Optimization of mycobacterial and HIV-1 *in vitro* co-infection

BACHELOR THESIS
For obtaining the degree

Bachelor of Science (BSc.)

submitted by
Nastasja Grdselloff

Vienna, July 2015
The practical work was carried out at the University of Miami, Florida, Leonard M. Miller School of Medicine, USA.

External Supervisor: Mario Stevenson, PhD

Internal Supervisor: Rohini Chopra-Dewasthaly, PhD

Reviewer: Ao.Univ.-Prof. Dr.med.vet. Dieter Klein
Acknowledgements

First of all, I would like to thank Mario Stevenson and Dieter Klein for giving me this great opportunity to do my practical work abroad. I gained a lot of experience – regarding my technical skills as well as social and personal skills.

Also, I would like to thank Moira for supervising my work, as well as Diogo, Mauricio, Jen and the whole Stevenson lab for welcoming me warmly and making my stay as wonderful as it was. Thank you for the unforgettable time we shared and for the support you have given me in all concerns. I am happy and thankful for the friends I found in you.

A very special thank you goes to Rohini for all the patience and guidance you have given me. This could never have been done without your efforts and positivity.

Most of all, I thank my parents, my family and my dear friends for always being there for me and supporting me through all the years. In those past months I have to specially thank Marlene, Kathi, Lena, Anita, Eva, Beni and Philipp, who never failed to motivate and push me not to abandon my goals.

This work is dedicated to everyone, who knows that this means much more to me than just a thesis. I am blessed to have so many loving people in my life who have always believed in me and given me strength whenever I struggled. This is the beginning of a new chapter of my life and I am happy to share it with you!
1 INTRODUCTION

1.1 HIV
1.1.1 HIV AND PROGRESSION TO AIDS 1
1.1.2 HIV SUBTYPES 3
1.1.3 HIV STRUCTURE AND GENOME 4
1.1.4 HIV LIFE-CYCLE 6
1.1.5 TRANSMISSION AND PHASES OF HIV INFECTION 7
1.1.6 DIAGNOSIS AND TREATMENT 8
1.2 MYCOBACTERIUM TUBERCULOSIS 10
1.2.1 MYCOBACTERIUM TUBERCULOSIS AND TUBERCULOSIS DISEASE 10
1.2.2 GENOME AND STRAIN VARIATION 11
1.2.3 MYCOBACTERIUM TUBERCULOSIS INFECTION 13
1.2.4 DIAGNOSIS AND TREATMENT 14
1.3 TUBERCULOSIS AND HIV-1 CO-INFECTION 15
1.4 RATIONALE 15
1.5 OUR HYPOTHESIS AND SPECIFIC AIMS 16
1.6 MYCOBACTERIUM SMEGMATIS AS A MODEL FOR MTB 17

2 MATERIALS AND METHODS 18

2.1 REAGENTS 18
2.2 MACROPHAGES 20
2.2.1 MACROPHAGE DERIVATION 20
2.2.2 CULTIVATION OF MACROPHAGES 20
2.2.3 MONITORING OF CELLULAR GROWTH 20
2.3 MYCOBACTERIA 21
2.3.1 PREPARATION OF MYCOBACTERIAL CULTURE 21
2.3.2 MYCOBACTERIAL INFECTION OF MACROPHAGES 21
2.3.3 MONITORING OF MYCOBACTERIAL INFECTION 22
2.3.4 QUANTIFICATION OF MYCOBACTERIAL INFECTION BY COLONY-FORMING UNITS COUNTING 23
2.4 HIV-1 23
2.4.1 HIV-1 STOCK DERIVATION 23
2.4.2 DETERMINATION OF VIRUS TITER BY REVERSE TRANSCRIPTASE ACTIVITY ASSAY 24
2.4.3 HIV-1 TITER DETERMINATION BY P24 ENZYME-LINKED IMMUNOABSORBENT ASSAY 25
2.4.4 HIV-1 INFECTION OF MACROPHAGES  
2.4.5 MONITORING OF HIV-1 INFECTION  
2.5 MYCOBACTERIUM SMEGMATIS AND HIV-1 CO-INFECTION AND MONITORING  

3 RESULTS AND DISCUSSION  
3.1 PLATING OF MACROPHAGES  
3.2 EVALUATION OF FILTERS FOR FLUORESCENCE PLATE READING TO QUANTIFY BACTERIAL INFECTION  
3.3 MYCOBACTERIAL CELL CULTURE  
3.3.1 DETERMINATION OF INOCULUM FOR MYCOBACTERIAL INFECTION  
3.3.2 EVALUATION OF GROWTH CONDITIONS AFTER MYCOBACTERIAL INFECTION  
3.4 VIRAL CELL CULTURE  
3.4.1 EVALUATION OF TRANSFECTION CONDITIONS  
3.4.2 DETERMINATION OF HIV INOCULUM FOR INFECTION  
3.5 CO-INFECTION OF MYCOBACTERIUM SMEGMATIS AND HIV-1  

4 CONCLUSION AND FURTHER STEPS  

5 SUMMARY  

6 ZUSAMMENFASSUNG  

7 ABBREVIATIONS  

8 REFERENCES  

9 TABLES AND FIGURES  
9.1 TABLES  
9.2 FIGURES
1 INTRODUCTION

1.1 HIV

1.1.1 HIV and progression to AIDS

In the 1980s, first incidents of patients suffering from the acquired immunodeficiency syndrome (AIDS) were reported and soon linked to homosexual individuals, drug users and patients who had previously received blood transfusions. However, it took two years to determine the human immunodeficiency virus (HIV) as major cause of this disease (BARRESINOUSSI et al., 1983). It is suggested that initial infection of patient zero ensued a zoonotic infection of bush meat hunters with simian immunodeficiency virus in Africa (MAARTENS et al., 2014).

Ever since the classification of AIDS as a global epidemic several strategic directions including improvement of prevention, diagnosis and treatment, were decided by the World Health Organization (WHO). The aim was a total eradication of the epidemic until 2015 – meaning zero new HIV-infections, zero HIV-related deaths and zero discrimination of people living with HIV (WHO, 2011). Even though this ambitious goal could not be achieved, it is still realistic to be reached until 2030, according to the UNAIDS statistics of the past few years. In light of these global directions, the numbers of new infections were diminished by 38 % since 2001. Especially in Africa, where most infections occurred, 44 % less people acquired HIV in 2013 than in 2001. Also, in South-East Asia a decrease by 33 % could be observed in the same period of time. In contrast to this positive development from a global point of view, there are some regions of the world where the opposite trend was observed. In the European region as well as the Mediterranean region, where the numbers of infected people were stable between 2001 and 2009, a subsequent rise of infections was observed. From 2009 to 2013, newly acquired HIV infections increased by 29 % in the Mediterranean and by 6 % in the European region. However, the introduction of antiretroviral therapy (ART) and the enhancement of its accessibility induced a global decline of HIV-related deaths (WHO, 2011; UNAIDS, 2013).
Figure 1 | Estimated number of new HIV infections globally, 2001 – 2013. The overall trend shows a worldwide decrease of new infections within the given timespan. Due to substantial progress in prevention of infection and increasing knowledge among people, a global decline of newly acquired infections could be achieved (2013 UNAIDS/WHO estimates).

Figure 2 | Estimated number of people dying from HIV-related causes globally, 2001 – 2013. After a steady increase of mortality among HIV-infected individuals reaching a peak in 2005, numbers have been falling annually (2013 UNAIDS/WHO estimates).

Thanks to ART, HIV-infection is not a sentence of death anymore and the lifespan of patients could be extended (ZAIDI et al., 2013). Because of this improvement, global HIV prevalence has increased, and HIV infected patients live longer under proper treatment, and global
incidence has also decreased from 3.3 million in 2002, to 2.3 million patients in 2012 (MAARTENS et al., 2014). HIV is a lentivirus and belongs to the family of retroviruses. Its main characteristics are the cone-shaped nucleoid and a small RNA genome encoding nine genes. Infections with lentiviruses usually show a chronic course of disease with phases of clinical latency and persistent viral replication. HIV can infect several cell types, such as T-cells, macrophages or dendritic cells. All of them express CD4, a receptor which interacts with the viral glycoprotein gp120. Additionally, co-receptors are required for successful HIV infection of target cells. Dependent on the co-receptor used for binding of the virus, HIV strains can be either M-tropic or T-tropic. When the chemokine receptor CCR5, which is mainly found on CD4\(^+\) memory cells, is targeted, virus strains are referred to as M-tropic. Alternatively, the co-receptor CXCR4 can be used. It is expressed on naïve T-cells, hence HIV strains are named T-tropic. A virus able to target both co-receptors, is known as dual-tropic strain. On grounds of the necessity of those receptors, a heterozygous mutation of the CCR5 receptor results in a delay of progression of HIV-1 infection to AIDS. This polymorphism is found in 13 – 20 % of Europeans, but could not be detected in Africans or Asians (HUSAIN et al., 1998). Transmission routes of the virus are clearly defined. Most commonly HIV infection is transmitted through transfer of secretion containing infectious virus in the course of unprotected sexual intercourse with an infected partner. Another infection route is via infected blood through injection or transfusion of contaminated blood products (e.g. during organ transplants, artificial insemination or using unsterilized injection equipment in drug abuse). HIV can also be passed from mother to the fetus during pregnancy, at birth or through breast-feeding. Alternative routes of HIV transmission as through insects, human saliva or everyday contact are explicitly excluded. Compared to other viruses like the influenza virus, HIV is not as easily transmitted, therefore prevention of HIV infection is feasible when adequate precautions, such as virus testing before blood donations or proper contraception, are made (ALBRECHT et al., 2007a).

