Analysis of CD4+ Th1 cells in immunity to intracellular *Mycobacterium avium* infection.

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ABSTRACT

Mycobacterial infections are considered a major health problem internationally. *Mycobacterium tuberculosis* (Mtb), the infectious agent causing tuberculosis, kills 1.4 million people each year. Other non-tuberculous species such as *Mycobacterium avium* (*M. avium*) are less pathogenic but cause opportunistic infections mainly in immune-compromised people. The current treatment for different mycobacterial infections is elaborate, expensive and ineffective and new targets for treatment are needed. *M. avium* is an interesting subject for research, both for finding targets for treatment towards *M. avium* infection but possibly also for treatment of tuberculosis as the two species show many pathogenic similarities. For mycobacterial infections, CD4+ Th1 cells secreting IFN-γ and TNF play a central role in host-defense.
In this project an *in vitro* assay was established and optimized for expansion of mycobacteria specific CD4+ Th1 cells from peripheral blood mononuclear cells (PBMCs) of healthy donors. The objective was to test the functions of the expanded T cells in a human antigen-specific setting with autologous macrophages generated from the same blood donor and infected with live *M. avium*.

The method was then used to study multiple aspects of CD4+ Th1 cell functions in anti-mycobacterial immunity. First, the (poly)functionality of the expanded mycobacteria-specific CD4+ Th1 cells was analyzed by multi-color flow cytometry. Next, the effect of CD4+ Th1 cells and IFN-γ on *M. avium* survival in infected monocyte derived macrophages (MDM) was analyzed. Finally, the effect of changes in MDMs on the MDM - CD4+ Th1 cell interaction by Kelch-like- ECH-associated protein 1 (Keap1) knock down (KO) was investigated.

CD4+ Th1 cells expanded with heat-killed *M. avium* were re-stimulated with *M. avium*-infected autologous macrophages and T cell effector responses were characterized by flow-cytometric staining. Analysis of the effector cytokine production of CD3+CD4+ T cells was performed by additional intracellular staining of IFN-γ, TNF and IL-2. After expansion the number of mycobacteria specific CD4+ Th1 cells was found to increase enormously compared to frequencies found directly in freshly isolated PBMCs. Poly-functionality of the CD4+ T cells was determined as detected by the simultaneous production of the effector cytokines IFN-γ, TNF and IL-2.

By testing the effect of IFN-γ, as well as the expanded *M. avium* specific CD4+ Th1- cells, on intracellular survival of *M. avium* in infected autologous macrophages, we found decreased *M. avium* survival only if both IFN-γ and expanded CD4+ T cells were added prior to *M. avium* infection of the macrophages. This was analyzed by measuring luciferase activity of the bacteria by a luminometer and confirmed by counting colonies from plating. These findings for *M. avium* survival are different from findings with MTb by other group.

Lastly, Keap1 was knocked down (KO) in macrophages prior to infection with live *M. avium* using siRNA technology. Infected macrophages +/- Keap1 KO were used to activate expanded T cells. Macrophages with Keap1 KO showed reduced CD4+ T cell activation as analyzed by flow-cytometry, where a decreased effector cytokine production was seen; less total cytokine producing cells and less polyfunctional cells were also seen. Phenotyping of the macrophages for expression of the co-stimulatory molecule CD80 and MHC class II revealed lower expression of these molecules in macrophages after Keap1 KO. This could provide an explanation for the reduced T cell activation from *M. avium*-infected Keap1 KO macrophages.

The established procedures to expand human mycobacteria-specific CD4+ T cells have been used to analyze different aspects of *M. avium* specific T cell effector functions *in vitro* in an autologous system. So far the assay has been used to investigate effector cytokine composition (polyfunctionality), T cell mediated killing of *M. avium* in macrophages, as well as analysis of the specific KO of a protein discussed in anti-mycobacterial immunity and mycobacteria-specific CD4+ T cell activation. The procedure can be further used to look at a variety of other factors and cells involved in antimycobacterial immunity and may provide a valuable tool for better understanding of the host-pathogen mechanisms in mycobacterial infections.

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1. INTRODUCTION

1.1 Mycobacteria and Mycobacterial infections

Mycobacteria species of the family *Mycobacteriaceae* are the cause of major public health problems and consists of several human pathogens including *Mycobacterium tuberculosis* (Mtb) causing tuberculosis in humans, which annually kills approximately 1.4 million people (WHO, 2012). Other pathogenic species are *Mycobacterium leperae* causing leprosy, another human disease and *Mycobacterium bovis* causing bovine tuberculosis (Gengenbacher and Kaufmann, 2012). Less pathogenic is the non-tuberculous *Mycobacterium avium-intracellulare* complex (MAC) including *Mycobacterium avium*, though it still often causes severe illness in immunocompromised individuals as acquired immunodeficiency syndrome (AIDS) patients (Inderlied *et al*., 1993).

Mycobacteria are gram-positive, aerobic and non-motile with either slow or rapid growth. Some species may be recognized by a considerably long reproductive cycle. Common for the mycobacteria species are the characteristic cell wall, with a thick and waxy layer and a hydrophobic nature. This characteristic cell wall plays an important role in the virulence and prevents drugs from acting on the bacteria, also helping the bacteria avoid degradation (reviewed in Kleinnijenhuis *et al.*, 2011).

Mtb as the causative agent for tuberculosis was discovered in 1882 by Robert Koch. Mtb infections occur by the inhalation of aerosol droplets carrying bacteria. The bacteria enter the lungs and the bacilli are phagocytized by alveolar macrophages. When the macrophages detect an invader they produce cytokines and recruit several inflammatory cells to the site of infection. Macrophages are however, not always able to destroy the bacteria as is the case with Mtb. The macrophages normally launch a microbial attack that kills the bacteria, but Mtb avoids this mechanism and can survive inside the macrophage. There it can replicate until the macrophage bursts and a large number of bacilli is released into the surroundings. The macrophages are helped by a subset of T cells, the Th1 cells (Gengenbacher and Kaufmann, 2012). Mtb infection cause the formation of a granuloma, a characteristic

structure of tuberculosis and other mycobacterial infections. The infected macrophages and the Th1 cells represent the central cell types of the granuloma structure. The granuloma has a necrotic center made by the lytic enzymes released from active macrophages. When inside a granuloma, the bacilli will enter a latent stage of slow growth, unable to replicate under those conditions (Ulrichs and Kaufmann, 2006). Active tuberculosis most often occurs because of a reactivation of a previously existing dormant Mtb infection in the host. This often happens in connection with a compromised immune response (Gengenbacher and Kaufmann, 2012). An estimated number of 2.3 billion people are believed to carry a latent infection of Mtb, and 8.7 million new cases of tuberculosis were registered in 2011 (Dye and Williams, 2010; Gengenbacher and Kaufmann, 2012; WHO, 2012). Even if more than 36 million people have received successful treatment according to the World Health Organization (WHO), the disease is still a problem with 630 000 multidrug resistant incidences and approximately 1.4 million deaths in 2011 (WHO, 2012).

M. avium is a part of the *Mycobacterium avium-intracellulare* complex (MAC), which consists of several opportunistic mycobacteria species, all of which cause non-tuberculous pulmonary disease (Inderlied *et al*., 1993). The pathogenesis of Mtb and *M. avium* is somewhat different in that Mtb is a primary pathogen, while *M. avium* is not considered one, infecting primarily immune-compromised people where infection in AIDS patients are the most frequent (reviewed in Appelberg, 2006). *M. avium* is mainly an opportunistic species, but healthy people can also be infected (reviewed in Early *et al*., 2011). The *M. avium* family of MAC is typically genetically variable and the strain morphological variations are problematic concerning treatment. *M. avium* is naturally occurring in the environment and people encounter it on daily basis. *M. avium* enters the body through several pathways, with transmission through respiratory or gastrointestinal route as the most common. Here the bacteria will invade the respiratory or gastrointestinal mucosa before being transported to the lymph nodes. *M. avium* can, as Mtb and other mycobacterial species, survive inside the phagosomes of macrophages and will also result in granuloma formation (reviewed in Appelberg, 2006 and Early *et al*., 2011).

Mycobacterial species in general are difficult to treat and an increasing resistance towards different types of antibiotics is demonstrated with reports of a fully drug resistant type of Mtb (Velyati *et al*., 2009). The treatments of mycobacterial infections today are elaborate and expensive, and alternatives are needed. To be able to detect drug targets and develop new

treatment, an increased understanding of the mycobacterial species mode of infection and the host's immune response is needed (Russell *et al*., 2010). *M. avium* as a target in itself or as a model for the more pathogenic Mtb both make it an interesting species to study.

1.2 The immune system

The immune system is the host's defense mechanism against microorganisms and comprises two main parts, the innate and the adaptive systems that cooperate in protecting the body against potential pathogens.

The innate immune system consists of molecular, cellular and non-cellular mechanisms and is highly effective in prevention of most infections. The innate response is characterized by an immediate response, no memory and germline-encoded receptors only. The first lines of the innate defense are physical barriers like the skin and epithelial surfaces, together with chemical barriers like urine and sweat. When a pathogen breaks the physical and chemical barriers of defense, the innate cells, effector molecules, germline surface receptors and inflammatory signals become important. The complement system is also a part of innate immunity discovered by Jules Bordet in the late 19th century. It consists of small proteins circulating the body in an inactive state. When activated, these proteins are able to inflict damage on the pathogen, facilitating its clearance or destruction (reviewed in Murphy *et al*., 2012).

Monocytes, macrophages, neutrophils and eosinophilic granulocytes, together with natural killer cells (NK cells) and dendritic cells, are cell types central in innate immunity and participate in the process of triggering the immune system's antimicrobial defense mechanisms (reviewed in Murphy *et al*., 2012).

The main task of blood monocytes, macrophages and to some extent neutrophils is phagocytosis of the invading pathogen. The process of phagocytosis is a type of endocytosis, and is the ingestion of extracellular material by the cells. This process enables the cells to destroy the pathogen before it has a chance to cause damage by exerting microbial activity in the host. For infections by mycobacterial species such as Mtb and *M. avium* this is a crucial process, but also a process the mycobacterial species have learned to exploit to their

advantage, which will be discussed at a later point (Aderem and Underhill, 1999). The dendritic cells and antigen-presenting cells on the other hand play their main part in antigen-presentation, and in that way represent an important connection between innate and adaptive immunity. Cells of innate immunity have, as mentioned, no memory, but are nevertheless capable of recognizing pathogens and distinguishing self from harmful non-self. This is achieved through germline-encoded surface receptors on immune cells called pattern-recognition receptors (PRRs) and will be covered later (1.3.2) (reviewed in Murphy *et al*., 2012).

The adaptive immune response can be triggered by the innate immune system when it itself is not able to combat the infection. Characteristic for this subset of the system is its specificity and memory, together with self-non-self distinction. The adaptive response is slower than the innate, but has a stronger mode of action and is therefore able to eradicate infections more vigorously. The adaptive system's memory ability gives it an advantage when the host encounters a pathogen for the second time, giving a more rapid and stronger response than on first encounter. The adaptive immune system's main participants are the B and T lymphocytes, more commonly known as the B- and T cells (reviewed in Bonilla and Ottega, 2010). The antibody-mediated immune response is based on the B cells maturing in the bone marrow. Antibody mediated immunity is most important in combating infections of free antigens in the body and is thus assumed to play a minor role in the immune response against intracellular infections such as infections with mycobacterial species and will therefore not be discussed further (reviewed in LeBien and Tedder, 2008). It should be mentioned that later findings suggest that B cells play a role in immunity towards Mtb (reviewed in Abebe, 2009).

The T cell mediated response is based on T cells, which mature in the thymus. T cells are made up of two main subpopulations, distinguished by the expression of different glycoproteins, with one expressing the cluster of differentiation 8 (CD8) and the other expressing cluster of differentiation 4 (CD4). In addition, all T cells express the antigen-binding T cell receptor (TCR), which is unique to every T cell. The cluster of differentiation 3 (CD3) is co-receptor associated with the TCR and mediates TCR signals into the cytosol. Together, CD3 and the TCR represent the TCR complex. Different from the antibody-mediated response, the T cell mediated response is of central importance in the anti-mycobacterial immunity. When a T cell becomes activated, it will proliferate, making a population of identical T cells with the same specificity as the T-cell of which they originate.

The T cells can proliferate into different effector cell subsets (reviewed in Bonilla and Oettgen, 2010). This will be elaborated later (1.4.2), with focus on the CD4+ T cells and their role in anti-mycobacterial immunity.

1.3 Innate immune response to mycobacteria

The main participants in innate immunity are innate cells with pattern recognition receptors (PRRs), in addition to effector molecules and inflammatory signals.

1.3.1 Macrophages

Macrophages are, as mentioned earlier, central in the innate immune reaction against intracellular pathogens such as *M. avium* and Mtb. Alveolar macrophages are the first cells of the innate immune system to encounter Mtb (reviewed in Gengenbacher and Kaufmann, 2012). The macrophages differentiate from monocytes. In humans, monocytes can be separated into several subtypes based on co-receptor expression; as the classical monocytes with high CD14 and no CD16 expression and the non-classical monocytes expressing both CD14 and CD16 (Passlick *et al*., 1989). The classical monocytes are the major subtype in humans. It is the monocytes that circulate in the blood before they migrate into tissue where they replace the tissue-specific macrophages residing here (reviewed in Mosser and Edwards, 2008).

Macrophages need to become activated. An active macrophage will go through several changes. The expression of MHC proteins will increase, together with its antimicrobial activity and cytokine production. CD4+ Th1 cells activate the macrophage by display of the co-stimulatory protein CD40 ligand, binding to the CD40 receptor on the macrophage, together with secretion of IFN-γ, binding to IFN-γ receptors on the macrophage's surface, this process will be explained in 1.4.1. In addition to Th1 cells, NK-cells together with antigen presenting cells (APC) can also activate macrophages. Th1-cells and NK-cells mediate their effect by the cytokine IFN-gamma, while APC's use TNF secretion. The activated macrophages play an important role in host defense concerning intracellular pathogens (reviewed in Mosser and Edwards, 2008). However, some intracellular pathogens, such as mycobacteria avoid these anti-microbial functions of the macrophages and exploit

macrophages as their host, where they can survive the immune mechanisms (reviewed in Appelberg, 2006 and Gengenbacher and Kaufmann, 2012).

1.3.2 Recognition of mycobacteria and cells of the innate immune system

The immune system needs to detect an invader breaking the physical and chemical barrier of the body. This is achieved by a system of germline-encoded receptors; the PRRs recognizing bacteria by their conserved and characteristic pathogen associated molecular patterns (PAMPs), (Janeway, 1989), together with their signaling pathways. These downstream signaling pathways cause secretion of inflammatory cytokines and chemokines, in addition to antimicrobial peptides. The recognition of the PAMPs by the PRRs is therefore crucial in the initial control of an infection (reviewed by Kleinnijenhuis *et al*., 2011 and Kawai and Akira, 2011). There are several types of PRRs, which can be split into two main groups; Membrane bound and cytoplasmic PRRs. Toll-like receptors (TLRs), NOD-like receptors (NLRs) and membrane bound C-type lectin receptors (CLRs) are the most central PRRs in the recognition of mycobacteria (Kawai and Akira, 2011). PRRs involved in the recognition of infecting mycobacteria are found in figure 1.1.

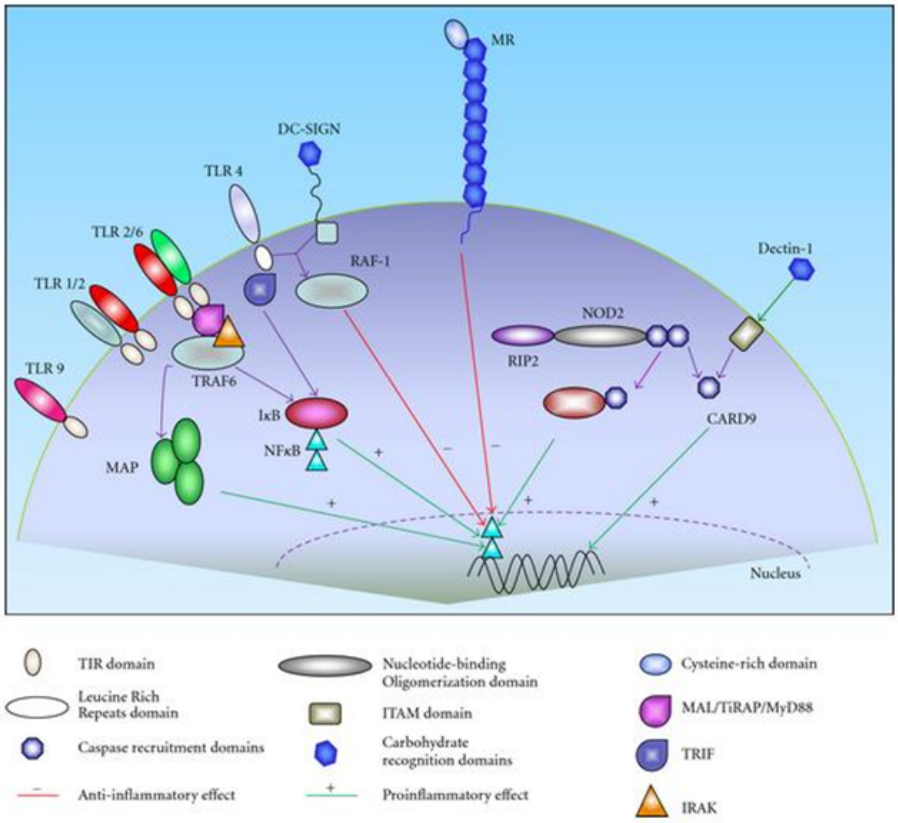


Figure 1.1: Mycobacterial species can be recognized by different pattern recognition receptors (PRRs) situated both extracellularly and intracellularly in the macrophage. The different PRRs interact with different signaling pathways. Common for all the PRRs involved in the recognition of mycobacteria is the resulting activation of the transcription of NF-κB. After transcription, the production of pro- and anti-inflammatory cytokines and chemokines are induced. Figure from Kleinnijenhuis et al., 2011.

1.3.2.1 Membrane bound PRRs

Toll-like receptors (TLRs) are transmembrane receptors recognizing both endosomal and extracellular PAMPs. As a result of interaction with PAMPs, the TLRs will induce the NF-κB signaling pathway, resulting in secretion of pro-inflammatory or type I interferon (reviewed in Kumar *et al*., 2011).

TLRs 1 to 10 are found to be present in humans. TLR1, 2, 4, 5 and 6 are situated in the outer membrane, while TLRs 3, 7, 8 and 9 are found on the surface of endosomes (reviewed in Kawai and Akira, 2011). TLRs 1, 2, 4, 5, 6, 7 and 9 are the central TLRs for recognizing bacterial components, but not all are known to be involved in Mtb or *M. avium* recognition. TLR2 is central in recognizing mycobacterial species in general, recognizing the

mycobacterial component lipoarobinomannan (LAM) (Underhill *et al*., 1999). For *M. avium*, TLR2 has been shown to be most important by investigating mice with TLR2 deficiency, and finding them to have an increased susceptibility to *M. avium* infection (reviewed in Appelberg, 2006). In addition TLR4, TLR8 and TLR9 recognizing unmethylated CpG motifs in the mycobacterial DNA are involved in *M. avium* and *M. tuberculosis* recognition. Also, the heterodimers of TLR2 and TLR1 or TLR6 are thought to play a role in recognition of the glycolipids in the mycobacterial cell wall's triacylated and diacylated lipoproteins, respectively. Several PAMPs are recognized by TLRs, together with the already mentioned LAM, also LM or 19-kD mycobacterial glycoprotein and phosphatidylmyo-inositol mannoisdes (PIM) are recognized (reviewed in Kleinnijenhuis *et al*., 2011 and Kumar *et al*., 2011).

