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The Role of KEAP1 in Regulating Inflammation and Antigen-Presentation in Response to *Mycobacterium avium* Infection in Macrophages

Master's Thesis

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Abstract

Mycobacterial infections are a health problem worldwide with *Mycobacterium tuberculosis*, the causative agent of tuberculosis, being responsible for over one million deaths every year. The opportunistic pathogen *Mycobacterium avium* is another member of the mycobacteria family, mainly causing disease in immunocompromised patients. The treatment of mycobacterial infections is extensive, and the emergence of mycobacterial strains resistant to the current treatments presents an urgent need for development of new treatments and effective vaccines.

KEAP1 is an oxidative stress sensor with several binding partners in the cell. Research in our group has shown that KEAP1 negatively regulates the induction of pro-inflammatory cytokines and autophagy in response to *M. avium* infection in primary human macrophages. The mechanism of how this regulation occurs is not understood, and has been under investigation in this thesis. HEK293 cells were tested out as a model for our investigation and were proved to be unsuitable for our purpose. The investigation of how KEAP1 negatively regulates inflammatory responses following *M. avium* infection was therefore carried out in primary human macrophages. Findings obtained throughout this study show that the E3 ubiquitin ligase complex proteins Cul3 and Rbx1 associated with KEAP1 might have an important role in the negative regulation of inflammation seen following *M. avium* infection.

Mycobacterial infections and innate immune receptor signalling can affect antigen-presentation. Since KEAP1 appears to be involved in anti-mycobacterial responses in primary human macrophages, we investigated the role of KEAP1 in regulation of antigen presentation and shaping of the following adaptive immune response. Using siRNA knockdown of KEAP1 in primary human macrophages, we obtained evidence suggesting that KEAP1 positively influences macrophage expression of the molecules CD80 and HLA-DR, both important for the activation of the adaptive CD4⁺ T cell response. How this alteration changes the following adaptive immune response is not known. We therefore wanted to establish an assay with expanded human mycobacteria-specific T cells in order to analyse whether or not KEAP1 affects the activation of mycobacteria-specific T_H1 cells upon stimulation with *M. avium* infected autologous macrophages. T cells survived the expansion and produced cytokines upon stimulation with a positive control (stimulation cocktail). However, preliminary experiments did not allow us to establish a solid protocol to analyse activation of

mycobacteria-specific T cells upon stimulation with primary human macrophages. More experiments are hence necessary to optimise this assay.

This thesis has increased our knowledge of how KEAP1 is involved in the innate immune response in primary human macrophages. Future experiments should aim to verify and further elaborate on the findings done in this thesis, as well as continue the work on investigating the links to adaptive immunity.

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Table of content

Abstract	iii
Acknowledgements	v
List of Figures	viii
List of Tables.....	ix
1 Introduction.....	1
1.1 Mycobacterial infections	1
1.2 The innate immune system in mycobacterial infection.....	3
1.2.1 Pattern recognition receptors.....	4
1.2.2 Autophagy	6
1.2.3 The KEAP1-NRF2 pathway.....	8
1.3 Adaptive immunity in mycobacterial infection.....	11
1.4 Cytokines in mycobacterial infection	13
2 Aims and objectives	15
3 Materials and methods	17
3.1 Isolation and seeding of peripheral blood mononuclear cells	17
3.1.1 Differentiation of monocytes to monocyte derived macrophages	17
3.1.2 Expansion of T cells.....	18
3.1.3 Restimulation of T cells	18
3.2 Cultivation and preparation of <i>M. avium</i>	19
3.2.1 Infection	19
3.2.2 Heat-killing.....	20
3.3 Human embryonic kidney 293 (HEK293) cells	20
3.3.1 Thawing of HEK293 cells.....	20
3.3.2 Maintenance and seeding of HEK293 cells	21
3.4 Transfection of cells <i>in vitro</i>	21
3.4.1 RNA interference	22
3.4.2 Transfection of plasmids into HEK293 cells <i>in vitro</i>	24
3.5 Real-time quantitative polymerase chain reaction.....	26
3.5.1 RNA extraction and cDNA synthesis.....	27
3.5.2 Real-time PCR procedure.....	28
3.5.3 Real-time PCR analysis.....	29
3.6 Flow cytometry.....	29

3.6.1	Staining of T cells for flow cytometry	31
3.6.2	Staining of macrophages for flow cytometry	32
3.6.3	Flow cytometric analysis of <i>M. avium</i> specific T cell activation.....	32
3.6.4	Flow cytometric surface marker characterisation on macrophages following KEAP1 knockdown	33
3.6.5	Processing of flow cytometry data	33
4	Results.....	35
4.1	Testing HEK293 cells as a model system for investigating the mechanisms behind how KEAP1 regulate inflammatory responses.....	35
4.1.1	KEAP1 knockdown decreases the TNF α production in HEK293 TLR2-GFP cells following <i>M. avium</i> infection	35
4.1.2	<i>M. avium</i> -induced NF- κ B activation decreases following KEAP1 knockdown in HEK293 cells	37
4.2	Cul3 and Rbx1 knockdown increases the inflammatory response to <i>M. avium</i> infection in primary human macrophages	40
4.3	KEAP1 knockdown downregulates the expression of macrophage surface proteins CD80 and CD86	42
4.4	Establishment of conditions for mycobacteria-specific T cell expansion and restimulation with autologous macrophages	44
4.4.1	Establishment of conditions for mycobacteria-specific <i>in vitro</i> T cell expansion 44	
4.4.2	Testing effector responses of expanded mycobacteria-specific T cells to autologous macrophages with different doses of <i>M. avium</i> infection	47
4.4.3	Kinetics of effector cytokine production from mycobacteria-specific CD4 ⁺ T _H 1 cells after restimulation with <i>M. avium</i> infected macrophages.....	48
5	Discussion	51
6	Conclusion	61
7	Future perspectives	62
8	References.....	63
9	Appendix.....	69

List of Figures

Figure 1.1: The stages of <i>M. tuberculosis</i> infection.....	2
Figure 1.2: TLR signalling pathways involved in mycobacterial recognition.....	5
Figure 1.3: General overview of autophagy.....	7
Figure 1.4: Domain structure of NRF2 and KEAP1.....	9
Figure 1.5: The immunological synapse.....	12
Figure 3.1: The principle of siRNA silencing.....	22
Figure 3.2: Establishment of siRNA treatment of HEK293 cells.....	24
Figure 3.3: The principle of real-time PCR TaqMan assay.....	27
Figure 3.4: Schematic overview of a flow cytometry setup.....	30
Figure 4.1: TNF α production by HEK293 TLR2-GFP cells decreases following KEAP1 knockdown and <i>M. avium</i> infection.....	37
Figure 4.2: NF- κ B activation following various stimulations decreases in HEK293 cells following KEAP1 knockdown.....	39
Figure 4.3: Increased inflammatory response following <i>M. avium</i> infection in KEAP1, Cul3, and Rbx1 depleted primary human macrophages.....	41
Figure 4.4: Decreased expression of CD80 and HLA-DR on the surface of primary human macrophages following KEAP1 knockdown.....	43
Figure 4.5: Cytokine response in CD4+ T cells expanded under different conditions and restimulated through co-culturing with <i>M. avium</i> infected autologous macrophages.....	46
Figure 4.6: CD4+ T _H 1 effector cytokine production following restimulation of mycobacteria- specific T cells with differently <i>M. avium</i> infected autologous macrophages.....	47
Figure 4.7: The frequency of CD4+ T cells producing the cytokines IFN γ , TNF α , or IL2 at different times of restimulation with autologous <i>M. avium</i> infected macrophages.....	49

List of Tables

Table 3.1: Temperatures and times for cDNA synthesis incubation.....	28
Table 3.2: The reaction cycle for real-time PCR using TaqMan probes.....	29
Table 3.3: Overview of flurochromes used for T cell staining for flow cytometry.....	31
Table 3.4: Overview of flurochromes used for macrophage staining for flow cytometry.....	32

Abbreviations

APC- Antigen presenting cell

BCG- Bacillus Calmette-Guérin

CD- Cluster of differentiation

CFU- Colony forming unit

CLR- C-type lectin receptor

Ct- Threshold cycle

Cul3- Cullin 3

DAMP- Damage-associated molecular patterns

DC- Dendritic cell

FRET- Fluorescence resonance energy transfer

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

GFP- Green fluorescent protein

HEK- Human embryonic kidney

HIV- Human immunodeficiency virus

IFN- Interferon

IKK- I κ B kinase

IL- Interleukin

IRF- Interferon regulatory factor

IP10- Interferon gamma induced protein 10

KEAP1- Kelch like ECH-associated protein 1

KIR- KEAP1 interacting region

LAM- Lipoarabinomannan

LC3- Microtubule-associated 1A/1B-light chain 3

LM- Lipomannan

LPS- lipopolysaccharides

MAC- *M. avium* complex

ManLAM- Manoylated lipoarabinomannan

MAPK- Mitogen activated protein kinase

MDM- Monocyte derived macrophage

MDR- Multidrug resistant

MHC- Major Histocompatibility complex

MOI- Multiplicities of infection

MyD88- Myeloid differentiation primary response gene 88

NF- κ B- Nuclear factor kappa-light-chain-enhancer of activated B cells

NK- Natural killer

NLR- NOD-like receptor

NRF2- Nuclear factor erythroid-2-related factor 2

PAMP- Pathogen-associated molecular pattern

PBMC- Peripheral blood mononuclear cell

PCR- Polymerase chain reaction

PRR- Pattern recognition receptor

Rbx1- Ring box 1

RCF- Relative centrifugal force

RLR- RIG-1 like receptor

ROS- Reactive oxygen species

siRNA- Small interfering RNA

TBK1- TANK- binding kinase 1

TCR- T cell receptor

T_H- T-helper

TLR- Toll-like receptor

TNF- Tumor necrosis factor

XDR- Extensively drug resistant

1 Introduction

1.1 Mycobacterial infections

Mycobacterial infections are a health problem worldwide, with *Mycobacterium tuberculosis*, the causative agent of tuberculosis causing 1.3 million deaths in 2012¹. Although the numbers of new cases and mortality rates have decreased for the last two decades, the global burden remains high with approximately 8.6 million new cases in 2012¹. This makes tuberculosis one of the leading causes of death worldwide and it is the main cause of death in human immunodeficiency virus (HIV) infected individuals living in Africa². *M. tuberculosis* most commonly affects the lungs, causing pulmonary tuberculosis, but can also affect other parts of the body. Infected individuals that do not show symptoms of disease are said to have a latent infection. In these individuals the immune response effectively inhibits disease, although the bacteria might not be eradicated. At this stage the disease is hard to diagnose. If an individual gets immune-compromised, the risk of reactivation increases, as exemplified in patients co-infected with HIV³.

The route of infection of *M. tuberculosis* is through inhalation of droplets containing the pathogen. Upon inhalation phagocytic cells at the epithelial surface of the lungs engulf the bacteria, mainly alveolar macrophages at this stage. Immune cells are activated and release chemokines to attract more immune cells, including dendritic cells (DCs), neutrophils, natural killer (NK) cells, and monocyte derived macrophages (MDMs). The immune cells are not able to stop the bacterial proliferation, and recruitment of innate immune cells allows the expanding mycobacterial population in the lung to spread from cell to cell. The innate immune response engages the adaptive immune response, specifically T cells, although there is a delay in this recruitment⁴⁻⁶. Once the adaptive immune response is established, there is a halt in the growth of the bacterium, but the infection is in most cases not eradicated. At this stage most people become asymptomatic, and are said to have a latent infection⁶. A granuloma is a characteristic feature of latent infection, and is formed during the immune response. The granuloma is an infiltrate consisting of an array of mononuclear immune cells, and gives a niche for the bacteria to reside in. It forms as a result of the immune cells not being able to eradicate the pathogen, and instead accumulate around the infection to avoid further spread. The granuloma is organised, and has a central core of macrophages, that is surrounded by lymphocytes, as well as DCs, endothelial cells, fibroblasts, and granulocytes^{3,5}. Within the granuloma, *M. tuberculosis* enters a state where replication is very low, but the

bacteria are still able to generate energy and hence survive⁵. If the immune system of the host is weakened, for reasons such as HIV infection or immunosuppressive treatment, the bacteria can get reactivated and return to a virulent state where it can once again cause disease⁶.

Figure 1.1 illustrates the stages of *M. tuberculosis* infection from inhalation to reactivation.

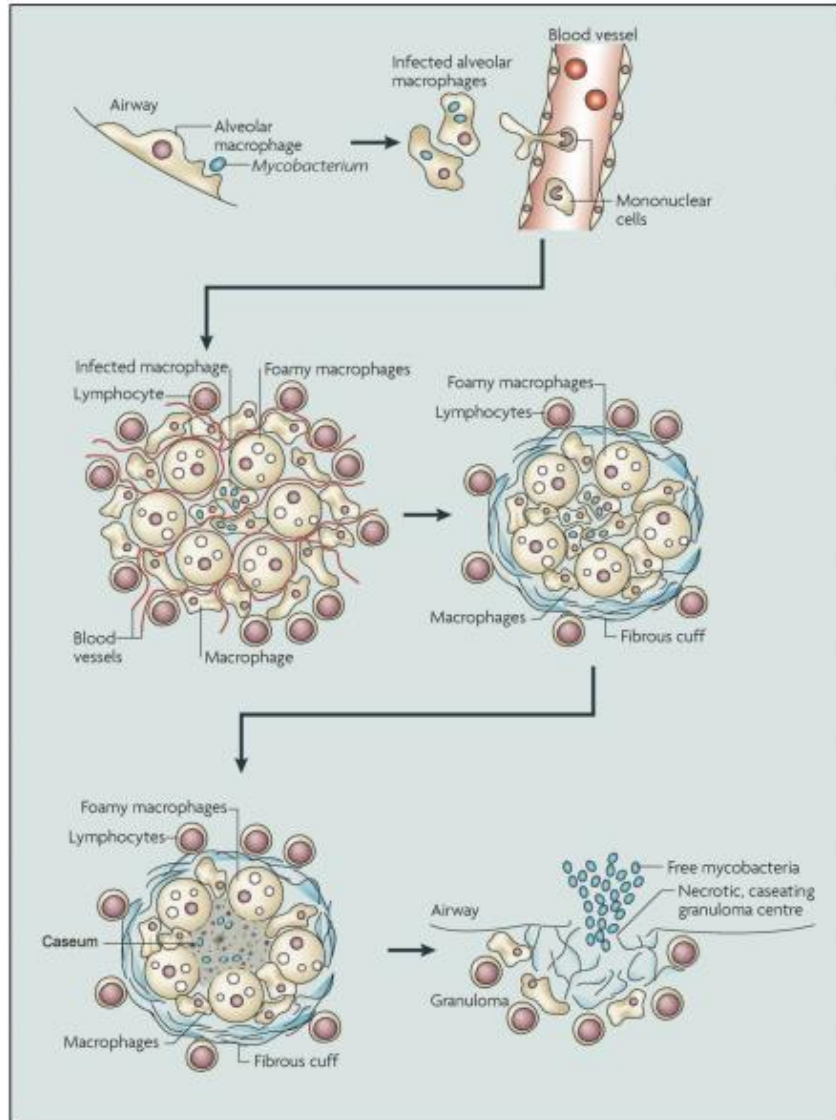


Figure 1.1: The stages of *M. tuberculosis* infection. Upon inhalation *M. tuberculosis* is engulfed by alveolar macrophages at the epithelial surface of the lung. The proinflammatory response triggered leads to recruitment of more immune cells. Macrophages in the forming granuloma can differentiate to epithelioid cells, multinucleated giant cells, and foam cells. A fibrous cap can form around the granuloma, in the same area where the lymphocytes are also mainly found. Upon reactivation the granuloma destabilises, and previously contained bacteria are released into the lung⁷.

Mycobacterium avium, a member of the *M. avium* complex (MAC), is another member of the mycobacteria family. This is a nontuberculous, opportunistic pathogen mainly causing disseminated infection in immunocompromised patients⁸. Lung infections with *M. avium* are not associated with immunodeficiency, but rather other lung conditions such as

chronic obstructive pulmonary disease and cystic fibrosis^{9,10}. As opposed to *M. tuberculosis*, *M. avium* is an environmental pathogen. The main source of exposure to *M. avium* is through water, in particular drinking water. Another source of exposure is through soil¹¹. Environmental bacteria, including *M. avium* and other nontuberculous mycobacteria have adapted to diverse and harsh conditions such as low oxygen tension, low nutrition levels, and acidic environments. Such adaptations often aid the virulence of these bacteria^{11,12}.

The current vaccine used against tuberculosis is the *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccine. BCG is efficient in protecting children against early manifestations of tuberculosis, but fails to protect against adult pulmonary tuberculosis¹³. The current treatment for tuberculosis consists of several antibiotics taken over a period of 6 months or more⁷. Multidrug-resistant (MDR) and extensively drug-resistance (XDR) strains have emerged, which are no longer susceptible to the current drugs used to treat tuberculosis¹⁴. Thus there is an urgent need for new and better vaccines and treatments than what currently exists.

To be able to develop new vaccines and treatments, we need a better understanding of how the mycobacterial pathogens interact and evades the immune system, and what can be done to overcome mycobacterial evasion mechanisms.

1.2 The innate immune system in mycobacterial infection

The innate immune system is the first line of defence against invading pathogens, including mycobacteria. It includes physical barriers such as the skin and mucosal surfaces, immune cells such as neutrophils, macrophages, NK cells, and DCs, as well as the complement system. The innate immune cells recognise foreign substances through germ-line encoded pattern recognition receptors (PRRs) which recognise and bind to structures common to many pathogens that are not normally present in our body. These structures are called pathogen-associated molecular patterns (PAMPs). Damaged self-cells can also be recognised, through damage-associated molecular patterns (DAMPs)¹⁵. The macrophage response to mycobacterial infections will be the focus of this thesis.

Macrophages play an important role in the defence against invading pathogens, in maintenance of tissue homeostasis, clearance of damaged self-cells, as well as in recruitment and instruction of cells of the adaptive immune system. They originate from myeloid progenitor cells in the bone marrow, which through several developmental steps become monocytes which are released into the blood stream. The monocytes can then migrate into

tissues where they further develop into long-lived tissue-specific macrophages¹⁶. When a macrophage encounters a pathogen, the invading pathogen is phagocytosed and processed, and antigens are presented on the cell surface via the major histocompatibility complex (MHC) class I and II. MHC class I molecules are present on the surface of almost all cells in the body, while MHC class II molecules are found on antigen presenting cells (APCs). Production of chemokines and presentation of antigens is required for the recruitment and activation of cells of the adaptive immune system¹⁷.

Upon stimulation, macrophages get activated and develop into effector macrophages. During a cell-mediated response, macrophages are activated through cytokines such as interferon γ (IFN γ) and tumor-necrosis factor α (TNF α). This activation leads to macrophages with increased microbicidal activity producing high levels of pro-inflammatory cytokines. The activating cytokines can thereby originate from other APCs or from T cells¹⁶.

M. tuberculosis can evade or even take advantage of the host's cellular response to an infection. Both adaptive and innate immunity can be evaded through mechanisms like inhibition of phagosome maturation, resistance to microbicidal and cytokine defences, and inhibition of antigen presentation¹⁸.

1.2.1 Pattern recognition receptors

PRRs on the surface of APCs include Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), RIG-1 like receptors (RLRs), as well as cytosolic DNA sensors¹⁹. In mycobacterial infection host macrophages and dendritic cells phagocytose the microbe at the site of infection. It is the PRRs on the surface of host APCs that recognise structures on the pathogen, leading to phagocytosis¹⁹.

The TLRs is a group of 10 PRRs in humans. They are found both on the plasma membrane and on intracellular membranes and play an important role in recognition of both intracellular and extracellular ligands¹⁹. Especially three TLRs; TLR2 and TLR4 which are found on the plasma membrane, and TLR9 located in endosomes, have been shown to play important roles in recognition and response to mycobacterial infection in macrophages¹⁹⁻²¹. Upon recognition, the TLRs rapidly induce intracellular signals leading to activation of the mitogen-activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) pathways, which both play important roles in the innate immune defence through the generation of antimicrobial effector molecules²⁰. The production of type I IFNs is also initiated, through activation of TANK-binding kinase 1 (TBK1) and interferon regulatory factors (IRFs)²². The

TLR signalling pathway is shown in **Figure 1.2**. The NF- κ B transcription factor is normally found in the cytoplasm bound to I κ B regulatory proteins. When the cell is activated, I κ B is degraded and the transcription factor is able to enter the nucleus and transcribe target genes. In most cases it is the I κ B kinases (IKKs) that targets I κ B for degradation. NF- κ B target many genes encoding pro-inflammatory cytokines and chemokines²³, including TNF α , and interferon gamma induced protein 10 (IP-10), while activation of IRFs leads to the production of IFN β ²⁴.