1.1.2 HIV subtypes

Due to the high mutation rate of the virus, there are various different strains of HIV, which can be classified based on their genetic similarities. There are two types of HIV: HIV-1 and HIV-2. Both have the same transmission routes and lead to AIDS. However, HIV-1 is worldwide predominant while HIV-2 is rarely found outside of West Africa (UNAIDS, 2004).
There are four groups of HIV-1 representing four separate introductions of simian immunodeficiency virus (SIV) into humans: the “major” group M, the “outlier” group O and two new groups, N and P. As suggested by the name, the majority with 90% of infections belongs to group M. Within this group, there are known to be at least nine genetically distinct subtypes (A, B, C, D, F, G, H, J and K) (WHO et al., 2011). Each of these subgroups is associated with a specific geographical region and they also differ in virulence. A study found that Ugandans infected with HIV-1 subtype D developed symptoms of AIDS sooner than patients infected with subtype A and the course of infection became lethal after a shorter period of illness (KIWANUKA et al., 2008). Eventually, two different HIV-subtypes can build a hybrid virus by mixing their genetic material when meeting in a cell of an infected individual (BURKE, 1997). These strains mostly do not survive for a very long time, but those, which infect more than one person are called “circulating recombinant forms” (CRFs). Due to this high variability of the virus, more subtypes of HIV-1 are expected to be discovered in the future.

1.1.3 HIV structure and genome

Viral particles have a diameter of around 120 nm and are surrounded by a lipoprotein membrane, which contains 72 glycoprotein complexes. Each of them is built of trimers of an external glycoprotein gp120 and a transmembrane protein gp41, which are forming spikes on the viral envelope. Also, host cell membrane proteins can be found on the virus’ surface, as it takes parts of a cell’s phospholipid layer including proteins, with it while budding from the host cell. The inner cone-shaped virus capsid consists of protein p24, whose level in blood serum is an indicator of HIV progression. In the capsid two copies of single-stranded HIV-RNA, stabilized by the p7 nucleocapsid proteins, are found.
together with necessary enzymes for replication: reverse transcriptase (RT), integrase and protease. At the length of 9.7 kbp the HIV genome consists of major genes for structural proteins that are found in all retroviruses: \textit{gag} (group antigen), \textit{pol} (polymerase) and \textit{env} (envelope). \textit{Gag} encodes the proteins to form the capsid, the RNA stabilizing capsid protein

\[ \text{p7} \] and the matrix proteins. The function of \textit{pol} is replication of virus, it encodes reverse transcriptase, integrase and protease. The \textit{env} gene encodes gp160, the precursor for gp120 and gp41 proteins (STEFFY \& WONG-STAAL, 1991). The HIV-1 genome encodes six additional accessory genes. The regulatory protein transcriptional transactivator (\textit{tat}) and the regulator of virion gene expression (\textit{rev}). They stimulate transcription of proviral HIV-1 DNA into RNA, promote RNA elongation, enhance transport of RNA to cytoplasm and are essential for translation. \textit{Tat} also plays a crucial role in viral replication. Negative factor (\textit{nef}) manipulates the host's cellular machinery to allow infection, survival and replication of virus. \textit{Vpu} (viral protein U) is involved in viral budding and enhancing virion release from the cell and also it is targeting CD4 degradation. The viral infectivity factor's (\textit{vif}) role is disrupting the antiviral activity of the human enzyme APOBEC. \textit{Vpr} plays a role in regulating the nuclear import of the HIV-1 pre-integration complex and also in arresting the cell cycle (STEFFY \& WONG-STAAL, 1991; ALBRECHT et al., 2007a; BARRÉ-SINOUSI et al., 2013; MOSS, 2013).

\textbf{Figure 5 | Genetic organization of HIV-1.} The structural scheme of a retroviral genome is: 5'LTR-gag-pol-env-LTR'3. Long terminal repeats (LTR) flanks both ends of the genome and are connected to the cellular DNA of the host cell after integration (ALBRECHT et al., 2007b)
1.1.4 HIV life-cycle

The first step to start the life cycle of a retrovirus is the entry of the virus into a target cell. For that, the HIV external glycoprotein gp120 binds to the target cell’s CD4 receptor. CD4 has been described as primary necessary receptor for HIV binding and is found on monocytes, macrophages, T-cells, eosinophils and dendritic cells (KLATZMANN et al., 1984). Binding of gp120 to the receptor induces conformational changes and allows attachment to the target cell’s co-receptors, CCR5 and CXCR4 (DRAGIC et al., 1996). Subsequently, fusion of the viral and the host cell membrane is promoted (ESTÉ & TELENTI, 2007) and the virus uncoats into the cell’s cytoplasm. Before entering the nucleus, viral RNA is converted to proviral DNA by reverse transcriptase (RT) and results in HIV cDNA with long terminal repeats (LTR) at both ends. Due to a lack of exonuclease activity of the viral RT, the reverse transcription is characterized by a high spontaneous mutation rate, sometimes as high as one mutation per 70 polymerized nucleotides (ROBERTS et al., 1988). This may allow the virus to evade the host's immune system or cause drug resistance. After the viral DNA is transcribed, a pre-integration complex containing several viral proteins (RT, integrase, matrix, Vpr) and the cDNA, can enter the host cell’s nucleus without rupturing the nuclear envelope by employing multiple nuclear localization signals (NLSs), which are integrated in the pre-integration complex’ proteins. Once in the nucleus, the viral DNA can be integrated into the host’s genome using the viral integrase. The viral DNA is then mostly integrated in transcriptionally active regions. After that, the provirus can be transcribed by the cellular RNA polymerase II, which is stimulated by transcription factors such as the nuclear factor ‘kappa-light-chain-enhancer’ (NF-κB), binding to the HIV-LTR region. First, regulatory HIV-1 proteins (tat and rev) are synthesized, and instantly tat stimulates transcription and the forming of long RNA transcripts. Rev promotes the expression of structural genes and enzymes, but also inhibits the synthesis of regulatory proteins, thus promotes the formation of mature viral particles. When transcription is completed, spliced as well as unspliced mRNA is exported from the nucleus. This transport is enabled by rev and necessary for translation of HIV-1 mRNA to proteins by the host cell’s cellular machinery. When this process is completed, viral proteins are processed in the endoplasmatic reticulum. Finally, HIV-1 envelope proteins gp120 and gp41 are inserted in the host cell’s plasma membrane, and some parts of it are taken by the virus, when it’s budding. The forming virion also contains the viral genomic RNA and several polyproteins, which are cleaved into functional HIV proteins during maturation.
With the new virions leaving the host cell, a new infection cycle starts (FURTADO et al., 1999; ALBRECHT et al., 2007a; WHO et al., 2011; BARRÉ-SINOUSSI et al., 2013).

**1.1.5 Transmission and phases of HIV infection**

Most commonly HIV infection is transmitted through transfer of secretion containing infectious virus in the course of unprotected sexual intercourse with an infected partner. Another infection route is via infected blood through injection or transfusion of contaminated blood products (e.g. during organ transplants, artificial insemination or using unsterilized injection equipment as is the case with drug abuse). HIV can also be passed on from mother to the fetus during pregnancy, at birth or through breast-feeding. Alternative routes of HIV transmission as through insects, human saliva or everyday contact are explicitly excluded.
(ALBRECHT et al., 2007b). In 40 – 90 % of infected individuals flue like symptoms would usually occur within few days to weeks after initial infection with HIV, while viral RNA is not yet detectable in plasma. That is why diagnosis of acute infection at this early state of infection is missed in most cases. It has been documented, that in around 80 % of diseases, successful HIV-1 infection was established by only one virus (KEELE et al., 2008). During acute HIV infection the virus replicates extensively in absence of detectable adaptive immune responses. Fitness of infecting virus, host genetic factors as well as host immune response can influence viral replication. Usually high plasma viremia and a marked decrease of CD4+ T-cells can be detected. Around this peak of plasma viral load, latent viral reservoirs are established. After weeks or months, an equilibrium called “steady state” between viral replication and the host immune response is reached and infection enters its chronic phase. Peripheral blood CD4+ T-cell counts recover and plasma viral loads are lower. A latency phase starts and clinical manifestations can stay away for an average period of 10 years until the onset of AIDS. At the end of this latency period immunological, dermatological and neurological symptoms and different opportunistic infections may appear due to continuously decreasing CD4+ T-cell counts, resulting in substantial lymphopenia (DOUEK et al., 2003). Progression to AIDS varies from host to host, long-term non-progressors represent only 5 % of all patients (ALBRECHT et al., 2007a; MOSS, 2013).

1.1.6 Diagnosis and treatment

Tests for detection of HIV are required to have high sensitivity and deliver accurate results. Therefore a combination of several testing methods is used to avoid false results. Patients’ blood, saliva or urine are tested for antibodies, antigens and viral nucleic acids. HIV infection has transformed from a fatal disease to a chronic manageable disease due to the development of antiretroviral therapy in the 1990s. Even though there is still no cure available, several drugs to block HIV replication to delay the onset of AIDS are at disposition. Current antiretroviral therapy combines two nucleoside reverse transcriptase inhibitors with non-nucleoside reverse transcriptase inhibitors, protease inhibitors or integrase inhibitors. The aim is to decrease the plasma viral load to concentrations below the limit of detection. This treatment also promises a positive CD4 response with a raise of cell counts. However, this is reached in only 71 % of patients while in the other 15 % of people under ART show only a CD4 response, whilst virological suppression is missing. Unfortunately, there are several side effects such as establishment of viral reservoirs in CD4 T-cells due to
administration of interleukin-7 (IL-7), which is known for promoting T-cell proliferation and growth. In many low-income countries, where viral load monitoring is not possible, only CD4 counts are used to monitoring of infection. Several studies show that therapy started early after HIV infection is most successful and slows disease progression (MAARTENS et al., 2014).

