from competitive antigen presentation in the respiratory tract associated lymph nodes [390–393]. Induction of Treg cells in the lungs and in the PLNs by MTB or co-localization of antigen presentation could help explain the inhibition of vaccine immunogenicity after i.n. delivery. However, due to the fact that immunogenicity of i.m. delivered vaccine was also diminished, is unlikely that there is only one mechanism causing this phenomenon.

It has been shown in studies of helminthes, that protozoa infection induces Th2 polarized responses during co-infection with malaria [394] . MTB is inducing a Th1 type of response and although it may be beneficial in the first phase of vaccination this could have and unwanted effect on the evolution of humoral response [395]. Studies of HIV-positive subjects have shown that antibody class-switching is associated with the ability to neutralize, rather than only opsonize, the virus in a chronic phase of the disease. Development of neutralizing antibodies is associated with class switch and a change from a Th1 to a Th2 type of cellular response. Thus, it could be hypothesized that MTB in our model mainly drives production of opsonizing IgG antibodies and thereby inhibiting the development of more specific neutralizing antibodies [395], [396]. Stopping class switch from IgG1 to IgG2a in MTB infected animals could be the reason why, despite high levels of IgG in serum, animals infected with MTB prior to the vaccination had much lower neutralizing ability than healthy counterparts.

In summary, we report for the first time, that chronic MTB-infection of mice prior to inoculation with an experimental HIV vaccine has a detrimental effect on vaccine-specific antibody and T cell responses. These results suggest that asymptomatic MTB-infection could also interfere with prospective HIV vaccination in humans, although the mechanisms behind these adverse effects are unknown.

SUMMARY

MTB infection changes humoral and cellular immunogenicity of HIV DNA/protein vaccine.

Characteristics of HIV specific immune response impairment in MTB infected animals are:

- Lower titers of IgA in vaginal secretions and IgG in serum after intranasal vaccination
- Lower neutralizing capability of serum after intramuscular vaccination
- Lowered or reversed IgG1/IgG2a ratio regardless of vaccination route
- Lower number of INFγ producing CD4+ and CD8+ cells regardless of vaccination route
- Diminished triple and double cytokine producing CD4+ T-cell numbers after intramuscular vaccinations

3.4 STUDY IV

A murine model of Mycobacterium tuberculosis/HIV co-infection for studies on pathogen-specific T cell responses in vivo

Current models of HIV and TB infection in humanized mice does not allow for easy genetic manipulation of the host and are unpractical in long-term infectious studies [140], [146]. The non-human primate provides a successful model of MTB/HIV co-infection, but it is a costly alternative, does not allow for easy genetic manipulation of the host and evokes additional ethical issues [397]. The growing need for a robust mouse model of MTB/HIV co-infection therefore becomes apparent. We decided to utilize the EcoNDK (MuLV/HIV-1 NDK isolate) chimeric virus developed by *Potash* et.al. and combined it with MTB aerosol infection of mice that are more or less susceptible to TB [48], [389].

3.4.1 EcoNDK does not influence the lung bacterial burden and health of MTB/EcoNDK co-infected mice.

To determine if Mtb/EcoNDK co-infection accelerates disease progression in MTB-susceptible DBA/2 compared to resistant BALB/c mice, we infected groups of these animals with MTB (Harlingen strain) via the respiratory route. Three weeks later, groups of mice were co-infected with the EcoNDK virus intraperitoneally. To assess the impact of EcoNDK on disease progression, we monitored the weight of the animals and plated lung homogenates on Middlebrook 7H11 agar to determine the bacterial load. To date, we have not observed any significant differences in body weight and bacterial burden between MTB-infected mice and MTB/EcoNDK co-infected animals.