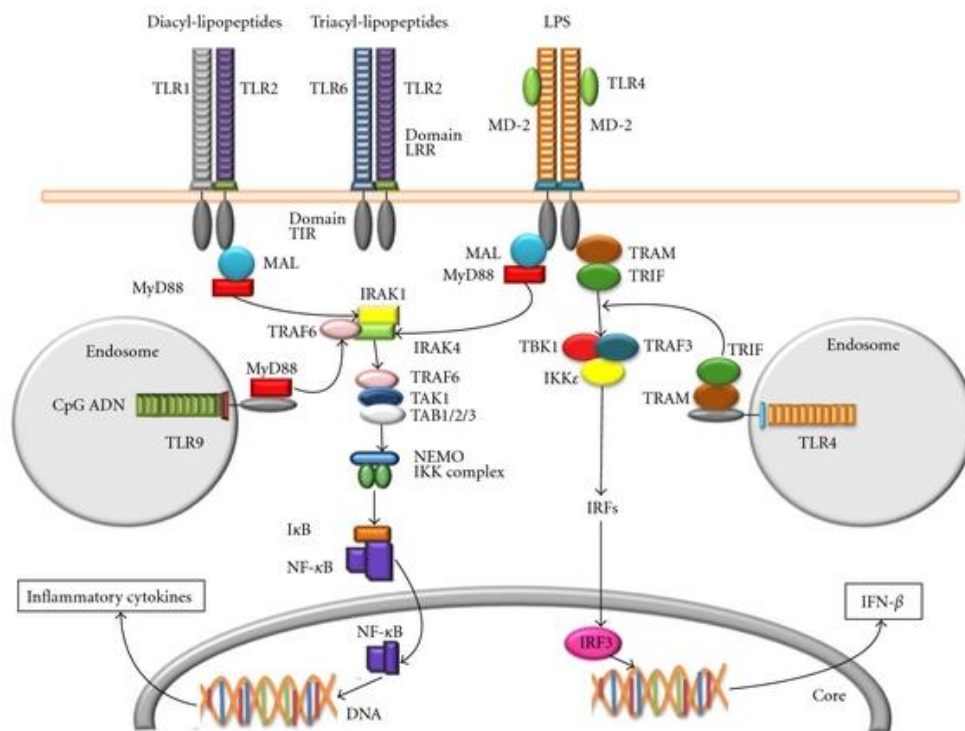


Figure 1.2: TLR signalling pathways involved in mycobacterial recognition TLR2 and TLR4 recognise ligands outside the cell, while TLR9 recognise bacterial DNA. Myeloid differentiation primary response gene 88(MyD88) is a common adaptor protein for all these TLRs and lead to activation of NF- κ B and transcription of inflammatory cytokines. TLR4 can also signal in a MyD88 independent way, through adaptor protein TRIF, activating IRFs and transcription of IFN- β ²⁵.

CLRs are membrane bound receptors that recognise carbohydrate-rich molecules and are important for phagocytosis of mycobacterial pathogens. The four CLRs mannose receptor, DC-SIGN, mincle and Dectin-1 are closely associated with responses to mycobacteria^{19,21}. The primary way of phagocytosis of *M. tuberculosis* in human macrophages is through recognition by mannose receptors, and this process is also associated with an anti-inflammatory program. The mannose receptors recognise lipoarabinomannan (LAM) and mannocylated LAM (ManLAM), which are glycolipids that are found on the cell wall of mycobacteria as well as on other microbes¹⁹. Dectin-1 has been shown to play an

important role in the recognition and ingestion of the fungal cell wall derivative β -glucan. Recognition of β -glucans by this receptor contributes to phagocytosis, reactive oxygen species (ROS) production, and cytokine production in macrophages^{21,26-28}.

Studies have shown that Dectin-1 and TLR2 can collaborate in the initiation of an inflammatory response, and that Dectin-1 expression enhances the activation of the NF- κ B pathway initially activated by TLR2, in response to β -glucan particles^{28,29}. The ROS production following recognition of these particles is independent of TLR2²⁹. Much of the research addressing Dectin-1 has been focused on immune responses to fungi, but the receptor is also involved in immune responses against mycobacteria. Yadav and Schorey²⁶ showed that Dectin-1 and TLR2 also cooperate in macrophage activation following mycobacterial infections in murine macrophages. Their data suggest that Dectin-1 serves to increase the TLR2-dependent inflammatory response, measured through MAPK activation and TNF α production in macrophages following infection with non-pathogenic mycobacteria. However, they did not see the same response following infection with pathogenic strains. Although evidence suggests that TLR2 is an important TLR in the recognition and defence against mycobacteria, the pathogen is also able to use this receptor for its own advantage and survival within host cells. Research suggests that *M. tuberculosis* can use TLR2 to decrease antigen presentation via MHC II, and hence decrease the adaptive immune response¹⁸.

Cytosolic DNA sensors have been shown to get activated upon *M. tuberculosis* infection in murine macrophages, as the bacteria can partially permeabilize the phagosomal membrane³⁰. This activation leads to signalling through the Sting/Tbk1/Irf3 pathway, and results in production of IFN β ³⁰. Emerging evidence also suggest a link between the sensing of cytosolic DNA and autophagy³¹. The mechanisms of inflammatory regulation in mycobacterial infections are starting to get unravelled, but much is still unknown.

1.2.2 Autophagy

Macroautophagy (hereafter referred to as autophagy) is a catabolic process where the cell wraps a double layered membrane around intracellular cargo, forming an autophagosome, which is then delivered to lysosomes for degradation. This is a process that is used by the cell to degrade cellular components as well as protection against intracellular pathogens³². The principle of autophagy is shown in **Figure 1.3**.

Autophagy has important roles in both the adaptive and innate immunity. It is involved in shaping the adaptive immune response through its role in thymic selection and homeostasis

of T cells, antigen presentation, and polarisation of T helper (T_H) 1 and T_H2 cells. In macrophages, autophagy protects the cells against bacterial toxins, aids in the activation of the cell, and can directly kill intracellular pathogens³². A common marker to recognise autophagosomes in mammalian cells is the microtubule-associated 1A/1B-light chain 3 (LC3). LC3 exists in two states, soluble LC3I, or membrane bound LC3II, and it is the latter which is found in the membrane of autophagosomes and is used as a marker for autophagy³³.

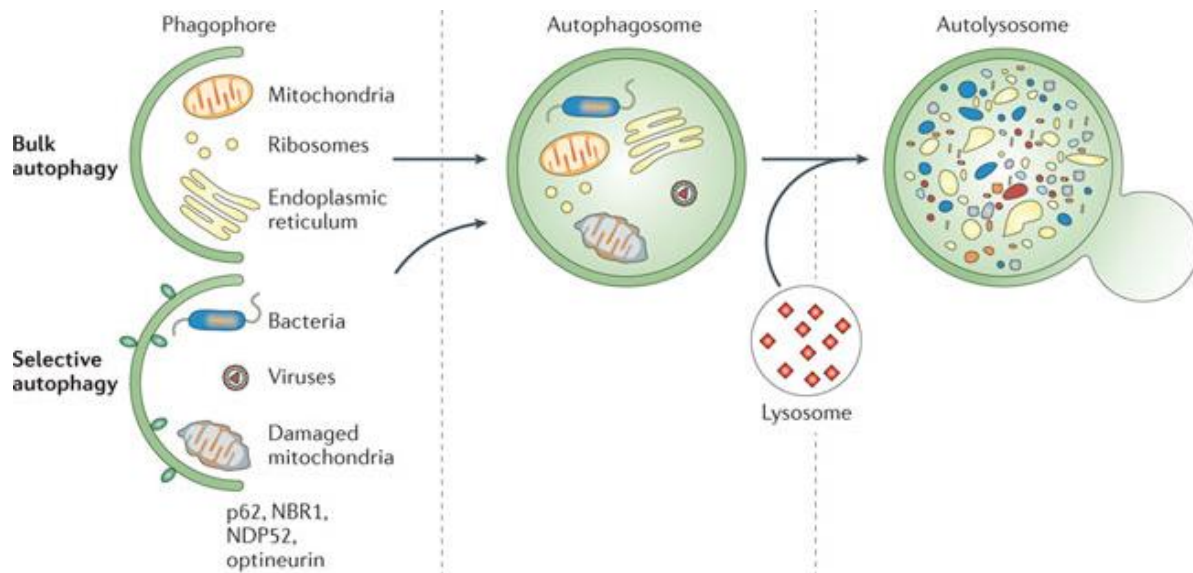


Figure 1.3: General overview of autophagy. Autophagy can break down both cellular components and invading pathogens. A membrane structure called a phagophore wraps around the target cargo, forming a double membrane autophagosome. The incorporation of cargo can be non-selective (bulk autophagy) or selective (selective autophagy). Selective autophagy is mediated through cargo receptors such as p62, NBR1, NDP52, and optineurin which bind substrates targeted for autophagy and bring them to the forming autophagosome through interaction with LC3 II. The autophagosome matures upon fusion with lysosomes, and the cargo is degraded. Figure modified from *Rubinsztein, et al.*³⁴.

Several bacteria, including mycobacteria, have developed mechanisms to avoid killing by autophagy. Some bacteria and viruses can even use the autophagosome for their own advantage, for replication and spread³⁵. *M. tuberculosis* can survive within autophagosomes in macrophages by inhibiting the phagosomal-lysosomal fusion³⁶. As autophagy can contribute to antigen presentation of peptides derived from intracellular pathogens³⁷, mycobacteria might thus effectively inhibit presentation of antigens and initiation of the adaptive immune response. Gutierrez, *et al.*³⁸ showed that induction of autophagy, either by starvation or pharmaceutically by rapamycin, led to phagosomal maturation, and co-localisation of the mycobacterial phagosome with LC3. Induction of autophagy was shown to decrease the intracellular survival of mycobacteria.

Jagannath, *et al.*³⁹ showed that induction of autophagy can enhance the vaccine efficacy against tuberculosis in a mouse model. They found that autophagy induced by rapamycin enhanced the presentation of the dominant mycobacterial antigen Ag85B via the MHC II pathway on APCs. The antigen is associated with the mycobacterial cell wall, but is also secreted into the cytosol of the infected host cell. This study also found that the viability of the mycobacteria was significantly reduced when autophagy was induced³⁹.

The interplay between autophagy and inflammation, and their regulation of each other has gained more attention in the later years. Proinflammatory cytokines can induce autophagy, while autophagy can positively or negatively regulate the transcription of proinflammatory cytokines⁴⁰. Evidence also suggests that antituberculosis drugs promote autophagy through the generation of ROS in the infected cells⁴⁰. However, the mechanisms of interaction between autophagy and inflammation are many and complex, and much is still to be learned.

1.2.3 The KEAP1-NRF2 pathway

Many mechanisms are applied by a macrophage infected with mycobacteria in order to kill the invading pathogen. Production of ROS is one such mechanism, although pathogenic mycobacteria are relatively ROS resistant^{41,42}. Uncontrolled levels of ROS can be damaging to cells, and the cell itself has strategies to modulate these levels. Kelch-like ECH-associated protein 1 (KEAP1) is a sensor for ROS, and plays an important role in protection against oxidative and xenobiotic damage through regulation of the transcription factor Nuclear Factor Erythroid-2-related Factor 2 (NRF2). NRF2 target genes are involved in glutathione synthesis, elimination of ROS, detoxification of xenobiotics, and drug transport. Under normal conditions KEAP1 is bound to NRF2, which is polyubiquitinated and rapidly degraded in a 26S proteasomal dependent manner^{43,44}. Generally, ubiquitination is carried out by a complex of 3 partners: E1, E2, and E3. E1 is the ubiquitin activating enzyme and E2 is the ubiquitin carrier protein (conjugating enzyme). The E3 ubiquitin-protein ligase enzyme serves to recognise the target substrate and is required for polyubiquitination⁴⁵. The KEAP1 protein in itself is not responsible for the ubiquitination of NRF2, but it is a substrate adaptor protein that recruits NRF2 to the Cullin3 (Cul3)-based E3 ubiquitin ligase system. In this system Cul3 is a subunit of the E3 ligase enzyme and its interaction partner ring-box 1 (Rbx1) is the E2 conjugating enzyme⁴⁶.

The KEAP1 protein has several cysteine residues which contain reactive thiol groups. Upon oxidative or electrophilic stress these thiol residues are modified, leading to a decrease

in the E3 ligase activity of KEAP1 and hence stabilisation of NRF2. This stabilisation allows the transcription factor to translocate into the nucleus and activate transcription of its target genes⁴⁴. Katoh, et al.⁴⁷ demonstrated the role of KEAP1 in NRF2 regulation by showing that under homeostatic conditions, the half-life of NRF2 went from 20.2 minutes in the absence of KEAP1, to 11.5 minutes in the presence of KEAP1. In a NRF2 mutant where the binding site for KEAP1 was altered, the presence or absence of KEAP1 did not affect the half-life of the transcription factor. The level of NRF2 mRNA was not significantly increased when the level of the protein increased, which suggests that it is the stabilisation of existing protein upon oxidative stress, rather than an increased transcription that leads to NRF2 accumulation in the cell^{48,49}. Other mechanisms suggested to contribute to regulation of NRF2 levels in the cell include autoregulation, microRNAs, phosphorylation/acetylation, protein stability, and cysteine modifications on NRF2⁵⁰.

Figure 1.4 shows the different domains on KEAP1 and NRF2 and the function the different domains have in the protein. KEAP1 is believed to function as a homodimer where two KEAP1 proteins interact with one NRF2 protein. The Neh2 domain on NRF2 binds to the DC domain of KEAP1⁴⁴. There are two motives on the Neh2 domain, DLG and ETGE which both binds to KEAP1 via the DC domain in a similar manner, but with different binding affinities. The hinge and latch hypothesis suggests that ETGE, which has a higher affinity, binds first and functions as a hinge, while DLG has lower affinity and functions as a latch in the binding of NRF2 to KEAP1⁵¹. Cul3 interacts with the IVR and BTB regions on KEAP1⁴⁶.

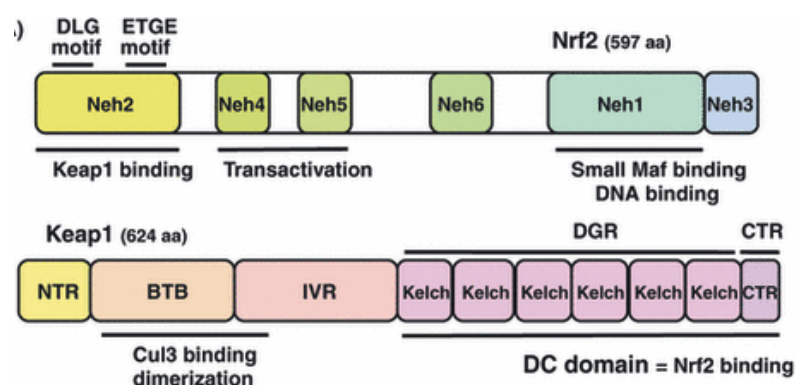


Figure 1.4: Domain structure of NRF2 and KEAP1. NRF2 consist of 6 NRF2-ECH homology (Neh) domains, where Neh2 is the binding domain for KEAP1. KEAP1 has an N-terminal region (NTR), a BTB region, a intervening region (IVR), a DC domain, and a C-terminal domain (CTR). The DC domain serves as the binding site for NRF2, and the BTB and IVR regions serve as binding site for Cul3⁴⁴.

Another substrate that interacts with KEAP1 is the mammalian sequestosome 1 (p62/SQSTM1, hereafter referred to as p62), which is able to disturb the binding between KEAP1 and NRF2, and hence activates transcription of NRF2 target genes⁴³. p62 protein has various roles in the cell and is amongst others involved in the selective autophagy where it serves as a cargo receptor. p62 binds ubiquitin on proteins that have been targeted for degradation, and brings them into forming autophagosomes via association with LC3II^{43,50}. Komatsu, *et al.*⁵² showed that p62 stabilises NRF2 in autophagy-deficient mice and hence leads to transcription of NRF2 target genes. p62 has a KEAP1-interacting region (KIR) that is conserved across species and binds to the DC domain on KEAP1, the same region where the NRF2 Neh2 region interact. The binding affinity of p62 to KEAP1 makes it able to compete with NRF2 for the binding site of the low affinity DLG motif, but not the high affinity ETGE motif⁵². When p62 binds to KEAP1, NRF2 is released and free to translocate to the nucleus and transcribe its target genes. The affinity of p62 to KEAP1 is also markedly increased when p62 is phosphorylated on S351, a serine residue found in KIR domain of the protein⁵³. This increased affinity leads to activation of NRF2 and degradation of KEAP1 by autophagy.

Much research on the KEAP1 pathway has focused on its implications in cancer, as mutations in KEAP1 have been found in various cancer types⁴⁴. These mutations can lead to the constant activation of NRF2, which in turn can lead to resistance to anti-cancer drugs as well as cell proliferation. Less focus has been given to the role of KEAP1 in other diseases, including inflammation and infection. However, recent research has started to focus more on the role of this pathway in relation to infectious diseases, including mycobacterial infections.

KEAP1 has been shown to have other roles in addition to the regulation of NRF2. Lee, *et al.*⁵⁴ showed that KEAP1 functions as a Cul3-based E3 ubiquitin ligase for IKK β and hence downregulates the NF- κ B signalling by targeting IKK β for proteasomal degradation. IKK β contains an ETGE motif, similar to other substrates that bind KEAP1, and interact with the DC domain on KEAP1⁵⁴. NF- κ B is a transcription factor whose target genes include genes involved in the immune response, cell proliferation, angiogenesis, cell survival, metastasis, and more.

Data from our group shows that knocking down KEAP1 increases the production of NF- κ B and IRF regulated cytokines as well as autophagy in response to infection with *M. avium* in primary human macrophages⁵⁵. The intracellular survival of *M. avium* decreased following knockdown of KEAP1, suggesting that the protein is beneficial for the survival of mycobacteria within the macrophage. The exact mechanism behind this KEAP1-mediated

regulation of mycobacterial infections and what interacting partners of KEAP1 are involved is not yet understood. Preliminary unpublished results from our group also indicate that KEAP1 may influence antigen-presentation and T cell immunity to *M. avium* infections.

1.3 Adaptive immunity in mycobacterial infection

The innate immune system is the first defence when we are exposed to pathogens. This response is in some cases enough to stop the infection and kill the microbe. In many cases, however, there is a need for the adaptive immune system to participate in the defence. Cells of the adaptive immune system are central in the control of mycobacterial infections⁵⁶. The innate immune cells phagocytose bacteria or other invading pathogens and recruit adaptive immune cells through cytokine production and antigen presentation. We can hence say that the adaptive immune response is shaped by the innate response.

The adaptive immune system consists of T-lymphocytes and B-lymphocytes, also referred to as T cells and B-cells. The T cells arise from hematopoietic progenitor cells in the bone marrow, before they migrate to the thymus where they develop into immunocompetent naïve T cells. In the thymus, T cell progenitors (thymocytes) go through a developmental and selective process where they develop T cell receptors (TCRs) that are specific for one antigen. All mature T cells express cluster of differentiation (CD)3 on the surface of the cell, and the major subsets in addition express either CD4 or CD8 (termed CD4+ and CD8+, respectively). CD8+ T cells recognise antigens presented on MHC class I, while CD4+ T cells recognise antigens presented on MHC class II. After development, T cells migrate to the secondary lymphoid organs such as the lymph node and spleen, where they can interact with antigens. Antigens are only recognised when presented by a MHC molecule on the surface of a host cell⁵⁷. T cell-APC interaction involves many molecules, and an immunological synapse is formed at the interphase between the two cells (**Figure 1.5**)⁵⁸. Two signals are required for a productive immune response to occur. The first signal is when the TCR recognises a peptide presented via a MHC molecule on APCs. The second signal occurs when the co-stimulatory molecule CD28 on the T cell interacts with co-stimulatory molecules CD80 or CD86 on APCs⁵⁹.