Owing to the relatively high mutation rate of the virus, resistance to antiretroviral drugs is not rare and poses a major threat to public health as resistant virus can be transmitted from one patient to another. In high-income countries prevalence of resistance to certain antiretroviral drugs has reached a plateau of 10 – 17 % of patients, while in low- and middle-income countries, prevalence is increasing from around 7 % in 2009 (MAARTENS et al., 2014). Since access to HIV testing and antiretroviral therapy is not granted in all regions of the world, especially in low- and middle-income countries, outcomes of treatment vary. Mortality rates during ART are much higher in resource-limited countries than in high-income countries. A US report showed that in 2010 only 51 % of patients who were diagnosed with HIV were held in care. These findings make clear, that there is still a lot of improvement needed in terms of public health management (MAARTENS et al., 2014).

Figure 7 | Comparison of the course of disease of untreated HIV infection and changes after ART. On the left graph, untreated infection is shown. The number of CD4 T-cells is steadily declining in blood while CD4 T-cells in the gastrointestinal tract (GIT) are rapidly depleted after a few weeks of infection. The graph on the right side illustrates changes of CD4 T-cell counts and HIV RNA after ART. In blood, T-cells are recovering under treatment, while numbers remain nearly unchanged in the GIT. HIV RNA clearly reduces under the limit of detection when HIV infection is treated (MAARTENS et al., 2014).
1.2 Mycobacterium tuberculosis

1.2.1 Mycobacterium tuberculosis and Tuberculosis disease

Tuberculosis (TB) is the second deadliest infectious disease worldwide, after the acquired immunodeficiency syndrome (AIDS). A third of the world population is estimated to be infected with the *Mycobacterium tuberculosis* (MTB), although only 3 – 15 % of infected people develop TB disease. In 2013, there were 9 million TB cases and 1.5 million reported deaths. Even though the numbers of new cases and deaths are declining annually, mortality is still unacceptably high. Due to improvements in diagnosis and treatment options, between 2000 and 2013 estimated 37 million lives were saved from TB infection and mortality reduced by 45 % since 1990. Unfortunately, access to health care and proper treatment is not granted in all regions of the world and TB remains a disease of people suffering from poverty and undernutrition (LAWN & ZUMLA, 2011). Thus, 56 % of all TB cases are found in South East Asia and the Western Pacific Regions, a quarter in the African Region and 11 % of all TB patients are documented in India and China (RUSSELL, 2011; WHO, 2014).

The WHO planned to halve the number of TB cases worldwide until 2015 and to eliminate the disease by 2030 (WHO, 2010). According to the WHO Global Tuberculosis Report 2014, this goal could not be achieved as planned, even though incidence declined steadily since the emerging outbreaks in the 1990's. It is thought that recent TB cases are rather a consequence of reactivation of latent infection than ongoing disease transmission (COMAS & GAGNEUX, 2009). The causative agents of TB are several species of obligate aerobic, facultative intracellular, acid-fast, non-motile and non-encapsulated tubercle bacilli or MTB complex. These species include obligate human pathogens, as well as mycobacteria adapted to other mammals. Their main characteristic features are slow growth with a doubling-time of 15 – 20 h, dormancy, complex cell envelope and intracellular pathogenesis (COLE et al., 1998). MTB is acquired by respiratory route and it primarily infects the lung tissue rich in oxygen supply, due to the pathogen’s highly aerobic properties. The main target cells of the mycobacteria are alveolar macrophages, where MTB is capable of arresting the microbial responses of their host. Once phagocytosed by its target cells, the mycobacteria
can spread via lymphatics and blood in order to cause extrapulmonary TB in skin, the genito-urinary system or bones (RUSSELL, 2011). However, MTB dissemination is dependent on the host’s immune status (MAZUREK et al., 2012).

1.2.2 Genome and strain variation

Analysis of the genome of MTB strain H37Rv was published in 1998 (COLE & BARRELL, 1998). It revealed a single circular chromosome with the size of 4 million basepairs, structured to 3959 genes. The function of 40 % of all genes has been characterized, while for the remaining genes 44 % specific functions were postulated. It was found that a remarkable number of 250 genes is involved in fatty acid metabolism of the pathogen. Therefore an evolutionary importance of this characteristic is recognizable for its survival as a pathogen. Further studies showed, that especially in the chronic phase of infection, MTB is capable of
using cholesterol as a major nutrient, in absence of other nutrients (COLE et al., 1998; GAGNEUX, 2008; WIPPERMAN et al., 2014).

Figure 10 | Lipid metabolism of *Mycobacterium tuberculosis*. Host cell lipids are degraded and provide precursors for many metabolic processes of the bacillus. They can be used for mycobacterial cell-wall constituents and provide energy for the bacteria. EchA1-21, fadE1-36, fadB2-5, fadA2-6, fadD1-36, accA1/accD1 as well as accA2/accD2 are genes encoding the synthesis or degradation of the fatty acids shown (COLE et al., 1998)

Significant phenotypic differences due to genetic diversity were found in clinical isolates of MTB. Various strains were linked to geographic regions such as an Euro-American strain or an East Asian and West African strain (GAGNEUX & SMALL, 2007). In total there are six main lineages of TB described as shown in figure 2-3.

The variations of the DNA sequence are mostly generated through mutation and recombination, while horizontal gene transfer is rare (HIRSH et al., 2004). New genetic variants can either become extinct or increase within a population as a result of natural selection and genetic drift. It was observed that genetic variants of MTB adapt to separate ecological, environmental and host genetic factors. Despite the challenges concerning diagnosis, the biggest threat to global health is still posed by drug resistance. Even though MTB has a rather long generation time, drug resistance mutants from the same progenitor strain can occur within one single infected person (SMITH et al., 2006). In general, resistance of MTB is classified into multidrug-resistant tuberculosis (MDR-TB) or extensively drug-resistant tuberculosis (XDR-TB). According to the Centers for Disease Control and Prevention (CDC), the multidrug-resistant strains do not respond to treatment with rifampicin
and isoniazid, while the XDR mycobacteria are additionally resistant to fluoroquinolone. However, the most important question is, if these phenotypic variations have an impact on the course of infection. Clinical studies revealed that genetic mutations of human TB differ in likelihood of reactivation, the disposition for extrapulmonary TB and the degree of cavitary disease (DRANCOURT, 2009). This dramatic impact of mutated TB-strains on patient survival was observed in KwaZulu-Natal, South Africa, in areas with high rates of HIV co-infection. All patients infected with XDR-TB, who were HIV co-infected died within 16 days after diagnosis (GANDHI et al., 2006).

1.2.3 *Mycobacterium tuberculosis* infection

Infection starts with the entrance of MTB into the host via the respiratory route. MTB binds to phagocytes by complement receptors (CR1, CR2, CR3, CR4) and mannose receptors. The bacterial cell wall glycoprotein lipoarabinomannan (LAM) mediates the binding. Expression of necessary receptors can be increased by through secretion of prostaglandin E2 and interleukin-4 (IL-4) of phagocytic cells. Especially macrophages react to the pathogen and phagocytose it thereby generating the superoxide burst as first microbicidal activity. The superoxides are produced by the NADPH complex assembled in the phagocytic cup.
Membrane proteins and cytosolic subunits generate toxic O$_2^-$ in the phagosome. Although most microbes can be eliminated this way, yet MTB is capable of avoiding the superoxide ions. In this context, different salvage routes have been documented for instance, via superoxide dismutase or scavenging properties of its cell wall lipidoglycans. Successful elimination of microbes within the phagosome is also characterized by acidification of the phagosome through lysosomal hydrolases. Due to complexes, which pump protons into the phagosome, a pH equilibrium at pH 4.8 – 5.0 is reached. This process can also be arrested by MTB, whilst pH remains at 6.2 – 4 when phagosomes contain MTB. Additionally, mycobacterial cell wall lipids have been demonstrated to modulate phagosome maturation as well as some proteins secreted by MTB. Despite the immune evasion of the pathogen, upon activation of macrophages through interferon gamma (INF-$\gamma$), antigen sampling and presentation is still possible. As mycobacteria are very immunogenic particles, they generate a very severe immune response even though immunogenicity is limited during early infection. The robust immune response is necessary for the bacteria to achieve late-stage tissue damage and necrosis (RAJA, 2004; RUSSELL, 2011). After the first immune response, a cell-mediated immunity develops within 2 – 6 weeks. The cell-based infection leads to an influx of lymphocytes and activated macrophages. Subsequently granulomas are formed, inside of which bacilli have the opportunity to grow exponentially in the disease center within apoptotic or dead macrophages. The bacteria can either remain in those granulomas, get activated and progress to active disease, or get discharged into the airways as soon as MTB has significantly increased in number. The last attempt of the immune system is formation of fibrosis surrounding a central area of necrosis and infection (RAJA, 2004).

1.2.4 Diagnosis and treatment

A majority of 90 % of MTB infections are found in low- and middle-income countries where diagnosis relies on sputum smear microscopy and chest radiology. Accurate and fast diagnostics are still missing in most affected countries. Over the past decade, progress has been made due to endorsement of the WHO. In high-income countries with solid health care systems, fluorescence microscopy and liquid culture systems represent the gold standard of diagnosis. Regarding drug resistance testing to optimize treatment, commercial line probe assays (LPAs) after positive sputum-testing have been approved by the WHO in 2008. The most promising method are nucleic acid amplification tests (NAATs) with high specificity. Also, real-time PCR technology is used to detect MTB and resistance mutations. Results of
PCR testing are reliable, but there is still need for a laboratory including expensive equipment. In summary, it can be said, that a reliable, sensitive and fast diagnosis method which can be used independently from laboratory infrastructures and irrespective of the country’s as well as the patient’s financial circumstances, still has to be developed (LAWN & ZUMLA, 2011). Treatment of TB is started right after diagnosis and comprises 6 month treatment with rifampicin. If patients return after relapsing from their first treatment course and no drug resistant strain could be diagnosed, isoniazid or fluoroquinololones are prescribed. In case there is still no effectiveness of treatment noticeable or a drug-resistance is found, further treatment gets prolonged and very costly. Successful treatment of MDR-TB is achievable in about two-thirds of patients. In contrast, XDR-TB is fatal in almost all cases (LAWN & ZUMLA, 2011).