C-type lectin receptors (CLRs) are another type of membrane bound PRR. Dendritic cell-specific intracellular adhesion molecule -3-grabbing nonintegrin (DC-SIGN) is one example, found on macrophages and dendritic cells that will recognize the mycobacteria by mannose-capped lipoarabinomannan (Man-LAM) (Geijtenbeek *et al*., 2000). DC-SIGN has been found to be central in tuberculosis infection. With tuberculosis active in the body, Tailleux *et al*., found that the DC-SIGN is up regulated in macrophages, and that it is these macrophages that are preferably infected by Mtb (Tailleux *et al*., 2005). The mannose receptors are another example found on macrophages and dendritic cells where they recognize mannose units, which will, when recognized, activate endocytosis and phagocytosis of the microbe by complement. Mannose is a recognition motif of several pathogens, such as mycobacteria (Schlesinger, 1993).

1.3.2.2 Cytoplasmic PRRs

The last group of PRRs is the cytoplasmic PRRs. Nod-like receptors (NLRs) are situated in the cytoplasma. NLRs recognize components in all bacterial cell walls, including those of *M. tuberculosis* and *M. avium*. Activation of NLRs results in activation of NF-κB or MAP-kinase, which in turn results in the production of inflammatory cytokines but also activation of the inflammasome (reviewed in Kleinnijenhuis *et al*., 2011). RIG-like receptors (RLRs) are the other main type of cytoplasmic PRRs, but RLRs are not found to be involved in mycobacterial recognition (reviewed in Kumar *et al*., 2011)

1.3.3 Mycobacteria entering the macrophage

After being recognized by macrophages, the bacteria are engulfed and phagocytized. Phagocytosis mainly by macrophages is an important part of the innate response and also has a role in coordinating the succeeding adaptive response, but neutrophils are also seen as professional phagocytic cells, and both are very efficient at internalizing particles. Eliè Metchnikoff was the first to describe phagocytosis and was awarded a Nobel Prize for this in 1908 (reviewed by Mosser and Edwards, 2008). The phagocytic process is initiated with the microbial particles interacting with the receptors on the surface of the macrophage. There are three main ways of phagocytosis mediation. First and most studied are the Fc-receptors. The Fc-γ receptors are the type that is involved in phagocytosis mediation and stimulate polymerization of actin and phagosomes formation. The Fc-receptors are continuously active (Aderem and Underhill, 1999). The next type is the Mannose-receptor mediated phagocytosis. The mannose-receptors are situated on the macrophages and recognize mannose and fucose on pathogen surfaces (Schlesinger, 1993); scavenger receptors also facilitate phagocytosis (reviewed in Crevel *et al*., 2002). Last are the complement receptors that opsonize the bacteria for phagocytosis. Several types of complement receptors (CR), CR1, 3 and 4 among others, are all expressed on macrophages and carry out the phagocytosis. In addition to the PAMP on the bacteria particle, the CR needs stimuli to be activated (Schlesinger, 1993).

The aim of the process of phagocytosis is to kill the bacteria. When the particle or pathogen is internalized by phagocytosis, the F-actin on the phagosomes will be depolymerized so that the phagosomes are available to the early endosomes. The phagosome will now transport the ingested particles through a pathway of membrane bound structures with increased acidity. First the early phagosome is formed by fusion with the early endosome, and then it fuses with the late endosome and, at last, the lysosome, forming the phagolysosome. The antimicrobial compounds made in the phagosome during this process, ensures the degradation of the phagocytized content. Reduced NADPH oxidase complexes make superoxide radicals from molecular oxygen, while nitric oxide synthetase produces NO radicals from molecular arginine (Kinchen and Ravichandran, 2008). Both Mtb and *M. avium* avoid the deleterious environment found in the phagosome by arresting the maturation and inhibiting the phagosomes-lysosome fusion. Mtb resides in the membrane bound vacuole where it resists the lysosmal fusion that normally would neutralize the bacteria. Also, the process of

acidification normally occurring in the phagosomes is greatly reduced, causing only mild acidification, which is also found to be the case for *M. avium* (Aderem and Underhill, 1999). Mycobacteria are also found to disrupt the cytoskeleton of actin microfilament (reviewed in Appelberg, 2006).

IFN-γ (1.4.3), mainly produced by CD4+ Th1 cells (1.4.2), plays an important role in activating macrophages and overcoming the inhibition of the phagosome-lysosome fusion by mycobacteria (Flesch and Kaufmann, 1987). IFN-γ mediated activation of the macrophage produces reactive oxygen and nitrogen species, which causes acidification of phagosomal compartments and promotes fusion of the phagolysosome. Despite being activated by IFN-γ, macrophages are unable to completely destroy the mycobacteria that inhabit the phagosomal compartments. For *M. avium* it has been found that the protective capsule makes it resistant towards lysosomal enzymes (reviewed in Flesch and Kaufmann, 1987 and Frehel *et al*., 1991). Not able to fully destroy the mycobacteria the host mechanisms are able to control the mycobacterial infection to a certain extent and the mycobacteria will enter a state of slow growth or dormancy, thus the host avoids active disease. This balance is kept until the host defense is somehow impaired (Kaufmann, 2001).

Although mycobacteria developed strategies to avoid the anti-microbial actions of the macrophage, they are still dependent on some factors for their survival. Mycobacteria need iron to survive inside the macrophage. Mycobacteria have evolved siderophores, which are molecules able to transfer molecules of iron to the cell wall of the mycobacteria gained from host proteins (Gobin and Horwitz, 1996). In response, the host developed defense mechanisms to compete with the mycobacteria for the iron supply: The protein lipocalin 2 (Lcn2) binds iron with a higher affinity than bacterial siderophores and as a result of those the host starves the bacteria of iron (Flo *et al*., 2004). However, Lcn2 endocytosed by macrophages has been found to localize to different compartments from the mycobacteria and thus has only limited effect on intracellular survival of mycobacterial species (Halaas *et al*., 2010)

1.3.4 Recognition and engulfment by autophagy

In addition to the presented phagocytic path of recognition and engulfment, the process of autophagy can also internalize the bacteria for destruction. Autophagy effectively eliminates intracellular bacteria (Levine and Deretic, 2007). Autophagy is a lysosomal degradation pathway and the process by which the cell can degrade components that are too large for degradation by the proteasome, such as damaged organelles and protein aggregates. The same process is used to degrade infectious agents such as bacteria and viruses when they infect intracellularly. The component marked for degradation is sequestered into an autophagosome. This autophagsome will fuse with a lysosome, which causes degradation of the content. The autophagic process is controlled by the autophagic proteins (Levine *et al*., 2011).

Autophagy has several modes of induction including starvation of the cells, immune signals such as different PAMPs and DAMPs, TLRs and NOD-like receptors, infection in general and cell-surface receptors as Fc-ɣ and reactive oxygen species (ROS). IFN-γ and its effector are also involved in activation of autophagy in macrophages. Microbes and microbial virulence factors, on the other hand, are able to suppress the process of autophagy (Singh *et al*., 2010 and Levine *et al*., 2011).

In host defense against Mtb as intracellular pathogen, autophagy has been found to be an important part of the antimicrobial defense to reduce the viability of the pathogen. For Mtb, IFN-γ induced autophagy causes the formation of a double membrane autophagososme, which can mature into an autophagolysosome resulting in inhibition of Mtb survival (reviewed in Early *et al*., 2011). Gutierrez et al. found autophagy to inhibit survival of the virulent Mtb H37Rv both in human and murine macrophage cell lines, indicating its important role in anti-mycobacterial defense. Thus the survival of Mtb in macrophages may depend on the ability to modulate autophagy (Gutierrez *et al*., 2004). For *M. avium*, the process of autophagy in macrophages is not fully understood, and little work has been done (reviewed in Early *et al*., 2011).

1.3.5 Reactive oxygen species (ROS) signaling and the Keap1-Nrf2 pathway

Reactive oxygen species (ROS) are oxygen containing free radicals such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). ROS are produced by the oxygen metabolism in the electron transport chain occurring in the mitochondria of the cell, or by several different cellular enzymes (Martinon, 2010). ROS signaling plays an important role in cell signaling and physiological processes. In the inflammation and immune response, phagocytes use ROS to create a ROS-dependent respiratory burst, which kills the invaders directly. However, the ROS are also potentially harmful to the cell if not suppressed when unneeded. Both tissue and cells can experience damage caused by oxidative stress. To avoid excessive ROS, several enzymes are expressed. The Kelch-like-ECH-associated protein (Keap1)- nuclear factor (erythroid-derived-2)-like 2 (Nrf2) system is important in controlling expression of genes involved in protection in the process of electrophilic and oxidative stress (Taguchi *et al*., 2011). The Keap1-Nrf2 pathway is found in figure 1.2.

Keap1 functions as a key repressor for Nrf2. Nrf2 is a critical regulating factor when the cells are under oxidative stress or stimulated with different chemo preventative compounds. Nrf2 will then be involved in regulating the cellular defense response. Keap1 negatively regulates Nrf2 by marking it for ubiquitination and subsequent proteasomal degradation (Zhang, 2006). While under stress, Keap1 is modified so its activity is inhibited. Then the Nrf2 is free to bind to cis –acting antioxidant responsive element (ARE), thus driving the transcription of several genes coding for cytoprotective enzymes (Kwak *et al*., 2003). Keap1 is a protein playing a role as an oxidative stress regulator in addition to being a binding partner for p62 and light chain 3 (LC3). It also has a key role when it comes to protecting cells from oxidative damage by cullin 3 (Cul3)**.** Keap1 is found to be recruited to the phagosomes by *M. avium* and play a role in regulation of inflammation, in autophagy and killing of *M. avium* infecting human macrophages (Awuh *et al*., unpublished).

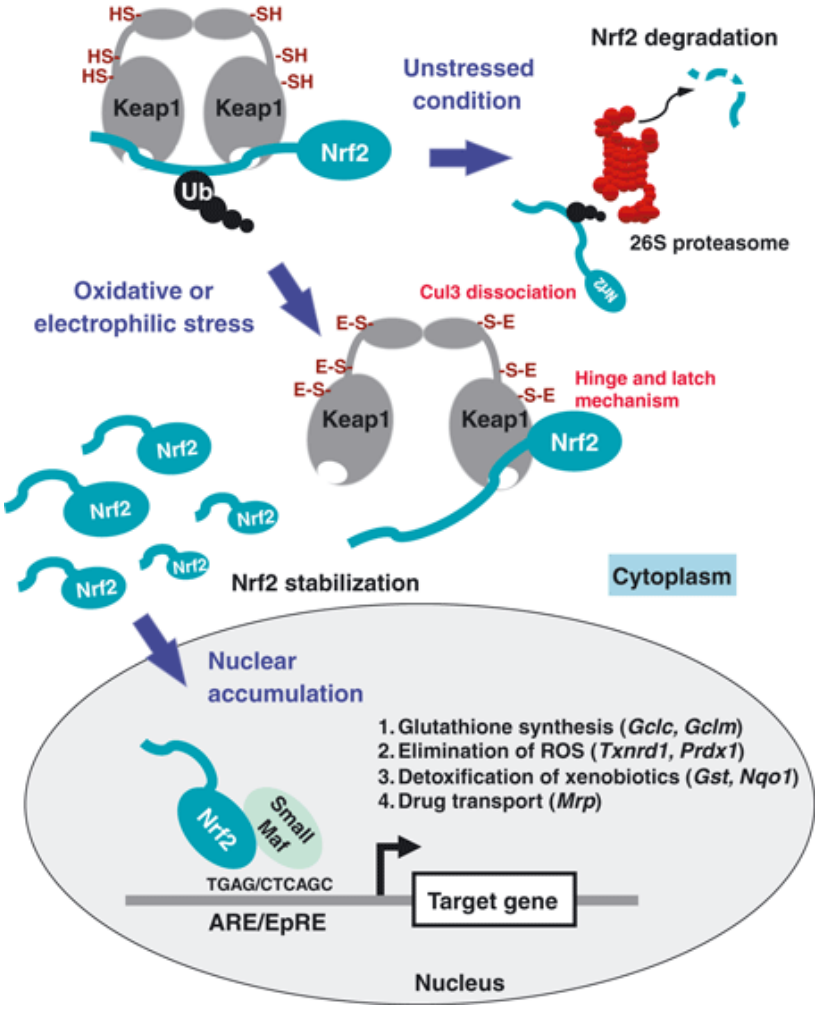


Figure 1.2: The Keap1-Nrf2 pathway. Kelch-like-ECH-associated protein 1 (Keap1) works as a key repressor for Nrf2. Nrf2 is a critical regulation factor when the cells experience oxidative stress or chemo preventative compounds. Keap1 is a cytoplasmic protein. Under unstressed conditions, Keap1 will ensure that Nrf2 is constantly degraded by the ubiquitin-proteasome pathway. Under oxidative or electrophilic stress on the other hand, the Keap1 will be inactivated. This enables Nrf2 to translocate into the nucleus where it activates target genes. Figure from Taguchi et al., 2011.

1.4 Adaptive immunity

The adaptive immune response is important in the defense against mycobacterial infections since innate immunity with macrophages as key players is not able to kill the mycobacteria itself. This is because the mycobacteria have developed several mechanisms that interfere with the antimicrobial defense as described above. The adaptive immune system depends on the innate immune system to become activated and antigen presenting cells such as dendritic cells and macrophages play a central role here. T cells with the CD4+ Th cell subset are

found to be the most central participants in the anti-mycobacterial immunity of the adaptive immune system (reviewed in Shiratsuchi *et al*. 2000).

1.4.1 Ag-presentation and T cell activation

For T cell activation, Major histocompatibility complex (MHC) -proteins, T cell receptors (TCR) and their co-receptors are required. MHC-proteins make up the receptors of antigen presenting cells (APCs), such as dendritic cells and macrophages, that present antigen to T cells (Swain, 1983). APCs can efficiently internalize antigen by phagocytosis or endocytosis followed by display of the fragment on MHC molecules. MHC class I (MHCI) is found on most cell types and presents endogenously derived antigens mainly to cytotoxic CD8+ T cells (Parham and Ohta, 1996; Rodgers and Cook, 2005). MHC class II (MHCII) is restricted to cells that take up foreign antigens and present to helper T cells (Th) and regulatory T cells (T_{reg}), and presents mainly exogenous derived antigens (Hughes and Yeager, 1998). The MHC-proteins will direct the T cells towards their appropriate target. TCRs are situated on the T cells and are what recognizes the antigen presented by the MHC-proteins. The TCR receives help from a complex of invariant transmembrane proteins, named CD3. The MHC/TCR/antigen complex needs further stabilization; this is provided through co-receptors, which bind to an invariant part of the MHC. The whole complex is shown in figure 1.3 (reviewed in Bonilla and Oettgen, 2010).

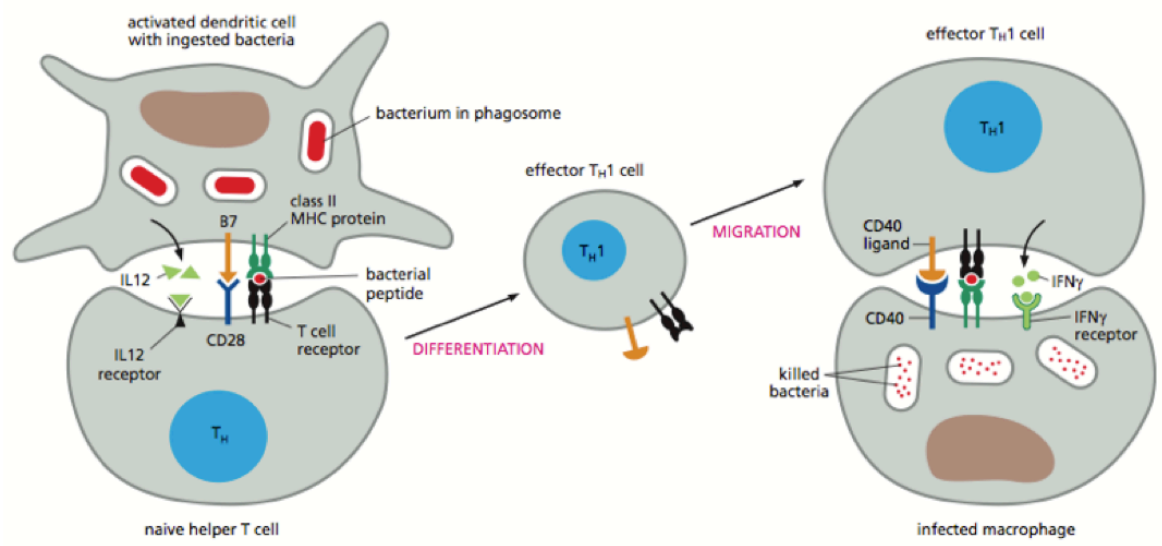


Figure 1.3: Naïve helper T cell becomes activated by interaction with antigen presenting cells (APCs), such as dendritic cells. The APCs ingest bacteria at site of infection and travel back to the peripheral lymphoid organs where they can induce the naïve Th cells to differentiate into Th1 effector cells. The activation is achieved by the interaction of the class II MHC protein –antigen complex with the T cell receptor in addition to co-stimulatory proteins, such as B7 interacting with CD28 on the naïve Th cell and interleukin 12 (IL-12) secreted by the dendritic cell. The differentiating Th1-cell will migrate back to the site of infection, where it activates macrophages by secretion of IFN-γ and expression of the CD40-ligand, binding to the IFN-γ receptor and CD40-receptor on the macrophage surface, respectively. The activated macrophage can kill intracellular bacteria residing in the macrophage's phagosomes. Figure adapted from Alberts et al., 2008.

Looking closer at the activation process; starting with the activation of the dendritic cell, which is initiated when the PRRs on the dendritic cell recognize the pathogen by PAMPs and ingest it together with its products. This initiates transduction pathways such as the NF-kB and Myd88-pathway. Now the dendritic cell will have an increased production of MHC and accessory proteins for T cell activation. The activated dendritic cell will migrate to the lymphoid tissue where it presents antigens to both Th cells through MHCII and cytotoxic T cells through MHCI molecules. In this way the dendritic cells are an important connection between the innate and adaptive immune system. In addition to MHC-proteins, dendritic cells express co-stimulatory proteins, which will bind to other receptors situated on the T cell surface, including B7 proteins that are recognized by the CD28 receptor on the T cell and CD40 receptors, which interact with the CD40-ligand on the T cell (reviewed in Bonilla and Oettgen, 2010). The APC bound Th cell will activate integrin adhesion proteins to increase the binding strength by binding to Ig-like ligand on dendritic cells. This is done to ensure that the T cell – dendritic cell binding is sustained sufficiently for the T cell to be fully activated.