3.4.2 Reduced numbers of antigen-specific CD8+ T-cells in the lungs of susceptible DBA/2 mice during pulmonary TB.

Because resistant BALB/c mice and susceptible DBA/2 mice express MHC class I of H-2^d, used the same MHC class I-multimer (H-$2K^d$) loaded with a MTB-derived peptide ($TB10.4_{20-28}$) to examine the antigen-specific CD8+ T-cell compartment in infected lung tissue in susceptible mice. After week three post infection, we observed that the number of antigen-specific CD8+ T-cells remained essentially unchanged in DBA/2 mice. In comparison, the number of antigen-specific CD8+ T-cells increased significantly after week 3 post infection in infected BALB/c lungs. This shows that recruitment of CD8+ T-cells is impaired in susceptible mice. We are currently conducting co-infection experiment with the use of these animals. Our MTB/EcoNDK co-infection model allowed us to extend these observations and to monitor how the TB10.4-specific CD8+ T-cell response was affected by EcoNDK co-infection.

3.4.3 Characterization of the MTB-specific CD8+ T-cell response during the early stage of MTB/EcoNDK co-infection.

To investigate the possible impact that EcoNDK infection may have on the early MTB-specific response, we identified total CD4+- and CD8+ T-cells, as well as TB10.4-specific CD8+ T-cells in the spleen and in the lungs. Two weeks after co-infection was established, the total number of CD4+ T cells and CD8+ T cells in the lungs and in the spleen was not significantly different in Mtb-infected mice compared to Mtb/EcoNDK co-infected animals. Analysis of the TB10.4-specific CD8+ T-cells in the spleen revealed that both the percentage and the absolute number of MTB-specific CD8+ T cells were significantly higher in MTB/EcoNDK co-infected mice. It is worth noting that the frequency of TB10.4-specific CD8+ T-cells in the spleen was 10-fold lower than in the infected lungs. Additionally, The number of TB10.4-specific CD8+ T-cell was not significantly different in the lungs. However, we did observe that lung antigen-specific CD8+ T-cells in co-infected animals expressed higher cell surface levels of the αβ T-cell receptor.

We examined the cell surface expression profile of Tim3 and PD1 on TB10.4-specific CD8+ T-cells to to start addressing how MTB/EcoNDK co-infection influences regulation of the MTB-specific T-cell response. Our results revealed different usage of PD-1 and Tim3 on CD4+- and CD8+ T-cells in MTB-infected mice. Up to 5% of the CD4+ T-cells in the MTB-infected lungs and in the spleen expressed Tim3. In comparison, over 15% of lung CD4+ T-cells and nearly 10% of splenic CD4+ T-cells expressed PD-1. We also noted that most $Tim3^{+}$ CD4+ T-cells in the lungs co-expressed PD-1.

Because infection with EcoNDK led to an increased number of TB10.4-specific CD8+ T-cells, we investigated the PD-1 and Tim3 expression profile on MTB-specific CD8+ T-cells. Strikingly, TB10.4-specific CD8+ T-cells in the lungs were enriched for Tim3+ (∼40%) and PD-1+ cells (∼20%). Also, TB10.4-specific CD8+ T-cells that were Tim3+ to a large extent co-expressed PD-1. In comparison, even though Tim3 expression was elevated on MTB-specific splenic CD8+ T-cells, the overall frequency of Tim3 expression in the spleen was lower than in the lungs. We did not detect any difference in Tim3 and PD-1 expression profile when we compared MTB-infected mice and in MTB/EcoNDK co-infected mice.

We analyzed the cytokine profile of antigen-specific CD8+ T-cells by intracellular cytokine straining. Similar to the PD-1 and Tim3 cell surface expression profiles, we did not detect any significant differences in cytokine production by T-cells isolated from MTB-infected mice or MTB/EcoNDK co-infected mice.

We used PMA and ionomycin to reveal the functional potential of both CD4+- and CD8+ T-cells. After polyclonal stimulation, around 20% of Tim3+CD4+ T-cells produced both TNF and IFNγ, and approximately 10% produced one of the cytokines. Among Tim3-CD4+ T-cells, the majority produced only TNF and only 10% of the cells were able to produce both TNF and IFNγ. Within the Tim3+CD8+ T-cell

subset, polyclonal stimulation resulted in ~40% of the cells producing both TNF and IFNγ. Approximately 30% of the Tim3+CD8+ T-cells produced IFNγ only and essentially no Tim3+CD8+ T-cells were single-producers of TNF.