1.3 Tuberculosis and HIV-1 co-infection

Co-infection with HIV-1 and MTB still presents the main burden of infectious diseases, especially in resource-limited countries such as Africa and Asia. It was observed that Tubercuosis (TB) incidence was much higher among HIV-positive patients. The risk of reactivation of latent TB is 20-fold higher in HIV infected individuals (PAWLOWSKI et al., 2012). In the past years, around 60 – 70 % of HIV infected patients developed TB. Co-infections result in a greater risk of extrapulmonary TB and disseminated disease together with a higher frequency of false-negative tuberculin tests as well as an increased rate of adverse drug reactions. This enhancement of both pathogens inevitably leads to death, if untreated (RAJA, 2004). Immune Reconstitution Inflammatory Syndrome (IRIS) may develop in TB/HIV co-infected patients who undergo anti-TB-treatment and antiretroviral therapy (ART) (LAWN et al., 2005). Patients suffer an exacerbation of symptoms of TB. Low CD4+ counts and high plasma viral load prior to initiation of ART are recognized predictors of IRIS.

1.4 Rationale

It is assumed that the basis of this enhancement of both diseases lies in the function of CD4+ T-cells. Activation of these cells as well as CD8+ T-cells is essential for control of active MTB in humans. Since the main feature of HIV infection is known to be depletion of CD4+ T-cells, cell-mediated immunity of MTB infection is reduced. It is indicated that similar receptor/ligand interactions are crucial for modulation of host immunity in both infections. T-cell exhaustion in
HIV-1* patients is marked by Programmed-Death 1 (PD-1) and T-cell immunoglobulin and mucin domain 3 (Tim-3), which plays a critical role in immune tolerance by triggering cell death. Two studies showed that Tim-3 is upregulated on virus-specific CD8* T-cells in patients with chronic HIV-1 infection as well as in patients with active TB (HASTINGS et al., 2009). Other mechanisms to facilitate infection with MTB in the context of HIV infection are an upregulation of bacterial entry receptors on macrophages (ROSAS-TARACO et al., 2006) manipulation of macrophage bactericidal pathways (SPEAR et al., 1990), deregulation of chemotaxis (WAHL et al., 1989), manipulation of granulomas (DIEDRICH & FLYNN, 2011) and impairment of TNF-mediated macrophage apoptotic response (PATEL et al., 2007). Additionally, epidemiological data shows that MTB infection has negative impact on the immune response to HIV and on progression to AIDS. The incidence and mortality rates for new opportunistic infections are higher if individuals with HIV are co-infected with MTB. It is thought that TB patients facilitate HIV infection by (i) increasing the co-receptors CXCR4 and CCR5 regulated by MTB products (ROSAS-TARACO et al., 2006), (ii) enhancement of HIV-1 replication within monocyte-derived macrophages (SHATTOCK et al., 1994), (iii) TNF production in response to MTB infection acting as an activator of HIV-1 replication in macrophages (KEDZIERSKA et al., 2003) as well as (iv) enhanced reverse transcription of viral cDNA and expression of HIV genes.

1.5 Our hypothesis and specific aims

It is often hypothesized that TB increases macrophage permissivity to HIV-1 infection and we aimed to determine the impact of Mycobacteria on macrophages infected with HIV, as well as the difference, if HIV-1 infection is present at first and TB appears later on. Further on, underlying mechanisms which increase macrophage permissivity to HIV-1 during co-infection should be studied.

The first step of this series of experiments was to find the appropriate cell culture conditions to ensure reliable and reproducible results. In this context, we tried to adapt standard laboratory methods to make veritable quantification and observation of HIV-1 and mycobacterial infection possible. To facilitate growth, easy handling and increased biosafety, it was decided to use Mycobacterium smegmatis (MSM) as a model for MTB in the first round of experiments later to be replaced by MTB if needed, to verify the obtained results.
1.6 *Mycobacterium smegmatis* as a model for MTB

As a category 3 human pathogen, use of MTB is very labor-intensive. It requires a Biosafety Level (BSL) 3 lab, substantial training prior to handling and still it bears a risk of accidental exposure. Additionally, with a doubling time of about 22 h MTB cultures grow very slowly making subsequent experiments quite time consuming. Therefore, less pathogenic mycobacterial model systems are used to facilitate handling. There are three common lab models of MTB: *M. smegmatis* (MSM), *M. marinum* and *M. bovis* and the latter is the most slow growing of all three as it also duplicates every 22 hours like MTB. It is a BSL 2 organism and 99 % of its genes are identical to MTB. *M. marinum* also belongs to BSL2 category, is an occasional human pathogen but grows faster than MTB with a duplication time of about 24 hours. Disease progression is very similar to TB, although *M. marinum* has a different cellular lifestyle. The only fast growing (~4 hours) avirulent model is the soil bacterium MSM that is generally regarded as a non-pathogenic microorganism though in very rare cases it may cause disease. It shares more than 2000 homologs with MTB and also has the same unique cell wall structure. It belongs to BSL 1 category and is amenable to genetic manipulation for experiments (REYRAT & KAHN, 2001). Due to all these factors, we chose to use MSM model organism in all our experiments. While working with model organisms it is important to remember that they are unique species themselves who have adapted to distinct environments. Results and interpretation of experiments with MSM cannot be directly and blindly applied on MTB, unless eventually verified with it.
2 MATERIALS AND METHODS

2.1 Reagents

Table 1

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Uptake Buffer</td>
<td>2.25 g glucose 50 mg calcium chloride 2.5 g bovine serum albumin 0.5 ml cold fish skin gelatin 500 ml PBS sterilized with 0.22 µm filter</td>
<td>All components from Life Technologies, Carlsbad, USA</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM)</td>
<td>Media for mammalian cell culture</td>
<td>Life Technologies, Carlsbad, USA</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 mg/ml in deionized water</td>
<td>Sigma-Aldrich Corp., St. Louis, USA</td>
</tr>
<tr>
<td>Lipofectamine©</td>
<td>Reagent for DNA and siRNA delivery</td>
<td>Life Technologies, Carlsbad, USA</td>
</tr>
<tr>
<td>Lysis Buffer</td>
<td>0.05 % SDS in H₂O, sterile</td>
<td></td>
</tr>
<tr>
<td>Macrophage Media</td>
<td>10 % True Pooled Human Serum from healthy blood donors 1 % L-glutamine 0.1 % Gentamicin DMEM</td>
<td>Life Technologies, Carlsbad, USA</td>
</tr>
<tr>
<td>Media A</td>
<td>10 % True Pooled Human Serum 1 % L-glutamine 0.1 % Gentamicin 6 ng/ml Macrophage colony-stimulating factor (M-CSF) DMEM</td>
<td>All components from Life Technologies, Carlsbad, USA – except serum.</td>
</tr>
<tr>
<td>Middlebrook 7H9 medium</td>
<td>Medium for Mycobacterial culture</td>
<td>Sigma-Aldrich Corp., St. Louis, USA</td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>OPTI-MEM®</td>
<td>Reduced Serum Media</td>
<td>Life Technologies, Carlsbad, USA</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>10,000 U/ml</td>
<td>Life Technologies, Carlsbad, USA</td>
</tr>
<tr>
<td>Phosphate-buffered Saline (PBS)</td>
<td>Isotonic water-based buffer solution</td>
<td>Life Technologies, Carlsbad, USA</td>
</tr>
<tr>
<td>RT assay Beta Plate Scint fluid</td>
<td>Scintillation fluid</td>
<td>PerkinElmer, USA</td>
</tr>
</tbody>
</table>
| RT assay Disruption Buffer | 100 mM Tris-HCl pH 7.9  
300 mM KCl  
10 mM DTT  
0.1% NP-40  
H₂O | Life Technologies, Carlsbad, USA |
| RT assay Reaction Mixture | 50 mM Tris-HCl pH 7.9  
150 mM KCl  
5 mM DTT  
15 mM MgCl₂  
0.05% NP-40  
10 ug/ml Poly A  
0.25 U/ml oligo dT pd(T)12-18  
10 uCi ³H-TTP | Life Technologies, Carlsbad, USA |
| Trypan Blue            | Cell stain to determine cell viability | Life Technologies, Carlsbad, USA |
2.2 Macrophages

2.2.1 Macrophage derivation

The macrophages used for infections were derived from elutriated monocytes. Cells were purified from healthy blood donor’s peripheral blood and supplied by the Nebraska Biobank (Elutriation Core Facility, University of Nebraska Medical Center, Omaha, USA) in a 50 ml conical centrifuge tube on ice. Monocytes were processed immediately after arrival, as follows. Elutriated cells were mixed by shaking the tube gently back and forth. To determine the amount of cells, 10 µl of Trypan Blue was added to 10 µl of diluted cells. Then cells were counted by hemocytometer under the microscope. To identify the necessary amount of cells to achieve a confluent monolayer for successive experiments, different amounts of monocytes were plated (4.5 x 10^5, 7.6 x 10^5 and 1.1 x 10^6 cells) according to the cell count. Cells were spun down and resuspended in Media A. Also two different 96-well plates were used to compare differences in growth, namely a clear plate (TPP®, Zellkultur und Labortechnologie, Switzerland) and a black Costar™ plate (Corning Inc. Corning, USA). The monocytes were incubated at 37 °C under 5 % CO₂. At day three 200 µl of macrophage media was added to each well. Half of the media volume was changed every 2 – 3 days. Monocytes were expected to have fully differentiated after one week (MUSSON, 1983).

2.2.2 Cultivation of macrophages

Macrophages were grown in macrophage media containing 0.1 % Gentamicin. Media was changed every second day, and 200 µl of supernatant were replaced with fresh media. Macrophages were kept in the incubator at 37 °C with 5 % CO₂ for a week, until they were used for infection experiments.