This together with the interaction of the receptors and co-receptors will cause the Th cell to proliferate and differentiate into effector Th cells. The T cells will now be able to stimulate their own proliferation and differentiation process through stimulating cells to produce IL-2. The T cells will at the same time synthesize high affinity receptors to bind the IL-2. When IL-2 binds, an intracellular signaling pathway is activated and the cell is differentiated into effector cells (reviewed in Hoyer *et al*., 2008). The activation of the Th cells also needs a control mechanism. This is ensured by a negative feedback loop. The T cells express CTLA4, which is similar to CD28 and binds to B7 with a higher affinity than CD28 does. The difference is, as CD28 activates the T cells, the CTLA4 inhibits the intracellular signaling that normally would activate the T cell by blocking the binding of CD28 (Krummel and Allison, 1995)

The active Th1 effector cell resulting from the interaction with APC will travel back to the site of infection, where it can activate macrophages by secretion of IFN-γ and expression of the CD40-ligand binding to the IFNγ - and CD40 - receptor on the macrophage surface, respectively (1.3.1). The active macrophage is now able to kill bacteria residing in the phagosomes and increases the expression of CD40, TNF-α while secreting TNF-α. This, in addition to the IFN-γ from Th1 cells, will increase the antimicrobial activity of the macrophages and the fusion of phagosomes with lysosomes. The fusion and the following events are the process by which macrophages kill pathogens. With mycobacteria, however, this is avoided by arresting the maturation and inhibiting phagosomes-lysosome fusion (1.3.1) (reviewed in Appelberg, 2006 and Gengenbacher and Kaufmann, 2012). The importance of CD4+ T cells in the antimicrobial effect against mycobacteria is well established, with depletion in the antimicrobial activity observed with a low number of CD4+ T cells (reviwed in Shiratsuchi *et al*., 2000).

1.4.2 T cells in anti-mycobacterial immunity

In the immune reaction against mycobacterial infections, the T cells play an important role. Mycobacteria activate both CD8+ and CD4+ T cells, with the CD4+ Th1 cells as the most important and the focus of this part (reviewed in Shiratsuchi *et al*. 2000).

The CD8+ cytotoxic T cells' key function is to induce infected target cells to kill themselves by apoptosis, either through perforin dependent or Fas dependent killing, where both result in the target cell undergoing apoptosis due to activation of a caspase cascade. The effector cells, cytotoxic T lymphocytes (CTLs), are activated by the interaction of the TCR with antigens presented on MHCI on an APC together with the co-stimulatory signals provided by the CD28 – B7 interaction and the signal induced by the IL-2 cytokine binding to IL-2 receptors on the cell surface. The involvement of CD8+ T cells in anti-mycobacterial mechanisms are still unclear, but indications that they, in fact, play a role in achieving optimal immunity have been found for Mtb infection (Flynn *et al*., 1992; van Pinxteren *et al*., 2000). MHCI presentation of intracellular mycobacteria may be possible through escape of mycobacteria into the cytosol and subsequent "cross-presentation" via MHCI. The opposite could also be the case, having cytosolic antigens entering through autophagsosmes and MHCII (reviewed in Cresswell *et al*., 2005).

CD4+ T cells can form T helper cell 1 (Th1) and 2 (Th2) (Mosmann *et al*., 1986), T helper cell 17 (Th17) (Weaver *et al*., 2006) and regulatory T cells (T_{reg}) (Sakaguchi *et al*., 1995). The different subsets are shown in figure 1.4. T cell subsets are characterized by their cytokine production. The distinct CD4+ T cell subsets have different functions and which effector cells the activated CD4+ T cells will differentiate into is influenced by the cytokines in the milieu of the naïve CD4+ T cell. An environment with high levels of IL-12 results in differentiation into Th1 cells, while high concentrations of IL-4 promote Th2 cell differentiation (Mosmann *et al*., 1986). Th cells are important in both extracellular and intracellular immune protection. When antigen is presented to the T cells, they become activated and go through the process of proliferation and differentiation. Th cells are considered one of the most important cells in adaptive immunity, being required for almost all adaptive immune responses. Th1 is central in immunity towards intracellular pathogens and involved in the activation of macrophages, cytotoxic T cells and B cells, while Th2 focus on extracellular pathogens by helping activate B cells to make antibodies. Th1 secrete IFN-γ and TNF-α, while Th2 secrete IL-4 and IL-10 (reviewed in Mosmann *et al*., 1986 and Bonilla and Oettgen, 2010).

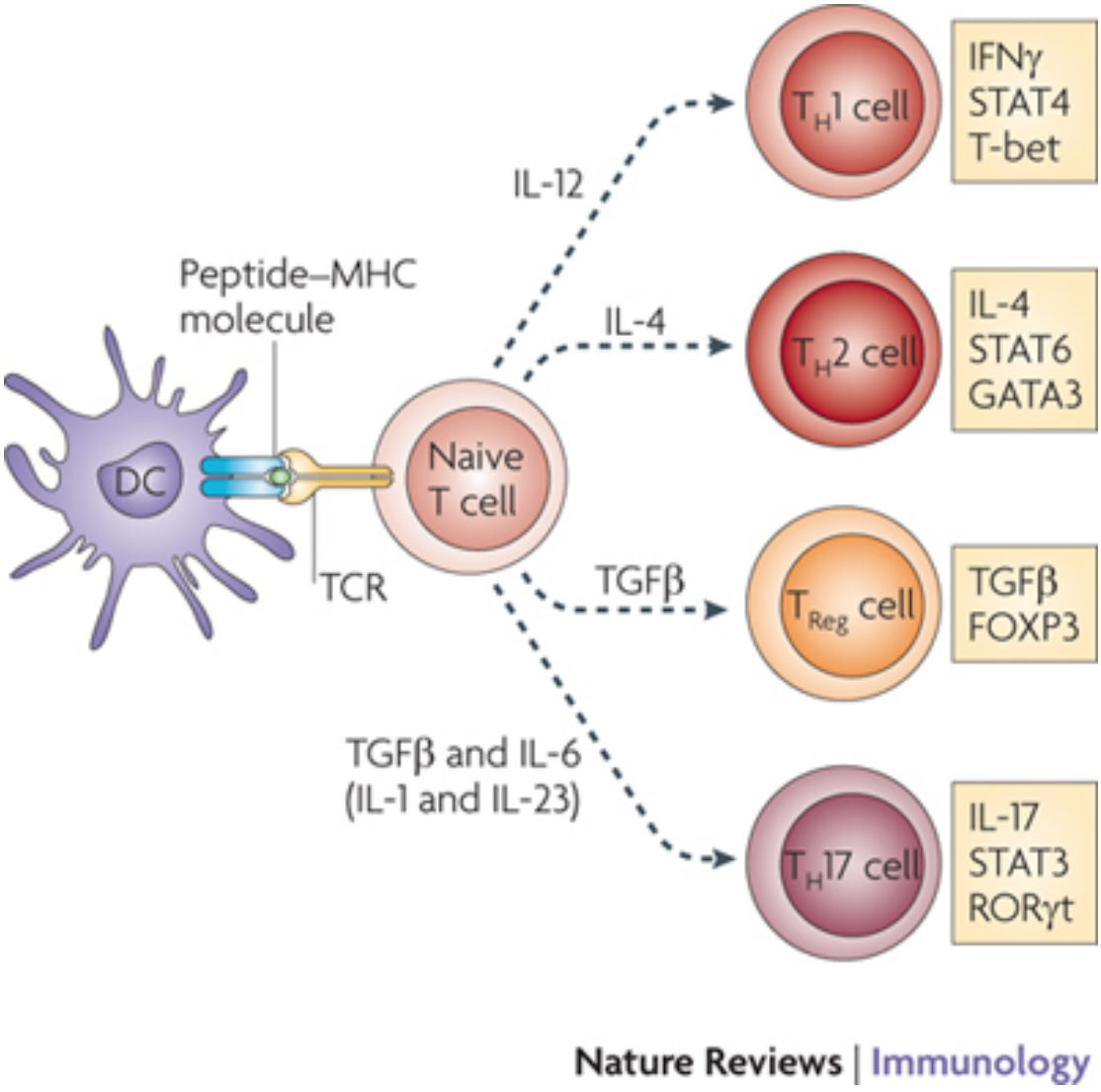


Figure 1.4: When a naïve CD4+ T cell is activated by an antigen presenting cell, such as a dendritic cell (DC), it can differentiate into four different types of T cells: T helper cell 1 (Th1), T helper cell 2 (Th2), regulatory T cells (Treg) and Th17. Cytokines decide which subset that is chosen and each type depends on a specific set of transcription factors to develop. In the presence of interleukin 12 (IL-12), the naïve –cell will differentiate into Th1 cells and in the presence of interleukin 4 (IL-4), Th2. The transforming growth factor - β (TGF-β) causes Treg cell differentiation and when combined with interleukin 6 (IL-6), the naïve T cell will differentiate into Th17 cells. Figure from Zou and Restifo, 2010.

The role of both T_{reg} and Th17 cell subsets in the immune response towards mycobacterial infections are unclear and thus only briefly introduced here, not being the focus of this study. T_{reg} are characterized by the expression of CD4 and CD25 in the outer membrane. The main task of T_{reg} cells is to suppress the activity of the other T cells and dendritic cells, and by that suppress the immune response. T_{reg} cells are thus an important control mechanism. In this way the T_{reg} cells help prevent an excessive T cell response from happening. Induction of T_{reg} cells requires transforming growth factor-β (TGF-β). It is, however, thought that T_{reg} has a role in antimycobacterial defense. T_{reg} cells normally suppress the effective CD4+ T cell anti-

mycobacterial reaction, and by that prevent the extinction of Mtb infection in mouse (Sakaguchi *et al*., 1995; reviewed in Zheng *et al*., 2004).

The last subset, Th17, is characterized by the secretion of interleukin 17 (IL-17). They work as a counterpart to the T_{reg}-cells and also have an important role in autoimmune diseases. Th17 require interleukin 6 (IL-6) and TGF-β for induction. Th17 cells mainly protect against extracellular bacteria and fungi, but also have a role in immunity against intracellular bacteria as Mtb, previously mentioned. Th17 cells inhabiting the lungs are found to produce chemokines inducing the recruitment of antigen specific Th1 cells that produce IFN-γ (Weaver *et al*., 2006; Khader *et al*., 2007).

1.4.3 Cytokines in mycobacterial infection.

Cytokines are low-molecular-weight regulatory proteins and glycoproteins that are important participants in immune function. They are secreted as a response to stimuli by white blood cells in addition to other cells of the body. This ensures communication between the many cells of the immune system. Cytokines are responsible for several important processes and their secretion coordinates the host response to infection, including proliferation, differentiation and the hematopoiesis of cells. In addition, cytokines also ensure recruitment and activation of inflammatory cells. Cytokines act by binding to specific receptors on the membrane of the target cell, and binding triggers subsequent signal transduction pathways (reviewed by Murphy *et al*., 2012). Here we will focus on cytokines in context of mycobacterial infections, in particular cytokines important in the Th1 response, as it is the most central cell subset in the defense against mycobacteria and also our work.

In Mtb infections, interaction with both innate and the adaptive immune cells will result in the production of cytokines. Tumor necrosis factor-α (TNF-α), interleukin-1 family cytokines, interleukin 12 (IL-12) and IFN-γ are considered the most important cyotkines, and lately also interleukin 2 (IL-2), all of which will be elaborated on further. IFN-γ and TNF-α are considered characteristic in protective anti-mycobacterial immunity. Both of these affect the macrophage bactericidal capacity and TNF-α is critical in granuloma formation. IL-12 is important because of its activation of the Th1 response. IFN-γ, TNF and IL-2 can all be secreted by mycobacteria specific Th1 cells. Th1 cells producing IL-2 are considered to have

the potential to proliferate (reviewed in Appelberg, 2006; Berrington and Hawn, 2007 and Cooper *et al*., 2011). If a T cell is capable of producing IFN-γ, TNF and IL-2 simultaneously it is considered a polyfunctional T cell. Activation of polyfunctional T cells are considered beneficial in infections with a number of pathogens and in vaccine development (Seder *et al*., 2008, Lindenstrøm *et al*., 2009). Other cytokines that have an important function in mycobacterial immune defense are IL-1, which as TNF-α is involved in granuloma formation and maintenance, and IL-6 and 10, which cause an increase and decrease in the effector response in target T-cells and macrophages, respectively. IL-22 and 23, both required for the Th1-response, are found to be of particular importance in the response towards intracellular pathogens such as Mtb and *M. avium* (reviewed in Appelberg, 2006; Berrington and Hawn, 2007 and Cooper *et al*., 2011).

1.4.3.1 Interferon-γ (IFN-γ)

IFN-γ is a macrophage activation factor produced by AP- and NK-cells at early points of infection, while at a later stage T cells are responsible for the main secretion (Flesch and Kaufmann, 1987; Garcia *et al*., 1999). It can induce the mononuclear phagocytes to kill or inhibit intracellular growth of mycobacteria (1.3.1). IFN-γ secretion is under the control of the pro-inflammatory cytokines 1L-12 and IL-18, formed by active macrophages causing the T-cells to increase the production. Negative control is ensured by the anti-inflammatory cytokines IL-4, IL-10 and TGF-b (Garcia *et al*., 1999).

The protective role of IFN-γ in Mtb infections is well established. For *M. avium* there are still some questions. Doherty et al. 1997 has shown IFN-γ to be important in *M. avium* infections by looking at mice deficient in IFN-γ expression. They found that gene knockout, severe combined immunodeficiency (scid) mice, together with animals having disruption in the IFN-gamma gene, had enhanced bacterial growth at 8 weeks past infection, compared to controls with no targeted disruption in the IFN-γ gene (Doherty and Sher *et al*., 1997). Toba et al. found that IFN-γ treatment of monocytes decreased the phagocytosis of *M. avium*, but did not have an effect on the intracellular replication (Toba *et al*., 1989). Carvalho de Sousa and Rastogi found that IFN-γ alone did not cause any antimicrobial action against *M. avium* in either monocytes or macrophages, which was the case with Mtb. They found that pretreatment with IFN-γ reduced the uptake of both mycobacteria species, but did not find the

same effect in macrophages, indicating that IFN-γ has to be presented at the right time during differentiation, before the monocytes turn into macrophages. These findings support Toba et al., in that *M. avium* seemed to be harder to control than Mtb in general. Carvalho de Sousa and Rastoga detected a ten-fold higher growth of *M. avium* was observed under the same conditions (Carvalho de Sousa and Rastoga, 1992). In addition, the effect of IFN-γ in macrophage infection seems to be dependent on variables such as which macrophages are infected and the strain morphology (Bermudez et al., 1991).

1.4.3.2 Tumor necrosis factor- alpha (TNF-α)

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine found to be up regulated early in a mycobacterial infection. TNF-α is mainly produced by macrophages but also other cells, as the lymphoid cells contribute. When TNF-α production is increased at a location, it will induce an inflammatory response. When a macrophage becomes activated, it will increase its expression of the TNF-α receptor together with its secretion of TNF-α, and activated T-cells also produce TNF-α. TNF-α has an important role in control and containment of the infectious bacteria by development and persistence of the characteristic structure of mycobacterial infections, the granuloma, as well as induction of chemokines (reviewed in Berrington and Hawn, 2007). The well-established effect of increased anti-mycobacterial activity in Mtb has also been detected in both MAC infected murine and macrophage cells (Bermudez *et al*., 1991).

1.4.3.3 Interleukin-2 (IL-2)

Interleukin 2 (IL-2) is found to have an important role in T cell growth, proliferation and differentiation. It is also a good marker of specific T cells. IL-2 is produced by active T cells, when an antigen binds to the TCR on the T cell surface (reviewed in Hoyer *et al*., 2008). An active T cell also increases expression of IL-2 receptors, and by this ensures further proliferation. T cells producing IL-2 together with IFN-γ and TNF are, as mentioned, considered polyfunctional and beneficial in infections with a number of pathogens including mycobacterial species (Seder *et al*., 2008, Lindenstrøm *et al*., 2009).

1.4.3.4 Interleukin-12 (IL-12)

The interleukin 12 (IL-12) cytokine is produced by macrophages and is a stimulating agent for the Th1 mediated response. Also, IL-12 is one of the major cytokines in macrophage activation. Thus, IL-12 is not a T cell cytokine but needed for the Th1 cells to develop. It is important both in the innate and adaptive immune response. IL-12 is thought to increase the resistance against tuberculosis by its role as a stimulating agent for Th1 cells, in addition to attracting macrophages to the site of infection by inducing the production of chemokines (reviewed in Appelberg, 2006 and Murphy *et al*., 2012). IL-12 is vital for CD4+ T-cells to develop and produce IFN-γ and is found to be important in not only the immune reaction against Mtb but also *M. avium*. If IL-12 is neutralized, a worsening of the *M. avium* infection is observed. Deficiency in the IL-12 gene makes mice susceptible to infection and IFN-γ response is found to be radically reduced (reviewed in Appelberg, 2006). Genetic susceptibility studies in humans have further revealed that defects in interleukin (IL)-12 increase susceptibility to mycobacterial infections (Altare *et al*., 1998)

1.5 Survival of mycobacteria - the life inside the macrophage

As reviewed (1.3.1), the mycobacterial species have developed several strategies for survival in cells while the immune system simultaneously attempts to counteract these measures. The survival of mycobacteria are thus dependent on several factors, both those provided by the bacteria itself to help survival, and the measures taken by the host to prevent infection and survival by the bacteria. Several factors have been identified during the last few years, but there are still mechanisms left to understand. The understanding of *M. avium* survival can reveal important targets for drugs and vaccines.

Inhibiting the host anti-mycobacterial action, the mycobacteria can enter a dormant state in the macrophage. The immune reaction in general is able to sustain the infection at a dormant state, but the host is not able to eradicate the infection, thus a large percentage (approximatly 90%) of those infected are known to carry Mtb in a latent state, breaking out only with severe immunosuppression (WHO, 20112). This containment of the infection is due to the immune processes described here. The T cells are very important, with polyfunctional Th1 cells making the central IFN-γ, TNF and IL-2 cytokines.

Th1 is important, but not sufficient for protection. The protective immunity depends on several mechanisms working together correctly, and this is still unknown for the different mycobacterial species and *M. avium* in particular. CD8+ CTLs, Th17 and antibody mediated immunity with B cells might also play a role together with innate immune responses such as PRR signaling and starvation of iron-supply by Lcn2, as well as the mentioned ROS and autophagic processes.

Our focus is still the activation of polyfunctional Th1 cells and the effect on mycobacterial survival, as well as the effect of Keap1, a protein found to be involved in ROS-signaling, inflammation and cytokine production in the macrophages in addition to the autophagy process.

2. AIM AND OBJECTIVES OF STUDY

The overall aim of the project was to establish *in vitro* procedures for expansion of mycobacteria specific CD4+ Th1 cells and generation of autologous macrophages from human peripheral blood mononuclear cells (PBMCs) from healthy donors. The expanded mycobacterial-specific Th1 cells and the autologous macrophages should then be used in an antigen-specific *in vitro* setting. Figure 2.1 illustrates the flow of the method and its applications.

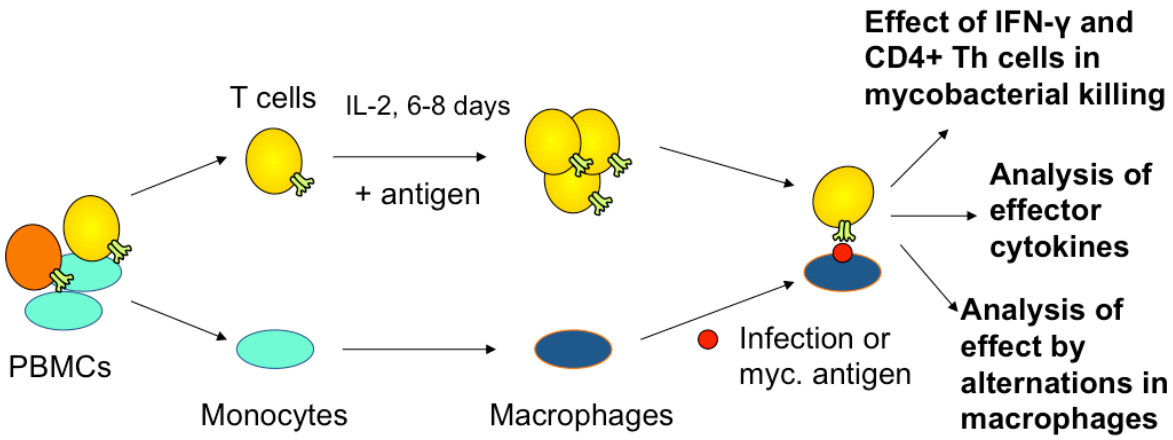


Figure 2.1: Isolated PBMCs from healthy human donors are stimulated for 6-8 days with mycobacterial antigen and the cytokine IL-2. Mycobacteria-specific memory T cells will be activated and start to proliferate due to the presence of IL-2 and the presentation of mycobacterial antigens from APCs in the PBMC culture. In parallel, autologous macrophages are generated from monocytes by incubation in 30% human serum medium. Monocytes were enriched from PBMCs by plastic adherence. On day 6-8, CD4+ T cells are purified from the T cell expansion culture, the macrophage culture will be infected with mycobacteria or stimulated with antigen. Purified CD4+ T-cells and the infected/stimulated macrophages will be co-cultured. From this method several readouts are possible, as macrophages will act as antigen-presenting cells and influence CD4+ T cell activation, while activated CD4+ T cells will also have an effect on macrophages and affect survival of mycobacteria inside the macrophages.