Antigen-specific stimulation of CD8+ T-cells isolated from the lungs revealed that the Tim3+ subset was enriched for cells producing pro-inflammatory cytokines. It is noteworthy that ~45% of the Tim3+CD8+ T-cells expressed both TNF and IFNγ, while only ~10% of the Tim3-CD8+ T-cells expressed both cytokines. Irrespective of Tim3 expression, TB10.4-peptide stimulated cells contained few single-producers.

3.4.4 Increased viral load in peripheral tissues and secondary lymphoid organs of Mtb/EcoNDK co-infected mice.

We isolated genomic DNA from the lungs and from the spleen, and performed quantitative PCR amplification of an EcoNDK-specific region of the *gag* gene to evaluate if MTB co-infection influenced the levels of the provirus in peripheral tissues and secondary lymphoid organs. Animals that were negative for the PCR product were excluded from the study.

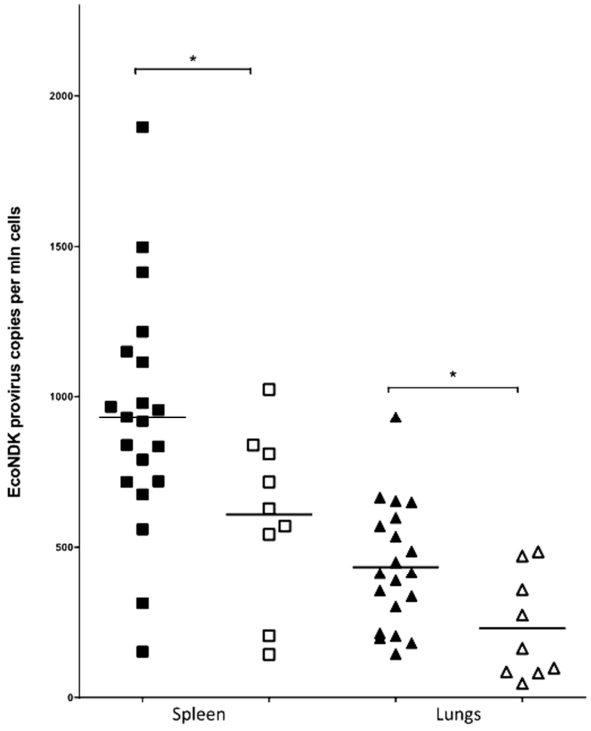

Figure 11. EcoNDK virus burden 2 weeks after infection. Black filled markings present mice infected with MTB 3 weeks prior to virus infection. Empty markings represent virus-only infected animals.

We observed productive viral infection in the spleen and in the lungs, with provirus levels averaging 600-650 infected cells/10^6 total cells in the spleen, and 150-250 infected cells/10^6 total cells in the lungs. Interestingly, in co-infected animals the levels of proviral DNA was significantly higher in both organs comparing to animals that were infected with EcoHIV only. The total number of cells increases dramatically

in peripheral tissues and in secondary lymphoid organs during pulmonary TB. Therefore, the total number of provirus in co-infected animals is dramatically higher than after EcoNDK infection.

These experiments clearly show that we were able to successfully infect immunocompetent wildtype with both EcoNDK and MTB, and thereby creating a unique small animal model of MTB/HIV co-infection. Additionally we showed that MTB infection significantly increased the viral burden during the early stage of co-infection. We are currently utilizing the MTB-susceptible DBA/2 strain to further delineate how the chimeric virus influences the functional potential of MTB-specific CD8+ T-cell responses, and assessing the HIV-specific immune response.