2.2.3 Monitoring of cellular growth

Cellular growth including confluence, cell size and adhesion to the well were examined visually under the microscope every day.
2.3 Mycobacteria

2.3.1 Preparation of mycobacterial culture

For infection, MSM was used as a model organism for MTB. All experiments were performed in cooperation with David Russell from Cornell University, who also supplied the bacteria for this project. The MSM strain we used was expressing the reporter protein mCherry to make it visible under the fluorescence microscope.

Mycobacteria were received as glycerol stocks. Due to high doubling time of three hours (SINGH & REYRAT, 2009), only a little part of the bacteria was scraped off the frozen stock, added to 2 ml of Middlebrook 7H9 medium and incubated overnight at 37°C with 200 rpm agitation. The next day 50 µl of bacterial suspension were transferred to 2 ml of Middlebrook 7H9 medium in a 14 ml polystyrene tube and incubated at 37 °C with medium agitation of 200 rpm. The following day the bacteria were used for infection of macrophages.

2.3.2 Mycobacterial infection of macrophages

Infection of macrophages with mycobacteria was tested with different inocula: MOIs 0.005, 0.01, 0.05, 0.01, 1.0 and 5. The amount of bacteria was calculated based on optical density at a wavelength of 600 nm (OD\textsubscript{600}) with the reference point of 0.1 OD\textsubscript{600} equaling 1x10\textsuperscript{7} bacteria per ml (BETTENCOURT et al., 2010). Ensuing from these numbers, multiplicity of infection (MOI) was calculated as ratio of bacteria to plated macrophages. To obtain the final MOIs, 5 ml of bacterial stock was centrifuged at 3000 rpm for 10 minutes and the pellet was resuspended in 1.0 ml warm Basal Uptake Buffer and transferred to another tube. In order to break bacterial clumps, the mycobacterial suspension was passed through a 26 G\textsuperscript{3/8} tuberculin syringe 10 – 12 times. Afterwards the suspension was diluted in DMEM (Dulbecco’s Modified Eagle Medium, Life Technologies, Carlsbad, USA) without antibiotics to measure the stock concentration with a spectrophotometer. OD at 600 nm was measured and compared to a DMEM blank. Concentration of bacteria was calculated as described above and stock dilutions were prepared to result in required MOIs.

Three different approaches were taken to have enough intracellular infection and avoid extracellular growth of bacteria, which had been observed in previous experiments.
1. Regular infection:

15 µl of each bacterial dilution was added to 35 µl of media in the corresponding wells and incubated for one hour at 37 °C under 5 % CO₂. After 3x washing with warm PBS, 200 µl of macrophage media was added either without antibiotics, with 0.1 % Gentamicin or with 1 % Penicillin/Streptomycin (Pen-Strep).

2. Gentamicin treatment:

In this approach, an additional incubation step with 1 % Gentamicin for 90 minutes was performed to the regular infection. 15 µl of each bacterial dilution plus 35 µl of media were added to the corresponding wells and incubated one hour in the incubator. Then, washed 3x with warm PBS. Then, 100 µl of 1 % Gentamicin media was added and incubated 90 min in the incubator. Washed 3x with warm media. Lastly, 200 µl of media was added either without antibiotics, with 0.1 % Gentamicin or with 1 % Pen-Strep.

3. Centrifuge-assisted infection:

To increase infection rate, plates were centrifuged during infection to facilitate cell entrance for bacteria in suspension (ALLAN & PEARCE, 1979). First, 15 µl of each bacterial dilution plus 35 µl of media were added to the corresponding wells. Then, the plate was centrifuged for 15 min at 730 g. After centrifugation the plate was incubated one hour in the incubator. Afterwards, washed 3x with warm PBS. Finally, 200 µl of media with 0.1 % Gentamicin was added to each well.

2.3.3 Monitoring of mycobacterial infection

The mCherry-tag of the bacteria allowed us to quantify the MSM infection with a Fluorescence Microplate Reader (BioTek, Winooski, USA). The protein was visualized using excitation and emission wavelengths 590 nm and 610 nm, respectively. Two filters were available for this experiment, whereas none provided the exact wavelengths. The following combinations of filters were compared to find which one had highest sensitivity to the reporter protein:

1. Excitation 530/25 and Emission 590/35
2. Excitation 575/15 and Emission 615/16
Additionally infected plates were examined under the fluorescence microscope to observe intracellular infection of MSM.

### 2.3.4 Quantification of mycobacterial infection by Colony-forming units counting

Colony-forming units (CFU) were counted from lysed macrophages infected with mycobacteria to quantify bacteria from cell culture. Media from infected cells was aspirated and cells were incubated with 100 µl of lysis buffer (0.05 % SDS in water) for 5 min at room temperature. Afterwards, the lysate was pipetted up and down for a few times to detach all cells and the whole content of every well was transferred to a tube. Dilutions with Middlebrook 7H9 media were prepared and plated on 7H10 plates (Thermo Fisher Scientific, Waltham, USA). Lastly, the plates were wrapped with disinfected aluminium foil and incubated at 37 °C and 5 % CO₂ for 2 – 3 days.

### 2.4 HIV-1

We used an HIV-1ADA strain, which is known for its macrophage-tropic (m-tropic) properties, meaning that macrophages are highly susceptible for its infection (WESTERVELT et al., 1991). This particular strain was also pseudotyped with vesicular-stomatitis-virus’ envelope protein G (VSV-G) driven by a human cytomegalovirus (CMV) promoter, which was used to increase infection rate of macrophages (MÁTRAI et al., 2010). Additionally, the strain was tagged with a green fluorescent protein (GFP) in place of nef to make infected cells visible by fluorescent microscopy. The combination of these components were hoped to provide high infection rates of HIV-1.

#### 2.4.1 HIV-1 stock derivation

Viral stock was obtained by transient transfection of Human 293T kidney fibroblast cells with pantropic pseudotyping.

Cells were transfected in T75 flasks under two different conditions: one was cultured with 10 ml DMEM without Pen-Strep and the other plate was kept with 10 ml DMEM with Pen-Strep to see if the antibiotic influenced the rate of infection. For transfection two tubes with 1 ml OPTI-MEM® each were prepared. To the first tube 40 µl of Lipofectamine® 2000 were added and to the second tube 11.6 µl ADA DNA (12 µg) and 2 µl CMV-VSV-G DNA (1.3 µg) were pipetted. After 5 minutes tubes one and two were
pooled together and incubated for 20 minutes at room temperature. Subsequently 1 ml of transfection mixture (Lipofectamine and DNA) was added to the flasks with 9 ml of media. Flasks were incubated for 24 hours at 37 °C, 5 % CO₂. Supernatants were collected and filtered with a 0.22 µm filter and again 10 ml of the corresponding DMEM were added into each bottle. Flasks were incubated for another 16 hours before collecting 5 ml of supernatants and filtering. One again, bottles were incubated until 48 h after transfection and 5 ml of supernatants containing viral particles were collected and filtered. Transfection efficiency at the three mentioned time points (24 h, 40 h and 48 h) was compared by Reverse Transcriptase (RT) activity assay. Supernatants were stored at 4 °C until the stocks were used for infection of macrophages or virus titer was determined.

2.4.2 Determination of virus titer by Reverse Transcriptase activity assay

Viral stocks were harvested at different time points (24 h, 40 h and 48 h) after transfection under two different conditions (treatment with antibiotics and without antibiotics). Differences in virus titer were measured by RT activity assay. Reverse transcriptase is a unique enzyme of retroviruses such as HIV-1. It performs DNA synthesis whereby single-stranded viral RNA is converted into double-stranded DNA, which is subsequently integrated into the host cell genome. Activity of RT can be measured by using radio-labeled thymidine. In this assay thymidine gets incorporated in the genome as DNA molecules are synthesized. The amount of radio-labeled thymidine is directly proportional to the concentration of RT and also the amount of virus.

Reaction mixtures were prepared as described by HOFFMAN et al., 1985; and LEE et al., 1987, while procedures were modified as stated below. Supernatants from transfected cells were collected at different time points and used to determine the virus titer. 5 µl of each viral stock was plated in duplicates on a 96-well round-bottom plate. OPTI-MEM® was used as negative control. Before incubating the plate at 37 °C for 10 – 15 minutes, 10 µl Disruption Buffer was added to each well. During the incubation, 12 µl of titrated thymidine (30 µCuries) was added to 3 ml of pre-aliquoted Reaction Mixture. After incubation 25 µl of Reaction Mixture with thymidine was added to each well. The plate was placed in the incubator at 37 °C and 5 % CO₂ over night. The following day 5 µl of each sample were placed onto a membrane filter and dried for 1 hour under a fan. The dry membrane was washed four times with 5 % Na₂HPO₄ while shaking at room temperature for 5 minutes. After this the membrane
was briefly rinsed with water and subsequently washed with 70 % Ethanol while shaking at room temperature for 5 minutes. Later the membrane was blotted dry with paper towels and dried under the fan again. Subsequently the membrane was placed in a plastic sleeve and sealed on three sides. Finally, 3.9 ml of BetaPlate Scint fluid (PerkinElmer, Akron, USA) was pipetted into the sleeve to cover the membrane. Air bubbles were removed and the sleeve was sealed completely. The sleeve containing the membrane was put into a cassette and RT-activity was measured in a microplate scintillation counter (PerkinElmer, Akron, USA).