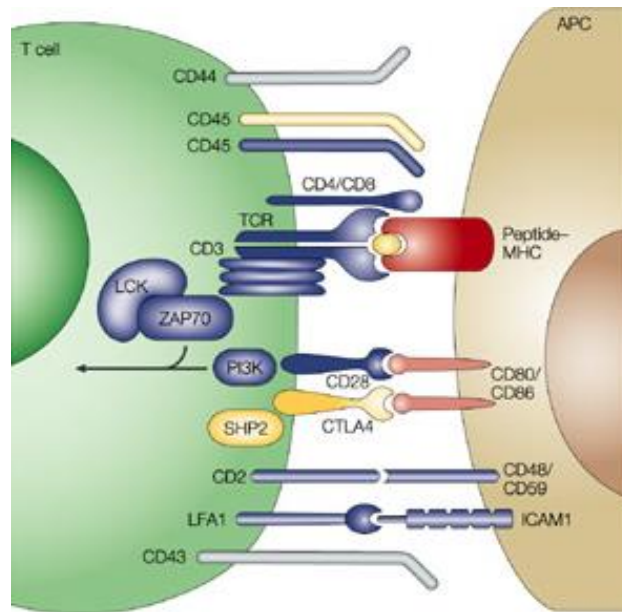


Figure 1.5: The immunological synapse. The interaction between a T cell and an APC involves many molecules, both stimulatory and inhibitory. The stimulatory molecules are shown in red, activating/co-stimulatory molecules are shown in red, and inhibitory molecules are shown in yellow. Figure modified from Huppa and Davis⁵⁸.

After activation by encountering a specific antigen, T cells differentiate into effector cells, and some cells survive as long lived memory cells. This memory allows for a rapid immune response upon subsequent exposure to the same antigen. CD4⁺ T cells have a helper function, and have been termed T_H cells. There are several subsets of T_H cells, differing in the panel of cytokines they produce. T_H1 cells produce cytokines that drive cell mediated responses such as activating mononuclear phagocytes, NK cells and cytotoxic T cells important for killing of intracellular pathogens. T_H2 cells produce cytokines enhancing the antibody production from B-cells. Other subsets include T_H17 which has roles in induction of inflammatory responses and autoimmunity, and T_{REG} cells which dampens the immune response⁵⁷. CD8⁺ T cells are circulating cells, and act to remove cells harbouring intracellular pathogens, cells infected with viruses, and cells that have been transformed. The CD8⁺ T cells mainly recognise cytosol-derived antigens⁵⁷.

T cells, in particular CD4⁺ T_H1 cells, are required for the protective immunity against pathogenic mycobacteria such as *M. tuberculosis*^{56,60}. T_H1 cells produce a range of cytokines, including IFN γ , TNF α , and IL2 aiding the phagocytic cells in killing pathogens⁵⁷. Upon infection with mycobacteria, a balanced activation of CD4⁺ as well as CD8⁺ T cells seems to be important to gain control of the intracellular bacteria⁶¹.

B-cells arise from hemopoietic stem cells in the bone marrow and are the source of humoral immunity. These cells develop into plasma cells that produce antibodies upon signals

from T cells or other immune cells⁵⁷. Most research on the role of the adaptive immune system in response to mycobacterial infections has focused on the role of T cells, as will be the focus of this thesis. However, there is also increasing interest in the role of B-cell mediated immunity in relation to mycobacterial infection^{62,63}.

1.4 Cytokines in mycobacterial infection

Cytokines produced both by innate and adaptive immune cells have varying roles in the response to mycobacterial infections, both in limiting and enhancing the growth and survival of bacteria. IFN γ is a type II IFN and is a pro-inflammatory cytokine. It is recognised by cells through IFN γ receptor (IFN γ R), and is one of the key activators of macrophages, with an important role in immune response to mycobacteria and other intracellular pathogens⁶⁴. This cytokine is produced by CD4+ T cells, as well as other cells in the body. Although other immune cells can also produce IFN γ , evidence suggests that the production from T cells is essential for long term control of mycobacterial infection⁶⁵. IL12 is produced by innate immune cells following stimulation of TLRs, and deficiency in this cytokine or its receptor is observed to result in an increased susceptibility to disease. It has been shown to be important for DC migration to the lymph node and T cell activation. Also, there is a reduction in T cells producing IFN γ in IL12 deficient mouse models⁶⁶. IP10 is a chemokine produced in response to IFN γ in many cell types, including macrophages. This cytokine works as a chemoattractant for other immune cells. Infusion of the type I IFN IFN β showed a decrease in bacterial survival in *M. avium* infection, but the opposite was seen in infections with *M. tuberculosis*⁶⁴. Production of IL10 on the other hand is beneficial for mycobacterial survival. This is an anti-inflammatory cytokine that aids the blockage of phagosomal maturation, seen both for virulent and attenuated strains of mycobacteria, and leads to enhanced bacterial survival and replication⁶⁷. It has also been shown that type I IFNs can induce the expression of IL10. This might explain why these IFNs can be beneficial to pathogenic mycobacteria^{64,68,69}. TNF α is a cytokine produced by both innate and adaptive immune cells, and is recognised by the TNF α receptor on the plasma membrane. TNF α plays several roles in a cell, including roles in apoptosis, activation, recruitment, and differentiation⁷⁰. Evidence for a role of this cytokine in protection against mycobacteria has been present for a long time. Infusion of TNF α has showed increased resistance to infection with both *M. tuberculosis* and *M. avium*, and both the cytokine itself and its receptor have been shown essential for protection against *M. tuberculosis* in mice⁷¹. TNF α also has a role in the granulomas. It is not needed for the formation of the granuloma, but for the stability through limiting bacterial replication within the macrophages and inhibition of necrosis⁷². IL2 is a type I cytokine, mainly produced by

CD4⁺ T cells after activation with specific antigens. IL2 mainly signals to T cells, B-cells and NK cells, where it leads to cellular growth, differentiation, survival, and activation induced cell death⁷³.

The production of cytokines from T cells is important in the control of mycobacterial infection, and a combination of cytokines can act together to produce a stronger immune response. Multifunctional T cells, T cells producing several cytokines, have been implicated to have an important role in mycobacterial protection. It has been seen that the presence of a large number of multifunctional T_H1-cells (producing IFN γ , TNF α , and IL2) in the lung correlates with a reduced burden of mycobacteria⁷⁴. In murine macrophages infected with *M. avium*, IFN γ stimulation alone was not enough to induce a strong bactericidal response, but inducing TNF α as a second signal gave acidification of the mycobacterial-containing phagosome and increased bacterial killing⁷⁵.

Many aspects regarding the host immune responses against infection with *M. tuberculosis* are well established, although much remains unclear. Much of the work carried out has been done in mice using different strains of *M. tuberculosis*, while less work has been carried out using the opportunistic pathogen *M. avium*. The oxidative stress sensor KEAP1 has been shown to be involved in regulation of inflammation and autophagy following *M. avium* infection, although the mechanism behind this regulation remains unclear. T cells undoubtedly have an important role in the host defence against mycobacteria, but the role of multifunctional T cells in *M. avium* infections in humans has not yet been established. If and how KEAP1 regulate antigen presentation and hence activation of the adaptive immune system is neither known.

2 Aims and objectives

M. avium infection induces an inflammatory response in primary human macrophages. KEAP1 appears to have a negative regulatory effect on this inflammatory response as knockdown of KEAP1 increases the inflammatory response following *M. avium* infection. The underlying mechanisms are not fully understood, neither are the consequences for shaping of the mycobacterium-specific adaptive immune response. The aims of this thesis are to investigate the mechanism in which KEAP1 regulate the inflammatory response in primary human macrophages following *M. avium* infection, and if KEAP1 affects antigen presentation and the following adaptive immune response.

Specific aims include:

1. Investigate if the mechanism by which KEAP1 regulates *M. avium* induced inflammation can be dissected using Human embryonic kidney (HEK) 293 cells as a model system.
2. Investigate the role of the ubiquitination-associated KEAP1 interaction partners Cul3 and Rbx1 in the regulation of inflammatory responses following *M. avium* infection in primary human macrophages.
3. Investigate if KEAP1 modulates antigen-presentation in response to *M. avium* infection in primary human macrophages, and subsequently affects the adaptive mycobacteria-specific CD4⁺ T cell response.

3 Materials and methods

3.1 Isolation and seeding of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) isolated from buffy coats from healthy donors were used for all experiments with human cells. The buffy coats were obtained from the blood bank at St. Olavs hospital, and the use of PBMCs from healthy blood donors has been approved by the Regional Committee for Medical and Health Research Ethics at NTNU. Isolation was done by density gradient centrifugation, where buffy coat diluted in 100 ml Dulbecco's phosphate buffered saline (PBS, Sigma-Aldrich) was layered on top of lymphoprep (Axis shield) in 50ml falcon tubes. The tubes were spun down at 645 RCF for 20 minutes without brakes and at 20°C. Lymphoprep has a density of 1.077 g/ml, which allows for the isolation of mononuclear cells which have a lower density. The mononuclear cells will be seen as a thin creamy layer on the interphase between the lymphoprep and the serum/PBS after centrifugation. Erythrocytes and granulocytes have a higher density and will pass through the solution and form a pellet at the bottom of the tube. The layer containing PBMCs was pipetted out and transferred to new tubes. No lymphoprep was transferred, as this is toxic to the cells. The cell suspension was then spun down again at 800 RCF for 10 minutes, now with brakes 7-7. The supernatant was removed by decanting, and the pellet resuspended in Hanks' balanced salt solution (Sigma-Aldrich) preheated to 37°C. The cells were spun down again, now at 127 RCF for 8 minutes. This step was repeated four times, in order to remove platelets, and purify the PBMCs as much as possible. After the fourth wash the cells were resuspended in 5 ml of RPMI-1640 (RPMI) (Sigma-Aldrich) media supplemented with HEPES (Gibco, life technologies) and 2mM L-glutamine (Sigma-Aldrich), followed by counting using a Countess automated cell counter (Invitrogen). Before using the Countess to quantify the number of cells, 10µl of the cell suspension (diluted 100:1) was mixed with 10µl of trypan blue (Life science). Trypan blue is a stain that is excluded by live cells, while dead cells are stained blue. The number of live cells/ml was used for further calculations.

3.1.1 Differentiation of monocytes to monocyte derived macrophages

For generation of macrophages from monocytes, the isolated PBMCs were diluted to a concentration of 5×10^6 cells/ml in RPMI with 5% human serum, and plated out in appropriate tissue culture plates (24 well or 96 well, Costar). Monocytes makes up approximately 10% of isolated PBMCs⁷⁶, and ten times the intended final number was therefore added per well. The cells were left in the incubator at 37°C and 5% CO₂ for 1 hour to allow monocytes to adhere to the bottom of the well. The wells were then washed at least three times with Hanks'

preheated to 37°C, in order to remove any non-adherent cells. Following the last wash, the Hanks' was replaced with RPMI media with 30% human serum. After washing, approximately 250 000 cells were estimated to be attached per well in a 24 well plate. Cells were incubated at 37°C and 5% CO₂. The media was changed to RPMI with 10% human serum after 4-5 days of incubation, when the monocytes had differentiated into monocyte derived macrophages (MDM). From this point, the MDMs were ready to use for further experiments.

3.1.2 Expansion of T cells

In the PBMC suspension there will be both APCs and lymphocytes. In the expansion step, the fraction of T cells specific to antigens of interest is increased compared to non-specific cells. Expansion activates the rare population of T cells that specifically recognise particular antigens (heat-killed *M. avium* in this thesis) presented by APCs (contained in the PBMC culture). The APCs will ingest heat-killed *M. avium* and present antigens to mycobacterium-specific T cells, which in turn will be activated. The activated specific T cells will proliferate, resulting in a higher fraction of specific T cells than what was present in the PBMCs after isolation. For the expansion of T cells, the PBMCs were diluted to a concentration of 3x10⁶ cells/ml in RPMI media containing 10% human serum, and 0.5 ml of the cell suspension was added per well in a 24 well plate. For the purpose of the experiments carried out in this thesis, mycobacterium-specific T cells were desired. To achieve this, heat-killed *M. avium* at desired concentrations was added to the suspension. IL2 (RnD Biosystems), which is important for the growth and proliferation of T cells, was added on day 2 at a concentration of 20U/ml. After 7-10 days of expansion, cells were used for experiments.

3.1.3 Restimulation of T cells

After 7-10 days of T cell expansion with heat-killed *M. avium*, the cells were restimulated with autologous macrophages generated from the same donor at day 0. Before restimulation the T cells were pooled into a 15ml tube, diluted in Hanks', and spun down at 528 RCF for 5 minutes. The supernatant was removed and the cells resuspended in pre-heated (37°C) RPMI media with 10% human serum. Cells were counted using the Countess, and T cells were added to the macrophages with a ratio of 10:1 of T cells to the estimated macrophage number of 250 000 macrophages per well (24 well plate). Prior to T cell restimulation macrophages were infected with *M. avium* for 4 hours (**Section 3.2.1**). T cells were restimulated for the desired time (varies between experiments) and for the last 4 hours of the restimulation time, Protein Transport Inhibitor Cocktail (eBioscience) was added to the cells. The transport

inhibitor contains Brefeldin A and monensin, which inhibits the secretory/transport pathway and results in accumulation of secreted proteins in the endoplasmic reticulum and Golgi apparatus (as described in the manufacturers' protocol). This accumulation later allows detection of intracellular cytokines, which would normally be secreted, using flow cytometry. As a positive control 1-2 wells with T cells were stimulated with a Cell Stimulation Cocktail (eBioscience) containing phorbol 12-myristate 13-acetate (PMA) and ionomycin. This cocktail activates the T cells to produce cytokines (as described in the manufacturers' protocol). These samples gave an indication of T cells functionality, and the functionality of the protocol for the flow cytometry staining. The T cells were restimulated with the stimulation cocktail overnight or as long as the remaining T cells were co-cultured with macrophages.

3.2 Cultivation and preparation of *M. avium*

A transformant of the virulent *Mycobacterium avium* clone 104 containing firefly luciferase, as described elsewhere⁷⁷, was used in this thesis.

The bacteria stock was stored in freezing media (bacterial suspension in 21% glycerol) at -80°C. To start a new culture, bacteria were thawed and 250µl of the stock suspension was added to 7ml of Middlebrook 7H9 complete media (Difco/Becton Dickinson) supplemented with glycerol, Tween 80, and albumin dextrose catalase. This media is commonly used for the cultivation of mycobacteria, and contains all nutrients needed for its growth. The culture was incubated on a shaker at 37°C for 3-6 days. The optical density (OD) for cultures used for experiments was between 0.3 and 0.6.

3.2.1 Infection

Bacterial culture for infection was prepared as described above. At the day of infection, the OD was measured using a spectrophotometer (Genesys 20, Thermo scientific) using a wavelength of 600nm. 1 ml of bacterial suspension was added to Eppendorf tubes, which was centrifuged at 9300 RCF for 2 minutes. The bacterial growth media containing Tween, which is toxic to human cells, was removed and the pellet was resuspended in 1 ml PBS. Sonication and vortexing was carried out to create a single cell suspension. Due to bacterial loss in the previous step, the OD was measured again. Knowing that OD 1=4.5x10⁸ cells/ml (calculated from serial dilutions and colony forming unit (CFU) counts) the number of CFUs per ml can be calculated from the OD measured. The CFU count is confirmed by plating the suspension on Middlebrook 7H10 agar (Becton Dickinson GmbH) at increasing dilutions, and the colonies were counted after approximately one week incubation at 37°C and 5% CO₂. Before

infection, the medium of the cells to be infected was changed, followed by addition of the bacterial suspension at the desired multiplicities of infection (MOI). After 4 hours of infection, the media containing bacteria were removed, and cells were harvested for analysis or used further in experiments.

3.2.2 Heat-killing

For heat-killing of *M. avium*, a culture was prepared as explained above.

Heat-killing of bacteria was done by incubating the bacterial suspension on a heat block (QBH2, Grant). To find optimal conditions to heat-kill bacteria, different temperatures and times were tested, followed by CFU counts and confocal microscopy. For this purpose *M. avium* dsRed (made in our lab) was used to allow visualisation of the bacteria in confocal microscopy. After treatment the bacteria were plated on 7H10 agar and examined by confocal microscopy to investigate the viability. The treatments tested were: 90°C for 30 minutes, 95°C for 20 or 30 minutes, and 72°C for 24 hours. The bacterial fluorescence was only visible in the bacteria subjected to 72°C for 24 hours. The bacteria were killed both at 72°C for 24 hours and 95°C for 30 minutes (data not shown). For the purpose of this thesis, 95°C for 30 minutes was used. After killing, the bacterial suspension can be stored at -20°C until needed.

3.3 Human embryonic kidney 293 (HEK293) cells

The HEK293 cells were cultured in Dulbecco's modified eagles medium (DMEM) (BioWhittaker) supplemented with 10% fetal calf serum (FCS) (Invitrogen) and 2nM L-glutamine. The cells were cultured in the presence of antibiotics, however, no antibiotics were present during experimental conditions. Two different HEK293 cell lines were used in this thesis: HEK293T (Thermo Scientific Open Biosystems) containing a T antigen and HEK293 TLR2-GFP cells (kindly provided by Nadra Nilsen) stably transfected with TLR2 tagged with green fluorescent protein (GFP). HEK293T was cultured in DMEM containing the antibiotic penicillin streptomycin (PenStrep) (Sigma-Aldrich) and the HEK293 TLR2-GFP cells in DMEM containing Geneticin (G418) (Gibco, Life technologies).

3.3.1 Thawing of HEK293 cells

The cell stock is stored in liquid nitrogen in freezing media, consisting of DMEM with 30% FCS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Before thawing the cells, all reagents needed were heated to 37°C. The cells were transferred to a 50ml tube containing 10ml preheated DMEM as soon as they were thawed, as DMSO is toxic to the cells. The cell suspension was centrifuged at 528 RCF for 5 minutes. The supernatant was removed by

decanting and the cells resuspended in 5ml preheated DMEM by careful pipetting. The cells were then transferred to a T25 tissue flask, and incubated at 37°C and 8% CO₂.

3.3.2 Maintenance and seeding of HEK293 cells

In order to split HEK293 cells, the adherent monolayer of cells was washed with preheated PBS before trypsin-ethylenediaminetetraacetic acid (TE) (Gibco) was added for approximately 2 minutes to detach the cells from the flask. The cell suspension was moved to a 50ml tube and the flask flushed with PBS again. The suspension was centrifuged at 528 RCF for 5 minutes, the supernatant removed, and cells resuspended in a small amount of DMEM. To avoid clumping, the tube was flicked gently and the cell suspension pipetted up and down. Cells were counted using the Countess automated cell counter, as previously described (**Section 3.1**). For further culturing, approximately 10ml DMEM (with appropriate antibiotics) containing 1×10^5 cells/ml were seeded out in a T75 tissue flask. Cells were incubated at 37°C and 8% CO₂. Splitting of the cell stock was carried out 2-3 times a week. For use in experiments the cells were resuspended in media without antibiotics following centrifugation, counted and seeded out in the appropriate concentration.

3.4 Transfection of cells *in vitro*

Cell transfection is a tool that can be used to investigate the role of proteins and genes within a cell through introducing foreign nucleic acids to the cells. Transfections can be either stable or transient, depending on the material introduced to the cell. In stable transfections the genetic material is incorporated into the cell's own DNA and is present also after the cell divides. Transiently transfected cells do not incorporate the nucleic acids into their genome, and are only functional for a limited period of time⁷⁸.

There are several ways cells can be transfected. These can broadly be categorised into biological, chemical, and physical methods. Biological methods include virus transfection, where a virus is used to introduce the foreign genetic material into the cell. Physical methods include microinjections, biolistic particle delivery, electroporation, and laserbased transfection. These methods involve the direct insertion of nucleic acids into the cell or disturbance of the cell membrane that allows nucleic acids to pass. The principle of chemical transfection is that positively charged chemicals bind to DNA, and this positively charged complex is attracted to the negative cell membrane and taken up in an unknown fashion⁷⁸. Chemical transfection was used throughout this thesis.

3.4.1 RNA interference

RNA interference (RNAi) is a gene silencing pathway which is triggered by double stranded RNA (dsRNA), and the interference occurs post transcription of messenger RNA (mRNA). Small interfering RNA (siRNA) is one type of dsRNA that causes post transcriptional gene silencing. Mature mRNA is exported from the nucleus to the plasma membrane, where the siRNAs have their effects. The principle of siRNA is shown in **Figure 3.1**. The siRNAs are derived from longer double stranded RNAs (dsRNAs) which can either be produced by the cell itself, or be introduced experimentally. dsRNA binds to the endonuclease enzyme Dicer, which cuts the siRNA into short fragments, approximately 21 nucleotides long. These short fragments then bind to the effector complex RNA-induced silencing complex (RISC). Here one strand is chosen as a guide strand, which continues to be bound to the complex, while the other strand is degraded. The guide strand directs RISC to complementary regions on target mRNA through Watson-Crick base pairing. Once bound the RISK complex catalyses cleavage of the mRNA by the RNaseH enzyme Argonaute (Slicer), degrading it. The degradation of mRNA before it is translated leads to reduced or depleted expression of the mature protein in the cell⁷⁹.