After establishment, this method should then be used to study multiple aspects of CD4+ Th1 cell functions in anti-mycobacterial immunity:

- *Hypothesis 1:*

  Polyfunctionality of CD4+ Th1 cells as measured by simultaneous production of several effector cytokines might play an important role in anti-mycobacterial immunity (1.4.3). However, there is still little knowledge about the functions of these cells in mycobacterial

infections in general and *M. avium* in particular. We hypothesize that polyfunctional *M. avium*-specific CD4+ T cells can be expanded from healthy human donor PBMCs. We wanted to establish a flow-cytometric protocol to detect several CD4+ effector-cytokine parameters (IFN-γ, TNF, IL-2) simultaneously after expansion, in order to analyze quantity and polyfunctionality of the CD4+ T cell response against *M. avium* in a human antigen-specific setting.

- *Hypothesis 2:*

  Some work has been done for Mtb showing an effect of CD4+ T cells and IFN-γ on activation of macrophages and intra-macrophage survival of Mtb in in vitro assays in a human system. It is not clear if these human in vitro assays also work with *M. avium* as mycobacterial agents. We hypothesize that *M. avium*-specific CD4+ Th1 cells can induce the activation of macrophages and subsequent killing of *M. avium* in the macrophages; T cell mediated killing might be promoted by IFN-γ and maybe also the CD40 ligand interaction (1.4.1). Our macrophage-T cell assay illustrated in Figure Y should be modified to enable us to test the effect of IFN-γ and *M. avium*-specific CD4+ T cells on killing of *M. avium* in macrophages.

- *Hypothesis 3:*

  Innate and adaptive immunity cooperate in combating infection. Triggering inflammatory signals on macrophages may have an effect on the antigen-presentation, the expression of co-stimulatory molecules and, as a consequence of that, on the T cell activation (1.4.1). We found that the Kelch-like ECH-associated protein (Keap1) has a role in the regulation of inflammation and autophagy response to *M. avium* infection (Awuh *et al*., unpublished). We hypothesize therefore that Keap1 can have an effect on the mycobacteria-specific CD4+ T cell activation. This should be tested by knocking down Keap1 in macrophages. The macrophages with and without Keap1 knockdown should be infected with *M. avium* and used as APCs to activate *M. avium*-specific CD4+ Th1 cells. T cell activation can then be detected by flow-cytometric analysis of effector cytokine production as for hypothesis 1.

1.Flow cytometric protocol for staining several effector-cytokine parameters (IFN-γ, TNF, IL-2) simultaneously after expansion, identifying polyfunctional T cells

2. Effect of Th1 cells, IFN-γ and maybe also the CD40 ligand (1.4.1) on activation of macrophages and mycobacterial killing.

3. Effect of an alternation in the macrophages by knockdown of the Keap1 protein on antigen presentation to CD4+ T-cells.

3. MATERIALS AND METHODS

In this project PBMCs are isolated and applied for different analysis, including macrophage and CD4+ T cell generation. The cell subsets are further used in several experimental setups investigating their role in anti-mycobacterial immunity towards *M. avium* infections. The main methods used are multicolor flow- cytometry and luciferase activity assay. Here we present the methodological overview of our work.

3.1 Applied reagents and kits

The kits and reagents with supplier used in this assignment are listed in table 3.1.

Table 3.1: Kits and reagents used in experiment listed with supplier.

Reagents	**Manufacturer**
Lymphoprep ™	Axis Shield PoC
Dulbecco's phosphate buffered saline (PBS)	SIGMA life science
Hanks balanced salt solution	SIGMA life science
RPMI 1640	SIGMA life science
Lysisbuffer	Promega
Luciferase assay substrate	Promega
Recombinant Human IFN-γ	R&D Systems
TaqMan® Gene Expression Assays	Applied Biosystems
TaqMan® Universal Mastermix	Applied Biosystems
Lipofectamine RNAiMAX transfection reagent	Invitrogen
Kit	**Manufaturer**
CD4+ isolation kit Dynal® CD4 Positive Isolation Kit	Invitrogen
CD4+ T Cell Isolation Kit	Miltenyi Biotec GmbH
High Capacity RNA-to-cDNA kit	Applied Biosystems
RNeasy Mini kit	Qiagen

3.2 Study participants

15-30 ml heparinized blood samples from healthy donors were obtained. Informed consent was given by all donors.

3.3 Peripheral blood mononuclear cell (PBMC) isolation

Blood samples were acquired according to guidelines at our institute and informed consent was obtained from all blood donors. Mononuclear cells were isolated from heparin-blood. Heparin blood is used because of heparins anti-coagulaing effect, preventing clotting of the blood for further experiments. Before separating the cells, the blood sample is diluted with phosphate buffered saline (PBS) to give a maximum yield. Based on the fact that mononuclear cells have a lower density than the erythrocytes, the cells were separated by centrifuging (1800 rpm, 20 minutes) the heparin blood layered on the density gradient media Lymphoprep ^{TM} designed for isolation of white blood cells. A gradient will form after of the centrifugation, with the heavy erythrocytes in the bottom and the low-density plasma on top. At the interface between these two, a ring of the white blood cells of interest will be formed. This cell layer is taken out with a sterile pippette, and washed in several steps (1. 800 rpm, 8 minutes, 2. 1400 rpm, 6 minutes) using Hanks balanced salt solution with 2% fetal calf serum (FCS). The cells were then resuspended in 10%A+/RPMI medium with10mM HEPES and 2mM L-glutamine before the cells were counted. HEPES is added to ensure effective buffering in a physiological range, while L-glutamine is an amino acid supplement (Crawfor and Cohen, 1985).

The cells can now be counted, first 10 µl sample were mixed with 10 µl trypan blue. This is transferred to non-gridded disposable Countess^{TM}-chamber slide and counted using Countess^{TM}-automated cell counter (Invitrogen^{TM}). Entering the chamber slide in the Countess^{TM} an image of the sample is visualized. By manual adjustments a clear image is visible, where live cells appear colorless and the dead cells as dark spots. The image based counter separates the live cells from the dead cells based on dye exclusion using 0.4% trypan blue dye, which only dead cells take up in their cytoplasm, while live cells exclude it because of its intact cell membrane (Life Technologies Cooperation, 2013). The counted cells are used in further experiments (3.5 and 3.6). For a complete protocol for PBMC isolation and cell count see Appendix I.

3.4 Cultivation and preparation of live and heat-killed *M. avium*

For growth inhibition assays, live *M. avium* was needed, while heat-killed *M. avium* was used for expansion of *M. avium* specific CD4+ T cells. All procedures were carried out according to standard operating procedure (SOP) in our laboratory for work with *M. avium*. In all experiments the *M. avium* strain 104 (fully sequenced) pMH109 C was used. This*M. avium* strain is stably transfected with pmH109C, which contains the mycobacterial optimal promoter (MOP). The pMH109C contains MOP and acr, a 16-kDa α-crystallin homolog, driving firefly luciferase and lacZ respectively. Clone C was obtained from Gerald Cangelosis laboratory, Seattle Biomedical Research Institute.

Aliquots of *M. avium* strain 104 pMH109 C were kept in -80°C bacteria-medium containing 10% glycerol. For each experiment new aliquots were used and prepared 4-5 days before usage. The aliquots were thawed quickly in warm water to avoid crystal formation in the cells and sonicated for 5 seconds to resuspend the cells. 250 µl bacteria aliquot was added to 7 ml Middlebrook 7H9 media supplemented with 0.05% Tween 80, albumin dextrose catalase (ADC) and glycerol in a 50 mL conical tube. The culture was grown at 37°C on a shaker incubator for 4-5 days.

On the day of the experiment, 1 mL of bacteria culture was added to each of three 1.5 mL tubes (screw cap), spun down and washed twice with 1 mL PBS, this was done to remove tween. One tube was put on a heating block set at 70°C for 30 minutes to generate heat-killed *M. avium*, while the second was kept at room-temperature and used for live *M. avium* infection purposes. The total volume of the last tube was added to a cuvette for optical density (OD) measurements. The OD of the culture was determined by a spectrophotometer (600nm). At 600 nm, OD= 1 represents $4.5x10^8$ bacteria per mL and based on this, the number of bacteria was determined. Bacteria culture was only used with OD value between 0.3 and 0.6 indicating that the bacterial growth is in log phase. When the OD was determined, the live and heat-killed bacteria culture was used for subsequent experiments with different multiplicity of infection (MOI).

3.5 Preparation of monocyte derived macrophages (MDMs)

Isolated PBMCs (3.3) were diluted with RPMI 10% A+ to a concentration of 2 x 10^6 cells/mL. The cell suspension was then plated on flat–bottomed 96-well plates, 100 µl per well and incubated for 1 hour at 37°C with 5% CO_2. The monocytes will now be the first cell component to adhere. After 1 hour of incubation, the cells were washed with pre-warmed Hanks solution to remove non-adherent cells before 100 µl of RPMI 30% A+ was added to each well. The cells were incubated for 5-7 days at 37°C with 5% CO_2. This was done to let the monocytes differentiate into macrophages. For a complete protocol for MDM preparation, see Appendix I.

3.6. Expansion of *M. avium*-specific CD4+ Th1 cells

Isolated PBMCs (3.3) were plated on a 24-well plate (3x10^6 PBMCs per well) and stimulated with heat-killed *M. avium* (3.4) at a MOI of 5:1. The cells were incubated at 37°C with 5% CO_2. On day 2, 20/mlU IL-2 were added to each well, being a important growth factor of T cells, and in inducing proliferation of the cells. After 6-8 days CD4+ T cells were isolated using in the beginning CD4+ isolation kit Dynal® CD4 Positive Isolation Kit- Invitrogen. Having problems with the kit's release agent not releasing all CD4+ T cells, the CD4+ T cell Isolation Kit from Miltenyi Biotec GmbH was used for the rest of the experiments. For both kits, the procedure were carried out according to manufacturers' protocol.

The isolated CD4+ T cells were then re-stimulated in most experiments for one more round with freshly prepared autologous monocytes prepared from PBMCs isolated (3.3) on the day of the CD4+ isolation from blood sample from the same donor. Monocytes for re-stimulation were prepared by dilution of the isolated cells as described in 3.5 and infected with heat-killed M. avium at a MOI of 5:1. 1-3 million CD4+ T-cells were then added to each well in 500 µl RPMI 10% A+. As for the first stimulation, the cells were incubated at 37°C with 5% CO_2. On day 2, 20/U IL-2 was added. After another 6-8 days the expanded mycobacteria-specific T cells were used for a T cell assay with in-vitro MDMs generated from PBMCs at the day of the CD4+ T cell restimulation (3.5). The CD4+ T cells were counted as described in 3.3 before use. For complete protocol for expanded *M. avium*-specific CD4+ Th1 cells preparation see Appendix I.

3.7 Growth inhibition assay

The ability of IFN-γ and heat-killed *M. avium* expanded CD4+ Th1 cells to inhibit the growth of live *M. avium* in MDMs was determined using a luciferase assay in addition to plating with CFU as control.

3.7.1 Experiment setup

For growth inhibition assay, MDMs (3.5), expanded *M. avium*-specific CD4+ Th1 cells (3.6) and IFN-γ (R&D Systems) were used. On day 5 of differentiation for the MDMs, the media was removed from the wells and the same amount of fresh media applied. Separate wells of MDMs were treated with the indicated concentration of IFN-γ or number of expanded *M. avium*-specific CD4+ Th1 cells both in in 100 µl 10% A+ /RPMI medium or left untreated as control (then added 100 µl 10% A+ /RPMI medium). All samples were incubated for 2 more days at 37°C with 5% CO_2. After 2 days of incubation the same wells were infected with live *M. avium* (3.4) at the desired MOI. After infection, the cells were incubated for 4 hours at 37°C with 5% CO_2 before non-adherent cells and excess bacteria were removed and fresh 100 µl 10% A+ /RPMI medium added. The cells were incubated again at 37°C with 5% CO_2. Luciferase activity was analyzed at timepoint 24 and 72 hours (3.7.3) post infection, combined with plating for CFU as control (3.7.4).

3.7.2 Luciferase activity assay principle

The luciferase assay system is based on the light that is produced with the conversion of chemical energy occurring by luceferin oxidation, resulting in the product, oxyluciferin, as shown in figure 3.1. Firefly luciferase catalyzes the steps from luciferin to oxyluceferin by the use of ATP and Mg2+ as a co-substrate. The process produces light with a wavelength of 560 nm (Luciferase Assay System, Promega).

The light intensity made in the reaction measure the rate of which the luciferase catalyzes the process. The luciferase enzyme is most effective at room temperature and must be equilibrated to this before being used, which is also required for the sample to be measured.

The emitted light of the reaction is quantified using a luminometer. The intensity measured by the luminometer should be proportional to the luciferase concentration. Thus, using Luciferase + bacteria, the luciferase concentration should be proportional to the number of live bacteria in our sample. The luminometer measures the intensity in relative light units (RLU) (Luciferase Assay System, Promega).

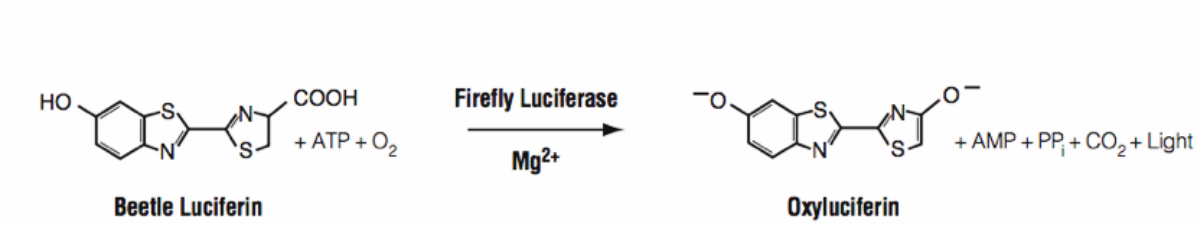


Figure 3.1: Bioluminescent reaction catalyzed by firefly luciferase.Firefly luciferase catalyze the steps from luciferin to oxyluceferin by the use of ATP. Figure from Luciferase Assay System, Promega.

3.7.3 Assay procedure

The cells from 3.7.1 were analyzed by the luciferase assay at different time-points. The non-adherent cells and excessive bacteria were removed from the adherent infected macrophages in the wells. The adherent infected macrophages were then washed twice with pre-warmed Hanks solution. 200 µl lysisbuffer (4:1 in sterile water, Promega) was added to each well by pipetting up and down 2-3 times. Then the cells were left to lyse for 5-10 minutes. 50 µl of the lysate for each sample was transferred in duplicates or triplicates to a luciferase assay plate (OptiPlex 96 well plate), in addition 10 µl were transferred to dilution plates for CFU control (3.7.4). Then 50 µl of firefly luciferase assay substrate (Promega) was added to each well before measuring luciferase activity within 10 minutes. The measurements were performed by a Victor 1420 Multilabel Counter.

3.7.4 Colony forming units (CFU)

Colony forming units (CFU) were used as control for the luciferase assay. 10 µl lysate were serially diluted 4 times in 90 µl PBS with 0.05 % Tween. Each dilution was plated in duplicates on Middlebrook 7H10 agar plates and incubated in polyethylene plastic bags at

37°C for until colonies were visible (1-2 weeks). Colonies was counted and used as control of luciferase assay result (3.7.3).

3.7.5 Processing of results from luciferase assay

Microsoft Excel (2011) and GraphPad Prism software version 5 (Software MacKiev, GraphPad) were used to analyze the data and for graphical display.

3.8 Flow cytometry

Expanded *M. avium* specific CD4+ Th1 cells were analyzed by multicolor flow-cytometry to detect presence of antigen-specific effector T cells by their cytokine production, and also detect and characterize polyfunctional cells producing IFN-γ, TNF and IL-2. At last, the multicolor flow-cytometry was used to compare expanded *M. avium* specific CD4+ Th1 cells experiencing MDMs with Keap1 knockdown compared to control MDMs without Keap1 knockdown.

3.8.1 Flow cytometry principle

Flow cytometry is the measurement of physical and chemical characteristics of cells and other particles**.** The flow cytometer consists of several components: the fluidics system, where the component of which the cells are injected into the sheath-fluid stream is found**.** The cells or particles are sent passed a light beam with one wavelength in a laminar flow. Within the flow cytometer hydrodynamic focusing produced a single stream of particles passing the laser beam, and each particle will be detected at certain angles. A schematic overview of a flow cytometer setup is presented in figure 3.2. The forward scatter (FSC) and sideward scatter (SSC) detector measure cell size and granularity respectively, while the fluorescence is measured by FL-1 to 4. This happens when cells marked with a fluorochrome pass the laser beam. The light scatter from the fluorochrome is measured, and the light is sent to the photo multiplier tube (PMT). The system of mirrors and filters will only allow certain wavelengths of lights to pass; the light that is allowed to pass ends up in the detection channels FL-1 to 4. The collected light energy of the measurements will be converted to

electrical energy, which in turn is converted to digital information. This is conducted by the electronics of the flow cytometer. Lastly, the data from the cytometer is collected on a computer for interpretation by the user (Rahman, 2006).

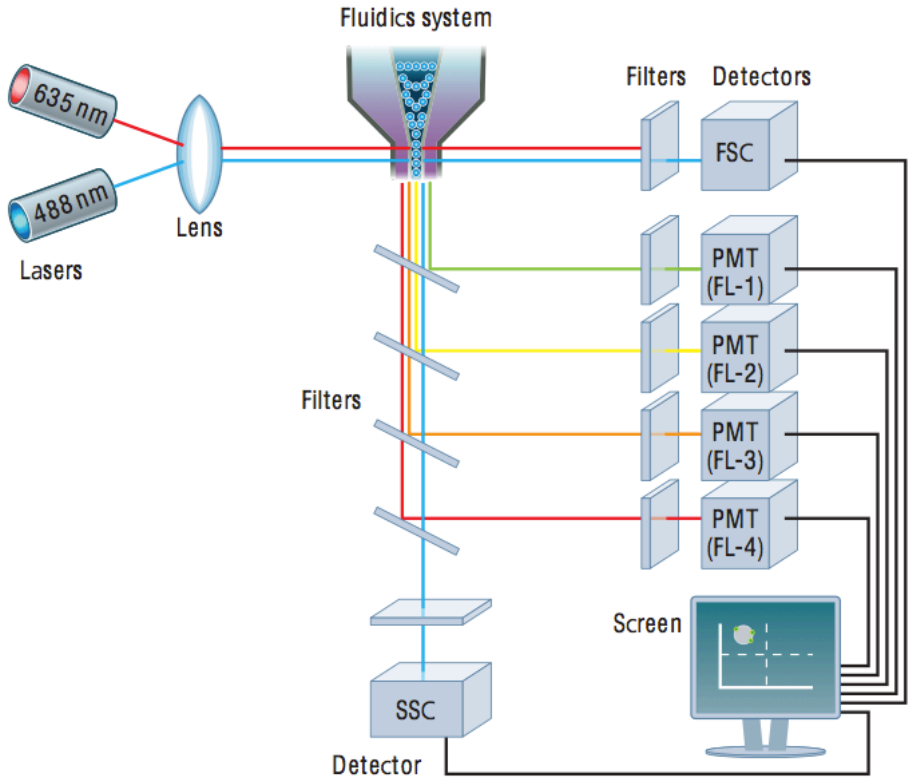


Figure 3.2: Schematic overview of a flow cytometer setup. The forward scatter (FSC) and sidward scatter (SSC) detector measure cell size and granularity respectively. The fluorescence is measured In FL-1 to 4. Mirrors and filters ensure that only certain wavelengths of light pass through to the different channels. The collected data are then transferred to a computer for user interpretation. Figure from Rahman, 2006.