2.4.3 HIV-1 titer determination by p24 Enzyme-linked immunoabsorbent Assay

In addition to RT-activity, HIV-1 titer was also determined by measuring HIV-1 core protein (p24) using an enzyme-linked immunoabsorbent assay (ELISA) (QuickTiter™ HIV Lentiviral Quantification Kit, Cell Biolabs, Inc., San Diego, USA). A mouse monoclonal p24 antibody coated microwell plate was used. P24 antigen contained in the sample or the standard binds to the coated plate and subsequently Fluoresceine Isothiocyanate (FITC)-conjugated antibody is used as secondary antibody that binds to p24 antigen captured by the first antibody. A Horseradish peroxidase (HRP)-conjugated mouse anti-FITC antibody binds to the FITC conjugated anti-p24 antibodies. Substrate solution reactive with HRP is added to the wells leading to the formation of a colored product proportional to the amount of p24. To determine quantity of p24 antigen we compared absorbance of samples with that of known recombinant p24 antigen standard curve. We performed the assay according to the manufacturer’s instructions. As antigen standard, a dilution series of p24 stock solution in the concentration range of 100 ng/ml – 1 ng/µl in Assay Diluent supplied with the kit was prepared. Supernatants of infected cells were diluted in fresh macrophage media 10 to 100 fold. 225 µl of each sample was transferred to microcentrifuge tubes containing 25 µl of Triton X-100 Solution and tubes were vortexed. Afterwards samples were incubated for 30 minutes at 37 °C. All reagents were mixed thoroughly before use. Samples were plated in duplicates and 110 µl of sample or standard were added to each well of the anti-p24 antibody coated plate. Later on, the plate was covered and incubated at 37 °C over night. The next day, wells were emptied tapping the microwell strips on a paper towel and washed 3 times with 250 µl 1x Wash Buffer. After the last washing step we removed excess 1x Wash Buffer thoroughly. In the next step 100 µl of diluted FITC-conjugated anti-p24 monoclonal antibody was added to each well. The plate was covered again and incubated for 1 hour on an orbital shaker. During incubation Substrate Stain Solution was warmed to room
temperature. After incubation the plate was washed as mentioned above and 100 µl of Substrate Solution was added to each well. The plate was put on an orbital shaker and observed for color change. After about seven minutes the reaction was stopped by adding 100 µl of Stop Solution into each well. Absorbance was measured immediately at 450 nm.

2.4.4 HIV-1 infection of macrophages

Before infecting, the macrophages were washed with macrophage media without antibiotics. To each well of the 24-well plate the following reagents were added:
- 300 µl of macrophage media with Gentamicin (0.1 %)
- 35 µl or 70 µl of viral stock (p24 cc: 2.5 µg/ml)

The HIV-1 inoculum equals 125 ng or 250 ng/10⁶ macrophages. Plates were incubated overnight. The following day infected wells were washed two times with PBS and afterwards 2 ml of fresh macrophage media was pipetted to each well.

2.4.5 Monitoring of HIV-1 infection

Viral infection was monitored by p24 ELISA as described above and also via the reporter protein GFP, control was possible under the fluorescence microscope at Excitation wavelength 395 nm and Emission wave length 509 nm.

2.5 Mycobacterium smegmatis and HIV-1 co-infection and monitoring

After determining the best MOIs for mycobacterial infection, as well as the best HIV inoculum for macrophages, co-infection was performed. Six different approaches were taken: Three plates were infected with HIV first, while macrophages from other plates were infected with MSM before viral infection. Each infection was performed as described above and according to previous results a bacterial suspension with an MOI of 0.1 was used for this experiment. For infection with virus, inocula of 125 ng/ml and 250 ng/ml were applied. In plates, which were infected with MSM first, viral infection followed 48 hours later, whereas macrophages incubated with virus first, were treated with mycobacteria 72 hours later. This was decided due to the fast bacterial growth that had been observed in earlier experiments. Detailed infection conditions and time points are summarized in table 2. On every 24-well plate where
co-infection was carried out, two wells with macrophages infected with mycobacteria only as well two wells with viral infection were plated as references. Also, negative controls with uninfected macrophages were kept in two wells per plate.

Table 2 | *Mycobacterium smegmatis* and HIV-1 coinfection

<table>
<thead>
<tr>
<th>Plate</th>
<th>First HIV (35 µl), 72 h later MSM (MOI 0.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate 2</td>
<td>First MSM (MOI 0.1), 48 h later HIV (35 µl)</td>
</tr>
<tr>
<td>Plate 3</td>
<td>First HIV (35 µl), 72 h later MSM (MOI: 0.1)</td>
</tr>
<tr>
<td>Plate 4</td>
<td>First MSM (MOI: 0.1), 48 h later HIV (35 µl)</td>
</tr>
<tr>
<td>Plate 5</td>
<td>First HIV (70 µl), 72hs later MSM (MOI: 0.1)</td>
</tr>
<tr>
<td>Plate 6</td>
<td>First MSM (MOI: 0.1), 48 h later HIV (70 µl)</td>
</tr>
</tbody>
</table>

Both infections were monitored the same way each infection was observed and quantified separately. On account of the reporter proteins, both pathogens could be observed by fluorescence microscopy. Quantification of HIV-1 infection was performed by p24 ELISA, as mentioned before. MSM was quantified by Fluorescence microplate reading at the same wavelengths as described in chapter 2.3.3.
3 RESULTS AND DISCUSSION

3.1 Plating of macrophages

To obtain a recommended cell confluency of 80% (BETTENCOURT et al., 2010) for the consequent infection experiments, different amounts of macrophages were seeded on a 96-well-plate. After the cells were processed as described in chapter 2.2.1 and counted with by hemocytometer, the following amounts of cells were plated: 456,000, 760,000 and 1,140,000 cells per well. Growth of macrophages was observed under the microscope daily and finally, on the sixth day after plating, a confluency of estimated 80% was observed in the wells with the highest amount of cells seeded. These results suggest that for infection experiments more than one million cells should be seeded, to obtain the right conditions for infection within one week. Also, two different plates were tested as mentioned in 2.2.2. In terms of cellular growth, no differences could be observed between the black and the transparent plate.

3.2 Evaluation of filters for fluorescence plate reading to quantify bacterial infection

Quantification of bacterial infection was performed by fluorescence plate reading by use of the fluorescent mCherry reporter protein of the MSM strain. Two different filters were used to compare their sensitivity, as both did not provide the exact excitation and emission wavelengths as required (see chapter 2.3.3). In general, an increase of fluorescence could be observed from higher to lower dilutions as well as in chronological progression of the infection. Concerning the filters, these graphs illustrate that the second filter (Ex 575/15 and Em 615/16) is clearly more sensitive to mCherry’s fluorescence spectrum. As a result, the four upper curves are easier to distinguish, show a steeper ascent and differences in concentration of the bacteria are more evident, thus quantity of bacteria is determined more precisely. In contrast, values of the three curves at the bottom, which display the results of the first filter (Ex 530/25 and Em 590/35), are not clearly distinguishable. There is no noticeable rise of fluorescence during the course of infection, and just slight differences between the curves are observed for decreasing dilutions. For further experiments, only the second filter combination was used. On day seven of the measurement, fluorescence was measured only with the second filter as the previous days had shown that those results are more accurate.
3.3 Mycobacterial cell culture

3.3.1 Determination of inoculum for mycobacterial infection

In this experiment macrophages were infected with different MOIs of MSM to observe infection rates and survival of cells. Non infected macrophages were used as negative controls. Mycobacterial infection was monitored under the fluorescence microscope daily for four days. As seen in figure 13 infection of macrophages with MSM worked very well. Cells were examined under the microscope right after infection to see if the bacteria had entered the macrophages. On day one and two after infection bacterial growth could be seen proportional to the dilutions. However, monitoring of the cell cultures became increasingly difficult from the third day onwards. In lower dilutions, such as MOI 5, 1 or 0.1 only a few parts of the wells could be examined due to bacterial aggregates floating in the media. All negative controls were bacteria-free. On day four after infection extracellular growth had increased drastically in all wells.
Figure 13 | *Mycobacterium smegmatis* infection under the fluorescence microscope, 200 x magnification. 
A) Macrophages right after infection with mycobacteria, picture taken without filters. B) OD_{600} 0.05, mCherry visible in macrophages right after infection. Only very few bacteria are visible. C) OD_{600} 0.01 on the fourth day after infection. There are a lot of extracellular bacteria and big bacterial clumps are floating in the media.

Fluorescence plate reading was performed until the fifth day after infection as illustrated in figure 14. This reading did not exactly reflect what had been observed during cell culture. Macrophages infected with an MOI of 0.1 showed less fluorescence than wells inoculated with an MOI of 0.05 and 0.01, while on the fifth day after infection the MOI of 1.0 showed the highest fluorescence. Also, there was no significant difference detected between the three lowest MOIs during the first three days of infection.

Figure 14 | Results of fluorescence plate reading of macrophages infected with different MOI of *Mycobacterium smegmatis*.

We would have expected a more linear increase of fluorescence during the first week of infection as seen in the filter testing experiment since the same bacterial stock was used in
both experiments. It is also unclear why the highest MOI showed a drop in fluorescence on day four of infection and had only the second highest results on the last day. Also in infections with MOI 0.005, a decrease of infection was measured on day four. Only MOIs 0.01, 0.05 and 1 provided parallel graphs and a consistent increase of fluorescence. According to these results and uncontrollable extracellular growth, which might have interfered with the reading, MOIs between 0.01 and 1 are suggested to deliver best results in the infection experiment. However, these results have to be seen in the context of the previous filter testing experiment, where the better filter was chosen, but still it did not provide the exact spectrum required for mCherry quantification.

### 3.3.2 Evaluation of growth conditions after mycobacterial infection

Extracellular bacterial growth posed a major problem in the first experiments and hindered quantitative monitoring of intracellular infection by fluorescence plate reading as well as observation of infection over a longer period of time. To solve this problem, three different treatments were tried as explained in chapter 2.3.2.