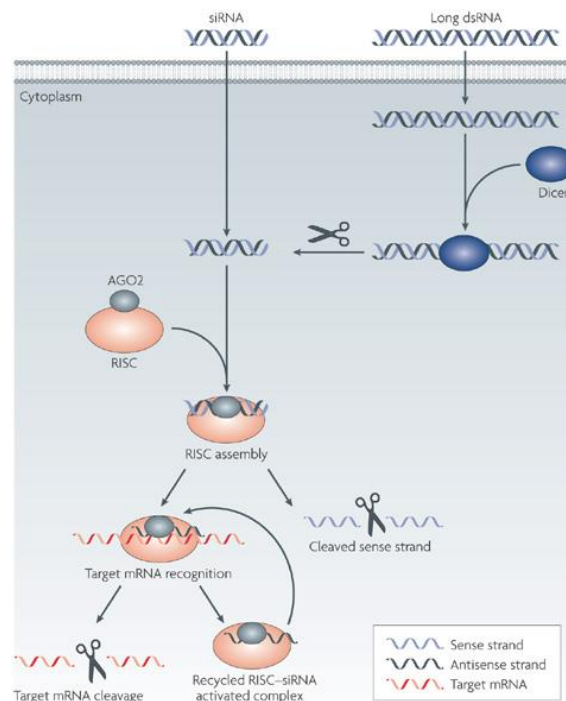


Figure 3.1: The principle of siRNA silencing Long dsRNA is introduced to the cell, and cleaved into shorter siRNAs by the enzyme Dicer. Alternatively the siRNA can be directly introduced to the cell. The siRNA then assembles with the RISC complex, where one strand (sense strand) is cleaved by argonaute (AGO2)/Slicer, while the guide strand (antisense) stays associated with the complex. The antisense strand is used to target out complementary mRNA, which is then cleaved. The RISC complex can be recycled, and target more mRNA⁸⁰.

3.4.1.1 siRNA transfection in vitro

Transient transfection of synthetically produced siRNAs (Qiagen) was used for altering of gene expression in cells throughout this thesis. The siRNA was chemically delivered via lipofection by the use of siLentFect lipid (Bio Rad), which is a proprietary mix of cationic compound and a co-lipid developed for the delivery of siRNA to mammalian cells in culture (as described in the manufacturers' protocol). Transfection of siRNA targeting KEAP1, Cul3, and Rbx1 were all carried out using siLentFect. The siRNA for any specific gene used was a pool of 4 different siRNAs specific for the same transcript (for details see **Appendix 1**). AllStar (AS) negative control (Qiagen) was used as a non-targeting negative control for the siRNA. Analysis of the effect of the treatment was done using real-time quantitative PCR (**Section 3.5**).

3.4.1.2 siRNA treatment of human monocyte derived macrophages

For siRNA knockdown in human primary macrophages, cells were seeded and differentiated as described in **Section 3.1.1**. After 5-7 days in culture, the media was changed to RPMI with 10% human serum, and the cells were transfected. Optimization of siRNA treatment of human primary macrophages has previously been carried out in our lab, and has shown that the best knockdown is seen after two treatments, at 0 hours and 48 hours, followed by further experiments at 72 hours. 0 hours is defined as the time where the treatment is started. Two solutions were prepared with OptiMem (Gibco, Life technologies) (50µl per well in a 24 well plate) plus either siLentFect (1.5µl per well in a 24 well plate) or siRNA (20nM). The two solutions were then mixed together through careful pipetting. The solution was left to incubate at room temperature for 15-20 minutes allowing siLentFect to form a lipid membrane around the siRNA. Following incubation, the solution was added to the cells, taking care to spread it evenly.

3.4.1.3 Optimisation of siRNA treatment of HEK293 cells

Different cell types differ in their response to various treatments, and protocols should be optimised to each cell type. An optimisation for siRNA treatment of the HEK293 cells was therefore carried out. HEK293 TLR2-GFP cells were prepared as described in **Section 3.3.2**, and left overnight. siRNA targeting KEAP1 was prepared as described in **Section 3.4.1.2**. Cells were either transfected 1 time and left for 48 hours in transfection media, or treated 2 times at 0 hours and 48 hours (as the primary human macrophages), and knockdown was measured after 72 hours. This experiment showed that the knockdown effect was high (more than 90% less expression of the mRNA of interest) already after 1 treatment and 48 hours, and

2 treatments did not increase the knockdown efficiency (**Figure 3.2 A**). For some experiments, the HEK293 cells were used for further transfections, and we therefore wanted to investigate the persistence of the knockdown effect after changing the medium containing siRNA. Cells were prepared as described above, and treated with siRNA (non-targeting (AS) or KEAP1) one time. The media was either changed after 24 hours, or left unchanged. This showed that the knockdown of KEAP1 in the cells where the media was changed after 24 hours was as good as in the cells left in siRNA containing media (**Figure 3.2 B**). This suggests that the media can be changed 24 hours after siRNA treatment, and the knockdown effect will persist for at least 96 hours from the start of the treatment. The mRNA levels were measured using real-time PCR.

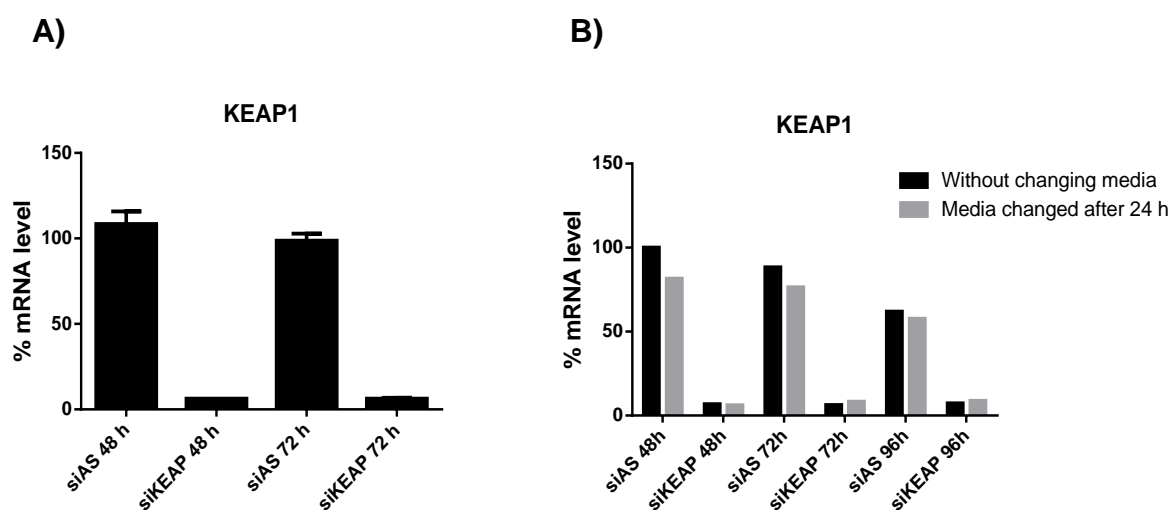


Figure 3.2: Establishment of siRNA treatment of HEK293 cells. A) Shows the knockdown effect of KEAP1 compared to non-targeting control (AS) in HEK293 TLR2-GFP cells, after 1 siRNA treatment and 48 hours in siRNA media, and 2 treatments after 0 and 48 hours and 72 hours of siRNA treatment. B) Shows the persistence of the knockdown effect of KEAP1 after 1 treatment in HEK293 TLR2-GFP cells. Comparing cells left in the siRNA containing media with cells where the media was changed after 24 hours of siRNA treatment. mRNA levels measured using real-time PCR, and normalised to GAPDH (see **Section 3.5**). Data in A) collected from one experiment in duplicates, and B) from one experiment and single wells.

3.4.2 Transfection of plasmids into HEK293 cells *in vitro*

To obtain expression of specific genes HEK293 cells were transfected with plasmids containing the genes of interest. For this transfection, GeneJuice Transfection reagent (Novagen) was used. This reagent is composed of nontoxic cellular proteins and a small amount of a novel polyamine (as described in the manufacturers' protocol).

3.4.2.1 Transfection of CD14 and pcDNA-cherry

The GeneJuice transfection method was used to introduce plasmids containing pcDNA-CD14^{81,82} or pcDNA-cherry. The protocol for GeneJuice transfection for a 24 well plate is described below.

1. The day before transfection, cells were seeded out at a concentration of approximately 150 000 cells/ml and 0.5ml was added per well. Cells that had previously been siRNA treated were also used. The cells should be approximately 50% confluent when transfected. Cells were left to incubate at 37°C and 8% CO₂.
2. For each well to be transfected, 25µl of serum free media (DMEM) and 3µl GeneJuice per 1µg DNA was mixed in a sterile tube. The solution was mixed thoroughly by vortexing.
3. The solution was incubated at room temperature for 5 minutes.
4. The desired concentration of plasmid per well was added to the above mixture and mixed by careful pipetting and incubated for 10-15 minutes at room temperature.
5. 25µl of the mixture was added per well, in droplets. The plate was rocked to ensure an even distribution.
6. The plates were incubated for 24-72 hours at 37°C and 8% CO₂.
7. Cells were now ready for stimulation and harvesting for analysis.

For pcDNA-CD14 and pcDNA-cherry, 0.15µg of the plasmid was added per well.

3.4.2.2 ELAM-Luciferase and IFNβ-Luciferase assay

We wanted to investigate the activation of NF-κB and transcription of IFNβ in HEK293 cells following stimulation. For this purpose we used a luciferase assay, where cells were transfected with plasmids containing the gene of interest and a reporter gene. ELAM-luciferase assay (Promega) was used as a reporter for NF-κB activation, and contains the promoter of Endothelial Leucocyte Adhesion Molecule-1 (ELAM) containing a NF-κB site, fused with the firefly luciferase gene. Upon NF-κB activation luciferase enzyme is produced, which can then be detected by adding luciferase substrate and measure fluorescence. The IFNβ reporter plasmid (provided by John Hiscott, McGill University, Montreal, Canada) functions in a similar fashion when IFNβ is produced. Cells were also transfected with TLR2^{81,82}, as HEK293 cells do not express this TLR. In order to see whether any activation was due to TLR2 stimulation, some cells were transfected with an empty control plasmid (pcDNA3, Invitrogen). These cells should show low activation compared to cells transfected with TLR2. The experimental procedure for a 96 well plate is described below. In addition to

the plasmids mentioned above, all cells were transfected with a plasmid containing Renilla luciferase (Promega). This was used as a constitutively active control and to measure the transfection efficiency and cell numbers.

1. On day 1, cells were seeded out at a concentration of approximately 1×10^5 cells/ml (100 μ l/well)
2. When cells were approximately 50% confluent (1-2 days) the cells were transiently transfected using GeneJuice. The following was added per well:
 - 50ng ELAM-luc/IFN β -luc
 - 1ng Renilla luc,
 - 10ng TLR2 plasmid
3. The plasmids were diluted in 10 μ l DMEM (with no additions) per well, and Gene Juice was added to a final concentration of 3 μ l/ μ g DNA.
4. The mixture was incubated for 5-15 minutes at room temperature, and 10 μ l was added per well. The cells were incubated until confluent (12-48 hours).
5. On the day of stimulation, the media was carefully removed and 90 μ l fresh media was added. Cells were stimulated with either *M. avium* at a MOI of 10:1, Lipomannan (LM) at 100ng/ml or 500ng/ml, or lipopolysaccharide (LPS) at 50ng/ml for 4 hours in a total of 10 μ l/well.
6. After stimulation, the supernatant was removed and frozen down at -80°C. The cells were then lysed using passive lysis buffer (50 μ l/well) (Promega), and incubated at -20°C overnight for improved lysis.
7. Cells were defrosted to room temperature for at least 2 hours for best lysis. Luciferase substrate (-20°C) and Renilla substrate (colentracine -20°C) were also defrosted to room temperature.
8. 15 μ l per sample lysate was added to two separate optiplates. 35 μ l luciferase substrate was added per well to 1 plate and luciferase fluorescence was measured by Victor 3 luminometer (PerkinElmer). There should be no more than 5 minutes from addition of luciferase substrate until fluorescence measurement. 35 μ l Renilla luciferase substrate was added per well to the other plate and measured in the same manner.

3.5 Real-time quantitative polymerase chain reaction

In order to investigate the efficiency of siRNA treatment and expression of genes of interest, real-time quantitative polymerase chain reaction (real-time PCR) was used. This is a method that allows for quantification of nucleic acids in a sample. DNA templates are replicated

exponentially, giving a relative relationship between the amount of starting target sequence and the amount of PCR product produced per cycle. For the purpose of this thesis, the real-time quantitative TaqMan assay (Life technologies) was used. *Thermus aquaticus* (Taq) is a DNA polymerase with 5' to 3' exonuclease activity. The TaqMan probe has a fluorescent reporter dye on its 5' end and a quencher dye on the 3' end (**Figure 3.3**). As long as the TaqMan probe is intact, the fluorescent reporter and the quencher are in close proximity. This inhibits any fluorescence emitted from the reporter to be detected, as any fluorescence emitted is absorbed by the quencher, following the fluorescence resonance energy transfer (FRET) principle. When a target sequence is present, the specific primer binds to the sequence, which has a TaqMan probe downstream of the primer binding site. The TaqMan probe is cleaved by the 5' nuclease activity of the Taq polymerase, which allows the reporter fluorophore to move away from the quencher, and a fluorescent signal can be detected. For each cycle of the PCR reaction more reporter substrate is cleaved from the probes and the fluorescent signal is therefore proportional to the amount of target sequence in the sample⁸³.

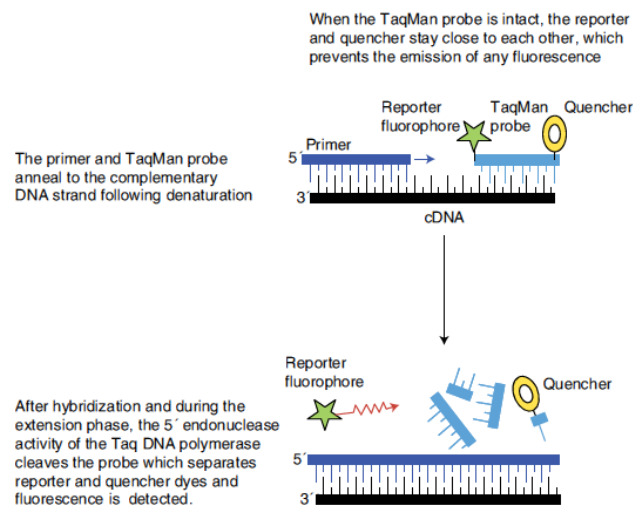


Figure 3.3: The principle of real-time PCR TaqMan assay. When the TaqMan probe is intact no fluorescence is emitted due to the receptor fluorophore and the quencher being in close proximity. When the target sequence is present, a primer binds and transcribes the strand. The TaqMan probe is cleaved, and the fluorophore emits a signal⁸³.

3.5.1 RNA extraction and cDNA synthesis

Cells for real-time PCR analysis were lysed and harvested using 350µl buffer RLT (Qiagen) containing 0.1% β-mercaptoethanol (Sigma-Aldrich). β-mercaptoethanol was added to inactivate RNases in the lysate. The lysate was then added to 2ml sample tubes RB (Qiagen), and frozen down at -80°C until RNA isolation. RNA isolation was carried out using RNeasy

mini kit and QIAcube (Qiagen) including DNase, following the manufacturers' protocol. 350µl cell lysate gives RNA suspended in 50µl water after isolation. The RNA concentration was measured using NanoDrop1000 spectrophotometer (Thermosience) at 260nm. The RNA was then used to produce complementary DNA (cDNA) using High Capacity RNA-to-cDNA Kit (Applied Bioscience). This is a reverse transcription kit which gives high performance with the TaqMan gene expression master mix, among other PCR enzymes. The kit contains an RT buffer mix (2x) and an RT enzyme mix (20x), and is optimised for a 20µl reaction. For every reaction 1µl RT enzyme mix, 10µl RT buffer mix, and 9µl of the previously isolated RNA was mixed. The reaction is incubated as described in **Table 3.1** using a thermal cycler (BioRad).

Table 3.1: Temperatures and times for cDNA synthesis incubation

°C	Time (minutes)
37	60
95	5
4	Forever

The 37°C incubation is the step at which reverse transcription occurs, and the reaction is stopped when heated to 95°C. The cDNA can then be stored at 4°C until further use. The remaining RNA can be stored at -80°C for further use.

3.5.2 Real-time PCR procedure

Real-time PCR was carried out subsequent to cDNA synthesis, for investigation of the expression of genes of interest at the mRNA level. The total volume per real-time PCR reaction should be 20µl and consist of 1-9µl of the sample, 10µl TaqMan Universal Mastermix (Life technologies), 1µl TaqMan Gene Expression Assay mix (contains two primers and a probe) (Life technologies), and water. We aimed to have a concentration of 9-10ng cDNA per reaction although lower concentrations were also used with successful results. Water was added to the cDNA samples to dilute them to a final concentration of between 1 and 10ng/µl. 1-9µl of the diluted sample was then added to each reaction tube depending on the cDNA concentration. A final volume of 20µl was reached by addition of water. For convenience, the total amounts of master mix, assay mix, and water needed per target were first mixed together in a 1.5ml Eppendorf tube, and then allocated to the reaction tubes. The master mix is glycerol based, while the assay mix is water based, therefore the reaction mix was pipetted up and down 5-10 times to ensure proper mixing. MicroAmp

Optical 96-well reaction plates (Applied biosystems) were used for the real-time PCR run. The premixed reaction mix was added to the appropriate wells of the 96 well plate and the samples were then added directly to the plate. The plate was sealed with optical adhesive cover, and centrifuged at 528 RCF for 2 minutes. StepOnePlus (Applied Biosystems) real-time PCR system was used for the PCR run. The location of targets and samples on the plate was specified on the software, and the reaction cycle set as shown in **Table 3.2**.

Table 3.2: The reaction cycle for real-time PCR using TaqMan probes

Degrees	Time (Sec)	
50	120	Hold Stage
95	20	
95	1	Cycling stage (40 cycles)
60	20	

The cycling stage is where the PCR reaction occurs. In the cycling stage the 95°C step denatures the cDNA, and the 60°C step allows for the Taq polymerase to anneal and transcribe its target sequence.

3.5.3 Real-time PCR analysis

From the real-time PCR run we get a threshold cycle (Ct) value, which is defined as the number of cycles required for the fluorescence to be greater than the minimal detection level. The detection level is set by the computer, but can be changed manually if needed. Relative quantification (RQ) or $2^{-\Delta\Delta Ct}$ was used in our analysis, which gives the expression level of a specific gene relative to a reference gene. The $\Delta\Delta Ct$ is equal to $(\Delta Ct \text{ sample}) - (\Delta Ct \text{ reference})$, and the ΔCt values were derived from the Ct value of the reference subtracted from the Ct of the sample⁸³. In our experiments, the glycolipid enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. All samples are normalised to the reference gene to allow to correct for variations such as different amounts or quality of the starting mRNA, as well as different efficiencies in the PCR amplification⁸³.

3.6 Flow cytometry

Flow cytometry is a method that is widely used both within research and diagnostics. It provides advantages such as analysis of cell populations on a cell to cell basis, rapid analysis, and it is able to measure several parameters of one single cell simultaneously. There are three main systems in flow cytometry; the fluidics, the optics, and the electronics. In addition to this, a computer is directly connected to the flow cytometer to allow for specific settings, as well as data analysis and storage. The fluidics system orders samples into a stream of single

cells, which can then be detected individually. The sample is injected in a narrowing central core, which is surrounded by sheath fluid. The sheath fluid moves faster than the sample, and creates a drag effect on the sample. This creates a velocity change in the fluid in the central core, where the velocity is higher in the centre, and 0 on the sides. This principle is called hydrodynamic focusing, and creates the flow of single cells. The single stream of cells does not mix with the sheath fluid under optimal conditions (laminar flow). This flow of single cells is passed through several beams of light, and is detected by specific channels when it has passed through the cell. Forward scattered light is detected by the forward scatter channel (FSC) and says something about the size of the cell. Side scatter is measured at approximately a 90° angle to the excitation line and is detected by the side scatter channel (SSC). This parameter tells about the granularity of the cell. Monoclonal antibodies can be tagged with fluorescent dyes, which allow for detection of specific cell properties. These fluorescent dyes (fluorochromes) are excited by a specific wavelength and emit light at a different specific wavelength. The emitted light is detected by different fluorescence (FL-) channels, and the data is sent to a computer for further analysis⁸⁴. **Figure 3.4** gives an overview of a flow cytometry setup.