When performing flow-cytometry, there is a possibility of spectral overlap. This may be avoided by using fluorochromes emitting in different ends of the spectrum, this is however often not possible. Fluorescence compensation is a method to work around the spectral overlaps. When applied during data analysis the %-interference of a fluorochrome in other channels than target channel will be determined and the "spillover" can be corrected for (Rahman, 2006). In our experiment compensation beads (BD Bioscience) stained with fluorescent antibodies were used for compensation in multicolor flow-cytometry analysis.

3.8.2 Procedure for extra- and intracellular staining for flow-cytometry analysis

Before the cells could be stained for flow cytometric analysis, a protein transport inhibitor cocktail (500x eBioscience) is used to prevent cytokine release form the cells, which is important for the later, intracellular staining (3.8.2.2). 4 µl of 10:1 diluted protein transport inhibitor cocktail were added 4 hours pre staining.

3.8.2.1 Extracellular staining

Cells were transferred from wells to flow tubes containing 500 µl PBS with 2 % FCS and 0.05% NaN_3 (wash buffer, NaN_3 ensures killing of *M.avium*) and the tubes were washed at 1500 rpm for 6 min. The supernatant were discarded before surface antibodies were added (1 µl/test) and tubes vortexed. The tubes were incubated on ice for 15 minutes, protected from light. After staining the tubes were washed once with 1 mL wash buffer before being fixated by resuspending cells in 500 µl PBS with 2% paraformaldehyde (PFA). This fixation procedure preserve the cells in a fixed state by terminating the cells biochemical reactions. The cells were then incubated for 20 minutes at room temperature protected from light before they washed twice in 500 µl wash buffer at 1500 rpm for 6 min. The fixed cells were either stored over night in 500 µl wash buffer, or intracellular staining (3.8.2.2) was completed the same day. For macrophage analysis, extracellular staining was followed by flow cytometric analysis (3.8.4). For complete procedure for intracellular staining see Appendix II.

3.8.2.2 Intracellular staining

The cells were washed twice in in 500 µl wash buffer at 1500 rpm for 6 min. Subsequently the cells were resuspended in 500 µl with 0.5% saponin and 1 % FCS and incubated for 5 min at room temperature. This is to permabilize the cells, to be able to stain the cells contained intracellular cytokines. Tubes were then centrifuged at 1500 rpm for 6 min, supernatant discarded and intracellular antibodies or isotypes added (1 µl/test). The cells were then incubated at room temperature for 30 minutes protected from light. After incubation the tubes were washed twice and resuspended in 500 µl wash buffer followed by flow cytometric analysis (3.8.3). For complete procedure see Appendix II.

3.8.3 Analysis of expanded *M. avium* specific CD4+ Th cells

Multicolor flow-cytometry was used to characterize polyfunctional T cell responses to mycobacterial antigens. To identify the CD4+ T cell subset, the samples were stained extracellularly with anti-human CD4 (FITC, eBioscience) and Brilliant violet 785™ anti-human CD3 (Biolegend). To analyze effector function by cytokine production, the cells were intracellularly stained with IFN-γ (PE, Biolegend), IL-2 (APC, eBioscience) and TNF-α (eFluor450, eBioscience). The cells were gated as follows:

1. Lymphocytes were identified and gated based on FSC and SSC properties
2. T cells in general were identified and gated based on CD3
3. From the CD3 positive population, CD4 positive cells were identified and gated
4. The CD3+CD4+ cells were then analyzed for IFN-γ, IL-2 and TNF-α by Boolean gating technique (3.8.3.1)

3.8.3.1 Boolean gating

Boolean gating is a gating technique that allows identification of all possible combinations of cytokine producing CD3+CD4+ T cells (FlowJo software, 3.8.5). With the measurement of three cytokines (IFN-γ, IL-2 and TNF-α) there are seven different types of effector CD3+CD4+ T cells. Triple producers (IFN-γ+IL-2+TNF-α+), double producers (1.IFN-γ+IL-2 +TNF-α-, 2. IFN-γ+IL-2 -TNF-α+, 3. IFN-γ-IL-2 +TNF-α+) and single producers (1.IFN-γ+IL-2 -TNF-α-, 2. IFN-γ-IL-2 -TNF-α+, 3. IFN-γ-IL-2 +TNF-α-) were so identified. For the complete protocol for Boolean gating, see Appendix III.

3.8.4 Analysis of macrophages with and without Keap1 knockdown

Multicolor flow-cytometry was used to characterize macrophages with and without Keap1 knockdown (3.9.1). The macrophages were dissociated from the well bottom using cell-dissociation buffer (Sigma), 100 µl was added per well and left for 10 minutes in room temperature. The cells were then stained according to extracellular staining procedure (3.8.2.1). The following antibodies were used: CD14 (PE, BD Bioscience), HLA-DR (APC), CD11b (eFluor 710, clone ICRFF44), CD80 FITC, and live/dead stain (Fixable Viability Dye

eFluor® 780, eBioscience) . The macrophages were identified and gated by CD14 and CD11b expression. The CD14+CD11b expressing cells were then gated for live/dead stain to identify the viability of macrophages (figure 4.7 b) in the different samples. For characterization of the macrophage, cells were gated for CD14 and CD11b expression. The CD14+CD11b expressing cells were then gated for HLA-DR and CD80 (figure 4.7 c).

3.8.5 Processing of results from Flow cytometry

FlowJo software 3.8.5 was used to analyze flow-cytometric data. Microsoft Excel (2011) and GraphPad Prism software version 5 (Software MacKiev, GraphPad) were used to further analyze the data and for graphical display.

3.9 siRNA transfection of MDMs and knockdown analysis by q-PCR

3.9.1 siRNA transfection of MDMs and co-culturing with heat-killed *M. avium* expanded CD4+ Th1

Human MDMs were cultured in wells of flat-bottomed 96-well plates (3.5). The process of transfection was performed according to manufacturers' protocol with Lipofectamine RNAiMAX transfection reagent (Invitrogen) at day 5 and day 7 of culture. The cells transfected with non-target control siRNA (siNTC) were used as control. AllStar Negative Control (Qiagen) was used as siNTC, while Hs_Keap1_pool HP Validate siRNA (Qiagen) was used for targeting Keap1 mRNA. On day, 8 the media of the cells was changed and the cells were incubated for 1 more hour before infection with live *M. avium* (3.4) at the indicated MOI. Infected cells were incubated for 4 hours at 37°C with 5% CO_2. The non-adherent cells and excess bacteria were removed from the adherent infected macrophages in the wells. Heat-killed *M. avium* and expanded CD4+ Th1 cells were then added, approximately 400 000 cells per well. The MDM-CD4+ T cell co-culture were kept over night and analyzed by flow-cytometry the next day as described in 3.8.3.

3.9.2 Freezing samples for quantitative real time-PCR (qPCR)

Human MDMs (3.5) were transfected as described in 3.9.1. Three wells with non-target control and another three with Keap1 siRNA. The media of the transfected MDMs were removed and 150 µl RLT buffer + 2 mercaptoethanol were added to each well and left for 5 minutes before the three wells with the same transfection were added to one cryotube and stored in -80°C until usage (3.9.3)

3.9.3 RNA extraction and quantitative real time-PCR (qPCR) for mRNA level assessment.

The gene knockdown of Keap1 was determined by quantitative real time-PCR (qPCR). QIAcube robot (Qiagen) in combination with RNeasy Mini kits including DNAse digestion (Qiagen) to eliminate DNA contamination were used to extract RNA from the cells. RNA concentration and quality was measured on a NanoDrop ND-1000 (Thermo Scientific) spectrophotometer at 260 nm. Revers transcription generating complementary DNA (cDNA) was performed according to provided protocol by the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). The cDNA is generated by the enzyme reverse transcriptase together with DNA polymerase and nucleotides. The C1000 Thermal Cycler (BioRad) was used to ensure optimal through the generation process (25°C for 10°C minutes, 37°C for 120 minutes, 85°C for 5 minutes and hold at 4°C).

The qPCR was performed by the StepOnePlus TM Realtime PCR System, using TaqMan® Gene Expression Assays, and TaqMan® Universal Mastermix (Applied Biosystems). Each sample was run in duplicates with 20 µl sample volume in each sample well. Housekeeping gene GAPDH were used as endogenous control. The samples were processed using the quantitative ΔΔCT program at the following settings: 50ºC for 2 minutes, 95 ºC for 20 seconds and 40 cycles of 95 ºC for 1 second and 60 ºC for 20 seconds. Gene expression of the target sample is normalized to the endogenous control gene and to a control (calibrator) to determine the samples relative quantification (RQ).

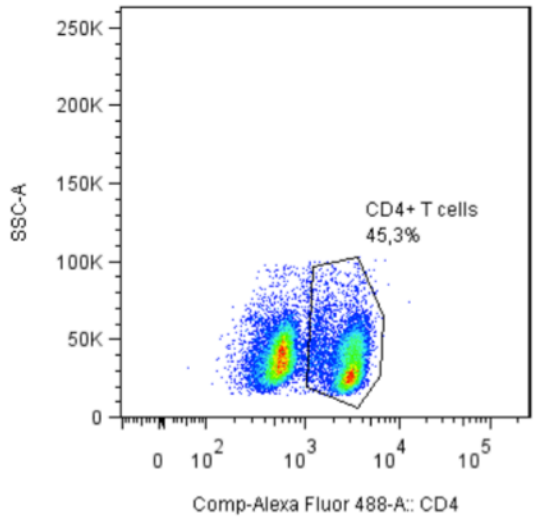
4. RESULTS

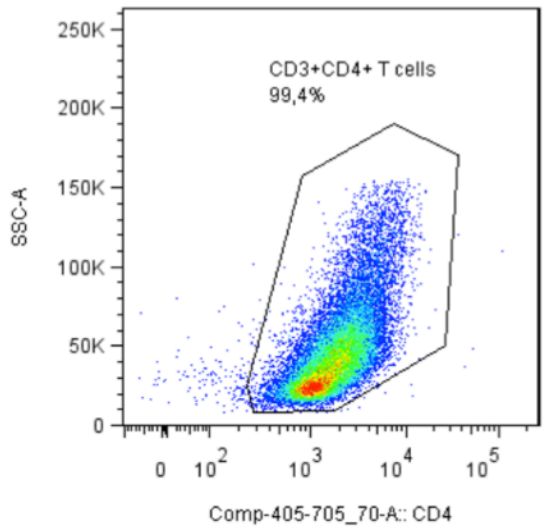
An *in vitro* assay for analyzing the interaction between monocyte derived macrophages (MDMs) and mycobacteria specific expanded CD4+ Th1-cells was established (figure 2.1). Others have found that polyfunctional T-cells may play an important role in anti-mycobacterial immunity (Seder et al., 2008; Lindestrøm et al., 2009). We established a flow cytometric protocol for the staining of several effector-cytokine parameters simultaneously, enabling the identification of this polyfunctional CD4+ Th1 cell subset. These cells were further used in the subsequent analysis (hypotheses 2 and 3). The established assay was then used to study the interplay between participants of the immune system, such as the known macrophage activator IFN-γ and Th1 cells also playing an important role in macrophage activation. Finally, the assay was used to look at the interaction between macrophages and T cells when the macrophages underwent alterations in terms of Keap1 knockdown.

4.1 *M. avium*-specific CD4+ Th1 cells can be expanded from healthy human donors in vitro and are polyfunctional (produce several effector cytokines simultaneously) (Hypothesis 1).

We isolated CD4+ T cells using a CD4+ isolation kit, first Dynal® CD4 Positive Isolation Kit- Invitrogen. However, this kit included a release agent that seemingly only released the cells partially from the beads. We changed to use the CD4+ T Cell Isolation Kit from Miltenyi Biotec GmbH, which seemed to work well. The isolated CD4+ T cells were then used in the bacterial-survival assay. Figure 4.1 (a) illustrates the flow cytometric characterization of CD4+ T cells, with and without CD4+ T cell isolation. Figure 4.1 (b) illustrates the different percentage of CD4+ T cells present with and without CD4+ isolation. Without CD4+ T cell isolation, 56.3% of the cell population is found to be CD4+ T cells, while after isolation, 98,4% is CD4+ T-cells.

a)





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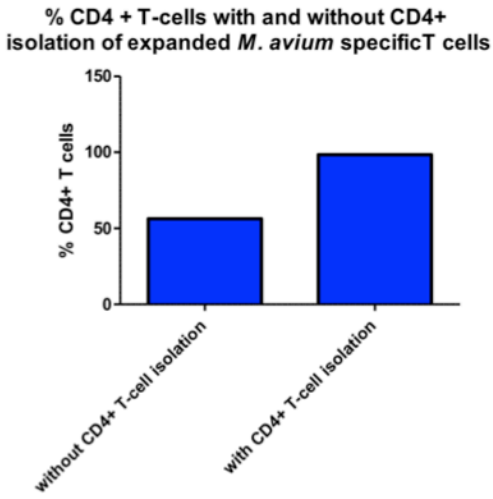


Figure 4.1: a) Flow-cytometric characterization of CD4+ T cells. The percentage of CD4+ T cells before and after CD4+ isolation with CD4+ T Cell Isolation Kit from Miltenyi Biotec GmbH. The CD4+ T-cells are identified based on the surface markers CD3 found on T cells in general and CD4 specific for the CD4+ T-cells. b) Percentage of CD4+ T cells with and without CD4+ isolation with CD4+ T Cell Isolation Kit from Miltenyi Biotec GmbH.

We established a flow cytometric protocol for staining several effector-cytokine parameters (IFN-γ, TNF, IL-2) simultaneously after expansion, identifying the polyfunctional CD4+ Th1 cell subset. The CD4+ Th1 cells obtained by expansion of PBMCs from 6-8 days infection with heat killed *M. avium* at MOI 5:1 and isolation of CD4+ T cells (4.1.4.1) were characterized by this flow cytometric

protocol. We were able to expand 20-30% specific T cells, whereas in fresh cells the percentage is only about 0.5 % (data not shown, also see master thesis Gunn Broli). Figure 4.2 illustrates the characterization of the CD4+ Th1 cells expanded from healthy human donors in vitro by flow cytometry, and indicates that we have been able to expand CD4+ Th1 cells and that there are T cells present producing IFN-γ, TNF, and IL-2. The expanded T cells produce more cytokines than the not expanded CD4+ T cells (approximately 0-3%).

This characterized polyfunctional CD4+ Th1 expanded cell subset was used in cell survival experiments (hypothesis 2) and antigen-presentation studies with Keap1 (hypothesis 3)

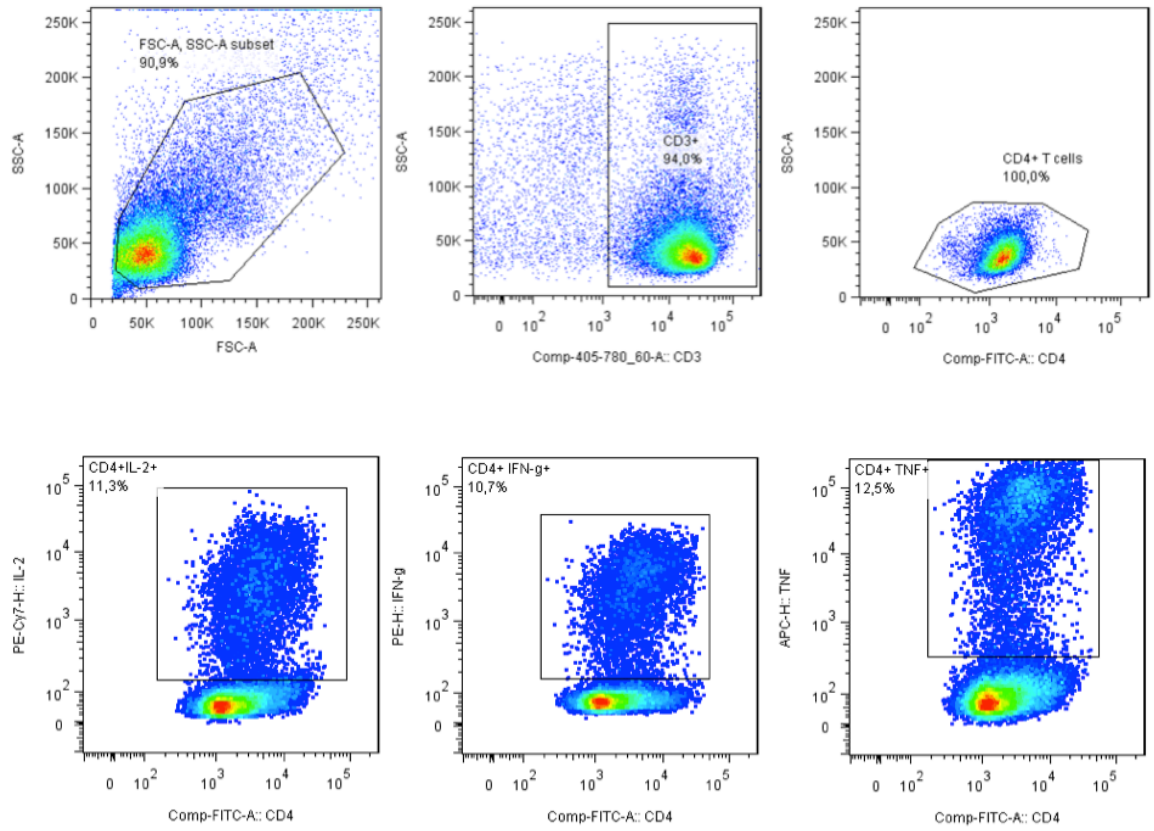


Figure 4.2: Flow-cytometric characterization of CD4+ T cells expanded by heat killed M. avium. PBMCs were stimulated for 6-8 days with heat-killed M. avium (MOI 5:1) to induce the expansion of specific T cells. CD4+ T cells were isolated using CD4+ T Cell Isolation Kit from Miltenyi Biotec GmbH. The isolated cells were stained extracellularly for CD4 and CD3 markers; this is to identify the CD4+ T cells. Following subsequent fixation and permeabilization, cells were intracellularly stained for three effector cytokines, IFN-γ, TNF and IL-2 for analyzing the CD4+ T cells cytokine production. The extracellular and intracellular staining procedure followed the protocol in (3.8.2). Cells were gated for CD3+CD4+ T cells, then CD4+ IL2+, CD4+IFN-γ + and CD4+ TNF+.