1. **Regular infection:**
   After regular infection, cells were kept in media in three different compositions. Wells with media containing either 0.1 % Gentamicin or 1 % Pen-Strep had no extracellular bacterial aggregates floating on the culture surface. However, only estimated 20 % of macrophages were infected when kept with Gentamicin. In wells where infected macrophages were cultured with 1 % Pen-Strep in the media, only around 10 % of cell contained fluorescent bacteria. Over-all, macrophages with no antibiotic supplement in the media showed highest intracellular infection with more than 80 % of cells holding MSM inside. Unfortunately those high infection rates were coupled with macroscopically visible bacterial clumps.

2. **Gentamicin pretreatment:**
   The second approach was to treat the cell culture for 90 minutes with media containing 1 % Gentamicin right after infection. Afterwards the same three media compositions were added as mentioned above under 1. Cells kept in media without antibiotics after the Gentamicin pretreatment, showed the highest infection rate of
10%. In macrophages, where antibiotics were added to the media after the Gentamicin pretreatment, hardly any bacteria could be seen inside the wells.

3. Centrifuge-assisted infection:
In order to facilitate bacterial entry into the cells and to obtain an evenly distributed infection, macrophages were centrifuged with the bacterial inoculum. Even though, spinning cells during infections is not recommended by all authors (BETTENCOURT et al., 2010), we obtained good infection results. According to visual observation under the microscope, around 50% of macrophages were infected with mycobacteria and no extracellular bacteria were detected.

In general, these results show that treatment with antibiotics is associated with a drop of infection rate, but most helpful in avoiding extracellular growth of MSM. Of both antibiotics, Pen-Strep was more drastic and left only a maximum of 10% of cells infected in all three approaches. Gentamicin, on the other hand, allowed the bacteria to infect around 20% of macrophages during the regular infection and a promising 50% during the centrifuge-assisted infection. An infection rate of 50% is the highest percentage of infection achieved in all tested conditions. As these percentages of infections were estimated based on visual quantification, fluorescence plate reading would be necessary to verify the results for more precise outcomes in following experiments. Fluorescence microscopy was performed on day three or four after initial infection. On day six, when it was planned to monitor the infection, no bacteria were visible under the microscope, except for the wells where no antibiotic treatment was given at all. Hence, it seemed that day 6, post infection onwards the bacteria did not survive antibiotic supplements to the media. To determine more specifically, if there were no bacteria inside the cells they were plated on agar plates for CFU estimation. No bacterial colonies were observed on agar plates corresponding to the macrophages treated with antibiotics. Only lysates from cells with no antibiotic supplements, and those that were pretreated with Gentamicin and further handled without antibiotics revealed colonies. Cells from colonies were put on glass slides and observed under the microscope. During this inspection, we observed that almost 90% of the bacteria had lost their fluorescence as shown in figure 15.

Figure 15 | Cultivation of Mycobacterium smegmatis from lysed cells.
Lysates of cells with no antibiotic treatment over the whole course of infection were used for plating. Only very few bacteria are fluorescing.
3.4 Viral cell culture

3.4.1 Evaluation of transfection conditions

Human Embryonic Kidney 293T-cells were transfected with HIV and CMV-VSV-G DNA under two different conditions: without antibiotics or with 1% Pen-Strep. Supernatants were collected 24 hours, 40 hours and 48 h after transfection to find the best time span to harvest the virus. Reverse transcriptase activity was measured in collected supernatants.

Figure 16 | Comparison of transfection efficiency using the Reverse transcriptase activity assay. Transfection was performed with and without antibiotics in the media. Virus was harvested 24 h, 40 h and 48 h after transfection. Subsequently reverse transcriptase activity was measured in counts per minute (cpm) per microliter.

There was no considerable difference in RT activity based on the presence or absence of antibiotics. The best yields were obtained at 40 hours after transfection, while at 48 hours there were already a lot of dead cells visible under the microscope. As media without antibiotics was thought to be better for mycobacterial co-infection, yields at the points of time 24 h and 40 h from cultures without Pen-Strep were aliquoted for macrophage infection.

3.4.2 Determination of HIV inoculum for infection

Macrophages were infected with GFP-tagged HIV-1ADA clone in 96-well plates with different p24 amounts: 8 ng, 16 ng, 32 ng, 49 ng, 64 ng and 81 ng per 300,000 cells. Uninfected
macrophages were plated as negative controls. All infections worked fine, although only very few infected cells could be detected in the wells with an inoculum size of 8 ng, 16 ng and surprisingly even with 64 ng of p24. Fluorescence plate reading (see figure 18) revealed that unfortunately negative controls also delivered positive results and appeared infected, even though no infection was visible under the fluorescence microscope. It is possible, that due to the transparency of the plate, readings of different wells interfered. Perhaps the black Costar™ plates (described under 2.2.2) would have given better results but they were not tested in this experiment. It still has to be tested, if such a non-transparent plate would avoid false-positive results. For better quantification of infection, Reverse Transcriptase (RT) activity assay was performed. Results are summarized in figure 19.

Figure 17 | Infected macrophages under the fluorescence microscope. All pictures were taken on the third day after infection. A) Picture taken without a filter, showing a confluency of almost 90 %. B) HIV-1 visible with a green fluorescence filter. Cells were infected with 8 ng p24 per 300,000 cells. C) Infection with 32 ng p24. C) Highest inoculum with 81 ng p24.

Both methods confirmed that the infection was successful in all wells at all amounts of p24. Also, the unexpected infection in the non-infected controls was evident in the RT-activity
assay and is hereby confirming the results of the fluorescence plate reading. However, it is rather weak and in combination of the microscopy results, where no fluorescence was visible, it is suggested that this is a false-positive result. The RT activity assay may deliver positive results generated by cellular polymerases, which cannot be discriminated from RT with this assay (CHANG et al., 1997). In this case, the experiment has to be repeated and obtained results should be validated with a more specific method, for example involving a RT specific polymerase chain reaction (PCR) step, which is 10 times more sensitive to RT then conventional assays (LUGERT et al., 1996). A closer look at the results of the two quantification methods clearly shows the highest infection in the 32 ng inoculum, even though on the fifth day of infection fluorescence dropped to the level corresponding to 81 ng. In contrast, the lowest p24 of 8 ng at infection shows surprisingly high fluorescence compared to higher inocula (e.g. 64 ng), while its RT-activity remains the lowest at all points of time. The main difference between the results of both assays is the general trend during the course of infection. In the fluorescence plate reading, values drop after the third day of infection, whereas RT activity is continuously rising until it reaches a slight plateau phase on the fifth day after infection. As already explained, both methods would need verification. The fluorescence plate reading has to be repeated with a non-transparent plate and the RT assay needs evaluation by a more specific method to exclude false-positive results.

Figure 18 | Reverse transcriptase activity of HIV-infected macrophages with different amounts of p24 per 300,000 cells. On the vertical axis, radioactivity is displayed in counts per minute per microliter. The horizontal axis shows the days of infection when supernatants were collected.
3.5 Co-infection of *Mycobacterium smegmatis* and HIV-1

Macrophages were infected either with HIV-1 or MSM first and after a certain period of time with the other pathogen as described in section two. As co-infection was performed in 24-well plates, more cells were plated than in the inoculum testing experiments. For the inocula of 125 ng and 250 ng about $10^6$ cells were seeded in each well. Unfortunately, the necessary confluency of at least 80 % could not be achieved. Instead, plate coverage was estimated around 60 %. During the course of infection mycobacterial growth was monitored visually under the microscope, while HIV-1 infection was measured later on by p24 ELISA.

![Figure 20](image)

*Figure 20 | Monitoring bacterial growth during co-infection.* Black aggregates of mycobacteria were microscopically attached to the cell surfaces as well as floating in the media. Picture was taken six days after MSM infection of macrophages followed by HIV-infection after 48 h.

![Figure 19](image)

*Figure 19 | Fluorescence plate reading of HIV-1 infected macrophages during the course of infection.* The graphs show the development of the infection in connection with the amount of p24 that was used for initial inoculation. Each concentration of p24 per 300,000 cells is assigned a different color as explained in the legend on the right side of the graph. The horizontal axis shows the days after infection, while the vertical axis represents the measured fluorescence.

![Figure 20](image)

*Figure 20 | Monitoring bacterial growth during co-infection.* Black aggregates of mycobacteria were microscopically attached to the cell surfaces as well as floating in the media. Picture was taken six days after MSM infection of macrophages followed by HIV-infection after 48 h.
The co-infection was stopped seven days after infection due to uncontrollable extracellular growth of the mycobacteria. Maximum extracellular bacterial growth was clearly seen in the co-infected wells. From all infected wells, p24 was measured.

The standard curve worked as expected, except the highest p24, which was supposed to be 100 ng/ml, resulted only in 75 ng/ml in the ELISA. Disregarding this value, the correlation coefficient is greater than 0.99 and the curve can be used as reference. Since the co-infection was not carried out as planned due to shortage in bacteria, p24 of as few as two plates with macrophages infected with both pathogens were performed. In addition, co-infection was observed only in cells first inoculated with MSM. Supernatants for p24 ELISA were collected from two co-infected wells and in HIV positive controls. Results are summarized in the table below (Table 3).

<table>
<thead>
<tr>
<th>ng/ml</th>
<th>abs 450</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.895</td>
</tr>
<tr>
<td>50</td>
<td>0.596</td>
</tr>
<tr>
<td>25</td>
<td>0.355</td>
</tr>
<tr>
<td>12.5</td>
<td>0.202</td>
</tr>
<tr>
<td>6.25</td>
<td>0.112</td>
</tr>
<tr>
<td>3.125</td>
<td>0.073</td>
</tr>
<tr>
<td>0</td>
<td>0.046</td>
</tr>
</tbody>
</table>

**Figure 21 | Standard curve of the p24 measurement after co-infection.** The graph on the left side shows the absorbance of standard samples with known p24 amounts in ng/ml at 450 nm on the vertical axis, while the horizontal axis displays the amounts of p24. A straight line was put through the results and a standard curve was calculated. Exact results are listed in the table on the right, where absorbance at 450 nm was converted into the amount of p24 in ng/ml.
### Table 3 | Results of p24 ELISA during course of HIV-1 and MSM co-infection.