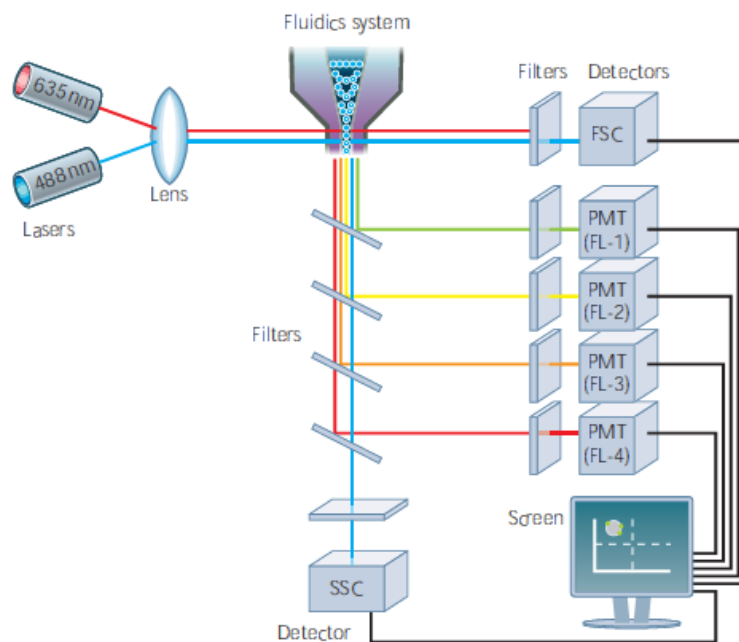


Figure 3.4: Schematic overview of a flow cytometry setup. Beams of light (from a laser) are passed through a lens, and through the stream of single cells. Emitted light is detected by the FSC, SSC, or different photomultiplier tubes (PMTs). Optical filters and mirrors ensure that only certain wavelengths are passed through different filters. The combination of lasers, filters, and detectors allows for the detection of a combination of fluorochromes in a single cell. The collected data is lastly sent to a computer for further analysis⁸⁴.

When several fluorochromes are used in the same experiment, there might be some spectral overlap, or “spill-over” where one fluorochrome will contribute to a signal on several detectors. By choosing fluorochromes that emit light at opposite sides of the spectrum one can avoid this, but this is often not possible when several fluorochromes are used. To correct for the spectral overlap, compensation is carried out. Here, the signal in detectors not assigned to the specific fluorochrome is subtracted from the total signal in that detector⁸⁵. Compensation beads (BD bioscience) stained with the fluorescent antibodies were used to carry out compensation in the experiments presented in this thesis.

Flow cytometry was used for analysis of T cell activation and for investigation of surface marker changes on macrophages in this thesis. The analysis was done using the LSR II (BD) flow cytometer.

3.6.1 Staining of T cells for flow cytometry

Following T cell expansion and restimulation, as described in **Section 3.1**, cells were stained and fixed for flow cytometry analysis. T cells were stained extracellularly using antibodies specific for the surface proteins CD3, CD4 and CD8, to allow recognition of the cells of interest, before fixation using 2-4% paraformaldehyde (PFA) (VWR). Following fixation, the cells were permeabilised using 0.5% saponin (Sigma-Aldrich). The permeabilisation is done to allow intracellular staining of T cell effector cytokines, which accumulate within the T cells due to the addition of the transport inhibitor for the last 4 hours of the stimulation time. The cytokines investigated in this thesis were IFN γ , TNF α , and IL2. **Table 3.3** gives an overview of the flurochromes used for extra- and intracellular staining of T cells. For a detailed staining protocol with extra- and intracellular markers see **Appendix 2**.

Table 3.3: Overview of flurochromes used for T cell staining for flow cytometry

Antibody	Flurochrome	Laser	Filter	Brand
CD3	FIT-C	488	525/50	eBioscience
CD4	Alexa 700	633	710/50	eBioscience
CD8	BV 605	405	610/20	BioLegend
IFNγ	APC	633	670/14	eBioscience
TNFα	eFluor 450	405	450/50	eBioscience
IL2	PE	561	780/60	eBioscience

After staining, the cells were analysed using the LSR II flow cytometer.

3.6.2 Staining of macrophages for flow cytometry

Flow cytometry was also used to investigate macrophages. The adherent macrophages were dislodged using a cell scraper in 24 well plates, or a non-enzymatic Cell dissociation solution (Sigma-Aldrich) in 96 well plates. When using the dissociation solution, the media was first removed, and cells washed with preheated Hank's balanced salt solution. 100µl of the solution was then added per well, and the plate was incubated at room temperature for 15-20 minutes for cells to dislodge. There was some trouble in dislodging the primary human macrophages using this solution, and pipetting up and down around the well was also required. Once cells were dislodged, extracellular staining was carried out using the same protocol as for extracellular staining of T cells (see **Appendix 2**). In addition to the extracellular staining, a viability stain (eBioscience) was used for the macrophages. This is an exclusion dye, where dead cells are stained because they have damaged membranes, while live cells exclude the dye and will not be stained. After step 4 (**Appendix 2**), the samples were washed twice with PBS (no FCS) by centrifugation, and 0.5µl viability stain diluted in 500µl PBS was added to each sample. The samples were incubated for 30 minutes on ice, protected from light. After this, the rest of the steps for extracellular staining were carried out. **Table 3.4** gives an overview of the antibodies and flurochromes used for investigation of macrophages.

Table 3.4: Overview of flurochromes used for macrophage staining for flow cytometry

Antibody	Flurochrome	Laser	Filter	Brand
CD14	eFluor450	405	450/50	eBioscience
CD11b	perCP eFluor710	561	710/50	eBioscience
HLA-DR	APC	633	670/14	eBioscience
CD80	PE	561	780/60	Immunotech
	Viability stain	639	780/60	eBioscience

3.6.3 Flow cytometric analysis of *M. avium* specific T cell activation

Multicolour flow cytometry was used to investigate the cytokine response in T cells following expansion and restimulation with *M. avium* infected autologous macrophages. The following gating procedure was used to identify the T cells and their cytokine production

1. Lymphocytes were identified by using the FSC and SSC.
2. T cells were identified and gated by their CD3 positive staining.
3. From the CD3 positive population, cells positively stained for CD4 were gated.
4. From the CD3+CD4+ population, each cytokine (TNF α , IFN γ , and IL2) was assessed individually.

3.6.4 Flow cytometric surface marker characterisation on macrophages following KEAP1 knockdown

Multicolour flow cytometry was used to assess activation marker changes following KEAP1 knockdown in primary human macrophages. The following gating procedure was used to identify the macrophages and their surface marker expression:

1. Macrophages were identified by CD14 and CD11b positive expression.
2. Viable cells were identified by no staining with the viability stain.
3. Viable CD14+CD11b+ cells were assessed for HLA-DR and CD80 expression, two molecules that are involved in antigen presentation and T cell activation.

3.6.5 Processing of flow cytometry data

For analysis of flow cytometry data, FlowJo X was used. Microsoft Excel 2010 and GraphPad Prism were used for further processing of the data and for graphical display. For statistical analysis unpaired Student's T-test was used.

4 Results

4.1 Testing HEK293 cells as a model system for investigating the mechanisms behind how KEAP1 regulate inflammatory responses

Recent results from our group indicate that KEAP1 suppresses the inflammatory response in primary human macrophages following infection with *M. avium*⁵⁵. The mechanism behind this regulation is still unknown. As KEAP1 has many interaction partners, including the Cul3/Rbx1 E3 ubiquitin ligase complex, the selective autophagy receptor p62, the inflammatory signalling protein IKK β , and the transcription factor NRF2, a whole variety of mechanisms may be influenced by KEAP1. One of the aims of this thesis was therefore to investigate the role of Cul3 and Rbx1 in the regulation of *M. avium* induced inflammatory responses. HEK293 cells were chosen as a starting point for these experiments as HEK293 cells are known to be easy to transfect, and due to no donor variations it is easier to obtain reproducible results compared with the use of primary human cells. Because of these advantages, we wanted to investigate whether or not the HEK293 cell line was suitable as a model system for dissecting the role of KEAP1 in regulating inflammatory responses in mycobacterial infection.

4.1.1 KEAP1 knockdown decreases the TNF α production in HEK293 TLR2-GFP cells following *M. avium* infection

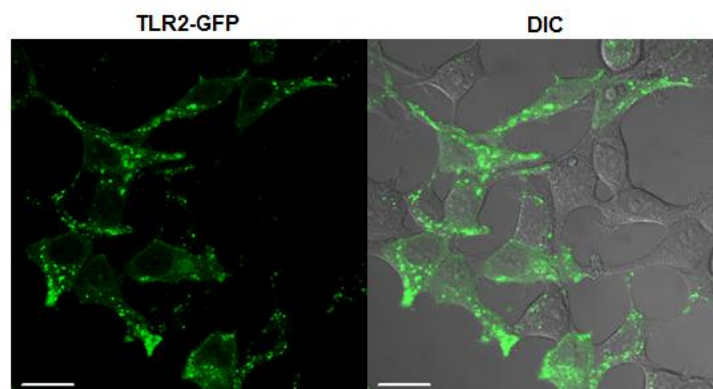
TLR2 is one of the TLRs that have been shown to recognise mycobacterial antigens¹⁹⁻²¹. HEK293 cells do not express TLR2, and we therefore used HEK293 cells stably transfected with TLR2 and GFP. We first confirmed that TLR2 was present in the cell using confocal microscopy to look for the GFP-tagged TLR2 protein. **Figure 4.1 A** shows that GFP was detected in confocal microscopy, and we can therefore assume that TLR2 was present in the HEK293 cells used in our experiments.

HEK293 TLR2-GFP cells were treated with KEAP1 targeting siRNA (siKEAP1) or non-targeting siRNA (siAS) as described in **Section 3.4.1.3**, followed by a 4 hour infection with *M. avium* at a MOI of 10:1. The knockdown of KEAP1 was more than 90% as measured by real-time PCR (**Figure 4.1 B**). The transcription of TNF α 4 hours post infection with *M. avium* was also measured for siAS and siKEAP1 treated cells using real-time PCR (**Figure 4.1 C**). The fold change in TNF α expression between infected and uninfected samples is shown in **Figure 4.1 C**. In contrast to what has been observed in primary human macrophages, *M. avium* induced TNF α production decreased when KEAP1 was knocked down in the HEK293 TLR2-GFP cells. High Ct values (above 30) were obtained, indicating

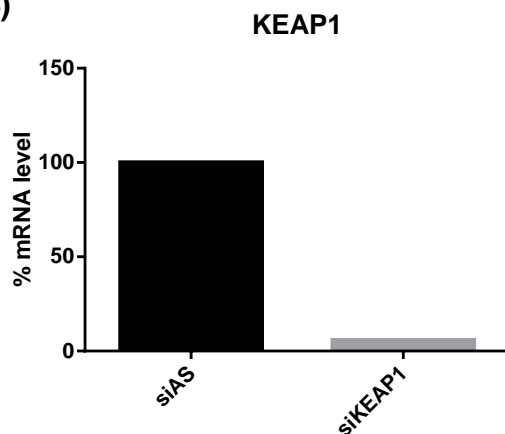
that the level of the target sequence was very low and the results may not be reliable (data not shown).

Due to low TNF α expression in the HEK293 TLR2-GFP cells, we tried to additionally transfect the co-receptor CD14 into the cells (as described in **Section 3.4.3.1**) to investigate if this increased the response to *M. avium* infection. pcDNA-cherry was used as control for transfection efficiency and visualised using fluorescence microscopy (**Figure 4.1 D**). It was found that transfection was effective already 24 hours post addition of transfection reagents (**Figure 4.1 D**). For infection experiments, cells were transfected with CD14 or pcDNA-cherry, 48 hours later the medium was changed and cells infected with *M. avium* at a MOI of 10:1 for 4 hours. Comparing the TNF α mRNA levels for HEK293 TLR2-GFP cells with and without CD14 showed only minimal effect for the co-receptor expression on the TNF α response to *M. avium* in these cells (**Figure 4.1 E**). Also, in this experiment the Ct values obtained from real-time PCR were above 30, indicating that the level of the target sequence was low (data not shown).

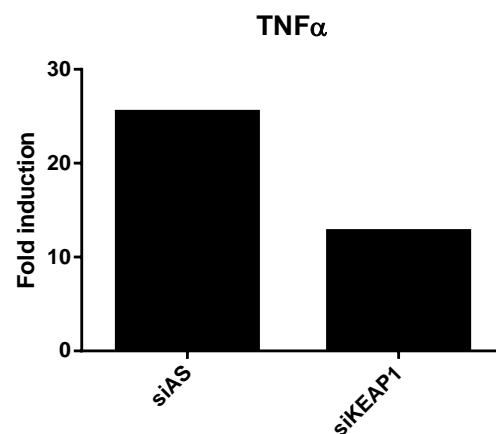
A)



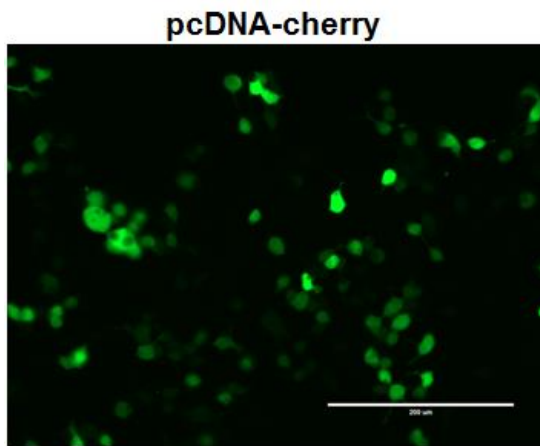
B)



C)



D)



E)

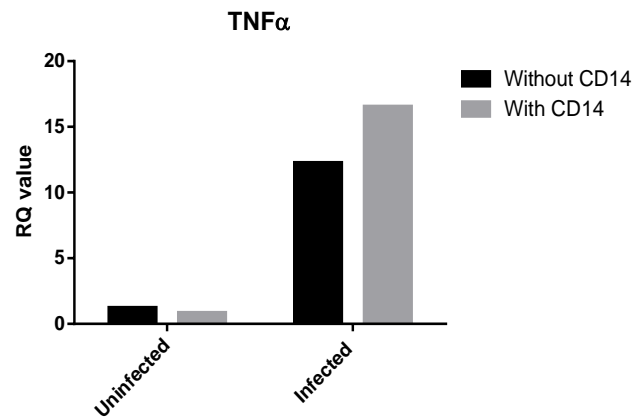


Figure 4.1: TNF α production by HEK293 TLR2-GFP cells decreases following KEAP1 knockdown and *M. avium* infection A) Confocal image confirming the presence of TLR2 tagged with GFP in stably transfected HEK293 cells. Scale bar represents 20 μ m (imaged in cooperation with Jane Awuh). B) The knockdown efficiency following KEAP1siRNA treatment of HEK293 TLR2-GFP cells compared to the non-targeting siRNA control AS. C) The fold change in TNF α mRNA level measured by real-time PCR for HEK293 TLR2-GFP cells treated with non-targeting siRNA (AS) or KEAP1 siRNA after 4 hour infection with *M. avium* at a MOI of 10:1. D) Fluorescence microscopy image of HEK293 TLR2-GFP cells 24 hours post transfection with pcDNA-cherry, demonstrating that transfection was efficient. Scale bar represent 200 μ m (imaged in cooperation with Jane Awuh). E) The relative quantification of TNF α expression at the mRNA level in HEK 293 TLR2-GFP cells transfected with co-receptor CD14, and untransfected cells after a 4 hour infection with *M. avium*. Data collected from one experiment done in duplicates.

4.1.2 *M. avium*-induced NF- κ B activation decreases following KEAP1 knockdown in HEK293 cells

The TNF α induction following *M. avium* infection was found to be low in the HEK293 TLR2-GFP cells. We therefore chose to test out a different method for measuring inflammatory activation, namely NF- κ B activation. We also included a different HEK293 cell line (HEK293T), where TLR2 was transiently transfected in, as described in **Section 3.4.2.2**. HEK293T cells were transfected with either pcDNA+ELAM-luciferase+Renilla luciferase, or TLR2+ELAM-luciferase+Renilla luciferase. In addition, the HEK293 TLR2-GFP cells were transfected with pcDNA+ELAM-luciferase+Renilla luciferase to investigate the TLR2 functionality. 48 hours after transfection, cells were stimulated for 4 hours with *M. avium* at a MOI of 10:1, lipomannan (LM, 100ng/ml), or lipopolysaccharides (LPS, 50ng/ml) (**Figure 4.2 A**). LM is a glycolipid derived from the cell wall of *Mycobacterium tuberculosis*, and is a known TLR2 ligand¹⁸. LPS is a cell wall protein found on gram negative bacteria that is not a TLR2 ligand but is recognised by TLR4.

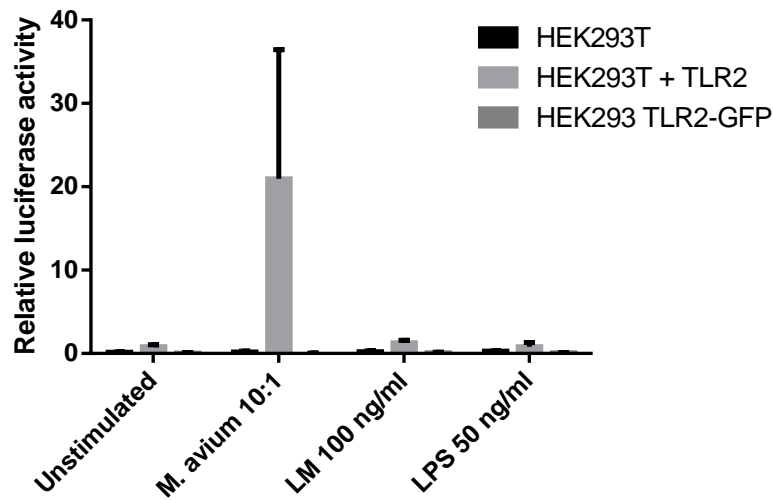
As displayed in **Figure 4.2 A**, HEK293T cells transfected with an empty plasmid showed low NF- κ B activation following all the stimulations, as demonstrated by low

luciferase activity. The HEK293T cells transiently transfected with TLR2 showed NF- κ B activation as displayed by increased luciferase activity following *M. avium* stimulation. Little activation was seen with stimulation with LM and LPS compared to the unstimulated sample. The HEK293 TLR2-GFP cells showed activation at the same level as the HEK293T cells lacking TLR2, indicating that the TLR2 in the stably transfected cells was non-functional (**Figure 4.2 A**).

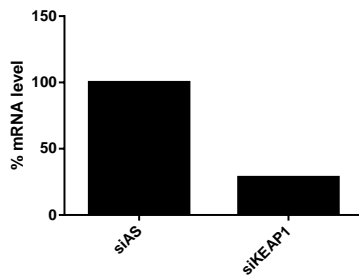
As the stably transfected HEK293 TLR2-GFP cell line did not appear to get activated following stimulation, we chose to proceed with the HEK293T cells and transiently transfect these with TLR2 for the individual experiments. We next wanted investigate the activation of NF- κ B following KEAP1 knockdown using the ELAM-luciferase assay. The HEK293T cells were given siRNA (KEAP1 or AS) treatment for 24 hours, followed by transient TLR2 transfection for 48 hours. The KEAP1 knockdown efficiency was measured 72 hours post knockdown using real-time PCR, and was above 70% (**Figure 4.2 B**). Transiently TLR2 transfected cells +/- KEAP1 knockdown were then stimulated as described above and the induction of inflammation was analysed by measuring luciferase activity as readout for NF- κ B activation (**Figure 4.2 C**). Due to low activation of NF- κ B with LM at 100ng/ml (**Figure 4.2 A**) we chose to increase the dose of LM to 500ng/ml. For all cells transfected with empty plasmid the NF- κ B activation was relatively low, as would be expected (**Figure 4.2 C**). Both *M. avium* and LM appear to activate NF- κ B, with LM giving the highest activation (**Figure 4.2 C**). Activation following stimulation with LPS was no higher than the unstimulated sample, as expected. Comparable to the TNF α measurements with the stably transfected HEK293 TLR2-GFP cell line (**Figure 4.1 C**), KEAP1 knockdown led to decreased *M. avium* induced inflammation measured by NF- κ B activation also in HEK293T cells transiently transfected with TLR2 (**Figure 4.2 C**). Similar results were seen with LM. We also investigated the IFN β induction using IFN β -luciferase with the same stimulations as above. We could not detect activation of the IFN β -luciferase in this assay (data not shown).