4.2 Influence of human mycobacteria-specific CD4+ Th1 cells and IFN-γ on growth and survival of *M. avium* inside of autologous macrophages (hypothesis 2).

Cytokines are important participants in the immune function and are secreted as a response to stimuli by white blood cells in addition to other cells of the body. IFN-γ is a macrophage activation factor produced by AP- and NK-cells at early points of infection while at a later stage T cells are responsible for the main secretion. It can induce the macrophages to kill or inhibit intracellular growth of mycobacteria. IFN-γ is the most central cytokine concerning mycobacterial infections. IFN-γ has been found to hasten the replication in MDM for *M. tuberculosis* in vitro; in vivo however, the cytokine is an important mediator of mycobacterial control. IFN-γ is also thought to induce autophagy (reviewed by Vogt and Nathan, 2011). Vogt and Nathan found the timing of cytokine addition had a clear effect on the outcome, with addition either at time of differentiation or activation. Also, different results were achieved for different donors indicating IFN-γ is donor-dependent.

Macrophages play an important role in innate immunity. In most cases, they are capable of ingestion and destruction of invading microorganisms. However, some types of microorganisms are able to counteract the anti-microbial measures of macrophages. The causative agents of mycobacterial infections such as Mtb and *M. avium* both resist destruction post macrophage ingestion. When the innate immune system and the macrophages are unable to destroy the pathogen, the adaptive immune system is put into action. Antigen specific T cells can activate the macrophage to kill the microorganism. Several cell subsets play a role in adaptive immunity. However, the CD4+ Th1 cells are found to be the most important cells in activating the macrophages to destroy intracellular infections by fusion of phagosomes with lysosomes and induction of nitric oxide and superoxide radicals (reviewed in Shiratsuchi *et al*., 2000).

In our set-up, macrophages were cultivated and infected with live *M. avium*. An assay to measure bacterial load and growth in the macrophages was established and used to look at factors thought to have an effect on bacterial growth. The first factor investigated was IFN-γ, known as a macrophage activator and thus a positive control. Further, the same was done for *M. avium* specific T cells differentiated from the same donor as the macrophages of the assay. We used human primary cells, thus the assay had the advantage of being close to the nature

of what we wanted to look at, *M. avium* infections in humans, compared to what a model organism as mouse or cell lines could tell us.

4.2.1 Luciferase-assay with firefly transfected M. avium 104 as a measure of mycobacterial growth.

We tested the growth of freshly prepared bacteria from frozen aliquots kept in culture for 5 days against bacteria kept in culture for more than one week for luciferase count, RLU. The results display a clear advantage of using bacteria freshly prepared from the freezer, as bacteria seem able to lose luciferase activity during culture. Therefore all experiments were done using a fresh aliquot of bacteria from -80°C and cultured to log phase growth for 4-5 days (250 µl bacteria aliquot + 7 ml culture medium). Results are shown in figure 4.3, with significantly higher counts for bacteria fresh from -80°C cultured in bacteria-medium containing 10% glycerol, with an average RLU value of 22865 compared to the culture kept for more than one week giving an average RLU value of 272. This indicates that Luc+ *M. avium* may lose its luciferase-activity when cultivated for one week or longer. Therefore it was decided to use bacteria prepared fresh from the freezer followed by a maximum of up to 5 days in culture medium for experiments measuring luciferase-activity of *M. avium* from infected macrophages.

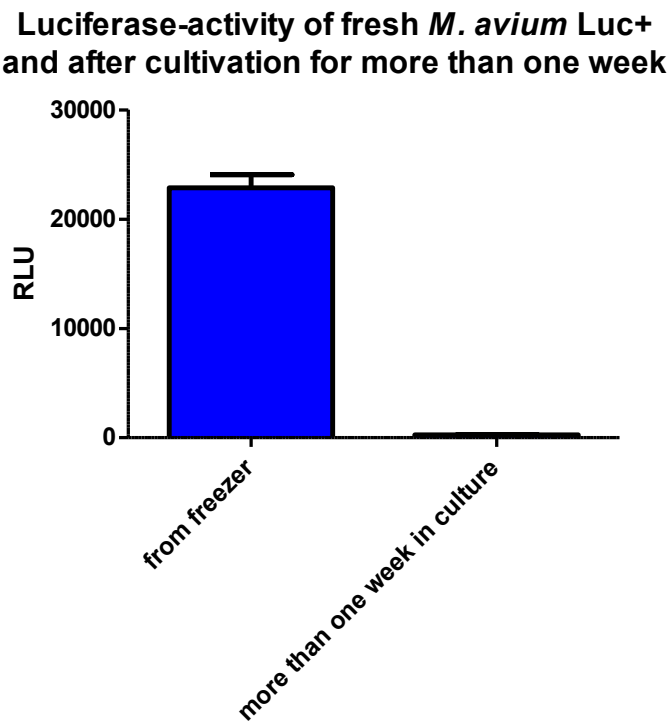


Figure 4.3: Luciferase-activity of M. avium Luc+ kept in culture for 5 days and after cultivation for more than one week. The lysate from fresh aliquot of bacteria from -80 and cultured to log phase growth for 2-3 days (250 µ bacteria aliquot + 7 ml culture medium) and compare to bacteria kept more than one week in culture. 50 µl luciferase substrate + 50 µl bacteria lysate is measured as the amount of emitted light (RLU) by a luminometer. RLU should be directly proportional to the number of bacteria. . Values are based on the mean RLU of 4 replicates with standard deviation.

The luciferase assays reliability was tested comparing bacterial counts from lysate of *M. avium* infected macrophages measured by the luminometer in RLU and plating for CFU. Serial dilutions of the luciferase lysates in PBS with 0.05% tween were plated on agar-plates in triplicates and incubated for three weeks. The relative value for CFU and RLU are both presented in figure 4.4. The trend shows that there is a good correlation between results obtained with the CFU based bacteria-counting method and results from measuring bacterial activity with the luciferase. The values are compared using the relative increase from 24 hours to 72 hours for RLU and CFU respectively.

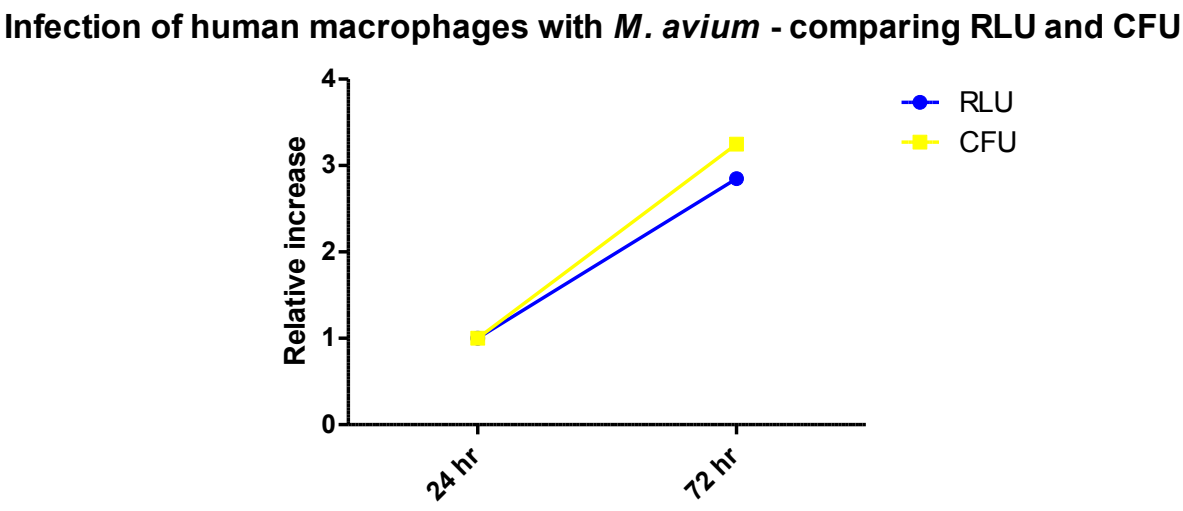


Figure 4.4: Infection of human macrophages with M. avium Luc+ comparing RLU from luciferase assay with colony forming units (CFU). Bacteria count in human macrophages infected with Luc+ M. avium for 4 hours was measured at time point 24 hr and 72 hr. The bacteria growth in the macrophages is measured as the amount of emitted light (RLU) by a luminometer. RLU is directly proportional to the count of bacteria. In addition, serial dilutions of the same luciferase lysates were plated on agar-plates in triplicates and incubated for three weeks at 37°C. The plated bacteria were counted and the actual bacteria count set. The CFU and RLU values show the relative values for the 72 hours timepoint compared to 24 hours. RLU values are based on the mean RLU of 4 replicates with standard deviation, the CFU values are based on mean value of triplicates with standard deviation.

4.2.2 Establishing an in-vitro assay to analyze the IFN-γ and mycobacteria-specific CD4+ T cell effect on *M. avium* survival in human macrophage.

Mtb has been the main target for earlier work concerning survival and interactions of the different factors of the immune system. It is expected to be an easier target to destroy compared to *M. avium*, which is mirrored in the treatment regime for *M. avium* compared to Mtb with longer treatment period and stronger antibiotics. This is supported by Caravalho and Rastogi. *M. avium* seemed to be harder to control than Mtb in general, as a ten-fold higher growth of *M. avium* was observed under the same conditions (Caravalho and Rastogi, 1992.).

In initial experiments testing the effect of IFN-γ and antigen-expanded CD4+T cells on mycobacterial growth in infected MDM we added IFN-γ and the antigen expanded CD4+T cells 4 hours post-infection of MDMs. These conditions were found to have an effect on T

cell-mediated mycobacterial killing in a similar set-up using Mtb (Petruciolli et al, 2011). However, using *M. avium* instead of Mtb, we found that the effect of both IFN-γ and the T cells on mycobacterial survival in macrophages was absent or very variable when added 4h post infection (data not shown). Finding in literature that there are strain differences in terms of IFN-γ effect on *M. avium* (Shiratsuchi et al., 1991) and that the effect of IFN-γ on survival of Mtb in macrophages may be dependent on being added at the right time as early as when macrophages are in differentiation (Vogt and Nathan, 2011), we decided to add IFN-γ 2 days pre-infection. Based on this, the assay using antigen-expanded CD4+T cells was conducted in the same way, adding the T cells 2 days prior to infection with *M. avium*. Performing the assay in this way with pre-treatment of MDMs with either IFN-γ (4.2.3) or *M-avium*-specific CD4+ T cells (4.2.4), we found both IFN-γ and mycobacteria specific CD4+ T cells to have an effect on intra-macrophage survival of *M. avium* (figure 4.5).

4.2.3 Pre-activation of MDMs with IFN-γ inhibits intracellular *M. avium*.

In order to determine the ability of IFN-γ to inhibit the growth of live *M. avium* bacteria by activation of the macrophages, the MDMs were given IFN-γ after 5 days of differentiation and kept until infection with live *M. avium* 2 days post experiencing IFN-γ. Infected MDMs without IFN-γ were used as a positive control.

To find dose and multitude of infection (MOI) for optimal IFN-γ function, we tested two different concentrations: 400U/mL and 1000U/mL, and four different MOI, 0.1:1, 1:1, 5:1 and 10:1. We were able to reduce the bacterial count using a high dose of IFN-gamma (1000U/mL) while a low dose (400U/mL) exposed little or no effect. For both MOI 5:1 and 10:1, a reduction with IFN-γ was detected, with some donor difference at 5:1 so we proceeded with an MOI of 10:1. MOI 1:1 and 0.1:1 had little or no effect (results not shown).

The decrease of intracellular bacteria was measured as decrease in intensity of light (RLU) in a luciferase assay, method described in 3.7.3. The emitted light value, RLU, is directly proportional to the number of bacteria. The results for two repeats are presented in the diagram of figure 4.5 (b). Each dataset represents mean RLU and standard deviation from four replicate wells. To ensure the credibility of our method, sample lysates were plated. The

correlation between colony forming units (CFU) and RLU are shown as relative values in figure 4.5 (c).

4.2.4 *M. avium* specific CD4+ T cells inhibit intracellular *M. avium* growth in MDMs.

CD4+ Th1 cells are assumed to be the most important cells in activating the macrophages to destroy intracellular infections. Th1 cells activate the macrophages by secretion of IFN-γ and expression of the CD40-ligand binding to the IFNγ - and CD40 - receptor on the macrophage surface respectively. The activation by the Th1-cell helps the macrophage to combat intracellular infections that it is not able to destroy itself (1.4.1). Knowing this, we wanted to establish a system to analyze the effect of specific CD4+ Th1 cells on *M. avium* strain 104 growth in autologous MDMs, which has previously not been shown in a human antigen-specific system for *M. avium*.

PBMCs were stimulated for 6-8 days with heat-killed *M. avium* to induce the expansion of specific T cells as described in 4.1. Petruccioli et al. did a similar expansion of T cells and co-culturing with macrophages looking at autophagy and Mtb infection. However they did not isolate CD4+ T cells. This is a drawback as the number of CD4+ T cells added to the macrophages is unknown (Petruccioli *et al*., 2011).

We isolated CD4+ T cells from the PBMC culture stimulated with heat-killed *M. avium* and IL-2 as described in 4.1. This allowed us to ensure that we added pure CD4+ T cells to the macrophages (other cell-types such as CD8+ T cells in the PBMC culture could also have an effect) and that we know the exact number of added CD4+ T cells (CD4+ T cell frequencies in the expanded T cell cultures are variable).

In addition to purification of CD4+ T cells we have characterized the T cells with flow cytometry to detect if there are antigen specific T cells present. This was analyzed by the production of the effector-cytokine from purified CD4+ T cells in response to overnight stimulation with infected macrophages, as described in 4.1. Presence of mycobacteria-specific T cells was ensured in this way as a control in all survival experiments, and a representative sample is shown in figure 4.5(a). To determine antigen-specificity of the CD4+ T cells, purified expanded CD4+ T cells were co-cultured overnight with MDM, both

infected (MOI 10:1) and uninfected cells were characterized with flow cytometry, illustrated in figure 4.5 (a)). Specific T-cells recognized by expression of CD3 and CD4 together with IFN-γ, TNF and/or IL-2, here shown with TNF and IFN-γ as an example. For unstimulated T cells, 0.055% produced IFN-γ and TNF, for stimulated with live *M. avium*, 14. 4%.

Figure 4.5 shows the effect of antigen-specific CD4+ Th1 cells on *M. avium* growth in comparison to the effect of IFN-γ analyzed in two separate experiments with identical set-up. *M. avium* growth was analyzed with both RLU and CFU-count methods (4.2.1). From day 5 post isolation, MDMs were co-cultured with CD4+ T-cells purified from the PBMC culture stimulated with heat-killed *M. avium* + IL-2 (T cell: Macrophage ratio 5:1) or IFN-γ (1000 U/ml). A third fraction of the MDMs remained untreated. On day 7, macrophages were infected with live luciferase-positive *M. avium* (MDM: *M. avium* MOI 10:1, 4 hours). At 24 and 72 hours post *M. avium* luc+ infection, bacterial growth was analyzed by luciferase-activity measurement in untreated MDMs as well as the MDMs pre-treated with T cells (5:1) and IFN-γ (1000U). Results of the luciferase assay are shown in fig. 4.5 b).

Enhanced killing of live *M. avium* by the MDMs was observed when MDMs were co-cultured with expanded *M. avium* specific CD4+ T-cells compared to infected MDMs without addition of specific T cells, shown in figure 4.5 b). The decreased number of bacteria was detected by measuring the decrease in intensity of light (RLU) in a luciferase assay, method described in 4.2.1. To ensure the credibility of our method, sample lysates were plated. The correlation between colony forming units (CFU) and RLU is shown as relative values in figure 4.5 c). CFU analysis indicated slightly lower values, but overall the same trend is seen comparing the increase/decrease in RLU and CFU between the different stimuli, IFN-γ, and *M.avium* specific CD4+ T cells.

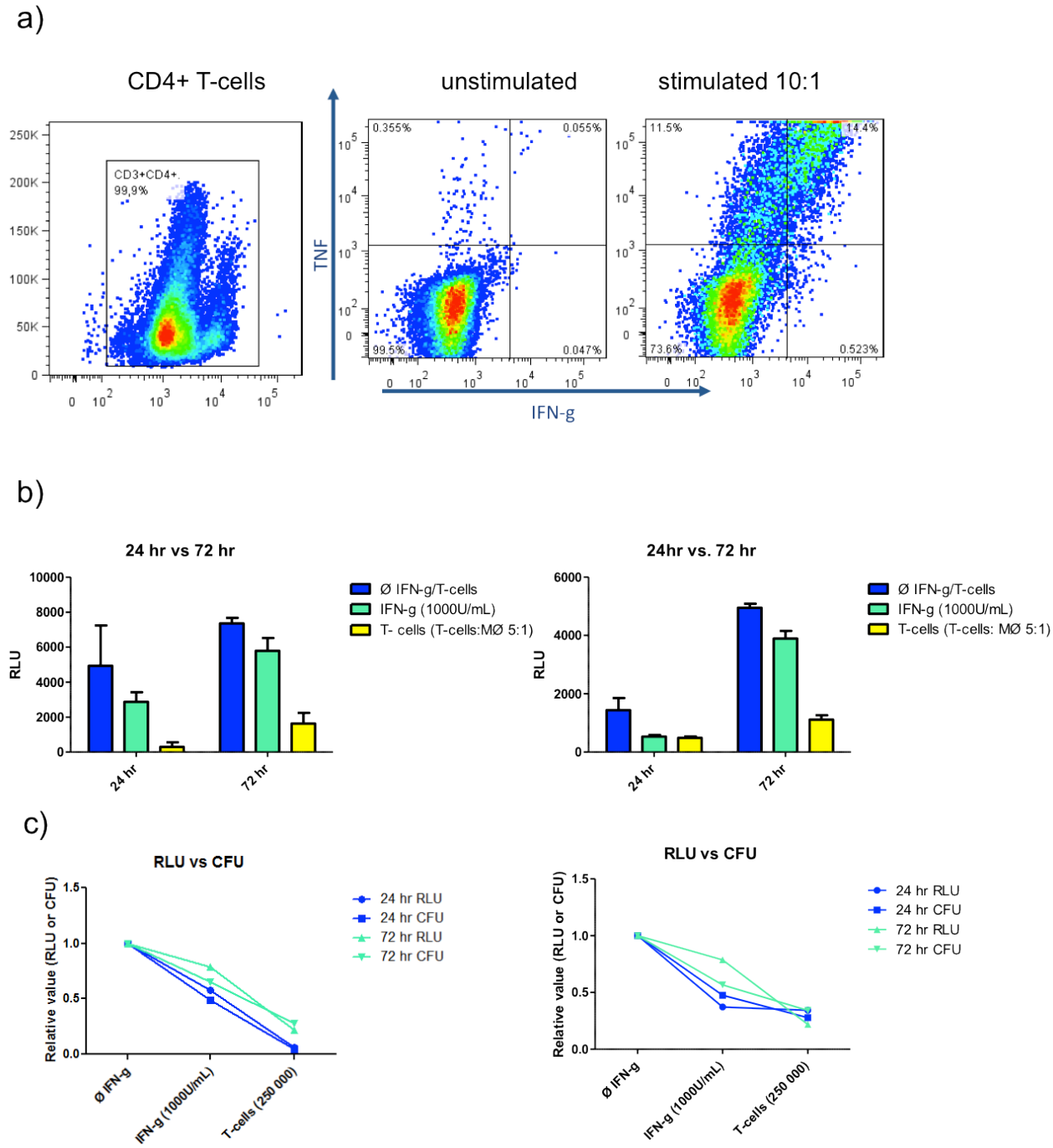


Figure 4.5: a) Flow-cytometry detection of specific CD4+ T cells characterized by increased cytokine production. CD4+T cells identified by the CD3 and CD4 surface-marker. CD4+ T cells were analyzed for the cytokine production comparing unstimulated CD4+ T cells and CD4+ T cells stimulated with heat-killed M. avium in a MOI of 10:1. The samples were analyzed for TNF, IL-2 and IFN-γ, here illustrated by TNF and IFN-γ, though a similar increase is observed in IL-2. b) Growth inhibition by IFN-γ and heat-killed expanded CD4+ T cells. MDMs were treated with IFN-γ 2 days pre-infection. Another set of wells containing MDMs were co-cultured with heat-killed M. avium expanded CD4+ isolated T cells 2 days pre-infection in the ratio T cells: MDMs 5:1. Both sets were infected with live M. avium MOI 10:1.The inhibition of growth in the macrophages is measured as the amount of emitted light (RLU) by a lumniometer. RLU is directly proportional to the count of bacteria. The bacterial count was measured at timepoint 24 hours and 72 hours. Values are based on the mean RLU of 4 replicates with standard deviation. MDMs infected with live M. avium at MOI 10:1 without IFN-γ or T cells are used as control. c) CFU is used as a control for the bacterial count. Serial dilutions of the luciferase lysates were plated on agar-plates in triplicates and incubated for three weeks. The relative value for CFU and RLU are both presented in the diagram.