<table>
<thead>
<tr>
<th>Infection</th>
<th>p24 amount of viral stock (ng/ml)</th>
<th>Absorbance</th>
<th>p24 amount according to ELISA (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td>125</td>
<td>0.122</td>
<td>6.45</td>
</tr>
<tr>
<td>HIV</td>
<td>125</td>
<td>0.050</td>
<td>0.02</td>
</tr>
<tr>
<td>HIV</td>
<td>250</td>
<td>0.041</td>
<td>-0.78</td>
</tr>
<tr>
<td>Coinfection</td>
<td>250</td>
<td>0.052</td>
<td>0.19</td>
</tr>
<tr>
<td>HIV</td>
<td>250</td>
<td>0.064</td>
<td>1.27</td>
</tr>
<tr>
<td>Coinfection</td>
<td>250</td>
<td>0.053</td>
<td>0.29</td>
</tr>
<tr>
<td>HIV</td>
<td>125</td>
<td>0.057</td>
<td>0.64</td>
</tr>
<tr>
<td>HIV</td>
<td>250</td>
<td>0.057</td>
<td>0.64</td>
</tr>
<tr>
<td>Coinfection</td>
<td>250</td>
<td>0.055</td>
<td>0.46</td>
</tr>
<tr>
<td>HIV</td>
<td>250</td>
<td>0.108</td>
<td>5.20</td>
</tr>
<tr>
<td>HIV</td>
<td>125</td>
<td>0.055</td>
<td>0.46</td>
</tr>
<tr>
<td>Coinfection</td>
<td>250</td>
<td>0.053</td>
<td>0.29</td>
</tr>
<tr>
<td>HIV</td>
<td>250</td>
<td>0.053</td>
<td>0.29</td>
</tr>
<tr>
<td>HIV</td>
<td>125</td>
<td>0.081</td>
<td>2.79</td>
</tr>
</tbody>
</table>

All calculations were made in accordance with the standard curve in figure 21.

As outlined in table 3, HIV-1 infection did not work properly. Even though p24 of the viral stock had been measured before, the collected supernatants contained no virus, except two samples (6.45 ng/ml and 5.20 ng/ml). Since the p24 amount was obviously much lower than it was thought to be. The results of the co-infection experiment are not significant.
4 CONCLUSION AND FURTHER STEPS

The first aim of this work was the establishment of an in vitro model to gain deeper insights into the underlying processes to the enhancement of co-infection with HIV and MTB with the use of the mycobacterial model organism MSM. Despite thorough review of already established methods for MSM culture, we did not succeed to optimize cell culture protocols to gain reliable and reproducible results. Thus, the basis for further research cannot be given with the approach we made within the given time. However, it can be thought of several improvements to provide the foundation to find answers to the essential question, how macrophage permissivity to HIV-1 infection is increased during co-infection. Reviewing all the data and results that were obtained, optimization of experiments is needed. As a first step, fluorescence filters according to the fluorescence spectrum of mCherry have to be used to gain more accurate results. Also, it has to be proven that the transparent cell culture plates do not interfere with the fluorescence plate reading of mCherry and GFP. Even when quantification of mycobacteria by fluorescence plate reading is optimized, it should be thought of methods to verify the results. For determination of stock concentrations as well as concentrations during progression of infection, quantitative Real-time PCR (RT-qPCR) (PATHAK et al., 2012) could be used. As already mentioned previously in the discussion, HIV stock concentration and infection could also be quantified by RT-PCR (SHAN et al., 2013). Application of this method delivers more precise results and is less time consuming as well as free from radioactive substances. Due to the fast growth of MSM, it should be considered using a different model organism. Especially in this case, where it is assumed that both pathogens enhance each other and infection progresses faster With these inputs and the knowledge from previously performed experiments in terms of infection procedures, another attempt can be made to reach the aims that were set.
5 SUMMARY

HIV and *Mycobacterium tuberculosis* infections are one of the world’s greatest public health concerns. The introduction of antiretroviral therapy (ART) in 1995 and increased awareness among people has helped in reducing the number of newly HIV-1 infected individuals as well as in lowering the number of AIDS-related deaths (UNAIDS, 2013). As stated in the Global Tuberculosis Report 2013, Tuberculosis are also declining due to improved diagnosis and treatment (WHO, 2013). However, Tuberculosis still poses a big threat to AIDS patients and remain the leading cause of death, killing one of three co-infected patients (RAVIGLIONE et al., 1995). HIV-1 and *Mycobacterium tuberculosis* infections are known to exacerbate each other in clinical studies (DIEDRICH & FLYNN, 2011; PAWLOWSKI et al., 2012), although the underlying processes are yet not so clear.

In this work, we hypothesize that *Mycobacterium tuberculosis* might influence macrophage permissivity to HIV-1 infection. To study the role of mycobacteria, we used *Mycobacterium smegmatis*, which is often used as a model organism for Tuberculosis (REYRAT & KAHN, 2001). Our aim was to establish *Mycobacterium smegmatis* as a co-infection model for cell culture studies and observe the progression of infection with mycobacteria and HIV-1 as compared to parallel separate infections of each. According to the hypothesis, we expected to see an enhancement of both infections during co-infection and hoped to study the basic mechanisms causing this effect. As a first step monocyte derived macrophages and human embryonic kidney 293 cells (293T) were cultured. Viral stocks were obtained by transient transfection of 293T cells with a pseudotyped virus whose envelope contained glycoprotein G from vesicular-stomatitis-virus and glycoprotein 120 from the wildtype HIV (pantropic VSV-G : wt ADA) to enable the virus to enter various cell types. Both *Mycobacterium smegmatis* and HIV-1 were labelled with separate fluorescence tags, to allow visual inspection of infection under the fluorescence microscope. For better quantification of infection, HIV-1 titer was measured using p24 ELISA while *Mycobacterium smegmatis* infection was determined via fluorescence plate reading. Establishment of cell culture conditions and testing of various methods for optimal and reproducible macrophage infections with both pathogens turned out to be a challenging task and unfortunately the co-infections did not progress as expected. We were not able to completely finalize the optimization of combining standard bacteriological with virologigac cell culture methods to provide a reliable *in vitro* model. Further experiments need to be performed to gain better and more detailed knowledge concerning the exact role of mycobacteria during HIV-1 infection.
6 ZUSAMMENFASSUNG


### 7 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>APOBEC</td>
<td>apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>CRF</td>
<td>Circulating recombinant form</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CR1, CR2, CR3, etc</td>
<td>Complement Receptors</td>
</tr>
<tr>
<td>CXC4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>env</td>
<td>envelope</td>
</tr>
<tr>
<td>Ex/Em</td>
<td>Excitation/Emission</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluoresceine isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>Gag</td>
<td>Group antigen</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Gp120</td>
<td>Glycoprotein 120</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeats</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MSM</td>
<td>Mycobacterium smegmatis</td>
</tr>
<tr>
<td>MTB</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative factor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor 'kappa-light-chain-enhancer'</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signals</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonyl phenoxypolyethoxylethanol</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>oligo dT</td>
<td>Oligodeoxythymidylic acid</td>
</tr>
<tr>
<td>P24</td>
<td>Protein 24</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>Pen-Strep</td>
<td>Penicillin-Streptomycin</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of virion gene expression</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Tat</td>
<td>Transcriptional activator</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus glycoprotein G</td>
</tr>
</tbody>
</table>
8 REFERENCES


death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet, 368, 1575–1580.


immunodeficiency virus Type 1 (HIV-1) subtype on disease progression in persons from Rakai, Uganda, with incident HIV-1 infection. The Journal of infectious diseases, 197, 707–713.


UNAIDS, (2013): GLOBAL REPORT: UNAIDS report on the global AIDS epidemic 2013,


WHO, (2013): Global Tuberculosis Report 2013,


9 TABLES AND FIGURES

9.1 Tables
Table 1 | Reagents 18
Table 2 | Mycobacterium smegmatis and HIV-1 co-infection 27
Table 3 | Results of p24 ELISA during course of HIV-1 and Mycobacterium smegmatis co-infection 38

9.2 Figures
Figure 1 | Estimated number of new HIV infections globally, 2001 – 2013. 2
Figure 2 | Estimated number of people dying from HIV-related causes globally, 2001 – 2013. 2
Figure 3 | Relations between different HIV subtypes. 4
Figure 4 | Structure of an HIV virion particle. 4
Figure 5 | Genetic organization of HIV-1. 5
Figure 6 | The HIV life-cycle. 7
Figure 7 | Comparison of the course of disease of untreated HIV infection and changes after ART. 9
Figure 8 | Electron micography of Mycobacterium tuberculosis. 10
Figure 9 | Estimated Tuberculosis incidence rates, 2013. 11
Figure 10 | Lipid metabolism of Mycobacterium tuberculosis. 12
Figure 11 | Global distribution of the six main lineages of human Mycobacterium tuberculosis. 13
Figure 12 | Relative fluorescence measured with two different filters. 29
Figure 13 | Mycobacterium smegmatis infection under the fluorescence microscope, 200 x magnification. 30
Figure 14 | Results of fluorescence plate reading of macrophages infected with different MOI of Mycobacterium smegmatis. 30
Figure 15 | Cultivation of Mycobacterium smegmatis from lysed cells. 32
Figure 16 | Comparison of transfection efficiency using the Reverye transcriptase activity assay. 33
Figure 17 | Infected macrophages under the fluorescence microscope. 34
Figure 18 | Reverse Transcriptase-activity of HIV-infected macrophages with different amounts of p24 per 300,000 cells. 35
Figure 19 | Fluorescence plate reading of HIV-1 infected macrophages during the course of infection. 36
Figure 21 | Standard curve of the p24 measurement after co-infection. 37