The results in **Section 4.1** taken together indicate a decrease in *M. avium*-induced inflammatory response following KEAP1 knockdown in HEK293 cells. The findings from experiments using the HEK293 model system are in contrast to what has previously been seen in human primary macrophages. Due to these opposing results, we believe that the HEK293 cell line is not suitable to use as a model system for our investigation.

A)



B)



C)

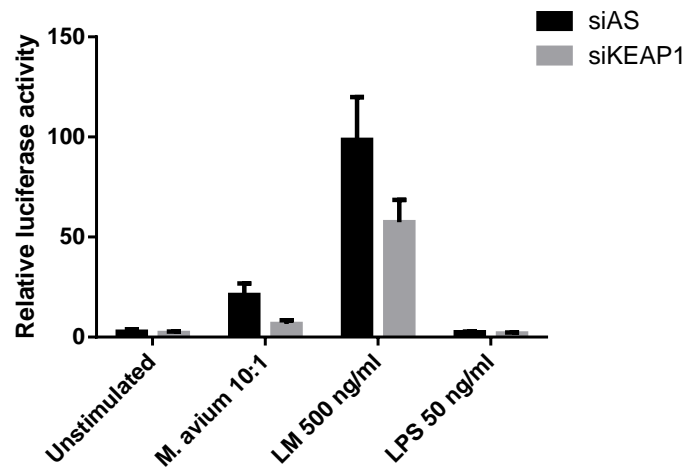


Figure 4.2: NF- κ B activation following various stimulations decreases in HEK293 cells following KEAP1 knockdown A) Shows the activation of NF- κ B in HEK293T cells transiently transfected with TLR2, or HEK293 TLR2-GFP cells, in response to 4 hour stimulation with *M. avium* (MOI 10:1), LM (100ng/ml) and LPS (50ng/ml). Activation was measured by the activity of ELAM-luciferase, and normalised using Renilla luciferase. B) The knockdown efficiency in HEK293T cells after one siRNA treatment (KEAP or AS) for 24 hours, followed by TLR2 transfection for 48 hours. Knockdown measured at the mRNA level using real-time PCR. C) The NF- κ B activation in HEK293T cells transfected with TLR2, in addition to ELAM-luciferase and Renilla luciferase. Cells were treated with either non-targeting siRNA (AS) or siRNA targeting KEAP1. Cells were stimulated for 4 hours with *M. avium* (MOI 10:1), LM (500ng/ml) and LPS (50ng/ml). NF- κ B activation measured by the activity of ELAM-luciferase, normalised to Renilla luciferase. Data collected from one experiment in triplicates.

4.2 Cul3 and Rbx1 knockdown increases the inflammatory response to *M. avium* infection in primary human macrophages

KEAP1 appears to suppress the inflammatory response following *M. avium* infections in primary human macrophages⁵⁵. As the HEK293 cells did not give the same results as previously obtained in primary human macrophages, we continued our work in primary cells, for more reliable results to study the effect of Cul3 and Rbx1. Cul3 and Rbx1 are two of the proteins known to interact with KEAP1, and are both part of the E3 ubiquitin ligase complex associated with KEAP1. In order to increase our understanding of how the negative regulation of inflammation associated with KEAP1 occurs, we wanted to investigate the roles of Cul3 and Rbx1. If it is the ubiquitination of proteins associated with KEAP1 that is important for its negative regulation of inflammation in *M. avium* infection, we expect the removal of Cul3 and Rbx1 to show increased inflammation, as we see for the removal of KEAP1.

PBMCs were isolated, and monocytes differentiated to macrophages as described in **Section 3.1**. siRNA treatment targeting KEAP1, Cul3, and Rbx1 was carried out as described in **Section 3.4.1.2**. After 72 hours of siRNA treatment, the cells were infected for 4 hours with *M. avium* at a MOI of 10:1. The knockdown of each of the proteins was measured using real-time PCR, and was above 75% in all samples (data not shown). The different siRNAs seemed specific to their target as the expression of KEAP1, Cul3, and Rbx1 were only affected by the targeting siRNA and not by the siRNAs targeting the other partners of the complex (**Figure 4.3 A**). The inflammatory response 4 hours after *M. avium* infection was measured by mRNA analysis for the cytokines IFN β , IP10, and TNF α by real time PCR (**Figure 4.3 B**). Consistent with previous results in our group⁵⁵, knocking down KEAP1 increased the inflammatory response following *M. avium* infection. The results also indicate that an increased inflammatory response is the case when knocking down Cul3 and Rbx1. The IFN β and IP10 responses were relatively low with less than a four-fold increase in expression following infection, compared to the TNF α response where the fold induction was more than 20 following knockdown of each of the three proteins KEAP1, Cul3 and Rbx1. The trend of increased cytokine production was, however, the same for all cytokines. The data in **Figure 4.2 B** is from two experiments. To further confirm these findings, one additional experiment was carried out to look at the effect on inflammation when knocking down the three proteins. This experiment showed the opposite result of our previous findings and is therefore shown separately in **Figure 4.3 C**. The knockdown cells had a lower induction of the cytokines TNF α , IFN β , and IP10 following *M. avium* infection, compared to cells treated with the non-targeting control (AS). The knockdown was more than 60% for all the proteins at the mRNA

level (data not shown). The last experiment was done in duplicates, and showed big variations in the cytokine expression between the samples within the same condition. This was especially true for the siAS and siKEAP1 treated cells.

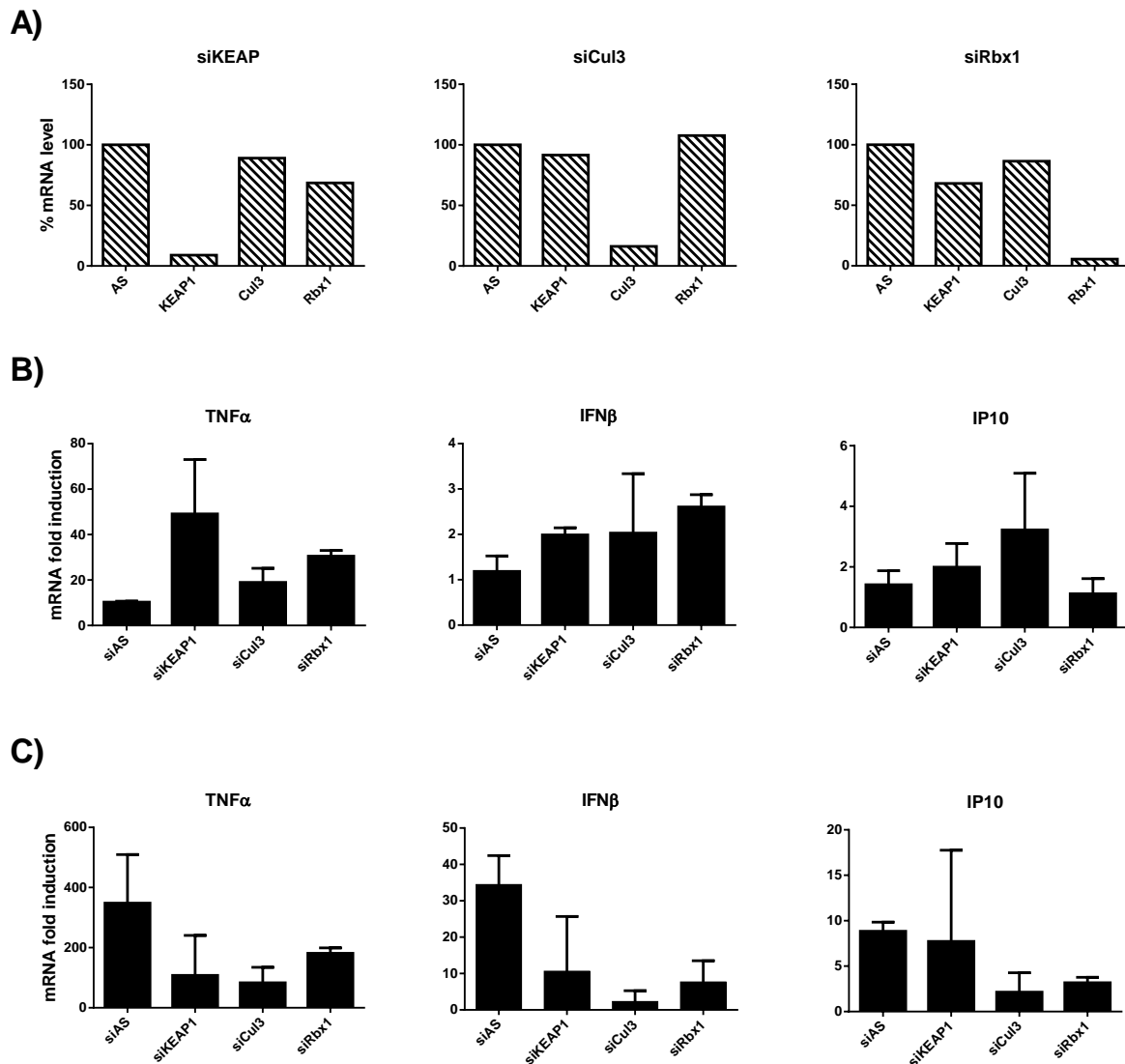
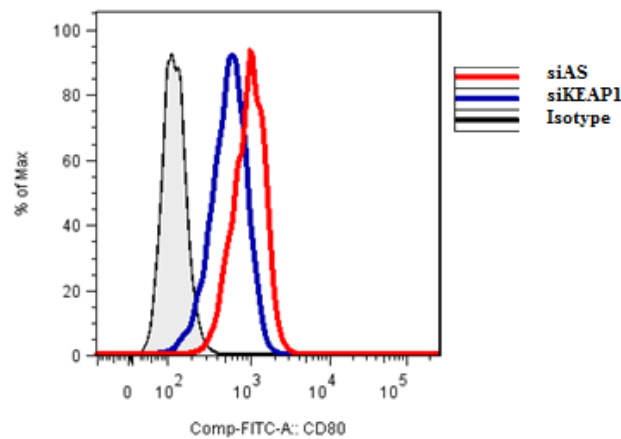


Figure 4.3: Increased inflammatory response following *M. avium* infection in KEAP1, Cul3, and Rbx1 depleted primary human macrophages A) Specific and efficient knockdown of mRNA levels of KEAP1, Cul3, and Rbx1 in primary human macrophages as assayed by real-time PCR. AS is non-targeting siRNA used as control. Example of results obtained from one experiment in single wells. B,C) Macrophages were treated with siRNA to KEAP1, Cul3, or Rbx1 as indicated on the X-axis prior to 4 hour infection with *M. avium* (MOI 10:1). Results are shown as fold induction of TNF α , IFN β , and IP10 mRNA levels in infected macrophages relative to uninfected controls. Results in B) are an average of two independent experiments with single well per condition, whereas results in C) are from one experiment with duplicate wells.

4.3 KEAP1 knockdown downregulates the expression of macrophage surface proteins CD80 and CD86

Results in our group have shown that decreasing the expression of KEAP1 in macrophages increases the inflammatory response and autophagy in response to *M. avium* infections⁵⁵. Research suggests that both autophagy³⁹ and NF- κ B⁸⁶ signalling are important factors in antigen-presentation. Knowing this we wanted to investigate if KEAP1 knockdown in human primary macrophages would affect the antigen-presenting capabilities of these cells. We therefore decided to investigate expression changes of the activation markers CD80 and HLA-DR (MHC class II) on the surface of macrophages following KEAP1 knockdown and *M. avium* infection. CD80 and HLA-DR are both proteins important for T cell activation⁵⁹. Macrophages were isolated, differentiated, and siRNA treated as previously described (**Section 4.2**). Cells were then infected for 4 hours with *M. avium*, followed by staining for flow cytometry as described in **Section 3.6.2**. Macrophages were positively identified by expression of CD14 and CD11b, and the mean fluorescence intensity for CD80 and HLA-DR fluorochromes were measured. **Figure 4.4 A** exemplifies the mean fluorescence intensity measurement for CD80 expression. The histogram shows a comparison of uninfected AS siRNA treated cells with KEAP1 siRNA treated cells as well as staining with an isotype control antibody. We decided to look at the relative expression of the surface proteins, using uninfected AS siRNA treated cells as 100%. The expression of both CD80 and HLA-DR was significantly decreased on the surface of uninfected macrophages compared to control cells (**Figure 4.4 B**) There was no significant difference between siAS uninfected and infected cells for either CD80 or HLA-DR, nor between siAS infected cells and siKEAP1 infected cells. For CD80 there was a significant decrease in expression between the siAS uninfected and siKEAP1 infected cells, a difference not seen for HLA-DR (**Figure 4.4 B**). KEAP1 knockdown was measured using real-time PCR, and was above 60% in all experiments (data not shown). These results indicate that removal of KEAP1 in human primary macrophages decreases the expression of both CD80 and HLA-DR, which are both important for T cell activation, and the decrease is independent of *M. avium* infection.

A)



B)

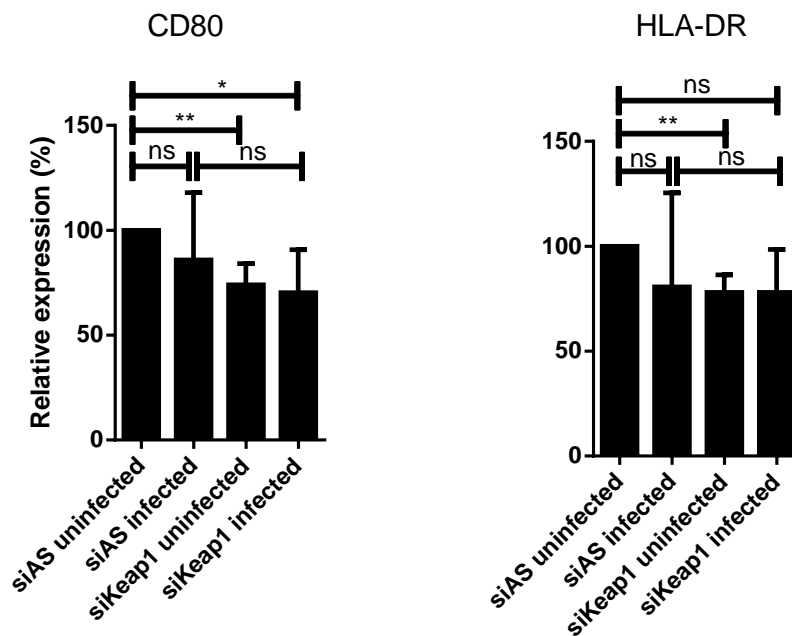


Figure 4.4: Decreased expression of CD80 and HLA-DR on the surface of primary human macrophages following KEAP1 knockdown The relative expression of CD80 and HLA-DR on the surface of primary human macrophages following KEAP1 knockdown and *M. avium* infection was analysed. Cells were infected for 4 hours with a MOI of 10:1. A) Uninfected primary human macrophages were treated with either AS or KEAP1 siRNA and stained for flow cytometry. Macrophages were identified by the expression of CD11b and CD14. An example of the fluorescence intensity measurement done to investigate the expression of CD80 on the surface of human primary macrophages following siKEAP or siAS treatment is shown. The black line represents isotype control, the red line represents siAS treated cells, and the blue line represents siKEAP1 treated cells. B) Summarizes results from 4 experiments analysing CD80 (left) and HLA-DR (right) expression on primary human macrophages +/- infection and +/- AS/KEAP1 siRNA. Mean fluorescence intensity of CD80 and HLA-DR expression was quantified from histograms (as shown in A). The bars represent % expression of CD80 and HLA-DR relative to uninfected cells treated with non-targeting siRNA (AS). Mean +/- SD of four experiments. * $p < 0.05$ ** $p < 0.01$.

4.4 Establishment of conditions for mycobacteria-specific T cell expansion and restimulation with autologous macrophages

Results presented in **Section 4.3** suggest a downregulation of the proteins CD80 and HLA-DR on the surface of human primary macrophages as a consequence of KEAP1 knockdown and thus might impact antigen-presentation and subsequent T cell activation. We wanted to investigate the activation of T cells following stimulation with *M. avium* infected autologous macrophages, and if KEAP1 knockdown in the macrophage would change this specific activation. For biological relevance it is desirable to use human primary cells, hence we first needed to establish a system to study mycobacteria-specific T cell responses to autologous macrophages. The assay involved PBMC isolation, T cell expansion, macrophage differentiation, and co-culturing of T cells and macrophages, all with cells isolated from the same donor.

4.4.1 Establishment of conditions for mycobacteria-specific *in vitro* T cell expansion

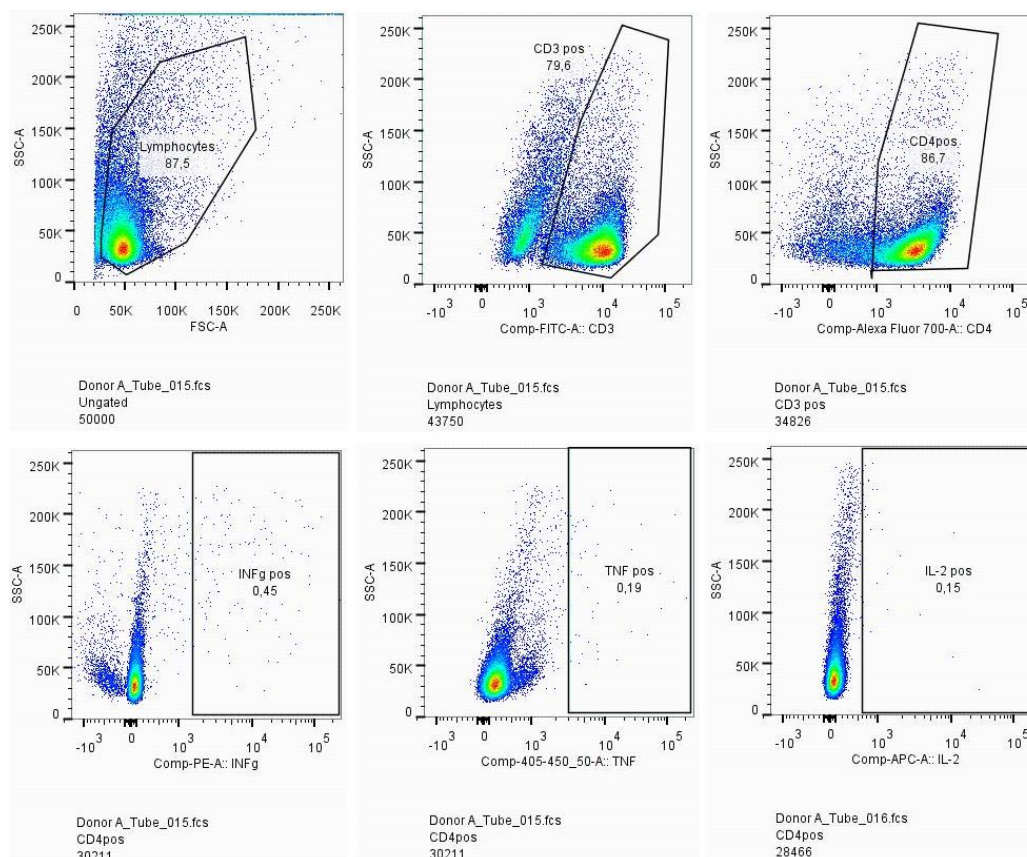
Mycobacteria-specific effector T cell responses take time to develop. For our investigation we used PBMCs from BCG vaccinated healthy donors, meaning some T cells should be mycobacteria-specific, and can also react with antigens from *M. tuberculosis* and *M. avium* (Markus Haug, unpublished data). The fraction of specific T cells however, is very low in PBMCs from healthy donors. In order to increase this fraction, activation and expansion of the mycobacteria-specific T cell fraction is needed. This was done by cultivating PBMCs with heat killed *M. avium* as described in **Section 3.1.2** for 7-10 days. In parallel, autologous macrophages were generated from monocytes as described in **Section 3.1.1**. Following the expansion, specific T cell responses were investigated by co-culturing the expanded T cells with the autologous macrophages infected with *M. avium*, as described in **Section 3.1.3**.