4.3 Effect of alterations (Keap1 knock-down) in the MDMs on antigen-presentation and *M. avium* specific- CD4+ T cell activation (Hypothesis 3).

As we had investigated the survival of *M. avium* in macrophages with addition of *M. avium* specific T cells and established a luciferase assay for this, we wanted to see if a change in the macrophages could affect the antigen presentation and thereby the T cell response as measured in cytokine production.

Keap1 is a protein of interest due to its many roles and poor understanding of its functions. The group is already involved in research on Keap1 knockdown in inflammation and autophagy, we therefore chose to look at how Keap1 knockdown would affect the antigen presentation and thereby the T cell responses in terms of cytokine production being infected with live *M. avium*. Knockdown was measured using qPCR.

4.3.1 Effect of Keap1 knock-down in MDMs on antigen-presentation and *M. avium* specific- CD4+ T-cell activation

The experiment was conducted 4 times but one experiment was rejected due to low knockdown (20%) .The remaining results consistently show a lowered percentage of both triple-producing CD4+ T cells and total cytokine production, using Boolean-gating (Appendix III) in Keap1 knockdown compared to control siRNA-treatment (Allstar), illustrated in figure 4.6.

The Keap1 gene was knocked down using small interfering RNA (siRNA) transfection (kit). RNA was isolated using QIAGEN RNeasy Mini Kit and QIAcube (Qiagen) and complementary DNA (cDNA) was generated by the use of High Capacity RNA-to cDNA Kit (Applied Biosystems). The percentage of knockdown was predicted by qPCR using StepOnePlus qPCR. The percent knockdown for each of the 3 experiments is found in figure 4.6 a), experiment 1, 94%; experiment 2, 86%; and experiment 3, 68%. Each of the sample data are based on duplicates. The MDMs with Keap1 knockdown and control AllStar were co-cultured with expanded *M. avium* specific CD4+ T cells. The T cells were characterized with flow cytometry. The T cells of interest were detected using CD3 and CD4 markers followed by markers for IFN-γ, TNF and IL-2. Using Boolean gating (Appendix III) we then

detected the single, double and triple producers, producing one, two or three of the cytokines respectively. Example dot-plots are illustrated in figure 4.6 b) and the percentage of triple producers in each of the three experiments in 4.6 c). All samples were tested in duplicates and the values are the average value of the two. Lastly, comparing the total cytokine production in the Keap1 knockdown of the three repeats with the total cytokine production in AllStar control for all three repeats, 4.6 d) indicates the total cytokine production by MDM AllStar control T cells relative to Keap1 knockdown MDM T-cells. The results from all experiments consistently indicate an up to two-fold higher total production of cytokines in T cells experiencing MDM without Keap1 knockdown than for T cells experiencing MDM with Keap1 knockdown, indicating Keap1 plays a role in the antigen presentation by MDM to T cells.

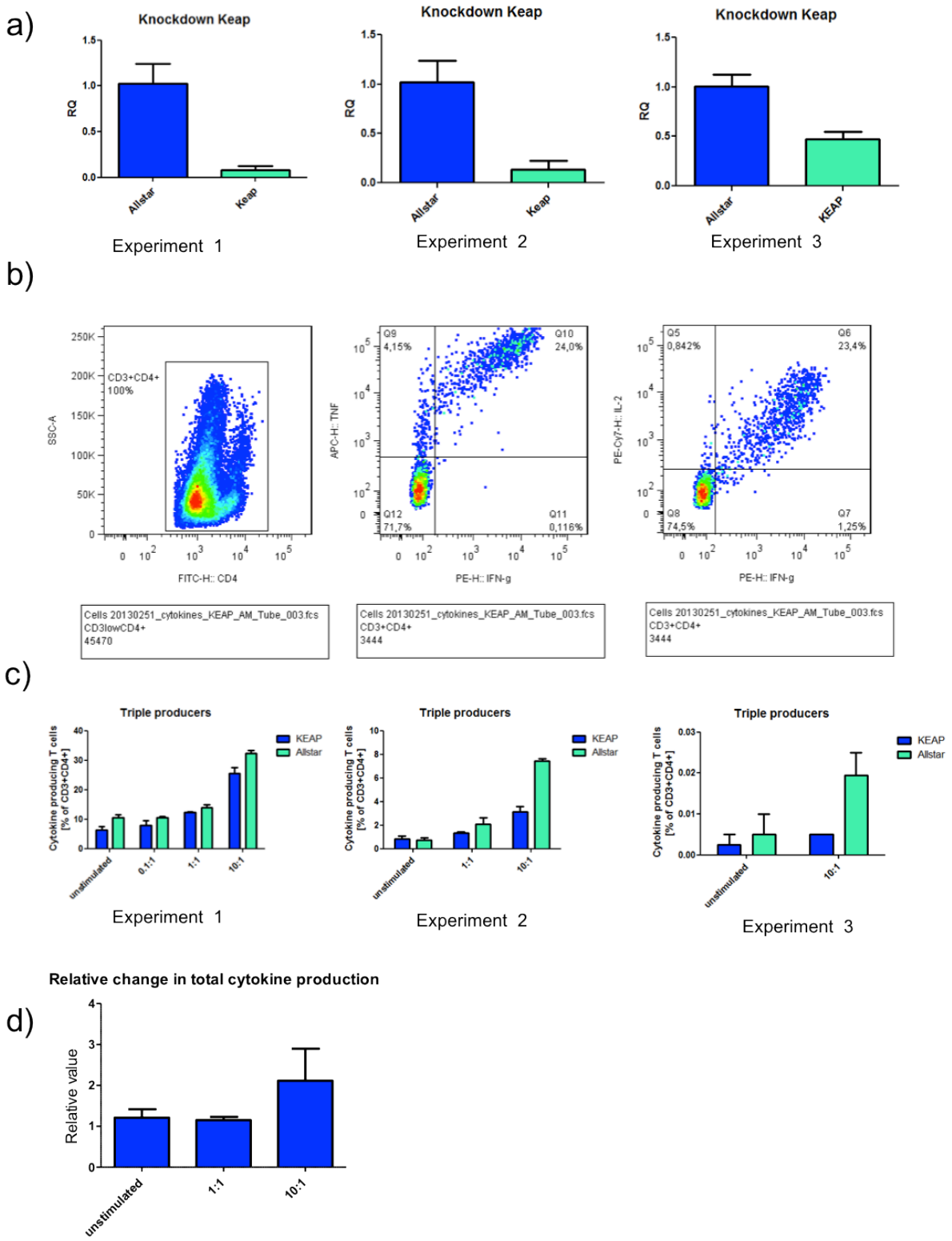


Figure 4.6:a) Percentage knockdown of the Keap1 gene. The keap1 knockdown was done using small interfering RNA (siRNA) transfection (kit). RNA was isolated using Qiagen RNeasy Mini Kit and QIAcube (Qiagen) and complementary DNA (cDNA) was generated by the use of a High Capacity RNA-to cDNA Kit (Applied Biosystems). The percentage of knockdown was predicted by qPCR using StepOnePlus qPCR. b) Flow cytometric characterization of CD4+ T cells by cytokine production. Representative dot plots for CD3+ CD4+ T cells. These were analyzed for each cytokine TNF, IL-2 and IFN-γ, followed by Boolean gating detecting the single, double and triple producers. Representative dot plots for IFN-γ and TNF, together with IFN-γ and IL-2. c) Cytokine production by heat killed- M. avium expanded CD4+ T cells. Autologous MDMs with knockdown of Keap1 and control AllStar were used for antigen presentation. The MDMs both knockdown Keap1 and controls

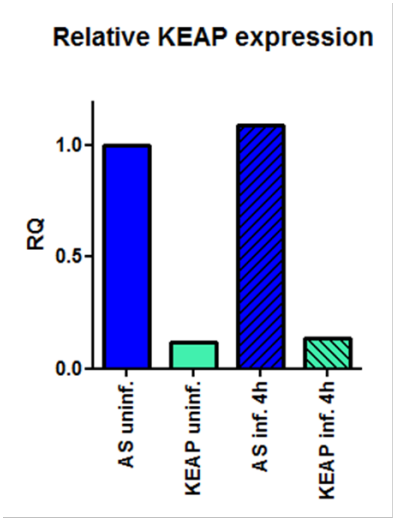
were infected with live M. avium in increasing doses, including a non-infected control. The results are shown as the percentage of triple producing T cells CD3+ CD4+ subset. 0.1:1, 1:1, and 10:1 refer to the MOI live to MDM in a 96 well plate, unstimulated refers to uninfected macrophages. KEAP refers to macrophages with Keap1 knockdown (KO Keap1), AllStar to control. d) Total cytokine production by heat killed M. avium expanded CD4+ T cells presented antigen by MDMs control AllStar relative to KO Keap1. 1:1 and 10:1 refer to the MOI live to MDM in a 96 well plate.

4.3.2 Analysis of macrophages viability and expression of surface markers with Keap1 knockdown compared to control AllStar.

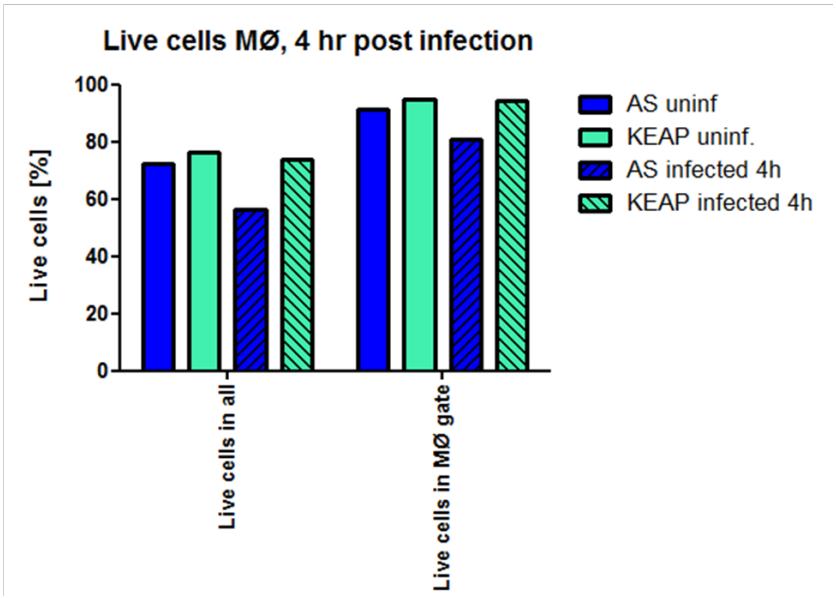
To assess the similarity of the macrophage number between wells with knockdown of Keap1 and without, macrophages in parallel wells were dissociated from the well bottom using cell-dissociation buffer (Sigma, 100 µl/well, 10 min room temperature). The knockdown was obtained as previously explained (4.3.1) and the percentage knockdown determined using qPCR (figure 4.7 a)). The samples were then stained for CD14 and CD11b marker to identify macrophages in addition to live/dead stain. We found that the percentage of live macrophages was similar in Keap1 knockdown and the control AllStar (figure 4.7. b)) The results show that the macrophages have comparable viability with Keap1 knockdown as with AllStar control, ensuring that the effect on the T cells of Keap1 knockdown macrophages is not caused by cell-death in the macrophages due to Keap1-siRNA treatment.

Further, we analyzed the expression of several surface markers on the macrophage to detect if this could change with a Keap1 knockdown. Expression of CD11b, CD14, CD80 and MHCII was analyzed. We found macrophages to have a lower expression of both CD80 and MHCII after Keap1 knockdown (figure 4.7 c)). Whereas CD11b and CD14 did not show any apparent change from Keap1 knockdown (results not shown). The decrease in CD80 (T cell co-stimulatory molecule) is found both for infected and uninfected Keap1 keap knockdown, MHCII seem to be low in uninfected cells with knock down, but might change after infection. The changes in CD80 and MHCII might result in less co-stimulation as well as antigen-presentation from Keap1 siRNA treated macrophages. This represents a possible explanation for the decrease in triple cytokine producing T cells as well as the overall T cell cytokine production. However, this is a preliminary result, though with a very good knock- down (90%). The viability was tested twice, while the HLA-DR and CD80 expression characterization was only conducted once.

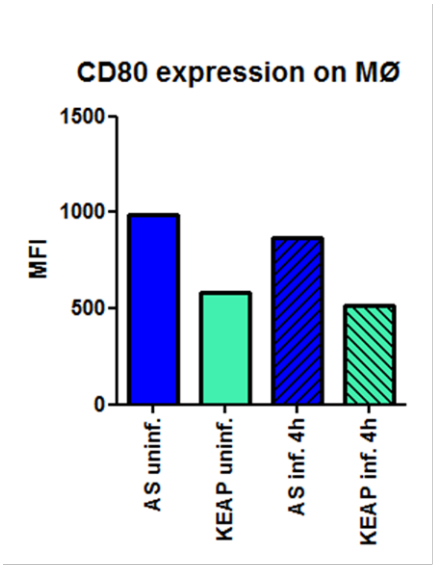
a)



b)



c)



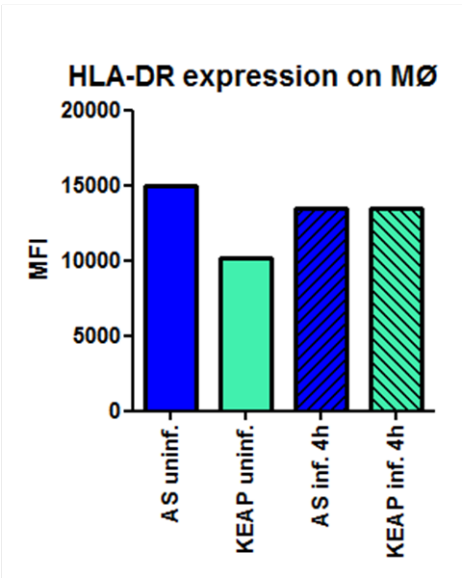


Figure 4.7: a) Percentage knockdown of the Keap1 gene in macrophages. The keap1 knockdown was done using small interfering RNA (siRNA) transfection (kit). RNA was isolated using QIAGEN RNeasy Mini Kit and QIAcube (QIAGEN) and cDNA were generated by the use of High Capacity RNA-to cDNA Kit (Applied Biosystems). The percentage of knockdown was predicted by qPCR using StepOnePlus qPCR. b)Percentage of live macrophages in wells with Keap1 knockdown compared to control AllStar. The percent of live macrophages was identified by staining for the surface marker CD14 in addition to live/dead stain. The cells were subsequently analyzed by flow cytometry. c) The expression of surface marker CD80 and HLA-DR (MHCII) on macrophages with Keap1 knockdown compared to control AllStar, both uninfected and infected with live M. avium for 4 hr. The macrophages were obtained by differentiation of PBMCs for 6-8 days in a 96-well flat-bottomed plate. The differentiated macrophages were released by using cell-dissociation buffer (Sigma, 100 µl/well, 10 min room temperature) and stained for the surface markers CD80 and HLA-DR in addition to CD14 and CD11b to identify the macrophages. The stained cells were analyzed by flow cytometry.

4.3.3. The effect of Keap1 knockdown on the bacterial growth of *M. avium*

Further work has been done looking at the effect of Keap1 knock down on *M. avium* survival. The survival was measured after lysing the live Luc + *M. avium* infected macrophages at the different time points, 4, 24, 48 and 72 hours. The survival was then measured as the amount of emitted light (RLU) by a luminometer as described in 4.2.1. RLU is directly proportional to the count of bacteria. From 24 hours after infection, the bacterial count was found to be higher in AllStar control than in Keap1 knock down (figure 4.8), indicating that Keap1 is somehow involved in the inhibition of *M. avium* survival. The effect of Keap1 knock down on survival of mycobacteria was only analyzed once in this study but was performed several times by other members of the "Keap1" project group and found to be even more prominent in some experiments at 72h post infection.

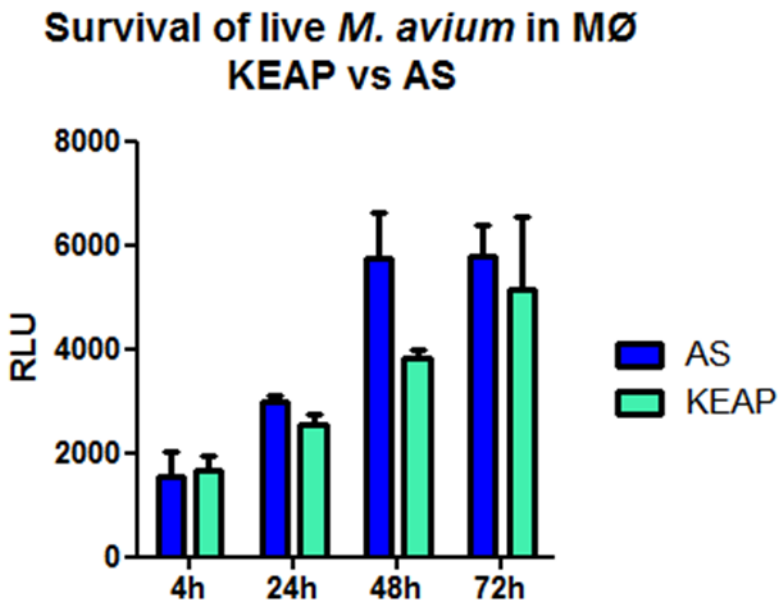


Figure 4.8: Survival of live M. avium in macrophages with Keap1 KO compared to control treated macrophages (AllStar, AS). The survival was measured after lysing the live Luc + M. avium infected macrophages at the different time points, 4, 24, 48 and 72 hours. Both sets were infected with live M. avium MOI 10:1.The inhibition of growth in the macrophages is measured as the amount of emitted light (RLU) by a lumniometer. RLU is directly proportional to the count of bacteria.

5. DISCUSSION

In 2011 approximately 1.4 million people died of tuberculosis caused by Mtb (WHO, 2012). Mycobacteria infections remain a challenge in global health. The vaccine for tuberculosis today, is only applicable during childhood, and only gives limited protection (Gengenbacher and Kaufmann, 2012).