3.4.5 Discussion

We have successfully established an MTB/HIV co-infection model in inbred, non-modified mouse strains. Modeling HIV-1 infection in rodents presents numerous obstacles described in detail in the 1.2 section of the introduction. One approach to overcome the inability of HIV to infect murine cells is to modify the virus itself. Developed by *Potash* et al., the chimeric MuLV/HIV (EcoHIV and EcoNDK) viruses were shown to successfully infect conventional, immunocompetent WT mice. Recently is has been shown that this virus is establishing a chronic infection [154]. Although modification of the virus changes the binding receptor from CD4 to mCAT-1, it productively infects activated murine splenocytes *in vitro*, including up to 10% of CD4+ T-cells [48], [398]. Similarly to the previous studies we have been able to infect mice after i.p. administration of the purified virus, reaching an infectivity rate of 80% [48], [154]. The levels of provirus detected in EcoNDK-infected animals were slightly lower comparing to other studies which could be a result of lower quality of the virus preparation, or slightly lower inoculum [152]. Importantly, we could detect the virus in animals co-infected with MTB and EcoNDK. The frequency of provirus in lung tissue and spleen was 1.5 to two times higher in co-infected animals compared to animals that were infected with EcoNDK only. Due to proliferation and recruitment of both myeloid and lymphoid cells into the lungs and spleen during MTB infection, the overall amount of provirus is several fold higher in co-infected animals. This finding is in agreement with clinical observations by Nakata et. al. on bronchoalveolar lavage (BAL) from MTB/HIV co-infected individuals. Lungs infected by MTB contained higher levels of HIV-1 branched DNA (bDNA) than samples collected from MTB-free samples. Interestingly, in these studies the viral load in the lungs was higher than in plasma of co-infected patients. Because the viral load in the lungs of the patients decreased after antibiotic treatment this suggest a close connection between viral load and bacterial burden [313]. Therefore, we are currently investigating how co-infection affects the viral burden in MTB susceptible DBA/2 mice, which harbor much higher amount of bacteria in the lungs [389]. On the other hand, in a recent macaque co-infection study, no increase in peripheral

virus replication was described during co-infection. A small number of animals could be responsible for lack of statistical significance in the obtained data [317].

As expected, acute infection with EcoNDK did not change the CFU or health condition of MTB/EcoNDK co-infected mice. The results presented here were obtained from mice that were sacrificed only two weeks after virus infection. If the bacterial load is affected during later timepoints of co-infection needs to be determined.

CD8+ T-cells are required for optimal host immunity and protection during pulmonary tuberculosis [399], [400]. We used MHC class I-multimer technology to monitor the MTB-specific CD8+ T-cell response in MTB/EcoNDK co-infected mice. After co-infection with EcoNDK we observed an increased frequency of TB10.4 specific CD8+ T-cells in spleens, and a similar trend in the co-infected lung tissue. This also correlated with an increased absolute numbers of antigen-specifc CD8+ T-cells. We showed that large fraction of these cells expressed Tim3 and PD-1, and about 20% of the cells co-expressed these two markers. In studies of chronic viral infections, and to some extend in clinical observations from TB patients, these proteins are considered to be markers of cell exhaustion [103], [105], [110], [111], [401], [402]. If PD-1 and Tim3 can be used as markers for antigen-specific T-cell exhaustion in our co-infection model needs to be determined. At the early stage of co-infection, we showed that Tim3 and PD-1 expression, as well as ability of these cells to produce cytokines, is similar for MTB and in MTB/EcoNDK co-infected animals. Still, co-infection induced a higher frequency of splenic TB10.4 antigen-specific CD8+ T-cells. Therefore co-infection induced a higher number of antigen-specific Tim3+ IFNγ- and TNFα-producing cells. Further investigations during later stages of co-infection are needed to determine if MTB- or EcoNDK-specific T-cells acquire an exhausted phenotype in our murine model. Also, we report increased viral replication in animals co-infected with MTB. How viral-specific host immunity is affected during MTB/EcoNDK co-infection also needs to be determined.

SUMMARY

Using chimeric MuLV/HIV virus and low-dose aerosol MTB infection we established new murine model of MTB and HIV co-infection in conventional BALB/c mice:

In our preliminary data co-infected animals comparing to MTB only infected controls had:

- Higher virus burden in lungs and spleens
- Same bacterial burden and physical fitness
- Significantly increased frequency of MTB specific CD8+ T-cells in spleens and elevated frequency of these cells in the lungs
- Significant percentage of MTB specific CD8+ T-cells route expressing Tim3 or/and PD1 in the lungs
- Almost half of MTB specific Tim3+ CD8+ T-cells produces IFNγ and TNF

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