We first tested out different concentrations (1:1 and 3:1) of heat killed *M. avium* with the T cells during the expansion step. After 7-10 days of T cell expansion autologous macrophages were infected for 4 hours with *M. avium*, and the expanded T cells were added and left for restimulation overnight.

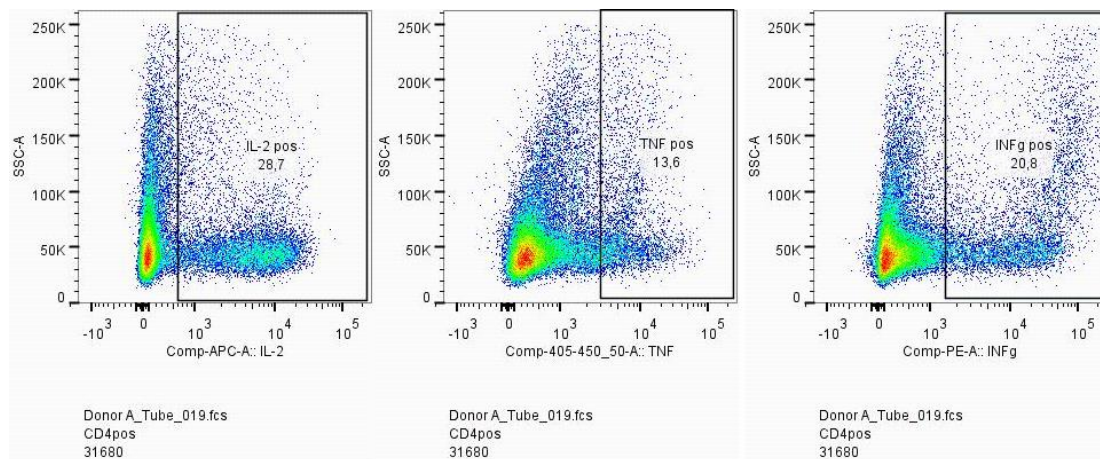
The activation of mycobacteria-specific CD4⁺ T_H1-cells was analysed by measuring the production of the T_H1 effector cytokines TNF α , IFN γ , and IL2 from CD4⁺ T cells using multicolour flow cytometry (**Section 3.6**). An example of the gating used to assay the cytokine responses in CD4⁺ T cells is shown in **Figure 4.5 A**, starting with identification of lymphocytes, followed by gating for CD4⁺ T cells through CD3⁺ cells and then CD4⁺ cells.

In the CD4⁺ population the production of each cytokine was assessed individually. Using this gating strategy, **Figure 4.5 B** demonstrates the CD4⁺ T cell production of the cytokines IFN γ , TNF α , and IL2 following stimulation with Cell Stimulation Cocktail overnight. The frequency of CD4⁺ T cells producing TNF α , IFN γ , and IL2 following restimulation with *M. avium* infected autologous macrophages is presented in **Figure 4.5 C**. The response for the CD4⁺ T cells expanded both 1:1 and 3:1 with heat-killed *M. avium* was low, with less than 0.5% of CD4⁺ T cells producing either cytokine for all the conditions tested. However the response in the T cells expanded 3:1 was higher in the majority of the conditions tested. Higher concentrations of heat killed *M. avium* (5:1) in the expansion step was also tested, but it seemed that the T cells got activated already before restimulation as seen by cytokine production by the unstimulated cells (data not shown). Due to these findings we chose to continue with an expansion ratio of 3:1 from here.

A)



B)



C)

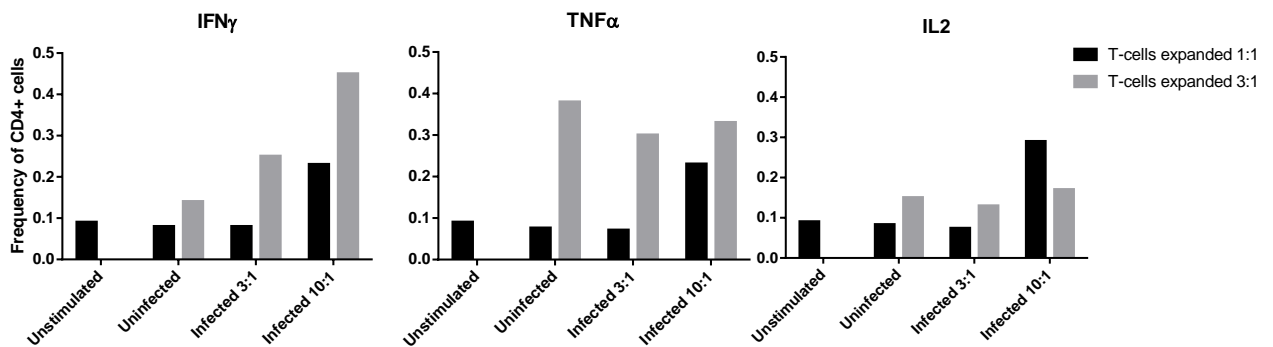


Figure 4.5: Cytokine response in CD4+ T cells expanded under different conditions and restimulated through co-culturing with *M. avium* infected autologous macrophages. PBMCs were isolated, and T cells were expanded with heat-killed *M. avium* at a ratio of 1:1 or 3:1 for 10 days and restimulated overnight with autologous macrophages uninfected or infected with *M. avium* at different MOIs. A) An example of the gating used in FlowJo to identify the CD4+ T cells, and investigate their cytokine production. Lymphocytes were gated using the FSC and SSC, followed by gating for CD3 and CD4. CD3+CD4+ T cells were then investigated for the production of IFN γ , TNF α , and IL2. B) T cells stimulated with Cell Stimulation Cocktail overnight, demonstrating T cell cytokine response and functional staining protocol. C) Shows the frequency of CD4+ T cells producing the cytokines IFN γ , TNF α , or IL2. CD4+ T cell effector cytokine production was analysed from unstimulated (no macrophages) 10 days expanded T cells, as well as overnight incubation with autologous macrophages that were uninfected, infected 3:1, or infected 10:1 with *M. avium*. Protein transport inhibitor was added for the last 4 hours of incubation to prevent cytokine excretion. Bars represent % of cytokine-producing cells within the gated CD3+CD4+ population as explained in the example in A). Black bars represent T cells that were generated from PBMCs expanded 1:1 with heat-killed *M. avium*, and grey bars show the results for T cells expanded 3:1. Results obtained from one experiment.

4.4.2 Testing effector responses of expanded mycobacteria-specific T cells to autologous macrophages with different doses of *M. avium* infection

Following establishment of mycobacteria-specific T cell expansion, we wanted to test which macrophage MOI that would result in the highest CD4⁺ T_H1 effector cytokine production. Macrophages were differentiated and T cells expanded as previously described. On day 7-10 macrophages were infected with live *M. avium* for 4 hours with different MOIs, and T cells were added for restimulation. The T cells were co-cultured with the macrophages overnight, and then stained and fixed for multicolour flow cytometry as described in **Section 3.6**. The frequency of CD4⁺ T cells producing each of the cytokines IFN γ , TNF α , and IL2 was measured and the results presented in **Figure 4.6**. Also here the fraction of CD4⁺ cells producing cytokines was relatively low, with the highest fraction around 0.5%. We experienced a big variation between different donors, as well as within each experiment. Despite the big variation, the trend was that a MOI of 10:1 gave the highest response, and we therefore chose to proceed with this MOI in further experiments.

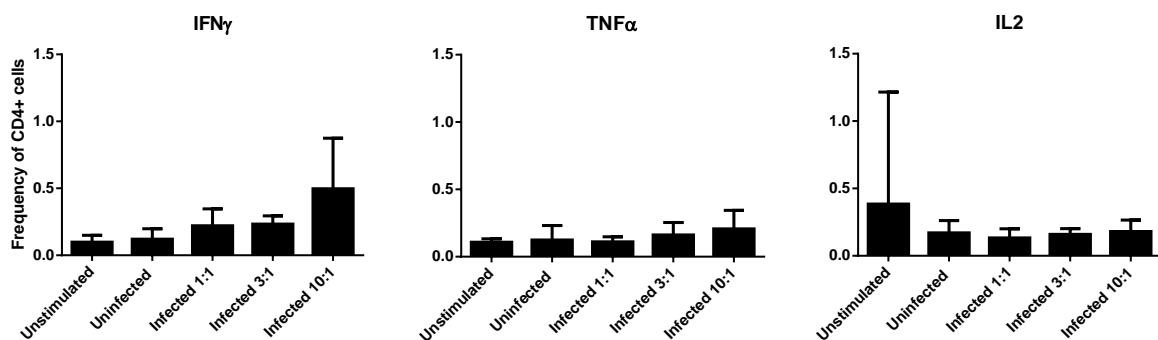


Figure 4.6: CD4⁺ T_H1 effector cytokine production following restimulation of mycobacteria-specific T cells with differently *M. avium* infected autologous macrophages PBMCs were isolated, and T cells were expanded with heat-killed *M. avium* at a ratio of 3:1 for 10 days. Mycobacteria-specific T cells were restimulated overnight with autologous macrophages uninfected or infected with *M. avium* at MOI 1:1, 3:1, and 10:1. The frequency of CD4⁺ T_H1 cells producing each of the effector cytokines TNF α , IL2, and IFN γ , after exposure to macrophages infected with live *M. avium* at different MOIs overnight. The results are an average of 2-4 experiments using different donors, with each experiment having 1-3 replicates. Results are given as mean +/- standard deviation.

4.4.3 Kinetics of effector cytokine production from mycobacteria-specific CD4⁺ T_H1 cells after restimulation with *M. avium* infected macrophages

As the cytokine response was low compared to expected results after overnight restimulation of the T cells, we wanted to investigate if earlier time-points allowed us to detect cytokines at a higher level. One experiment was carried out to investigate this. The macrophages and expanded mycobacteria-specific T cells were prepared as previously described. T cells were expanded with heat-killed *M. avium* at a concentration of 3:1 for 10 days, and macrophages were infected with *M. avium* at a MOI of 10:1 for 4 hours before T cell were co-cultured with the macrophages for restimulation. The time points tested for restimulation of mycobacteria-specific T cells were 4, 8, 16, and 24 hours, followed by staining and analysis by multicolour flow cytometry. The protein transport inhibitor was added for the last 4 hours of the stimulation time. The results obtained from this experiment are presented in **Figure 4.7**. We see a higher cytokine production from CD3⁺CD4⁺ T cells (frequency of more than 1) of the T_H1 cytokines IFN γ , TNF α , and IL2 at the earlier time points (4 and 8 hour restimulation time), followed by a decrease in production towards the previously used 24 hour restimulation time point. The frequency of CD4⁺ T_H1 cells producing cytokines is also higher at these early time points compared to what has been obtained in previous experiments. T cells not restimulated show low cytokine expression, as expected (**Figure 4.7**). We would not expect T cells restimulated with uninfected autologous macrophages to show as high activation as those stimulated with infected macrophages. However, as seen in **Figure 4.7**, T cells restimulated with uninfected macrophages also induced a strong cytokine response (frequency of CD4⁺ cells of up to 7% for the 16 hour time point for IFN γ production), albeit somewhat slower than T cells exposed to infected macrophages. We thus cannot be sure if the cytokine response we see is mycobacteria-specific, or if the T cells are activated in response to other molecules on the macrophage surface. For previous experiments IFN γ has been the most produced cytokine; however, in this experiment TNF α had the highest induction. IL2 induction had the lowest expression, which is consistent with previous experimental data (**Figures 4.5 and 4.6**).

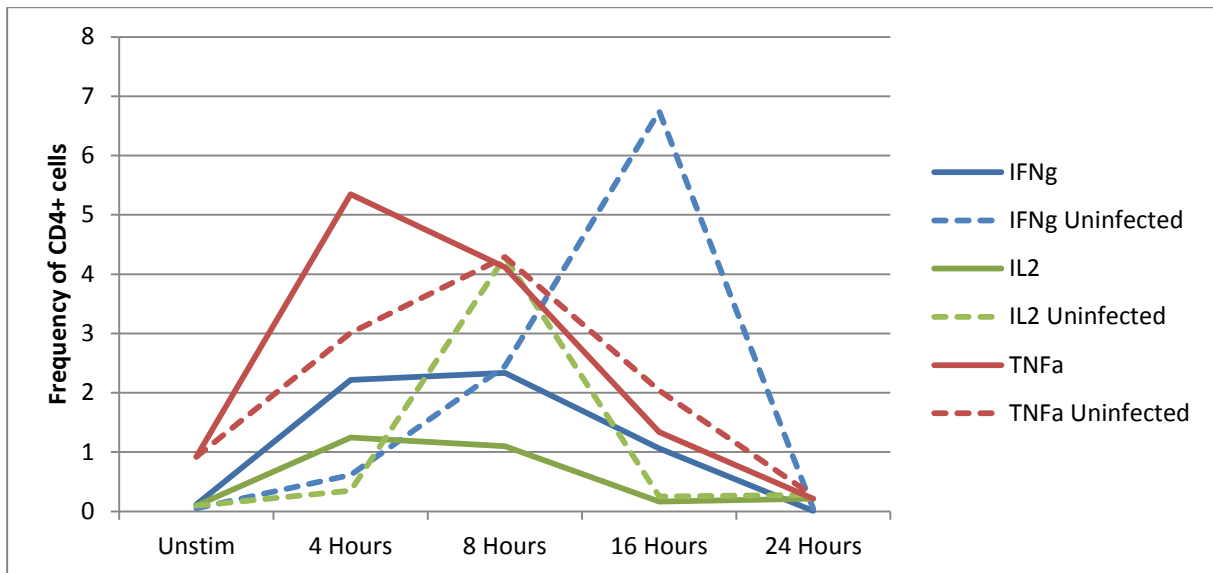


Figure 4.7: The frequency of CD4+ T cells producing the cytokines IFN γ , TNF α , or IL2 at different times of restimulation with autologous *M. avium* infected macrophages. PBMCs were isolated, and T cells were expanded with heat killed *M. avium* at a ratio of 3:1 for 10 days and restimulated overnight with autologous macrophages uninfected or infected with *M. avium* with a MOI of 10:1. Protein transport inhibitor was added for the last 4 hours of the total stimulation time. The solid lines represent T cells that have been restimulated with infected macrophages, and the dotted lines represent T cells that have been restimulated with uninfected macrophages. Production of the CD4+ T_H1 effector cytokines IFN γ (blue), TNF α (red), and IL2 (green) was analysed 4, 8, 16, and 24 hours after co-culturing with autologous macrophages. The unstimulated sample represents T cells that have not been restimulated with macrophages. Results obtained from one experiment with duplicate wells. Large variations were seen within each condition.

Since a higher frequency of cytokine producing CD4+ T_H1 cells was seen at 4 hours than at 24 hours (**Figure 4.7**) one experiment was carried out where the T cells were restimulated for a total of 4 hours with macrophages infected with live *M. avium* with MOIs from 0.1:1 to 100:1. We did not observe a higher frequency of CD4+ T_H1 cytokine producing cells after 4 hour stimulation than after 24 hour stimulation (data not shown).

5 Discussion

Findings from the work carried out in this thesis suggest that Cul3 and Rbx1 are important for the KEAP1 mediated inflammatory regulation in *M. avium* infection in primary human macrophages, as knockdown of both Cul3 and Rbx1 resulted in an upregulation in inflammatory cytokines following *M. avium* infection. We also found that KEAP1 knockdown downregulated the expression of HLA-DR and CD80 on the surface of primary human macrophages, independent of *M. avium* infection. These results implicate that KEAP1 might play a role in regulating co-stimulation and antigen-presentation to CD4+ T cells.

The oxidative stress sensor KEAP1 has various roles in the cell, including downregulation of NF- κ B signalling⁵⁴, links to autophagy^{52,87}, and a role in inflammation⁵⁵. KEAP1 has been shown to negatively regulate the inflammatory response and autophagy in response to infection with the non-tuberculous opportunistic pathogen *M. avium* in primary human macrophages⁵⁵. The mechanism of how KEAP1 negatively regulates the inflammatory response to *M. avium* is still unclear, and was under investigation in this thesis. In particular, the roles of the E3 ubiquitin ligase complex proteins Cul3 and Rbx1 in regulation of inflammation in response to *M. avium* infection and possible roles of KEAP1 in antigen presentation were investigated.

The transcription factor NF- κ B is negatively regulated by KEAP1 through KEAP1 dependent ubiquitination and hence degradation of IKK β ⁵⁴. There is conflicting evidence about how IKK β is inhibited. Proteasomal degradation, degradation through autophagy, and inhibition of phosphorylation have all been suggested as possible mechanisms^{54,88}. Results from our group show that primary human macrophages produce ROS in response to infection with *M. avium*. We have also seen that knockdown of KEAP1 leads to an increased inflammation in response to mycobacterial infection, indicated through measurements of a range of cytokines produced both by activation of NF- κ B and IRFs. Autophagy was also induced by *M. avium* infection, and autophagy increased in response to KEAP1 knockdown. This is the first time the role of KEAP1 in regulating inflammation and autophagy in response to *M. avium* has been reported⁵⁵.

Primary human cells are often time consuming and difficult to work with. Cell lines are often faster to work with, cells can be available at any time, and it is easier to get reproducible results independent of donor variations. HEK293 cells are easy to transfect and can be used to express proteins of interest⁸⁹. We wanted to dissect the mechanism of how KEAP1 regulates the inflammatory response following mycobacterial infections. KEAP1

interacts with several proteins (including NRF2, p62, and the Cul3/Rbx1 complex), and a way to determine the role of each of these binding partners in inflammation is through systemic siRNA knockdown of each protein. HEK293 cells can in addition be transfected with plasmids containing mutated KEAP1 constructs where the interaction site of various proteins is removed to further dissect the mechanisms involved in inflammatory regulation by KEAP1. In addition, fewer experiments would most likely have to be carried out to obtain conclusive results, compared to work in primary human macrophages, as there are no donor variations that have to be taken into consideration. An example of how donor variation may affect results is seen in the experiments carried out in **Section 4.2** where two experiments showed increased inflammation following *M. avium* infection when knocking down KEAP1, Cul3, or Rbx1, while one experiment showed a decreased inflammation following knockdown, although with a great variation between the duplicates. All of these advantages would make it easier to unravel the mechanism of the KEAP1 regulated inflammatory response to *M. avium* using a cell line.

We show in this thesis that siRNA treatment of HEK293 cells is effective already after 48 hours, and these cells only require one siRNA treatment, compared to two treatments in primary human macrophages, saving both time and reagents using this system. HEK293 cells naturally express few TLRs on their surface, and we therefore chose to test a cell line that was stably transfected with TLR2 tagged with GFP. TLR2 was chosen, as it has been shown necessary for induction of NF- κ B in response to mycobacterial infections⁹⁰. Using different methods for testing both the induction of TNF α and activation of NF- κ B in HEK293 cells showed a decreased inflammatory response in KEAP1 knockdown cells compared to controls following *M. avium* infection (see **Section 4.1**). A decreased inflammatory response following KEAP1 knockdown is the opposite of what has been shown in primary human macrophages, where a range of pro-inflammatory cytokines are more highly induced in KEAP1 knockdown cells compared to controls following *M. avium* infection⁵⁵. Due to different inflammatory responses following *M. avium* infection in HEK293 cells and primary human macrophages, we believe that HEK293 cells are not a good model system for dissecting the mechanisms of how KEAP1 regulates inflammation in *M. avium* infection. One possible explanation for the different responses is that primary human macrophages express a range of PRRs, both on the plasma membrane and on intracellular membranes, many of which are important for both the recognition and phagocytosis of mycobacteria¹⁹, while some of these receptors may not be present in the HEK293 cells. Although TLR2 is shown to be important for NF- κ B activation in response to mycobacteria⁹⁰, many other receptors are also involved in the recognition of

mycobacterial antigens, and some of these may be responsible for the responses we see. This underlines the importance of always confirming findings in primary cells, as model systems might respond differently than the primary cells to various treatments.

siRNA is a helpful method to study genes of interest, but there is also a risk of off-target effects using this method, both at mRNA and protein levels⁹¹. The induction of interferon-stimulated genes has also been detected in response to siRNA, but there is contradictory evidence of this^{92,93}. Different siRNAs targeting the same genes can induce divergent effects on off-target control genes⁹³. Cleavage of the target mRNA occurs when there is a complete match between the siRNA and the target strand. However, there can be translational blockage of mRNA with less than perfect base pairing, which might also be a reason for off-target effects observed with some siRNAs⁹³. Due to these effects, one needs to be careful when analysing siRNA data. We tested how siRNA treatment for each of our genes of interest (KEAP1, Cul3, and Rbx1) affected the expression of the untargeted genes (**Figure 4.3.A**). At the mRNA level there was little off-target effect, and the siRNA to one partner of the complex did not influence the expression of the others. Although the siRNA treatments against KEAP1, Cul3, and Rbx1 did not appear to influence each other, we cannot say if the siRNA affects other genes related or unrelated to the inflammatory response. We observe an increase in the inflammatory response in uninfected cells treated with the non-targeting siRNA (AS), compared to untreated controls, suggesting that the siRNA on its own may induce some inflammatory response (data not shown). To try to correct for the unspecific siRNA induced inflammation, we chose to present data as the fold induction of cytokine expression of uninfected against infected cells given the same siRNA treatment. This would correct for the inflammation caused by each of the siRNAs.