Mycobacterial species of the *M. avium* complex (MAC) are less pathogenic relatives of Mtb, known to cause disease mainly in AIDS infected humans. With this study we aimed to increase the understanding of the host response to mycobacteria infections in humans in general, and *M. avium* infections more specificly. *M. avium* strains have previously been found to have an infection model comparable to that of Mtb infections (Kondratieva *et al*., 2010; Haug *et al*. 2013 (in press)). Still, Mtb and *M. avium* are different species and in general, *M. avium* seems to be harder to control than *M. tuberculosis* as a ten-fold higher growth of *M. avium* was observed under the same conditions in human peripheral blood monocytes and in cultured macrophages (Carvalho de Sousa and Rastoga, 1992). This is consistent with the treatment regimen for the two different species, with *M. avium* demanding a longer treatment period.

The overall aim of the project was to establish *in vitro* procedures for expansion of mycobacteria specific CD4+ Th1-cells and generation of autologous macrophages from human peripheral blood mononuclear cells (PBMCs) from healthy donors. The established protocol gives the opportunity to investigate the interactions between macrophages and CD4+ Th1-cells in mycobacterial infections and how other factors can affect these interactions. Mycobacterial survival and replication depends on a complicated interplay between various host and mycobacterial factors. We elucidated in this project the effect of IFN-γ and expanded antigen-specific CD4+ Th1 cells on the survival of *M. avium* in infected macrophages. The survival of *M. avium* was determined using a luciferase assy. The luciferase assay was chosen because it is easy to perform and has advantages compared to CFU-plating and counting concerning timespan and workload. As an alternative, a qPCR method previously established in our lab could have been used to quantify the number of cells by DNA from host macrophages. The disadvantage of the qPCR method is that it is also quite time-consuming and was originally optimized for tissue samples. However, in parallel with the luciferase assay we also plate the sample lysates as a control to our counting method.

5.1 The effect of IFN-γ on *M. avium* infected MDMs

The effect of IFN-γ in mycobacterial defense is well established for Mtb, but timing and concentration seems crucial (Carvalho de Sousa and Rastogi, 1992; Vogt and Nathan, 2011). For *M. avium* the effect is more debatable, but IFN-γ is believed to play a positive role in bacterial destruction and removal of *M. avium* infection. It has been demonstrated that murine cells are more likely to become infected by *M. avium* if they are IFN-γ deficient or lack IFN-γ receptors (McGarvey and Bermudez, 2002; Danelishvilli *et al*., 2003). However, others have found IFN-γ to have no effect on *M. avium* (Carvalho de Sousa and Rastogi, 1992). Toba et al, found the uptake of *M. avium* in human monocytes to be inhibited by IFN-γ but not the replication of the intracellular bacteria (Toba *et al*., 1989).

We found in this study that the IFN-γ has an effect on infected macrophages when added 2 days prior to infection and with a certain concentration but not when added post infection. This is in accordance with the findings by Vogt and Nathan for Mtb, showing an effect for IFN-γ when added during the time of macrophage differentiation (Vogt and Nathan, 2011). We tested several doses of IFN-γ and multiplicities of infection (MOI) with *M. avium*, finding that a high dose of IFN-γ (1000U/mL) in combination with MOI 10:1 had the most pronounced effect on *M. avium* survival when donor differences were ruled out. Vogt and Nathan also observed donor differences in IFN-γ response for *M. tuberculosis* but used lower IFN-γ concentrations. This could be a result of different suppliers or possibly also donor differences as observed within our experiments (Vogt and Nathan, 2011). The exact function of IFN-γ is not known. IFN-γ initiates the expression of several thousand genes and is, for example, an important stimulator of NO and enhances superoxide anion production. However there are indications that NO does not have a profound effect in *M. avium* destruction, as it seems to not have a toxic effect on most strains (McGarvey and Bermudez, 2002). The effect of IFN-γ is therefore most likely due to its ability to activate macrophages and its antimicrobial activity, with increased TNF-α production (Rocco and Irani, 2011). Even if we observe an effect in our experiment, *M. avium* was never removed completely by the use of IFN-γ. This could be supported by the findings of ten-fold higher growth of *M. avium* under the same conditions (Carvalho de Sousa and Rastoga, 1992

5.2 Effector cytokine production from *M. avium* specific CD4+ Th1 cells

The importance of CD4+ T cells in antimicrobial immunity against mycobacteria is well established, with depletion in the antimicrobial activity observed with a low number of CD4+ T cells (reviewed in Shiratsuchi *et al*., 2000). Recently, the function of poly-functional CD4+ T cells has been emphasized with the discovery that not only does the quality of the T cell response matter, but the quality and ability of co-expressing multiple cytokines also seems important in an effective response (Darrah *et al*., 2007; Kannanagat *et al*., 2007; Seder *et al*., 2008; Lindenstrøm *et al*., 2009). Little work has been done on *M. avium*, and none on *M. avium* strain 104 concerning the effect of CD4+ Th1 cells. *M. avium* 104 is interesting because it is a human-pathogenic strain isolated from an HIV patient and the effect of specific CD4+ Th1 cells effect on *M. avium* strain 104 growth in autologous MDMs has not been shown before in a human antigen-specific system for *M. avium*. We expanded T cells from PBMCs by stimulation with heat-killed *M. avium* for 7-9 days, which was based on findings of very low frequencies of responding CD4 T cells after short-term stimulation with the antigen (Chonchoro, 2012). IL-2 was added on day two after stimulation to expand antigen specific cells. From the expanded T cells, CD4 T cells were isolated. We established a flow cytometric protocol for staining several effector-cytokine parameters (IFN-γ, TNF, IL-2) simultaneously after expansion to identify poly-functional T cells. This characterized poly-functional CD4+ Th1 expanded cell subset was used in our subsequent cell survival experiments.

5.3. The effect of expanded *M. avium*-specific CD4+ T cells on survival of *M. avium* in macrophages

We found that the poly-functional CD4+ Th1 expanded cell subset reduced the survival of *M. avium* in MDMs when added pre-infection compared to survival of *M. avium* in infected MDMs without T cell co-culture. The reduction was observed as decreased luciferase-activity with the luminometer. We observed highly variable results when the T cells were added post-infection or at the time of infection. The decrease also depended on the number of added T cells in relation to the number of macrophages. Activation of the macrophages might not happen when the number of T cells is below a certain limit. This might be because not all macrophages are able to interact with a T cell if there are not enough T cells present. Finding a reduced *M. avium* survival in MDMs only when adding the CD4+ Th1 cells pre-infection could also indicate that the polyfunctional CD4+ Th cells mainly inhibit the mycobacteria

from entering the MDMs and do not help with killing the bacteria when they have already entered. Petruccioli *et al*. did a similar assay with Mtb, co-culturing MDMs with T cells to look more closely at the T cells' ability to restore the autophagic flux (Petruccioli *et al*., 2012). However, they did not isolate CD4+ T cells, and this is a drawback in terms of not knowing how many CD4+ T cells are added to the macrophages. We wanted to look at the effect of CD4+ Th cells in particular and thus, knowing the number is an advantage. In contrast to our observations, Petruccioli *et al.*, observed reduced survival of Mtb when T cells were added post-infection. This could be caused by several differences in our experiments, mainly the different species (*M. avium* and Mtb) but also that they did not isolate CD4+ Th cells and the observed difference could be caused by other cell subsets in the stimulated PBMCs.

5.4. Effect of Keap1 knock-down in macrophages on M. avium-specific CD4+ T cell activation

Results from Awuh *et al*, show that both Mtb and *M. avium* induce autophagy independent of inducers, and that the process may be controlled by Keap1 through LC3 recruitment to Keap1-positive mycobacterial phagosomes within MDMs (Awuh *et al*., unpublished). Keap1 is also found to be involved in the oxidative stress response, interacting with Nrf2, a key-regulating factor in the oxidative stress response (Taguchi *et al*., 2011). We show here that Keap1 might also play a role in the interaction between macrophages and CD4+ Th1 cells in terms of antigen presentation. We found that the CD4+ Th1 cells have a decreased presence of triple producers, which are cells producing IL-2, TNF and IFN-γ. Excluding the possibility that the observed effect in the CD4+ Th1 cells is a result of macrophage cell-death, together with the knowledge that the interaction between macrophages and T cells largely depends on receptors and co-receptors, we characterized the expression of surface receptors on the macrophages with and without Keap1 knockdown. We found that the expression of MHCII and CD80 were down-regulated on Keap1 KO macrophages. This could correlate with less antigen-presentation and co-stimulation and provide possible explanations as to why we find a decreased CD4+ Th1 response with Keap1 knock down. With polyfunctional T cells known to play important role in immunity towards intracellular pathogens (reviewed in Seder *et al*., 2008; Lindenstrøm *et al*., 2009), the decrease of triple producers is expected to increase the survival of *M. avium* in the macrophages. This was not possible to test during the course of this study but can be tested using our established assay.

5.5 Limitations

Our studies do not precisely reflect the immune system *in vivo*, and cannot be directly transferred to account for the mechanisms *in vivo*. But breaking the processes down to their subparts makes it possible to look at the complex process of immunity one step at a time. *In vivo*, the macrophages and T cells would actively interact with other cells of the immune system. However, establishing *in vitro* assays using human primary cells might resemble relatively closely what is happening *in vivo*. Also, primary human cells have the disadvantage of showing larger donor-to-donor variations when compared to cell lines or animal models. Establishing these assays using human primary cells can be very valuable tools for a better understanding of the pathogenesis of mycobacterial infections

6. CONCLUSION

In this study we have established *in vitro* procedures for expansion of mycobacteria specific CD4+ Th1 cells and generation of autologous macrophages from human peripheral blood mononuclear cells (PBMCs) of healthy donors. The expansion of mycobacteria specific CD4+ Th1 cells was confirmed in each experiment with multicolor flow-cytometry, which also detected the presence of polyfunctional T cells. With this procedure, the effects of IFN-γ, expanded CD4+ Th1 cells and changes in the infected macrophages by the knockdown of Keap1 were analyzed. IFN-γ and CD4+ Th1 cells were both found to decrease the survival of *M. avium* when added pre-infection. It could, however, also be CD40L interactions causing the decrease. Future studies should test if T cell-macrophage contact is necessary for killing of *M. avium.*

The knockdown of Keap1 led to a decrease in the triple producing CD4+ Th1-cells as well as the total cytokine production, indicating that Keap1 knockdown is directly or indirectly involved in the antigen presenting process of MDMs. Possible explanations were preliminary analyzed, looking at expression of co-stimulatory molecules on macrophages after Keap1 knock down as well as the effect of Keap1 knock down on survival of *M. avium* in macrophages. Survival was found to be similar in knock down and control, while the expression of CD80 by macrophages was found to decrease. This indicates lower co-stimulatory interaction as one explanation for the change in CD4+ Th1 response. HLA-DR expression was also changed in uninfected MDMs, indicating that a change in antigen presentation also might play a role in CD4+ Th1 response, though this difference in expression is seen to even out when the macrophages are infected.

7. FURTHER PERSPECTIVES

In this study, we have established an *in vitro* tool enabling a closer look at the immune response towards mycobacterial species in terms of macrophage and CD4+ T cell interaction and factors playing a role in here. Using the established assay we have been able to elucidate some aspects of this complex cooperation, including the ability of IFN-γ and CD4+ Th1 cells to activate macrophages to kill intracellular infecting *M. avium*. Still, there are several factors left to investigate including other cytokines and cells that are involved in the functional immune response and the tool that we have established could be a basis for these studies. Our assay, for example, could be used for looking more closely at CD40 ligand or IFN-γ to see if they play important roles in the killing of the *M. avium*.

The negative effects of Keap1 knock down on CD4+ T cell activation was found consistently in several experiments. However, we present only preliminary results for possible explanations of the change in the CD4+ Th1 cell response with Keap1 knock down. This should be confirmed with several repeats and the mechanisms behind this effect further elucidated as Keap1 acts on a multitude of important processes in mycobacterial immunity (autophagy, inflammation) and its role in these should be further elucidated. The assay used in this study can in later studies also be used to look at the effect of many other proteins involved in anti-mycobacterial processes such as Toll-like receptors or other autophagic proteins. It should also be possible to establish antigen-specific T cell assays with other pathogens such as *Staphylococcus aureus* or *Salmonella typhi*, which will be of interest to our group for other projects.

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APPENDIX I

Detailed procedure for PBMC isolation, processing and generation of monocyte derived macrophages and M. *avium* specific CD4+ T-cells.

PBMC isolation

Authorized personnel collect heparin-blood samples from helthy donors.

1.Lymphoprep and Phosphate buffered saline (PBS) are heated to room temperature in a 37°C water bath.

2.Transfer the blood to 50 mL centrifuge tubes, approximately 15 ml blood per tube and dilute 1:1 with preheated PBS

3. Distribute 15 ml lymphoprep to 2x50 mL centrifuge tubes.

4. Layer the blood/PBS mixture carefully onto the lymphoprep using a 25 ml pipette, making a layer on top of the lymphoprep. Add slowly and pipette towards the wall of the tube.

5. Centrifuge blood+PBS+lymphoprep at 1800 rpm in 20 min with breakforce set to 7-3

6. Pipette the white layer of mononuclear cells with a 10 mL pipette and transfere to 2x50 mL centrifuge tubes.

7. Centrifuge at 2000 rpm for 10 min with breakforce set to 9-9

8. Discard supernatant.

9. Add 1-2 mL of Hank's with 2% FCS and resuspend the pellet. Add Hank's up to 25 mL

10. Centrifuge at 2000 rpm for 10 min with breakforce set to 9-9

11. Discard supernatant.

12. Add 1-2 mL of Hanks with 2% FCS and resuspend the pellet. Add Hank's up to 25 mL

13. The tubes are centrifuged at 800 rpm for 8 min with breakforce set to 9-9

14. Discard supernatant.

15. Add 1-2 mL of Hanks with 2% FCS and resuspend the pellet. Add Hank's up to 25 mL

16. Centrifuge at 1400 rpm for 6 min with breakforce set to 9-9

17. Discard supernatant.

18. Resuspend pellet in 10%A+/RPMI medium with10mM HEPES and 2mM glutamine. Add medium up to 3 mL.

19. Count cells on Invitrogen™, Countess™ automated cell counter by mixing 10 microliter tryphanblå+10 microliter cell suspension. Add 10 microliter of mixture to a slide and count. Multiply the total number of cells by 3, since the sample is diluted in 3 mL of medium.

The cells can now be grown for different purposes:

a) Isolation of monocytes and generation of macrophages

1. Dilute cell suspension to 2 x 10^6 cells/mL in RPMI 10% A+ with10mM HEPES and 2mM glutamine

2.Plate 100 µl of cell suspension per well on a 96-well plate

3. Incubate the plate for 1 hour at 37°C. Monocytes will adhere

4. Using a multichannel vacuum suction device, carefully remove the supernatant from the wells.

5. Add 100 µl of pre-warmed Hank's.

6. Discard supernatant

7. Repeat step 5-6.

8. Add 100 µl RPMI 30% A+ with 10mM HEPES and 2mM glutamine to each well

9. Incubate for 5-8 days at 37°C to generate macrophages
10. Add 50 µl fresh RPMI 30% A+ to each well on day 4

b) Mycobacteria specific T cells

1. Dilute cell suspension to 3 x 10^6 cells/mL in RPMI 10% A+
2. Plate 1 mL of cell suspension per well on a 24-well plate.
3. Stimulate with freshly prepared heat-killed *Mycobacterium avium* at a MOI of 5:1.
4. Incubate cells for 8-10 days at 37°C
5. Add 20U/mL of IL-2 on day 2
6. Cells may be split on day 4, and then IL-2 should be added again

The in-vitro generated macrophages and the expanded mycobacteria-specific T-cells are further used for the T cell assay. T cells could also be restimulated.

Restimulation of T cells

1. Add 500 µl fresh cell suspension of isolated mononuclear cells (2 x 10^6 cells/mL in RPMI 10% A+) per well to the needed amount of wells on a 24-well plate.
2. Add wanted number (1-3 x 10^6 cells/mL in RPMI 10% A+) of isolated/non isolated T-cells directly to the cell suspension.
3. Stimulate each well with heat-killed *M. avium* MOI 5:1
4. Incubate cells for 8-10 days at 37°C
5. Add 20U/mL of IL-2 on day 2
6. Cells may be split on day 4, and then IL-2 should be added again

Infection of macrophages and T cell addition

1. Using a multichannel vacuum suction device, remove differentiation medium.
2. Wash 1-2x with prewarmed Hanks solution
3. Add 100 µl warm RPMI 10% A+
4. Stimulate the macrophages with live bacteria solution with desired MOI for 4 hours
5. After 4 hours, remove medium with bacteria and add 100 µl warm RPMI 10% A+
6. Keep the cells in the incubator until T-cells are added.
7. Add wanted number of T-cells per well.
8. a) Cells for luciferase assay- measure at 24 hr and 72 hr timepiont after T-cells added
   b) Cells for flow cytometry-
   1. Add 4 µl of 1:10 diluted 500x protein transport inhibitor cocktail (eBioscience) to each well
   2. Incubate for 4 hours
   Samples are now ready for extra- and intracellular staining for flow cytometry

APPENDIX II

Detailed procedure for extra- and intracellular staining for flow cytometry.

1. Transfer cells from wells to flow tubes containing 500 µl PBS with 2 % FCS and 0.05% NaN_3 (wash buffer).
2. Wash tubes at 1500 rpm for 6 min.
3. Discard supernatant
4. Add surface antibodies
5. Vortex tubes
6. Incubate for 15 min on ice, protected from light.
7. Add 1 mL wash buffer
8. Vortex tubes
9. Wash tubes at 1500 rpm for 6 min
10. Discard supernatant
11. Resuspend cells in 500 µl PBS with 2% paraformaldehyde (PFA)
12. Vortex tubes
13. Incubate for 20 min in room temperature, protected from light.
14. Centrifuge tubes at 1500 rpm for 6 min
15. Discard supernatant
16. Add 1 mL wash buffer
17. Vortex tubes
18. Wash tubes at 1500 rpm for 6 min
19. Discard supernatant
20. a) Resuspend in 500 µl wash buffer and store overnight at 4°C, proceed with step 18. the next day.
    b) Proceed with intracellular staining for effector cytokines, step 18.
21. Add 1 mL wash buffer
22. Vortex tubes
23. Wash tubes at 1500 rpm for 6 min
24. Discard supernatant
25. Resuspend in 500 µl with 0.5% saponin and 1 % FCS
26. Incubate for 5 min at room temperature
27. Centrifuge tubes at 1500 rpm for 6 min
28. Discard supernatant
29. Add intracellular antibodies or isotypes
30. Incubate for incubate at RT for 30 minutes protected form light.
31. Wash x2. Add 1 mL wash buffer, vortex tubes, wash tubes at 1500 rpm for 6 min, discard supernatant.
32. Resuspend cells in 500 µl wash buffer and proceed with flow cytometric analysis.

APPENDIX III

Detailed procedure for Boolean gating using FlowJo software, 3.8.5.

Open workspace with files of interest. Gate lymphocytes identified based on FSC and SSC properties followed by CD3+ for T cell population in general. The cells are gated for CD4+ to identify the CD4+ Th cells.

Identify the CD4+ cytokine production one cytokine at the time. Cytokine on y-axis and CD4+ on x-axis, gate the positive population and name IFNg, TNF and IL-2 respectively. Mark the IFNg, TNF and IL-2 analysis in the overview field and click tools ->create combination gates. Now, mark all analysis from sample one, and add to all samples in workspace. For sample 1 mark the resulting triple, double and single producers in addition to the triple negatives and click on ”Open table editor”. Choose batch to add the rest of the samples, and click the green X, choose Excel file format, and save. The Excel file will open with the percent of the different type of cells, triple, double and single producers in addition to the triple negatives.