The mechanism of how KEAP1 is involved in regulating the inflammatory response is still unclear. After concluding that the HEK293 model system was not an appropriate system to investigate the mechanism behind how KEAP1 regulate inflammation in response to *M. avium* infection, the investigation was continued in primary human macrophages. KEAP1 functions as an adaptor for Cul3-based E3 ubiquitin ligase⁴⁶, and we wanted to investigate what role the E3 ubiquitin ligase proteins Cul3 and Rbx1 played in the inflammatory regulation following *M. avium* infection. This was carried out through knockdown experiments targeting each of the three genes KEAP1, Cul3 or Rbx1 as presented in **Section 4.3**. In agreement with previous results the knockdown of KEAP1 gave higher induction of the cytokines TNF α , IFN β , and IP10 compared to control samples following *M. avium*

infection in two independent experiments (**Figure 4.3 B**). The trend of higher inflammatory response appears to be the same for the cells with reduced expression of Cul3 and Rbx1. One experiment shows the opposite result, where the silencing of all three genes of interest showed lower cytokine induction following *M. avium* infection compared to control samples, although there were big variations within duplicates in this experiment. Variations in the inflammatory response to infection between experiments may in part be explained by the use of primary human macrophages (**Figure 4.3 C**). The use of primary cells means that every donor is different, and hence the variation between experiments can be expected to be bigger than with the use of a cell line. Several repeats were also required to allow our group to conclude upon the role of KEAP1 in inflammation⁵⁵. The use of primary cells is still beneficial because it gives us more reliable insight into what actually happens in the human body, compared to the use of cell lines or mouse cells where the results may not always be transferrable to primary human cells. Previously the effect of knocking down NRF2 or p62 has been investigated in our group. Since KEAP1 serves as a negative regulator for the transcription factor NRF2^{44,46}, one can speculate that a changed immune response following KEAP1 knockdown and infection might be due to the increased activity of NRF2. However, knockdown of NRF2 gave an inflammatory response comparable to control cells following *M. avium* infection⁵⁵. The selective autophagy substrate p62 can bind and inactivate KEAP1⁵², and KEAP1 is indicated to be degraded through autophagy in a p62 dependent manner⁹⁴. p62 knockdown has shown a decreased induction of inflammation in response to *M. avium* infection (unpublished data). We can speculate that KEAP1 accumulates in p62 depleted cells due to less inactivation and degradation, and hence the inflammatory response is decreased. However p62 has also been reported to have a direct negative regulatory effect on activation of NF- κ B⁹⁵, which would suggest a higher inflammatory induction in the absence of p62.

The preliminary findings presented here indicate that knockdown of Cul3 as well as Rbx1 gives the same results as knockdown of KEAP1. It can be speculated that the ubiquitination carried out by the E3 ubiquitin ligase complex is an important factor in the regulation of the inflammatory response. We cannot conclude that knocking down Cul3 or Rbx1 gives the same increase in inflammatory response as knocking down KEAP1, and more repeats of the experiment is needed to determine this. There is however reason to believe the outcome is the same, as the two experiments showing expected results for KEAP1 knockdown also showed increased inflammatory response in Cul3 and Rbx1 knockdowns (**Figure 4.3 B**). Lee *et. al.*⁵⁴ focused on the role of KEAP1 in cancer, and saw that there was a high percentage of genome loss or mutations in the genes encoding KEAP1, Cul3, and Rbx1

in cancers that did not degrade IKK β . They suggest KEAP1 induction may be a target for drug development as it influences the expression of both IKK β and NRF2, which unregulated may lead to cancer or chemotherapy resistance. However, in infection with *M. avium* KEAP1, Cul3, and Rbx1 all seem to be beneficial for the bacteria and not the host, as decreasing the expression of these genes increases the inflammatory response. KEAP1 also decreases the intracellular survival of the bacteria⁵⁵. These results taken together suggest that the ubiquitin machinery associated with KEAP1 is important both in the regulation of cancer development and in regulation of inflammatory responses. Using KEAP1 as a target for drug development might be promising, however it is important to take into consideration that KEAP1 appears to be beneficial for the survival of some pathogens, as demonstrated with *M. avium*.

For the detection of cytokines following stimulation of cells, we measured the mRNA level using real-time PCR. This gives us information about the transcriptional level of genes, which might not always correlate with protein levels. Studies show varying results regarding this issue, and different cytokines show different correspondence between cytokine mRNA and secreted protein levels in animal models^{96,97}. A study using human cells suggests a good correlation between mRNA and protein levels of some cytokines (including IFN γ , TNF α , and IP10), while other cytokines did not show correlation⁹⁸. These data suggest that the expression measured at the mRNA level does not always reflect the protein expression. A comparison of the mRNA levels and protein expression measuring cytokine responses following *M. avium* infection has previously been carried out in our lab, and showed good correlation. Due to these previous results suggesting good correlation between protein and mRNA levels only real-time PCR was carried out to measure cytokine expressions in this thesis. Other methods that could be used to confirm results from the mRNA analysis include measuring protein levels by enzyme-linked immunosorbent assay (ELISA) or western blotting.

Measurements of knockdown efficiency were also done by real-time PCR. Also here there might not be concordance between the levels measured at the mRNA level and the actual presence of proteins. KEAP1 has been measured to have a half-life of 12.7 hours in resting cells, which goes down following electrophilic stress in an autophagy dependent manner⁹⁴. The siRNA treatment in the primary MDMs lasted for 72 hours, and we can therefore assume that low mRNA levels reflect low protein levels. Previous results from our group have also showed a good correlation between mRNA and protein levels following

knockdown with siRNA. Real-time PCR is a good method when starting an investigation of new proteins, as it is relatively quick and can give an indication of the protein expression.

HLA-DR is a MHC class II protein and CD80 is a T cell co-stimulatory molecule, both found on the surface of APCs. Their expression becomes upregulated when APCs comes into contact with antigens. Both of these molecules are required for the stimulation and activation of specific CD4+ T cells⁵⁹. Since KEAP1 has been shown to play a role in inflammation following *M. avium* infection⁵⁵, and *M. tuberculosis* has been shown to downregulate the expression of MHC class II molecules on the surface of macrophages¹⁸, we wanted to investigate if KEAP1 influenced the antigen-presentation following *M. avium* infection in primary human macrophages. Any changes in the surface markers might influence the activation of the adaptive immune system, specifically CD4+ T cells. It appears that the knockdown of KEAP1 alone decreased the expression of both HLA-DR and CD80 with approximately 20% (**Figure 4.4 B**). In the presence of *M. avium* infection, the expression of both CD80 and HLA-DR was somewhat increased in the KEAP1 knockdown cells. These results suggest that KEAP1 has a positive role on expression of molecules associated with antigen presentation. The mechanism behind how KEAP1 influence the antigen presentation and how *M. avium* affects this is not known, but there can be several possibilities as KEAP1 is a molecule with many interaction partners involved in various cellular mechanisms. We might speculate that autophagic processes are involved.

Autophagy is a defence mechanism that is shown to contribute to antigen presentation, decrease intracellular survival, and increase the adaptive immune response to *M. tuberculosis* infections^{35,38,39}. Our group has provided evidence suggesting that *M. avium* induces autophagy⁵⁵. Autophagy was induced already at 4 hours of infection, and sustained up to 72 hours post infection. As autophagy is induced by *M. avium*, and induction of autophagy has been shown to increase peptide presentation and T cell responses to *M. tuberculosis*³⁹, we might expect the expression of HLA-DR and CD80 to increase in response infection with this pathogen. The presence or absence of infection did appear to affect the levels of HLA-DR and CD80 to some degree, by decreasing the difference in expression seen between control cells and KEAP1 knockdown cells, however no significant difference was seen between control cells (siAS) and infected control cells (siAS infected) (**Figure 4.4 B**). 4 hours of infection has been shown to be enough to induce autophagy, but it is possible that more time is needed to see the change in antigen-presentation. Further experiments should aim at investigating CD80 and HLA-DR expression at different time points post *M. avium* infection +/- knockdown of

KEAP1. KEAP1 has previously been reported to have a positive role in autophagy regulation for ubiquitin aggregate clearance⁸⁷. Results from our lab, however, suggest that KEAP1 may negatively regulate autophagy in *M. avium* infected in human macrophages⁵⁵. These conflicting results make it hard to draw any conclusions.

We are looking at isolated pathways when investigating the effects of knocking down KEAP1, and many factors outside of our focus may influence our findings. Uncontrolled inflammation can be damaging to the cells, and mechanisms to control this are needed. Knocking down KEAP1 leads to increased pro-inflammatory cytokine production⁵⁵. It might therefore be possible that KEAP1 on the other hand downregulates the stimulatory molecules needed for T cell activation in order to avoid excessive inflammation. We do, however, not know how much the downregulation of HLA-DR and CD80 that we see would affect the following acquired immune response and CD4+ T cell activation. If KEAP1 positively regulates the expression of the two proteins HLA-DR and CD80, it would be interesting to see how induction of KEAP1 changed the expression of these two molecules. If an induction of KEAP1 leads to an increase in expression of HLA-DR and CD80 on the surface of primary human macrophages, this could be a possible target to increase vaccine efficiency through increased T cell activation. Again, it is important to take into consideration the positive role KEAP1 appears to have in survival of intracellular *M. avium*⁵⁵. In order to understand if and how KEAP1 affects the activation of mycobacteria-specific CD4+ T cells, experiments where mycobacteria-specific T cells were expanded, and restimulated with *M. avium* infected autologous macrophages were carried out.

The protection provided by mycobacteria-specific CD4+ T_H1 cells is essential for the host defence against pathogenic mycobacteria such as *M. tuberculosis*. It has also been shown that the presence of multifunctional CD4+ T cells simultaneously producing the cytokines IFN γ , TNF α , and IL2 reduce the burden of mycobacteria⁷⁴. Knowing this we wanted to investigate the activation and cytokine production of CD4 T_H1 cells specific to *M. avium*, in response to *M. avium* infected human primary macrophages. We also wanted to investigate if KEAP1 knockdown in the macrophages would affect the T cell activation, as we observed a downregulation of the surface proteins HLA-DR and CD80 on the surface of primary human macrophages following KEAP1 knockdown.

Before we could investigate the T cell activation, there was a need to establish the method for expansion and restimulation of specific T cells. Different conditions for both expansion and restimulation were tested out, trying to identify the optimal conditions for our

investigation. T cells were able to get activated and produce cytokines, as demonstrated using a positive control (**Figure 4.5 B**). This indicates that the T cells were alive and able to be activated upon stimuli. It also gives an indication that the staining protocol for flow cytometry was functioning, as cytokines were stained and detected in these samples (**Figure 4.5 B**). As demonstrated in **Section 4.4**, we were not able to obtain a cytokine response at the level we expected, and the response was relatively low with mostly around 0.5% of CD4⁺ T cells producing either cytokine tested. Stimulation of expanded T cells with uninfected macrophages gave comparable cytokine production as stimulation with *M. avium* infected macrophages in some experiments. In the kinetics experiment, where the cytokine response from T cells was tested at different time points, we saw that uninfected macrophages also induced a cytokine response, although this response is somewhat slower than with the infected macrophages. This unspecific activation may be due to unspecific interactions with the macrophages. In *in vitro* experiments, conditions are never optimal for the cells, and some cells will be in distress or dead. Signals from these cells may be enough to activate T cells. Cells were assessed by microscopy before the start of each experiment, and were not used unless they looked healthy. We also had problems with large variations both between different donors and within biological replicates from the same donor. The variation between donors can in some part be explain by the fact that we were working with primary human cells, and that cells from different donors will always vary in their response to different stimuli. There may also have been some differences in the number of macrophages per well, leading to different numbers of cells presenting antigens for T cell stimulation.

The low cytokine response may be due to an inability to expand the T cells specific for the pathogen of interest. As mentioned above, from unexpanded T cells only a low fraction of the cells would recognise antigens presented from the *M. avium* infected macrophages, which could explain the low fraction of CD4⁺ T cells producing cytokines. Several parameters were tested out to attempt to increase the expansion, without luck. Other parameters that could be tested out for a better expansion include changing the ratio of PBMCs for expansion, changing the ratio of T cells to macrophages upon restimulation, addition of less or no IL2 during the expansion step, longer expansion time, or using Purified Protein Derivative (PPD) from mycobacteria for expansion of specific T cells instead of heat killed *M. avium*. We could also measure the levels of cytokines in the supernatant using ELISA.

One of our aims was to look at the fraction of multifunctional CD4⁺ T_H1 cells producing all three cytokines IFN γ , TNF α , and IL2 in response to *M. avium* infected autologous

macrophages. However, due to the low response for each cytokine, the fraction of multifunctional T cells could not be addressed in this work. In order to carry out this investigation, the protocol for expansion and restimulation of specific T cells must be optimised further, to allow solid analysis of T cell activation and cytokine production in response to stimulation with *M. avium* infected autologous macrophages. If a functional protocol is established it would also be interesting to investigate the effect of mycobacteria-specific CD4⁺ T cells on the intracellular killing of *M. avium* in the infected autologous macrophages.

6 Conclusion

Looking at evidence obtained previously and results from this thesis, there is little doubt that KEAP1 plays a role in the host defence against the opportunistic pathogen *M. avium*. KEAP1 appears to play roles in the inflammatory response in macrophages following *M. avium* infection by downregulating the following cytokine response⁵⁵. The mechanism underlying the KEAP1 regulation of inflammatory responses and autophagy is still unclear.

We concluded that HEK293 cells are not a suitable model system for dissecting the mechanisms behind how KEAP1 regulates the inflammatory response following *M. avium* infection. Primary human macrophages were therefore used for the investigation. Findings from the work done in this thesis indicate that the ubiquitination machinery associated with KEAP1, including the proteins Cul3 and Rbx1, play an important role in the KEAP1-mediated regulation of inflammatory responses to *M. avium* infection.

Furthermore, the results presented here add evidence that KEAP1 might also play a positive role in antigen presentation. We found that the expression of CD80 and HLA-DR were approximately 20% downregulated when KEAP1 was knocked down in primary human macrophages. How KEAP1 positively influence the two molecules CD80 and HLA-DR and if this might have consequences for mycobacteria-specific T cell activation could not be determined in this study.

7 Future perspectives

HEK293 cells were not found an appropriate model system for dissecting the role of KEAP1 in inflammatory regulation in response to *M. avium*, as they responded differently compared to primary human macrophages. Other cell lines should be examined to establish a good model system for further analysis of KEAP1s involvement in inflammation. With a suitable model system that allows knockdown of endogenous KEAP1 as well as transfection with differently mutated variants of the gene, the dissection of how KEAP1 regulates inflammation in response to *M. avium* infection can be further investigated.

Through this project we have obtained preliminary results suggesting that the ubiquitin machinery (Cul3 and Rbx1) associated with KEAP1 is important for the regulation of inflammation following *M. avium* infection in primary human macrophages. Knockdown of these two genes prior to infection give a similar inflammatory response as knockdown of KEAP1. More repeats of the experiment should be carried out in order to conclude upon this. If these molecules are proved to be central in the regulation of inflammation, further research should be carried out to identify the targets for ubiquitination and how these are degraded. If KEAP1 regulates degradation of central inflammatory signalling molecules, this can also have implications for other systems. If this is the case, the regulation we see is probably not mycobacteria-specific, but can also be important for other infections and inflammatory conditions where the same signalling pathways are involved.

Evidence obtained here suggests that KEAP1 positively influences the expression of CD80 and HLA-DR, both molecules that are involved in antigen-presentation and activation of CD4+ T cells. How KEAP1 positively influences expression of these two molecules in human macrophages is not known and more research should be undertaken to investigate this.

It is not known if KEAP1's effect on expression of molecules involved in antigen-presentation, as well as its effect on inflammation in response to *M. avium* infection has an impact on subsequent activation of a mycobacteria-specific adaptive T cell response. In this work, we tried to establish a model system that would allow analysis of the activation of expanded mycobacteria-specific T cells in response to stimulation with autologous macrophages *in vitro*. More experiments are necessary to optimise this model system. So far we can only speculate that KEAP1 knockdown has an inhibitory effect on the antigen-specific CD4+ T cell activation, and future work will show how KEAP1 in innate immune cells affects the adaptive immune response.

8 References

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9 Appendix

Appendix 1: siRNA additional information

KEAP1 siRNA sequences

Name	Catalog no.
Hs_KEAP1_5	SI03246439
Hs_KEAP1_7	SI04267886
Hs_KEAP1_6	SI04155424
Hs_KEAP1_2	SI00451675

Cul3 siRNA sequence

Name	Catalog no.
Hs_CUL3_5	SI02225685
Hs_CUL3_8	SI03030160
Hs_CUL3_10	SI04379963
Hs_CUL3_2	SI00053347

Rbx1 siRNA sequence

Name	Catalog no.
Hs_RBX1_5	SI02654190
Hs_RBX1_6	SI02654316
Hs_RBX1_3	SI00700525
Hs_RBX1_9	SI04438469

Appendix 2: Staining for flow cytometry

Extracellular staining

All samples for flow cytometry were transferred to polystyrene tubes, where the staining and fixation steps occurred, followed by running the sample. Flow wash is a solution with PBS containing 2% FCS.

1. Label polystyrene tubes appropriately, and add 1 ml of flow wash to all tubes. To kill any live bacteria, 0.05% NaN₃ was added to the wash buffer at this first stage.
2. The appropriate sample was added to the labelled tubes.
3. The tubes were centrifuged at 528 RCF for 5 minutes, and the supernatant was discarded by decanting.
4. Extracellular staining step: 1 µl of each extracellular stain was added to each tube. For convenience, all extracellular stains were added to an Eppendorf tube and made up to a final volume of 10 µl using flow wash. One “colour mix” was made for all samples. 10 µl of the mix was added to each of the tubes, which were then vortex and left on ice for 15 minutes protected from light.
5. To remove any unbound stain, 1ml flow wash was added per tube, and centrifuged at 528 RCF for 5 minutes.
6. The supernatant was discarded by decanting, followed by fixation. 500 µl of 2% paraformaldehyde (PFA) per tube was diluted in PBS from a 16% stock. The tubes were vortexed and incubated at room temperature for 15 minutes, protected from light.
7. Samples were centrifuged at 528 RCF for 5 minutes, and the supernatant removed.
8. The samples were resuspended in approximately 1 ml wash buffer by vortexing. The extracellular staining was now complete, and the samples were further used for:
 - I) Flow cytometry analysis, or
 - II) Extracellular staining (see step 9 and on)

Intracellular staining

9. The samples that had been extracellularly stained were centrifuged at 528 RCF for 5 minutes.
10. The supernatant was removed and the samples were resuspended in 1 ml wash buffer, and centrifuged again, using the same settings as above.
11. The supernatant was removed by decanting. In order to be able to stain the cells intracellularly they had to be permeabilised. This was accomplished by using saponine. The samples were resuspended in 500 µl of flow wash with 0.5% Saponin.
12. Samples were centrifuged at 528 RCF for 5 minutes. The supernatant removed, and cells were resuspend by vortexing in the 0.5% saponin solution again, and incubated for 5 minutes at room temperature.
13. Samples were centrifuged at 529 RCF for 5 minutes the supernatant discarded.
14. Intracellular staining: A “color mix” was prepared as for the extracellular staining (see step 4). 10 µl of the mix was added per sample, and the cells left to incubate for 30 minutes at room temperature, protected from light.
15. The samples were then washed twice by addition of 1ml wash buffer followed by centrifugation at 528 RCF for 5 minutes and discarding the supernatant between.
16. The samples were resuspend in 0.5 ml of wash buffer and were now ready for flow cytometry analysis. The samples can be stored overnight at 4°C, protected from light.